THE IMPACT OF CYCLOPHOSPHAMIDE ON MALE GERM CELL QUALITY AND CONSEQUENCES ON EARLY POST-FERTILIZATION EVENTS

by

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This thesis is dedicated to…

…My Grandfather, whose wisdom and strength taught me the importance of determination, perseverance, honesty and pride.

…My parents, who have always encouraged and supported me with unconditional love, I could not have accomplished any of this without their guidance; to my sister, for reminding me to live life to its fullest.

…My husband, for always being there for me, and whose patience, love and optimism were essential elements through every step of the way.

… And to my daughter, whose beaming smile and endless energy inspired the completion of this work.

ABSTRACT

Paternal exposure to therapeutic drugs or environmental chemicals can alter the genomic integrity of male germ cells, and consequently, be a source of detrimental effects on embryo development. Preconceptional exposure of male rats to the anticancer alkylating agent, cyclophosphamide, resulted in increased embryo loss, malformations and behavioral deficits in the offspring; these drug effects can be transmissible to subsequent generations. The goal of the studies presented in this thesis is to gain an understanding of the manner by which altered spermatozoal genomic integrity, acquired during preconceptional exposure to a DNA damaging agent, may lead to developmental instabilities in the early preimplantation embryo. Spermatozoa must be functional in order to effectively transmit genetic information to the oocyte. Motility is one component contributing to the ability of the spermatozoon to achieve proximity to and penetration of the oocyte for fertilization. Chronic cyclophosphamide exposure had little effect on the kinematic parameters constituting spermatozoal motion patterns. Drug-exposed spermatozoa are motile, and may thus be at an increased risk of transmitting an altered genomic complement to the embryo. An intact paternal genome is essential for normal embryogenesis. Using the rat sperm Y-4 fluorescence in situ hybridization assay, we determined that 9 weeks of chronic cyclophosphamide treatment, but not 6 weeks, significantly increased the frequency of numerical chromosomal abnormalities in epididymal spermatozoa. Epigenetic marks, characterized by various posttranslational modifications, also play an intricate role in chromatin dynamics during spermatogenesis and embryogenesis. Early post-fertilization, histone H4 acetylation and DNA methylation were dysregulated in both parental pronuclei in zygotes sired by cyclophosphamide-treated males. Intriguingly, posttranslational modifications important for DNA damage recognition are rapidly activated following fertilization by drug-exposed spermatozoa. Phosphorylated histone H2AX was dramatically increased in a biphasic manner in the paternal genome; Interestingly, poly(ADP-ribose) polymerase-1 was markedly elevated in both the paternal and maternal genome in zygotes fertilized by cyclophosphamide-treated

males. Collectively, these studies demonstrate that male germ cell damage acquired during spermatogenesis can be transmitted to the embryo leading to aberrant epigenetic reprogramming and a dramatic induction of distinct modifications involved in DNA damage recognition in the 1-cell embryo. Importantly, we have highlighted the involvement of pronuclear cross talk as a unique phenomenon for both epigenetic reprogramming and DNA damage response in the zygote.

RÉSUMÉ

L'exposition paternelle à des traitements thérapeutiques ou à des produits chimiques contenus dans l'environnement peut modifier l'intégrité génomique des cellules germinales mâles, et par voie de conséquence, être la source d'effets négatifs sur le développement embryonnaire. Le traitement de rats mâles avec un agent alkylant anticancéreux, le cyclophosphamide, avant la conception, augmente le taux de perte embryonnaire, de malformations et de déficits comportementaux chez la descendance qui peuvent être transmis sur plusieurs générations. Le but de ce travail de thèse est de déterminer les mécanismes cellulaires et moléculaires par lesquels des anomalies génomiques contenues dans les spermatozoïdes, après exposition à un agent altérant l'ADN, peuvent induire des anomalies dans le développement embryonnaire préimplantatoire. Le spermatozoïde doit être fonctionnel afin de transmettre son information génétique à l'ovocyte. La motilité constitue un des critères de fonctionnalité des spermatozoïdes puisque qu'il permet la proximité de ces deux cellules pour la fécondation. Nous avons démontré qu'une exposition chronique au cyclophosphamide n'a que peu d'effet sur les paramètres de motilités des spermatozoïdes. Les spermatozoïdes exposés sont motiles et peuvent donc transmettrent des anomalies génétiques à l'embryon. L'intégrité du génome paternel est un autre paramètre essentiel pour l'embryogenèse. En utilisant la technique d'hybridation in situ Y-4 FISH, nous avons montré une augmentation de la fréquence d'un nombre anormal de chromosome par spermatozoïde prélevé dans l'épididyme après 9 semaines de traitement chronique au cyclophosphamide ; un traitement de 6 semaines n'ayant pas d'effet significatif. Les marques épigénétiques, caractérisées par diverses modifications post-traductionnelles, jouent un rôle dans les modifications de la chromatine pendant la spermatogenèse et l'embryogenèse. Chez les embryons issus de pères traités au cyclophosphamide , nous avons observé que peu après la fécondation, l'acétylation de l'histone H4 ainsi que la méthylation de l'ADN sont altérés dans les deux pronucléus parentaux. De plus, nous avons montré que les modifications post-traductionnelles nécessaires à la reconnaissance des

altérations de l'ADN sont rapidement augmentées chez ces zygotes: la phosphorylation de l'histone H2AX est augmentée de façon biphasique dans le génome paternel et la poly(ADP-ribose) polymérase-1 est augmentée dans les génomes paternel et maternel. L'ensemble de ces résultats montre que les altérations du génome des cellules germinales mâles pendant la spermatogenèse peuvent être transmises aux embryons, pouvant induire une programmation épigénétique anormale et des modifications dans la reconnaissance des anomalies de l'ADN chez le zygote. Enfin, cette étude a permis de mettre en évidence un rôle unique du dialogue entre les deux pronucléus dans la pre-programmation épigénétique et la réponse aux altérations de l'ADN chez le zygote.

TABLE OF CONTENTS

LIST OF FIGURES

Chapter 1:

Chapter 2:

Chapter 3:

Chapter 4:

Chapter 5:

LIST OF TABLES

FORMAT OF THE THESIS

This thesis is comprised of six chapters, three of which are in the format they were submitted for publication. Chapter I, the introduction, provides an overview of spermatogenesis, early preimplantation development and the ways by which paternal toxicant exposures may disrupt germ cell quality, and subsequently interfere with normal embryonic development. The mechanisms contributing to the DNA damage response during spermatogenesis and in the early embryo, as well as former work on cyclophosphamide are also discussed. Chapter I concludes with the rationale and research objectives set forth for the studies here within. Chapter II is a data chapter describing the effects of chronic cyclophosphamide exposure on spermatozoal motility parameters. Chapter III has been published in *Biology of Reproduction;* 69(4):1150-1157, 2003. Chapter IV has been published in *Proceedings of the National Academy of Sciences;* 102(22):7865-7870, 2005. Chapter V was submitted for publication to the journal, *Toxicological Sciences,* 2007. Connecting texts are included to ensure continuity in accordance with Thesis Preparation and Submission Guidelines for a Manuscript-based thesis. Chapter VI, the discussion, highlights the key findings of this work in the context of the field of reproductive medicine; this is followed by the List of Original Contributions.

CONTRIBUTION OF AUTHORS

For chapter III, all animal treatments, sample collection, slide preparation and analysis was done by the candidate. Sperm Y-4 fluorescence *in situ* hybridization was done by the candidate in collaboration with Francesca Hill of Lawrence Livermore National Laboratory, Livermore, California. The manuscript was written by the candidate with approval from co-authors.

For chapters II, IV and V, the candidate conducted all of the experiments under the supervision of Dr. Barbara Hales and Dr. Bernard Robaire.

ABBREVIATIONS

3AB	3-aminobenzamide
4-OHCPA	4-hydroxycyclophosphamide
A_{al}	$A_{aligned}$ spermatogonia
ABVD	adriamycin, bleomycin, vinblastine and decarbazine
ALH	amplitude of lateral head displacement
A_{pr}	A_{pairs} spermatogonia
A_s	A_{single} spermatogonia
ATM	ataxia telangiectasis mutated
ATR	ataxia telangiectasia mutated and Rad3-related
BCF	beat cross frequency
BEP	bleomycin, etoposide, cisplatin
BER	base excision repair
CASA	computer assisted sperm analysis
COPP	cyclophosphamide, vincristine, procarbazine and prednisone
DBCP	dibromochloropropane
DDT	dichlorodiphenyl trichoroethane
DL	dominant lethal
DNA-PK	DNA dependent protein kinase
DNMT	DNA methyltransferase
DSB	double strand break
EDC	endocrine disrupting chemical
ESC	embryonic stem cell
ESTR	expanded simple tandem repeats
FISH	fluorescence in situ hybridization
FS	fibrous sheath
HAT	histone acetyltransferase
HDAC	histone deacetylase
HR	homologous recombination
HT	heritable translocation
ICM	inner cell mass

ICSI	intracytoplasmic sperm injection
IVF	in vitro fertilization
LIN	linearity
MMR	mismatch repair
MOPP	mechlorethamine, vincristine, procarbazine and prednisone
MS	mitochondrial sheath
MZT	maternal to zygotic transition
NER	nucleotide excision repair
NHEJ	non-homologous end joining
NOVP	novanthrone, oncovin, vincristine, prednisone
NPB	nucleolar precursor body
ODF	outer dense fiber
PARP-1	poly(ADP) ribose polymerase11
PCB	polychlorinated biphenyls
PGD	prenatal genetic diagnosis
PI3K	phosphatidylinositol-3 kinase-like
PN	pronuclear stage
RLGS	restriction landmark genomic scanning
ROSI	round spermatid injection
SC	synaptonemal complex
SCSA	sperm chromatin structure assay
SEM	standard error of the mean
SLT	specific locus test
SSB	single strand break
SSBR	single strand break repair
STR	straightness
TE	trophectoderm
TGR	transgenic rodent mutation
TRC	transcription-requiring complex
TSA	trichostatin A

TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling
UDS	unscheduled DNA synthesis
VAP	average path velocity
VCL	curvilinear velocity
VSL	straight line velocity
WHO	World Health Organization
ZGA	zygotic gene activation
γH2AX	phosphorylated histone H2AX

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CHAPTER 1

INTRODUCTION

1.1 Male Gametogenesis

1.1.1 Spermatogenesis

Spermatogenesis is a highly ordered process by which diploid spermatogonial stem cells undergo multiple mitotic divisions (proliferative phase), meiotic reduction (meiotic phase) and post-meiotic modifications (differentiation phase) in order to produce spermatozoa. The developmental journey from spermatogonia to functional spermatozoon is characterized by a host of unique events implemental in nuclear repackaging and epigenetic reprogramming such that the male genome is competent to accurately participate in early embryogenesis (Sassone-Corsi, 2002; Kimmins and Sassone-Corsi, 2005).

1.1.1.1 Proliferation Phase

Spermatogonial proliferation and stem cell renewal sustain the constant requirement for germ-cell precursors; the mitotic activity of spermatogonia continuously replenishes the germ cell population supporting male gamete production. Spermatogonia are defined according to nuclear heterochromatin content and have been subdivided into type A (heterochromatin absent), Intermediate (heterochromatin sparse) and type B spermatogonia (heterochromatin abundant) (Clermont, 1962). According to the "single stem cell" theory, the A_{single} (A_s) spermatogonia are the most primitive cells of spermatogenesis; half of the population mitotically divide to produce A_{paired} (A_{pr}) spermatogonia while the remainder of the A_s cells undergo self-renewal division. The A_{pr} spermatogonia actively proliferate into chains of $A_{aligned}$ (A_{al}) spermatogonia which differentiate into A_1 spermatogonia; A_1 spermatogonia enter a phase of sequential divisions producing A_2 - A_4, Intermediate and type B spermatogonia giving rise to primary spermatocytes following the last mitotic division (Huckins, 1971; Oakberg, 1971; de Rooij, 2001).

At variance with the single stem cell model is the "reserve stem cell" theory which proposes that A_s and A_{pr} spermatogonia are nonproliferative in the normal testis and are collectively denoted as A_0 spermatogonia (Clermont and

Bustos-Obregon, 1968; Clermont and Hermo, 1975); A_0 cells only divide when needed, as occurs after spermatogonial depletion following irradiation (Dym and Clermont, 1969). In this model, A_1, A_2, A_3 and A_4 spermatogonia retain stem cell properties at a similar phase of differentiation. A few A_4 spermatogonia divide to replenish A_1 cells while the majority progress to form Intermediate and type B spermatogonia (Clermont and Bustos-Obregon, 1968; Clermont and Hermo, 1975). Together, there are approximately 5 -10 spermatogonial divisions in the rat prior to the formation of diploid primary spermatocytes (Huckins, 1971; Clermont and Bustos-Obregon, 1968).

1.1.1.2 Meiotic Phase

Each primary spermatocyte duplicates its DNA and subsequently undergoes two meiotic divisions to produce haploid spermatids. Meiosis involves several intricate chromosomal events necessary to ensure that each gamete possesses an accurate genetic complement. Preceding the two meiotic cell divisions, spermatocytes undergo a lengthy prophase I during which chromosomes must find each other, align and synapse (Cobb and Handel, 1998). Prophase I can be divided into substages with respect to chromosomal movements and positions; these stages include leptonema, zygonema, pachynema, diplonema and diakinesis. The landmark feature of meiotic prophase is the synaptonemal complex (SC). This proteinaceous structure provides the structural basis for chromosome pairing, chaperones reciprocal recombination events and ensures proper disjunction of chromosome homologues from leptotene to anaphase I of meiosis (von Wettstein, 1984; Heyting, 1996). Sister chromatids replicate and prophase I initiates at leptonema when chromosome axes condense; pairing and synapsis begins in early zygonema and is completed in pachynema, the stage during which homologous chromosomes undergo recombination. During diplonema, chiasmata form between homologues in the regions where crossing over occurred, chromosomes desynapse and begin to segregate apart in preparation for the meiotic divisions (Handel et al., 1999a; Cohen and Pollard, 2001).

Spermatocytes enter into metaphase I during which chromosomes align at the equatorial plate; chiasmata separate and chromosomes move to separate poles during anaphase I before proceeding to telophase I and undergoing cytokinesis. The second meiotic division is similar to mitosis; sister chromatids separate and cytokinesis II results in the formation of two haploid spermatids from each secondary spermatocyte (Figure 1.1).

Successful meiotic divisions of the spermatocyte are essential for the generation of the correct ploidy in gametes as well as facilitating genetic diversity in successive generations (Handel et al., 1999b; Hassold and Hunt, 2001).

1.1.1.3 Differentiation Phase

During post-meiotic male germ cell development, termed spermiogenesis, round spermatids undergo striking biochemical, morphological and physiological processes resulting in the formation of flagellated spermatozoa (D'Occhio et al., 2007).

Early in spermatogenesis, until the round spermatid stage, male germ cell chromatin structure remains in a somatic cell-like conformation. Nucleosomes (consisting of an octamer of the core histones H2A, H2B, H3 and H4) connected by linker DNA are organized into a supercoiled "beads-on-a-string" conformation. At the onset of nuclear elongation (elongating spermatid stage), nucleosomal chromatin is dramatically changed from a nucleohistone to a sperm-specific nucleoprotamine structure; facilitating this nucleoprotein exchange are chromatin basic transition proteins. During spermatid differentiation, transition proteins are the major chromatin component actively involved in DNA destabilization and nuclear condensation prior to protamine deposition (Meistrich et al., 2003). Later in spermiogenesis, transition proteins are replaced by protamines which are responsible for the final transformation of chromatin into a highly condensed toroidal structure. Protamines stabilize DNA by the formation of inter- and intra-molecular disulphide bonds via a phosphorylation-dephosphorylation dependent process; the number of disulphide cross-links determines the level of chromatin stability. Ultimately, the replacement of histones by protamine results in

transcriptional silencing of sperm chromatin (Kimmins and Sassone-Corsi, 2005; D'Occhio et al., 2007).

In addition to the dramatic chromatin changes, the differentiating spermatid also develops an acrosome produced from the golgi apparatus; this lysosomal-like granule contains the enzymes necessary for sperm-egg penetration and fertilization. The axoneme assembles in the cytoplasm and protrudes from the cell to yield the initial flagellum which is appended onto the nuclear membrane by the cytoskeletal connecting piece. The outer dense fibers, fibrous sheath and mitochondrial sheath are assembled subsequently, providing the necessary structural components to permit the acquisition of sperm motility. Prior to spermiation, the release of sperm into the lumen of the seminiferous tubules, the spermatids shed unnecessary cytoplasm and organelles; spermatozoa released into the lumen of the seminiferous tubule are immotile and unable to fertilize oocytes (Clermont and Tang, 1985; Fawcett, 1975).

1.1.2 Epigenetic Regulation during Spermatogenesis

Chromatin is exceptionally dynamic during spermatogenesis; it contains both genetic information, encoded in DNA, and epigenetic information, regulated by DNA methylation and post-translational histone modifications. The histone code hypothesis proposes that covalent histone modifications, acting sequentially or in combination, create signals for the docking of multisubunit complexes which are implicated in chromatin-related functions (Strahl and Allis, 2000). Accordingly, DNA and histone modifications are crucial in spermatogenesis, serving as essential interfaces in the regulation of structure and function at specific chromatin domains. While chromatin-DNA interactions are directed by combinations of various modulations of histone tails, including, acetylation, methylation, phosphorylation, ADP-ribosylation, ubiquitination, and sumoylation, for purposes of this thesis, discussion will be primarily focused on histone acetylation and DNA methylation.

1.1.2.1 Histone Acetylation and Methylation

Histone acetylation is regulated in phase-specific waves throughout spermatogenesis (Figure 1.2). Core histones H2A, H2B and H4 are acetylated in spermatogonia and preleptotene spermatocytes, become globally hypoacetylated during meiosis and remain unacetylated in round spermatids. Interestingly, histone H4 hyperacetylation re-emerges in early elongating spermatids in the absence of DNA replication; acetylated chromatin is dispersed in the nucleus until acetylated histones begin to progressively disappear in the final stages of elongation. The loss of acetylation progresses in an antero-caudal direction corresponding to the pattern of nuclear condensation in spermatids implicating a role in directing the first steps of genome compaction. Specifically, histone acetylation during spermiogenesis appears to be tightly associated with the replacement of testis-specific histones and protamine-dependent chromatin condensation (Hazzouri et al., 2000; Meistrich et al., 1992a). Identification of the testis-specific bromodomain-containing protein, BRDT, which induces large-scale chromatin reorganization in the presence of hyperacetylated histone H4, further emphasizes the importance of histone acetylation in nuclear remodeling during spermiogenesis (Pivot-Pajot et al., 2003; Govin et al., 2006).

Histone methylation is also crucial for accurate spermatogenesis; histone H3 lysine 9 (H3-K9) methylation is prominent from leptotene spermatocytes to round spermatids. Disruption of histone methytransferases (HMTs) Suv39h1 and Suv39h2, which are responsible for H3 methylation at lysine 9, leads to chromosomal instability, impaired homologous chromosome pairing and meiotic arrest (Peters et al., 2001). Interestingly, the timing of DNA and histone H3-K9 methylation correspond to spermatogenic phases that are deacetylated (Figure 1.2), together suggesting a collaborative effort in achieving global gene repression in spermatocytes undergoing meiosis as well as in regulating chromatin architecture (Rousseaux et al., 2005).

1.1.2.2 DNA Methylation

DNA methylation, a well characterized epigenetic modulator, occurs, within dinucleotide motifs, on the 5'-position of cytosine residues followed by a guanosine (CpG); CpG dinucleotides are 60-80% methylated (Trasler, 2006). During mouse male germ cell development, DNA methylation patterns begin to be acquired *in utero* (between gestational days 15.5 and 18.5)(Coffigny et al., 1999); DNA methylation continues during spermatogonial divisions through the meiotic phase of spermatogenesis (Figure 1.2) (Davis et al., 2000; Kerjean et al., 2000). A family of DNA cytosine methytransferases (DNMTs) catalyzes the transfer of a methyl group to DNA; identified DNMTs include Dnmt1, Dnmt2, Dnmt3a, Dnmt3b, Dnmt3L, and the oocyte specific form Dnmt1O. The most abundant DNA methyltransferase, Dnmt1, is required for the maintenance of established DNA methylation patterns while Dnmt3a and Dnmt3b are important *de novo* methytransferases; Dmnt3L acts collectively with the Dnmt3 enzymes to modulate *de novo* acquisition of DNA methylation patterns in male germ cell development (Trasler, 2006). The importance of the involvement of DNMTs during spermatogenesis is emphasized by the appearance of abnormal spermatocytes at meiotic prophase and spermatogenic arrest prior to meiosis, in Dnmt3a and Dnmt3L-knockout mice, respectively, leading to a complete lack of meiotic and post-meiotic germ cells (Kaneda et al., 2004; Bourc'his and Bestor, 2004; Webster et al., 2005). Recently, using Restriction Landmark Genomic Scanning (RLGS) to assess the genome-wide nature of male germ cell methylation, a highly unique testicular DNA methylation status was observed; an eight-fold increase in hypomethylated nonrepetitive, non-CpG-island sequences was correlated with regional chromosome features with proposed effects on structure. Accordingly, Dnmt3L-deficient mice, which display abnormal chromosomal organization, do not acquire the unique testis-specific DNA methylation pattern, emphasizing the role of a germ cell specific epigenetic state in the regulation and maintenance of chromosomal structure (Oakes et al., 2007).

The establishment of methylation patterns during male germ cell development is also essential for epigenetic silencing or activation of one parental allele of a gene, a phenomenon referred to as genomic imprinting. Paternal imprints are erased from the primordial germ cells during embryogenesis and must be re-established in male germ cells during the late fetal stages of development; methylation of imprinted genes is complete prior to meiosis. The sex-dependent pattern of gene imprinting must be maintained through fertilization and embryogenesis; genomic imprinting plays significant roles in the regulation of fetal growth, placental development and function as well as in postnatal behaviour. In humans, cancer as well as several genetic diseases causing neurodevelopmental, metabolic and psychiatric/behavioural disorders are due to abnormal imprinting (Trasler, 2006; Paoloni-Giacobino, 2007).

Faithful epigenetic reprogramming during spermatogenesis is necessary for the production of a highly specialized spermatozoal genome equipped with a developmental program competent to participate in normal embryogenesis.

1.1.3 Epididymal Transit

1.1.3.1 Spermatozoal Maturation

Spermatozoa are transported in testicular fluid to the epididymis where they become functionally mature. As sperm journey through the caput, corpus and cauda epididymal regions they acquire motility and the ability to naturally fertilize an egg (Yanagimachi, 1994). Structural alterations are also important during epididymal transit; the cytoplasmic droplet is shed, the acrosome acquires its final shape and the process of disulphide bond formation which began in the testis continues, thereby further condensing and stabilizing spermatozoal nuclei into compact DNA structures (Bedford and Calvin, 1974). Mature spermatozoa are stored in the cauda epididymidis until being mixed with prostatic and seminal vesicle fluids at ejaculation.

1.1.3.2 Motility Acquisition

The maturation of sperm motility patterns is one of the most prominent changes occurring during epididymal transit. Spermatozoa must acquire the potential for flagellation followed by the capacity to elicit a coordinated waveform flagellar beat; good sperm motility is essential for normal male fertility. The flagellar beat matures from an erratic circular movement pattern with little forward progression in the caput and proximal corpus epididymides to a mature progressive swimming pattern in the cauda epididymidis (Soler et al., 1994).

Numerous structural and biochemical changes influence the ability of the sperm to attain the characteristic forward propulsion required for fertilization. The flagellum is composed of the connecting piece, the midpiece, the principle piece and the end piece. The axoneme, which extends through most of the flagellum, is composed of 2 central microtubules surrounded by 9 microtubule doublets (referred to as the '9 + 2' array) and is the motor generating the force of the flagellum (Fawcett, 1975). ATPase activation of dyneins (motor proteins) located in the arms of the outer microtubule doublets results in flagellar bending (Milisav, 1998). Accessory structures, including the outer dense fibers (ODFs), fibrous sheath (FS) and mitochondrial sheath (MS), provide structural support, rigidity and supply fuel for sperm motility, respectively (Figure 1.3). Specifically, the MS of the midpiece contains mitochondria for ATP production required by the axonemal dyneins for flagellar motion (Storey and Kayne, 1980); the FS contains proteins implicated in motility signaling and metabolism-based pathways likely serving as an organizing center for cascades that are critical for the modulation of flagellar oscillation (Turner et al., 1999; Miki et al., 2002; Eddy et al., 2003). The cAMP/protein kinase A and calcium pathways play important roles in the initiation and maintenance of mammalian sperm motility. Furthermore, changes in intracellular pH have been demonstrated to alter the induction of flagellation and the forward progression of sperm movement.

Together, the complex interaction of proteins involved in spermatozoal structure, protein assembly, and signaling cascades, as well as the influence of the epididymal intracellular environment, ensures that spermatozoa have

acquired the functionality needed to swim in the female reproductive tract (Turner, 2006).

1.2 Fertilization

Once released into the female reproductive tract, the mature spermatozoon must complete three main processes for successful fertilization to occur. The spermatozoon must first locate, meet and bind to the extracellular matrix of the oocyte, the zona pellucida. Spermatozoal binding initiates the acrosome reaction, thereby providing accessibility to the enzymatic machinery required for zona penetration; completion of the acrosome reaction permits sperm penetration into the perivitelline space and subsequent fusion to the plasma membrane of the oocyte. Gamete plasma membrane fusion allows the entry of the sperm nucleus, mitochondria, centriole and flagellum into the oocyte; the message of male chromatin has thus been delivered to the recipient egg. This choreographed collision and fusion of the mature male and female haploid gametes during fertilization triggers genomic activation and chromatin remodeling that lead to the formation of the diploid zygote (reviewed in Storey, 1995).

1.2.1 Oocyte Activation

During meiotic maturation, the oocyte acquires histone-transfer activity (HTA) developed by protein synthesis-dependent mechanisms; HTA is essential for oocyte derived histone assembly onto sperm chromatin post-fertilization (Mclay and Clarke, 1997; Mclay et al., 2002). Oocyte factors, such as glutathione, the mammalian homologue of *Xenopus* nucleoplasmin, nucleoplasmin 3 (Npm-3), histone acetyltransferases (HATs), histone deacetylases (HDACs) and other proteins accumulate in the metaphase ooplasm for use in histone assembly and epigenetic regulation during initial sperm chromatin remodeling activities (MacArthur and Shackleford, 1997; Perreault, 1992; Mclay and Clarke, 2003; Mclay et al., 2002).

The ovulated oocyte is arrested in metaphase of the second meiotic division; fertilization initiates the completion of meiosis II and extrusion of one-

half of the maternal chromosomes in the second polar body (Schultz and Kopf, 1995). The ooplasm of the activated oocyte plays a specific and functional role in converting the spermatozoal genome into functionally competent DNA, enabling a coordinated interaction between parental genomes. Maternal control of early chromatin remodeling events is further evidenced by the ability of the oocyte to reprogram somatic nuclei in cloning (Wilmut et al., 1997; Loi et al., 2001; Yanagimachi, 2002).

1.2.2 Paternal Chromatin Remodeling

The DNA of the spermatozoa is delivered as a transcriptionally inert densely compacted package in need of dramatic remodeling to participate in further developmental events. In contrast to maternal chromosomes, which readily decondense into the female pronucleus following completion of meiosis II, the condensed sperm chromatin undergoes the reverse process of spermiogenic condensation; orderly condensation during spermiogenesis is necessary for orderly decondensation to take place after fertilization (Storey, 1995). Decondensation in the fertilized egg utilizes oocyte glutathione to reduce the protamine disulphide bonds, permitting unfolding of the intricately organized DNA loop domains (Perreault et al., 1984; Perreault, 1992; Perreault et al., 1988). Sperm protamine removal is complete by the end of oocyte anaphase II, coincident with a threefold increase in paternal chromatin dispersion. During telophase II, the sperm chromatin briefly recondenses, corresponding to nuclear envelope reconstruction from oocyte-derived membrane vesicles (Wright and Longo, 1988). Maternally supplied histones, H2A, H2B, H3 and H4, (Wiekowski et al., 1997) progressively assemble in an ATP-dependent manner onto the denuded sperm DNA during the recondensation phase (Wright and Longo, 1988; Mclay and Clarke, 1997) and are visible by immunofluorescence staining prior to the onset of zygotic replication (Nonchev and Tsanev, 1990; Adenot et al., 1997). Upon completion of the protamine-histone exchange and subsequent nucleosomal organization of the paternal genome, male and female pronuclear envelops form synchronously. Paternal chromatin dramatically decondenses

again within the male pronucleus, increasing the area of occupancy by 10-fold, as does the oocyte chromatin in the female pronucleus (Figure 1.4) (Wright and Longo, 1988; Mclay and Clarke, 2003).

1.2.3 Pronuclear Development

Parental pronuclei continue to swell and active mechanisms guide migration toward the center of the cell to restore diploidy of the zygote. In contrast to paternal centrosomal inheritance in other mammals, which lead to the formation of a single sperm aster for microtubule mediated movement (Reinsch and Karsenti, 1997; Reinsch and Gonczy, 1998), rodent spermatozoa shed both centrioles during spermiation and the centrosome is strictly maternally inherited (Woolley and Fawcett, 1973; Manandhar et al., 1998). Multiple egg cytoplasmic asters organize the microtubules that provide the motile force required for male and female pronuclear migrations (Schatten et al., 1985; Schatten et al., 1986). Microfilament-dependent events are further required for successful pronuclear apposition in rodents (Terada et al., 2000; Sun and Schatten, 2006). As the male and female pronuclei progressively enlarge and approach each other, the zygote can be developmentally staged from pronuclear stage (PN) 1 through PN5, based on pronuclear size, morphology and location in the cytoplasm; at PN5 large central male and female pronuclei are apposed (Figure 1.5) (Adenot et al., 1997). The synchronized development of parental pronuclei ends at syngamy, when male and female nuclear envelopes are lost so that chromosome condensation can occur to form a unique metaphase plate before the first round of embryo cleavage.

1.3 Early Preimplantation Development

1.3.1 Zygotic Development

1.3.1.1 The Zygote as an Experimental Model

The one-cell embryo is a unique model system in which the parent-specific genomes from the spermatozoon and oocyte are still physically separated within a common cytoplasm. The genomic separation within their respective pronuclei provides an excellent opportunity to study the highly coordinated molecular and cellular events that occur very early post-fertilization prior to the transition to embryonic control. Moreover, the uniqueness of the genome separation in the zygote provides us with an invaluable tool to (1) investigate the manner in which paternal exposures to genotoxic agents may deregulate reprogramming in the early embryo, (2) determine the mechanism(s) by which the parental pronuclei communicate the presence of genomic disturbances and (3) assess the implementation of various damage response pathways. Interestingly, a recent study has shown that pronuclear stage morphology and kinematic scoring in the zygote is an efficient system to select embryos with a high implantation potential in *in vitro* fertilization (Wittemer et al., 2000; Arroyo et al., 2007).

1.3.1.2 Epigenetic Modifications Post-fertilization

Nuclear reprogramming of epigenetic marks in the zygote is no exception to the distinct parent-specific events that are required to establish a precise developmental programme during early embryogenesis (Robertson and Wolffe, 2000).

1.3.1.2.1 DNA Methylation

DNA methylation in the zygote is a prominent epigenetic alteration, regulating mechanisms involved in transcriptional repression and structural integrity (Robertson and Wolffe, 2000; Morgan et al., 2005). Functional

differences between the paternal and maternal genomes are enhanced by the asymmetric methylation status observed in the zygote; this has been proposed to have an important impact on chromatin dynamics beyond the one-cell stage (Mayer et al., 2000b).

The maternal genome is highly methylated in the zygote and is gradually demethylated in a passive manner that is replication-dependent during ensuing preimplantation cleavage divisions (Rougier et al., 1998; Mayer et al., 2000a; Santos et al., 2002); exclusion of the DNA methytransferase, Dnmt1o, from the nucleus has been suggested to affect sequential demethylation (Cardoso and Leonhardt, 1999; Bestor, 2000; Howell et al., 2001). Preferential and exclusive binding of the heterochromatin protein HP1β, in association with histone H3 methylation at lysine 9 in the maternal genome, may also play a role in *de novo* DNA methylation (Arney et al., 2002; Santos et al., 2005).

In contrast with the maternal genome, the paternal pronucleus is rapidly and actively demethylated immediately following sperm decondensation, in the absence of DNA replication and transcription. Genome-wide epigenetic asymmetry has been extensively characterized using 5-methyl cytosine immunofluorescence and is highly conserved in eutherian mammals including cattle, pigs, rats, mice, humans (Barton et al., 2001; Dean et al., 2001; Fulka et al., 2004) and to a limited extent in sheep (Beaujean et al., 2004). Moreover, bisulphite sequencing confirmed that active demethylation of the paternal zygotic genome also affects single-copy DNA sequences (Oswald et al., 2000); centromeric heterochromatin, IAP retrotransposons and paternally methylated imprinted genes are resistant to demethylation at this stage (Rougier et al., 1998; Lane et al., 2003; Olek and Walter, 1997).

Despite efforts to elucidate the mechanisms and candidate enzymes and/or proteins involved in active demethylation of the paternal genome, the particulars of this event remain unknown. Three possible mechanisms have been put forth: 1) direct removal of the methyl group from the cytosine base in the major groove (Bhattacharya et al., 1999), 2) removal of the methyl cytosine by a glycosylase and repair or CpG dinucleotide removal by nucleotide excision

repair, 3) deamination of 5-methyl cytosine, conversion to thymine, followed by mismatch repair (Cedar and Verdine, 1999). Detailed analyses on active repair mechanisms in zygotic pronuclei will provide unprecedented information on these hypothetical mechanisms. Interestingly, recent studies have established a lack of involvement of previously characterized methyl CpG-binding domain (MBD) proteins with proposed demethylase activity (Bhattacharya et al., 1999; Hendrich and Tweedie, 2003). Examination of zygotes produced from reciprocal crosses of mice deficient in either the candidate gene Mbd2 or Mbd4 resulted in 5-methyl cytosine staining patterns that were indistinguishable from wild-type embryos (Santos et al., 2002; Santos and Dean, 2004). The search continues for zygotically relevant demethylase candidates. Regardless of the mechanism, the unique temporal methylation asymmetry appears to be essential for the transformation of specialized gametogenic patterns into embryonic epigenetic patterns responsible for temporal transcriptional repression of precise genes and genomic stability (Morgan et al., 2005).

1.3.1.2.2 Histone Acetylation

Histone modifications also play fundamental regulatory roles controlling gene accessibility during the early stages of preimplantation development. In particular, histone acetylation facilitates unfolding of the nucleosomal fiber rendering chromosomal domains accessible to transcriptional machinery (Eberharter and Becker, 2002); acetylation is widely correlated with an active chromatin configuration and interacts with other epigenetic codes such as DNA methylation (Strahl and Allis, 2000; Jenuwein and Allis, 2001).

In the newly fertilized embryo, acetylation of specific histone isoforms is temporally and spatially regulated in the zygotic chromatin. During sperm protamine-histone exchange and throughout most of G1, histone H4 is more highly acetylated in the paternal genome than in the female genome; the balance of deacetylase/acetyltransferase activities maintains appropriate levels of acetylation as early as G1 of the first cell cycle. Beginning in S phase and into G2, levels of hyperacetylated H4 are equivalent in both parental genomes; DNA

replication is not required for pronuclear acetylation symmetry (Adenot et al., 1997). In depth analysis of various acetylated histone H4 isoforms revealed that histone H4 acetylated on lysine 16 is homogeneously distributed throughout the zygotic nucleoplasm. Similarly, selected acetylated isoforms of histone H3, H2A and H2B do not show localized staining patterns in the 1-cell embryo (Stein et al., 1997). Intriguingly, histone H4 acetylation on lysines 5, 8 and 12 is enhanced at the pronuclear periphery during late G2 of the zygotic cell cycle; peripheral enrichment was DNA replication-dependent as treatment with aphidicolin (a reversible inhibitor of DNA replication) inhibited preferential localization (Worrad et al., 1995).

Modulation of acetylation levels in the zygote is crucial for chromatin remodeling during development; increased levels of acetylation in the early G1 paternal pronucleus appear to be implemental in the initial recruitment of transcription factors conferring a permissive chromatin structure (Adenot et al., 1997). During the first cell cycle, evolving histone modifications of the paternal genome seemingly produce a chromatin state analogous to that of the maternal genome (Morgan et al., 2005). Enhanced peripheral localization of select histone isoforms in both parental pronuclei late in G2 may represent transcriptionally permissive chromatin regions involved in early programming of the zygotic genome (Stein et al., 1997).

Together, the zygotic epigenetic marks dictated by post-translational modifications and the histone code (Strahl and Allis, 2000; Jenuwein and Allis, 2001) establish and maintain parent-specific regulation to ensure dynamic coordination of pronuclear development. Defects in early epigenetic programming may disrupt the epigenome and affect subsequent events during development (Robertson and Wolffe, 2000; De Rycke et al., 2002).

1.3.1.3 Role of chromatin structure in activational states of the zygote

In the zygote, male and female pronuclei display regions of condensed DNA surrounding the nucleolar precursor bodies (NPBs); NPBs are inactive embryonic nucleoli presenting as structures of compact fibrillar spheres (Flechon

and Kopecny, 1998). Centromeres and late replicating pericentric DNA are associated with NPB periphery while the rest of the chromosome extends radially toward the nuclear periphery. The 1-cell stage specific chromosomal organization has been denoted as the "cartwheel" configuration. Interestingly, this "cartwheel" arrangement appears identical in both the male and female pronucleus, highlighting the importance of underlying epigenetic mechanisms in the modulation of nucleosomal structure and activational states (Martin et al., 2006).

Post-translational epigenetic modifications contribute to the plasticity of chromatin structure and are tightly coupled to states of nuclear activity. The zygotic genome is compartmentalized in transcriptionally competent and incompetent domains determined through the interplay of protein complexes that associate with and epigenetically manipulate the nucleosomes (Patterton and Wolffe, 1996; Rountree et al., 2001). As a general rule, methylated, hypoacetylated, maternal chromatin is characterized by more heterochromatic structures, while unmethylated, acetylated paternal chromatin has more euchromatic structures, correlating with repressive and permissive states, respectively (Li, 2002). More recently, it has been demonstrated that the methylation pattern that is established during early embryogenesis serves as a global mediator of the sequence-independent genomic structural profile (Hashimshony et al., 2003). Lysine specific histone H3 methylation is significantly delayed in the paternal pronucleus despite the presence of polycomb group proteins with histone methyltransferase activity; demethylated paternal chromatin promotes accessibility to appropriate transcriptional machinery. The striking differences between male and female pronuclear staining for mono-, di- and trimethylated histone H3-K9 and H3-K27, as well as associated patterns for polycomb group proteins and heterochromatin protein 1 beta (HP1β) have begun to reveal a chromatin based understanding of the parent-specific epigenetic phenomena regulating developmental progression (Santos et al., 2005).

1.3.1.4 Replication

DNA replication is one of the first functional events occurring approximately 12 hours after fertilization in the one-cell mouse embryo (Ferreira and CarmoFonseca, 1997); protamine-histone exchange and male pronuclear formation must occur prior to DNA synthesis (Naish et al., 1987). Distinct patterns of DNA replication demonstrate precisely defined temporal and spatial S-phase progression in the zygote. Early patterns are characterized by diffuse nucleoplasmic foci which become localized in perinuclear and perinucleolar regions mid S-phase; only a few large intranuclear patches remain prior to G2 (BouniolBaly et al., 1997; Ferreira and CarmoFonseca, 1997). Although similar spatial patterns were observed in both male and female pronuclei, the appearance of each pattern was temporally distinct, indicating asynchronous DNA replication of the parental genomes. Male pronuclei initiate replication slightly earlier and S-phase is longer than in the female pronucleus (Ferreira and CarmoFonseca, 1997; Aoki and Schultz, 1999). Temporal differences in DNA replication may be a result of nucleoprotein exchange in the male genome which could provide a window of opportunity for the assembly of replication machinery onto paternal DNA. The mature nucleosomal structure of the maternal genome may initially impede replication factor binding, delaying S-phase in the female pronucleus (Ferreira and CarmoFonseca, 1997).

The first round of DNA replication is required for increased pronuclear concentration of transcription factors and is important to alleviate repression of some endogenous genes (including translation initiation factor eIF-4C and the transcription-requiring complex (TRC)) and for the reactivation of gene expression (Worrad et al., 1994; Davis et al., 1996). Accordingly, transcription is initiated during late replication patterns; co-localization studies found essentially no spatial relationship between sites of replication and transcription (BouniolBaly et al., 1997). Inhibition of the first round of DNA replication decreased BrUTP incorporation by ~35%, reflecting diminished transcription in the zygote (Aoki et al., 1997). Interestingly, high-resolution two-dimensional electrophoresis has determined the presence of both replication-dependent and replication-

independent polypeptides (Davis and Schultz, 1997), suggesting that a major role of zygotic replication is to disrupt the repressed chromatin state in order to restore transcriptional competence in a select subset of genes that are crucial for coordinated reprogramming in the preimplantation embryo (Forlani et al., 1998).

1.3.1.5 Transcription

Transcriptional activation is initiated during the late S/G2 phase in the one-cell mouse embryo; the male pronucleus is transcriptionally active prior to the female pronucleus and consistently supports levels of transcription 4 to 5 times greater throughout the first cell cycle (Ram and Schultz, 1993; Wiekowski et al., 1993; Aoki et al., 1997). The early remodeling of the paternal genome has also been proposed to provide enhanced accessibility and thus promote sequestration of maternally derived transcription factors in the male pronucleus. In support of this notion, a much higher concentration of transcription factors, Sp1 and TATA box-binding protein, TBP, were observed in the male pronucleus (Worrad et al., 1994).

The functional consequence of transcriptional activation in the one-cell embryo has been suggested to mark promoters for rapid reactivation following the first cell division. Specifically, transcription factors implicated in the generation of active transcription complexes at promoters remain associated with mitotic chromatin, thereby potentially providing a mechanism to reprogramme and maintain gene expression patterns during the first cleavage division (Christova and Oelgeschlager, 2002; Schultz, 2002).

1.3.1.6 Minor Zygotic Gene Activation

Genome activation is regulated in a time-dependent manner relative to fertilization. The zygotic clock controls the transition from transcriptionally incompetent parental genomes to a permissive chromatin state accessible to trans-acting factors required for transcriptional activation or repression. Embryonic chromatin remodeling and epigenetic modifications leading to transcription beginning in zygotic S-phase are referred to as minor activation of

the zygotic genome (Schultz, 1993; Wiekowski et al., 1991; Aoki et al., 1997). Minor activation results in the synthesis of a small subset of proteins that are transiently increased in the 2-cell stage embryo (Conover et al., 1991; Davis et al., 1996; Christians et al., 1995).

1.3.2 The Two-Cell Embryo

Using the power of bioinformatic tools, together with *in vitro* confirmation of *in silico* data, Evsikov and colleagues have begun to characterize the functionality of the 2-cell mouse embryo transcriptome (Evsikov et al., 2004). The 2-cell stage embryo is a complex biological system with multiple molecular networks acting in concert to guide the timely onset of the major zygotic gene activation. Analysis of ESTs from a cDNA library have established the presence of an abundance of genes encoding transcriptional regulators, DNA- and RNA-binding proteins, protein modification and degradation components, as well as genes involved in controlling the cell cycle, presumably for regulation of the zygotic clock. Also important to the function of the 2-cell stage embryo is the capacity to transport molecules between cytoplasmic and nuclear compartments; accordingly, exportin (*Xpo1*), which is involved in nuclear export, is expressed abundantly, possibly playing a role in controlling the nuclear pool of transcription factors (Evsikov et al., 2004).

Epigenetic modifications at this stage remain pivotal for the progression of embryonic development. 5-Methyl cytosine staining in 2-cell embryos reveals the persistence of asymmetrical DNA methylation patterns in parental genomes. In normal embryos, the paternal half of each blastomere is demethylated and the maternal half is highly methylated reflecting compartmentalization and continued parent-specific epigenetic reprogramming of the genome (Mayer et al., 2000a; Barton et al., 2001). Interestingly, paternal and maternal chromatin do not appear to immediately mix; rather parental genomes appear to remain segregated up to the 4-cell stage in the preimplantation embryo (Mayer et al., 2000b). Abnormal establishment and maintenance of appropriate genome-wide DNA methylation patterns in the 2-cell stage embryo are predictors of

reprogramming defects leading to early developmental failure (Barton et al., 2001; Shi and Haaf, 2002).

Dynamic regulation of histone acetylation is also significant at the 2-cell embryo stage. The preferential peripheral localization of histone acetylation that was observed in late G2 of the first cell cycle is retained after cleavage. Transient perinuclear enrichment was observed for histone H4 acetylated at lysines 5, 8 and 12 (Worrad et al., 1995), histone H3 lysines 9 and 18, as well as acetylated histone H2A. Restricted localization is absent in the 4-cell stage embryo and later cleavage stages (Stein et al., 1997). Interestingly, areas of enriched hyperacetylated histones colocalize with RNA polymerase II, suggesting that the nuclear periphery is a region of potentially high transcriptional activity. Transient sequestration of acetylated histones and transcription factors suggests that a specialized chromatin structure organized at the nuclear periphery in the 2-cell embryo likely is implemental in modulating genes that are crucial for embryonic development. Importantly, this spatial restriction correlates with the onset of zygotic genome activation and may be necessary to sustain gene expression for continued development (Worrad et al., 1995; Stein et al., 1997).

1.3.3 Major Zygotic Gene Activation

The major developmental transition activating the mammalian zygotic nucleus is the maternal to zygotic transition (MZT), or major zygotic gene activation (ZGA), which occurs in the 2-cell mouse embryo (Schultz, 1993). Interestingly, the timing of major genome activation is variable among other mammalian species, occurring by the 4- to 8-cell stage in bovine, porcine, ovine and human embryos and by the 8- to 16-cell stage in rabbit embryos (Telford et al., 1990; Kanka, 2003). One of the main functions of the MZT is the degradation of maternally inherited proteins and transcripts with subsequent replacement by zygotic transcripts. Additionally, major ZGA promotes a dramatic reprogramming in the pattern of protein synthesis and gene expression. ZGA reprogramming is predicted to be responsible for the transformation of the oocyte into totipotent

embryonic blastomeres and is essential for continued development (Latham et al., 1991; Schultz, 2002).

Coupled with major ZGA at the 2-cell stage in the mouse is the induction of chromatin-mediated global transcriptional repression of promoters requiring embryo-responsive enhancers for efficient gene expression (Henery et al., 1995; Martinezsalas et al., 1988; Martinezsalas et al., 1989); histone hyperacetylation relieves the requirement for an enhancer confirming repression via chromatin structure (Henery et al., 1995). The proposed role for the development of a repressive state is to oppose what appears to be a relatively promiscuous global activation of the genome in order to sculpt an embryonic gene expression profile compatible with further development. The net result of preferentially reducing gene expression, while concomitantly permitting continued expression of specific genes that are regulated by strong promoters/enhancers, is to establish directionality at the onset of mammalian development (Ma et al., 2001).

It is clear from the evidence cited above, that many complex processes occur during spermatogenesis and in the early embryo to contribute to the development of healthy offspring. However, due to the same complexity, male germ cell development is vulnerable to exogenous agents that lead to altered genomic quality, bearing impact on both male fertility and progeny outcome. The consequences of various toxicant exposures on male reproductive function, the possible mechanisms in place for DNA damage resolution and the effects of a damaged paternal genome on progeny outcome will discussed in subsequent sections.

1.4 Toxicant Exposures during Spermatogenesis

Male rodents serve as excellent experimental models to investigate germline mutagenicity and toxicity due to the distinctive features of male germ cell development. The defined kinetics of spermatogenesis allows the assessment of germ-cell stage specific susceptibility following toxicant exposure

(Figure 1.6). The continuous cycle of male germ cell production provides an abundance of cells for analysis (Russell et al., 1990). Furthermore, by mating animals following specific exposure regimens a large number of offspring may be produced, allowing assessment of the genotoxic effect on progeny outcome.

1.4.1 Male Germ Cell Stage Sensitivity to Toxicant Exposure

Classical analysis of male germ cell mutagenicity in the mouse was done using the specific locus test (SLT), the dominant lethal (DL) assay, and/or the heritable translocation (HT) assay to detect mutations, measure chromosomal aberrations and identify genotoxic effects on implantation success and embryo survival. Numerous chemicals have been tested and the germ cell stage susceptibility is a function of the compound being administered. Although a majority of the chemicals tested maximally damage germ cells in the later stages of spermatogenesis (spermatids and spermatozoa), there is now emerging evidence that an increasing number of mutagens induce peak effects in spermatogonia, spermatocytes and during spermiogenesis (Table 1.1). The capacity for DNA synthesis, repair and recombination, the nucleosomal protein association and chromatin conformation all influence the germ cell stage sensitivity to a particular class of chemicals with a specific mode of action (Singer et al., 2006; Wyrobek et al., 2007).

The diversity observed in the time specific genotoxic effects and their impact on progeny outcome further emphasize the need to carefully assess the reproductive hazards of chemicals on an individual basis to clearly identify class and/or compound specific effects. The use of new generation mutation assays (expanded simple tandem repeats (ESTR), transgenic rodent (TGR) mutation assays) (Singer et al., 2006), chromatin integrity assays (COMET, TUNEL and sperm chromatin structure assays (SCSA))(Codrington et al., 2004; Said et al., 2005; Evenson and Tritle, 2004), aneuploidy testing (fluorescence *in situ* hybridization (FISH))(Wyrobek et al., 2005) and assays for the identification of germ cell epigenetic alterations that are transmissible to the early embryo will aid

in the elucidation and clarification of male germ cell genotoxicity and the mechanisms of induction (Eichenlaub-Ritter et al., 2007).

1.4.2 Exposure to Occupational and Environmental Toxicants

The concept that male exposures to exogenous toxicants present in the workplace are a major factor contributing to reproductive dysfunction was highlighted in 1977 following a report of infertility in workers exposed to the pesticide dibromochloropropane (DBCP) (Whorton et al., 1977). Accordingly, DBCP has been classified as a potent male reproductive toxicant with demonstrated deleterious effects on human spermatogenesis (Whorton et al., 1979; Potashnik and Yanaiinbar, 1987). Since this finding, several epidemiological studies have identified positive associations between male occupational and environmental exposures and adverse reproductive and progeny outcomes.

Several paternal occupations have been correlated consistently with reproductive abnormalities and birth defects; these include janitors, painters, printers - occupations frequently exposed to organic solvents, as well as fire fighters and those involved in the agricultural industry (Chia and Shi, 2002; Jensen et al., 2006). Men exposed to organic solvents (glycol ethers, 2-bromopropane, styrene and aromatic solvents), heavy metals (lead, mercury, cadmium, chromium), radiation or pesticides experience a range of adverse effects on semen quality. Male reproductive dysfunction following exposure is compound specific; however, the range of effects include: deceased sperm counts, motility and viability, increased morphological abnormalities, higher frequency of asthenospermia, hypospermia and teratospermia, changes in reproductive hormones, testicular dysfunction, infertility, temporary or permanent sterility (sterility has been observed to occur only following high dose radiation exposure) (Welch et al., 1988; Welch et al., 1991; Kim et al., 1996; Kolstad et al., 1999; Tielemans et al., 1999; Apostoli et al., 1998; Bonde et al., 2002; Popschu, 1978; Dawson et al., 1998; Rowley et al., 1974; Clifton and Bremner, 1983; Whorton et al., 1979; Wyrobek et al., 1981; Abell et al., 2000). Of utmost

concern, is the evidence that paternal occupational exposures lead to an increased incidence of detrimental effects on the health and development of their children. Paternal toxicant exposure leads to an increase in spontaneous abortions, a plethora of congenital malformations (including hydrocephalus, cardiac defects, neural tube defects, organ anomalies, urogenital anomalies, circulatory and respiratory anomalies, musculoskeletal defects and Down's syndrome), neurological deficits in behaviour and learning and increased incidence of childhood cancers (Chia and Shi, 2002).

Clearly data on paternal occupational exposures have delineated a relationship between workplace toxicants and adverse reproductive outcomes. However, one must consider that there are evident limitations in epidemiological studies; outcomes may be influenced by compounding workplace and/or lifestyle factors, exposure assessment may be pertinent to a specific window during spermatogenesis, adverse outcomes may be due to a combination of paternal and maternal effects (Burdorf et al., 2006). Therefore, animal models are critical in the assessment of reproductive abnormalities following male exposures as well as in the elucidation of the mechanisms responsible for adverse paternal effects.

Although occupational exposures are generally higher than those encountered in the environment, persistent synthetic chemicals have emerged as an important example of environmental toxicants that disturb reproductive success. Humans may be exposed frequently, in everyday life, to a mixture of synergistically acting environmental chemicals. A major concern is that some of these chemicals can disrupt the endocrine system (known as endocrine disrupting chemicals or EDCs) and bioaccumulate, presenting the opportunity for long term detrimental consequences as a result of inappropriate hormone action. Included in the class of endocrine disrupting chemicals are polychlorinated biphenyls (PCBs), dioxins, dichlorodiphenyl trichoroethane (DDT), bisphenol A, pthalates and phytoestrogens (Yang et al., 2006). To date, the debate continues on the extent of reproductive effects that are attributed to current environmental exposures. Nonetheless, there are epidemiological data that warrant concern.

Males environmentally exposed to pesticides in rural areas (via drift during spraying season or through food consumption) exhibit reduced sperm concentrations and lower sperm motility; cryptorchidism and, possibly, hypospadias in boys living on farms have been attributed to pesticide exposure (Kristensen et al., 1997; Garry et al., 1996).

Additionally, EDCs have been shown to alter offspring sex-ratios. Hormonal levels at the time of conception are presumed to be associated with sex ratio; hormone disruption as a result of EDC exposure could lead to an unusual sex ratio. Accordingly, paternal organochlorine exposure was associated with elevated proportions of Y-chromosome bearing sperm, suggesting the likelihood of fathering a higher proportion of boys (Tiido et al., 2005). Conversely, elevated paternal serum levels of the dioxin, TCDD, were associated with higher proportion of female births (Mocarelli et al., 2000). Moreover, the proportion of male live births has been declining significantly in a First Nation Community located in close proximity to several large industrial plants near Sarnia, Ontario; this study clearly demonstrates the reproductive health concerns associated with environmental and occupational chemical exposures (Mackenzie et al., 2005).

Many human studies have shown that EDC exposure during fetal development is a critical factor in teratogenicity; male reproductive tract abnormalities and neurobehavioral disorders prevail (Yang et al., 2006). To further emphasize the significance of these findings, rodent studies convincingly demonstrate the paramount health risks associated with exposure to chemicals that interfere with normal endocrine function. The endocrine disruptors, vinclozolin (fungicide) and methoxyclor (organochlorine insecticide), dramatically affected spermatogenesis; remarkably, the male germ line effects of exposure to these toxicants were propagated across four generations. Altered DNA methylation patterns contribute to the transgenerational phenotype, accentuating the potential hazards of environmental toxicant exposure on multiple generations (Anway et al., 2005).

1.4.3 Exposure to Chemotherapeutic Agents

The human testis is a primary target for the harmful effects of therapeutic drugs. Chemotherapeutic regimes, routinely consisting of drug cocktails, severely compromise spermatogenesis; alkylating agents are most commonly implicated. Cytotoxic agents may produce permanent damage to the testicular germinal epithelium; azoospermia develops in the majority of patients between 8 and 12 weeks post treatment initiation as a consequence of spermatogonial germ cell destruction (Meistrich, 1986). The probability for spermatogenic recovery is dependent upon the agent(s) administered, the dose and the duration of the treatment. Treatment of Hodgkin's disease using combination chemotherapy regimes, including MOPP (mechlorethamine, vincristine, procarbazine and prednisone) or COPP (cyclophosphamide, vincristine, procarbazine and prednisone), result in oligozoospermia or azoospermia in 90% of patients (Mackie et al., 1996; Viviani et al., 1985). Multi-agent therapy with combinations that exclude alkylating agents, such as ABVD (adriamycin, bleomycin, vinblastine and decarbazine), are much less gonadotoxic; temporary oligozoospermia and azoospermia were observed, however, full recovery of gonadal function was achieved within 18 months (Viviani et al., 1985).

Ongoing research is aimed at developing strategies to preserve fertility in cancer patients. The observation that the prepubertal testis appears to be less susceptible to the cytotoxic effects of chemotherapeutics (Rivkees and Crawford, 1988) led to the proposal that gonadal suppression by manipulation of the testicular environment may protect spermatogenesis. So far, hormonal suppression of the hypothalamic-pituitary-gonadal axis for preservation of spermatogenesis has been successful in rodents (Kangasniemi et al., 1995; Meistrich et al., 1994; Meistrich, 1998); clinical studies on cancer patients have not been done. Currently, cryopreservation of spermatozoa is the only option available to patients concerned about future paternity (Tournaye et al., 1993); in many cases, pretreatment sperm quality is poor (Meirow and Schenker, 1995). Fortunately for the cancer patient, assisted reproductive technologies, such as intracytoplasmic sperm injection (ICSI), circumvent the common problems

associated with subfertility, low sperm counts and poor motility (Aboulghar et al., 1997). Unfortunately for the cancer patient, the use of assisted reproductive technologies raises concern about the transmission of genetic damage.

In cases of spermatogenic recovery, there is growing concern that cytotoxic chemotherapy introduces genetic defects to the germ cells that may be transmitted to the conceptus by natural conception or by assisted reproductive technologies. A number of studies have demonstrated a significant risk of chromosomal abnormalities in the spermatozoa both during and after BEP (bleomycin, etoposide, cisplatin)(Martin et al., 1999) and NOVP (novanthrone, oncovin, vincristine, prednisone) chemotherapy (Robbins et al., 1997); numerical chromosomal abnormalities were observed for several years after MOPP treatment for testicular cancer (Genesca et al., 1990; Brandriff et al., 1994). Sperm DNA damage, detected using single cell comet assays, was also present in patients during and after cancer treatment (Chatterjee et al., 2000). Although existing data do not provide evidence for an increased incidence of spontaneous abortions, low birth weight or genetically mediated birth defects in the progeny of cancer survivors (Byrne et al., 1998; Green et al., 2003), these data are not adequate to completely eliminate concern for an increased rate of congenital abnormalities and/or childhood disease in children following paternal cytotoxic exposure. Furthermore, the use of ICSI to allow previously infertile patients to father children may increase the associated risks. Conversely, animal studies provide clear evidence that paternal exposures to anticancer agents can adversely affect progeny outcome (Brinkworth, 2000; Hales et al., 2005; Vaisheva et al., 2007).

1.5 Chromosomal Abnormalities in Spermatozoa

A considerable amount of literature is available on the various structural and numerical chromosome anomalies that have been demonstrated to arise in male germ cells (McFadden and Friedman, 1997; Guttenbach et al., 1997; Egozcue et al., 2000; Kamiguchi and Tateno, 2002). Although *de novo* structural

reorganizations are of paternal origin in approximately 85% of cases (Olsen S.D. and Magenis, 1988), the overall incidence of these abnormalities, including deletions, duplications, translocations and inversions, are much less frequent than numerical aberrations in humans (McFadden and Friedman, 1997). Therefore, only an overview of numerical chromosomal abnormalities will be discussed.

Aneuploidy, referring to cells carrying the wrong number of chromosomes, is one of the most serious problems in human reproduction. At least 25%, with some estimates being as high as 50%, of all human conceptuses have the wrong chromosome number. Numerical aberrations are associated with developmental arrest, implantation failure, spontaneous abortions, severe congenital defects, infant mortality, infertility and genetic diseases, including cancer, in the offspring. Among fetuses that survive to term, 1 in 300 infants is aneuploid (Hassold and Hunt, 2001; Hunt and Hassold, 2002).

Molecular cytogenetic analysis estimated that 3% to 4% of human spermatozoa in healthy men are aneuploid (Martin et al., 1991; Shi and Martin, 2000). Although studies assessing the aneugenic potential of mutagenic agents in human sperm have produced divergent results, there is evidence that occupational exposure to pesticides, lifestyle habits and treatment with chemotherapeutic agents and radiotherapy induce time and dose-dependent increases in sperm aneuploidy (Martin, 2006; Pacchierotti et al., 2007). Interestingly, rates of numerical chromosomal abnormalities are unequivocally increased in the sperm of infertile men presenting with a normal 46 XY karyotype (McInnes et al., 1998; Aran et al., 1999; Nishikawa et al., 2000); this has raised particular concern for the use of ICSI to allow men with severe infertility to father children (Van Steirteghem et al., 2002).

Human epidemiological studies are laden with limitations, including small sample sizes and different exposures and treatment regimes analyzed at variable times post exposure. Therefore, animal models play an extremely important role in assessing aneuploidy induction in germ cells; the introduction of rodent sperm fluorescence *in situ* hybridization (FISH) with chromosome specific DNA-probes

has been an imperative tool for germ cell aneuploidy hazard evaluation in experimental animals. A number of reviews have been published which provide an overview of results from studies evaluating the chemical induction of aneuploidy in male germ cells (Adler et al., 2002; Pacchierotti et al., 2007).

1.5.1 Mechanisms of Aneuploidy Induction

Numerical chromosomal aberrations are generally the result of errors in meiotic chromosome segregation; specifically, nondisjunction of homologous chromosomes in meiosis I or II. A recurring theme in the induction of aneuploidy is aberrant recombination. Variations in the frequencies and distribution of chiasmata have been shown to influence the induction of both autosomal and sex chromosome aneuploidies (Hall et al., 2006). Errors leading to abnormal segregation in meiosis I include: (1) failure to pair and/or establish recombination chiasmata, resulting in the independent segregation of both homologues to the same MI spindle pole; (2) failure to resolve chiasmata between homologous chromosomes at anaphase I separation, resulting in the segregation of both homologues together; and (3) premature separation and segregation of sister chromatids from each other. Aneuploidy arising from meiosis II error involves segregation failure of the sister chromatids (Hassold and Hunt, 2001).

The development of mouse genetic models and transgenic animals has aided in the elucidation of new aneugenic susceptibility factors. Due to the complexity of meiosis and germ cell formation, there are numerous opportunities for errors to occur in the normal process of chromosome separation and distribution. Additional types of aberrations predisposing to abnormalities in meiosis have been identified recently. These include mutations affecting chromatin condensation and remodeling, the formation of functionally intact centromeres and kinetochores, cytoskeleton and spindle apparatus function and cell cycle checkpoint control. The currently established genetic models are beginning to provide essential information on the key molecular mechanisms responsible for meiotic errors and aneuploidy induction; however, further research is needed to determine the in depth molecular basis for aneuploidy

induction and to identify gender, stage and target sensitivity (Eichenlaub-Ritter, 2005).

1.5.2 Significance of Paternally Transmitted Chromosomal Damage

Sex chromosome aneuploidy is the most common chromosomal aberration in humans; numerical anomalies primarily consist of three sex chromosomes (XXX, XXY and XYY). Interestingly, the majority of sex chromosome anomalies originate in the paternal germ line. Paternal nondisjunction accounts for 100% of 47, XYY cases (XYY syndrome), 80% of 45, X cases (Turner syndrome), 50% of 47, XXY cases (Klinefelter's syndrome) and 6% of 47, XXX cases (Triple X syndrome)(Sloter et al., 2004). Although the frequencies of autosomal aneuploidies in conceptuses are high, most monosomies and trisomies are incompatible with fetal development and are therefore identified in spontaneous abortions. Autosomal aneuploidies that survive to term are trisomies for chromosome 13 (Patau syndrome), 18 (Edwards syndrome) and 21 (Down syndrome). These aberrations are associated with severe physical and mental retardation, vital organ and fertility impairment and reduced life expectancy (Pacchierotti et al., 2007). The phenotypic effects of numerical sex chromosome anomalies are less detrimental, permitting fetal development to term and birth of live born infants. Children with sex chromosome aneuploidy have behavioural difficulties, learning impairment, reduced intellectual capacities and fertility disorders (Egozcue et al., 2000; Martin, 2006). The prominence of chromosomal abnormalities of paternal origin, together with the spectrum of associated deficits, highlights the importance of assessing the risk of aneuploidy induction following genotoxic exposure.

1.6 Evidence for Lack of Selection against DNA Damaged Spermatozoa

The process of male germ cell development is highly sophisticated. In the event that the DNA damage imparted to the germ cell is sufficient enough to impede the competency of spermatogenesis and fertilization there is no

accompanied risk to the offspring following preconceptional paternal exposures. However, using male mice doubly heterozygous for two Robertsonian translocations, it has been shown clearly that aneuploid sperm are as competent as normal sperm for fertilizing mouse oocytes (Marchetti et al., 1999). Equivalent frequencies of chromosome 16 hyperhaploidy and hypohaploidy occurred in both metaphase II spermatocytes and during the first mitotic division after fertilization (Marchetti et al., 1999). Importantly, chromosome 16 trisomy in the mouse corresponds to Down syndrome in humans (Epstein, 1985). The concordance between the frequencies and categories of chromosomal aberrations during spermatogenesis and in the zygote provides direct evidence that spermatozoa carrying genetically abnormal chromosomal complements are not at a disadvantage for fertilization compared to normal spermatozoa.

Indirect evidence that chemically induced spermatozoal aberrations do not prevent fertilization was shown following paternal exposure to various mutagens. Following preconceptional drug exposures, first cleavage stage zygotes contained both structural (including fragments, translocations, insertions, breaks and exchanges) and numerical chromosome aberrations (Marchetti et al., 1997; Marchetti et al., 2001; Marchetti et al., 2004). These studies indicate that DNA damage accumulated during spermatogenesis does not influence fertilization capacity and may be transmitted to the zygote. The lack of selection against spermatozoa containing numerical and structural chromosomal abnormalities suggests that there would also be no fertilization restrictions or disadvantages for spermatozoa carrying alterations in chromatin structure and/or epigenetic programming. More subtle alterations of the genome are likely to produce viable offspring and, therefore, contribute to heritable aberrations in future generations.

1.7 Evidence for Paternally-Mediated Heritable Alterations in the Genome (germline instabilities)

To date, our knowledge of the genetic risks of human exposures to irradiation, therapeutic and environmental chemicals for germline mutations and

subsequent transgenerational effects is limited. Although evidence for human mutation induction remains controversial, germline hazard investigations on survivors of ionizing radiation accidents show significantly elevated minisatellite mutation rates (Yauk, 2004; Dubrova, 2003). Minisatellite DNA sequences are found predominantly in non-coding regions of the genome. Consisting of 10-60 bp long tandemly repeated units, they are the most unstable loci primarily restricted to mutations within the human germline (Jeffreys et al., 1994; Tamaki et al., 1999). Individuals inhabiting areas in the vicinity of the Chernobyl disaster, chronically exposed to internal and external radiation sources, displayed significantly increased paternal but not maternal mutation rates; a 1.6-fold increase was observed in children born subsequent to the accident, indicating elevated germ-line mutation rates of fathers following radioactive exposure (Dubrova et al., 1996; Dubrova et al., 1997; Dubrova et al., 2002b). Furthermore, radiation exposure as a result of nuclear bomb testing in Kazakhstan (1949 – 1989) increased mutation rates by 1.8-fold and 1.5-fold for first and second generations, respectively, compared to control cohorts (Dubrova et al., 2002a). Therefore, prior to conception, fathers acquired radiation-induced DNA damage in their germline which affected minisatellite loci stability.

Current risk assessment for heritable genomic instabilities in humans relies heavily on extrapolation from animal data. In mice the analysis of expanded simple tandem repeat (ESTR) loci, composed of up to 16 kb arrays of short (<10 bp) repeat units, has revealed that paternal radiation exposure significantly elevates germline mutation rates in multiple generations. Destabilization of the F1 germline occurs after fertilization independent of the target phase of spermatogenesis. Remarkably, the radiation-induced genomic alterations persist at similar frequencies in the germline of F2 progeny (Barber et al., 2002). Barber et al. (2002) have also shown that, in addition to transgenerational ESTR locus mutations, protein-coding genes (*hprt)* and spontaneous SSBs and DSBs (detected by alkaline Comet and γ-H2AX assays, respectively) are significantly elevated in the F1 offspring of irradiated males. The persistence of elevated mutation rates and DNA lesions in subsequent generations suggests that DNA-

dependent instabilities in spermatozoa may be inherited in an epigenetic manner. Interestingly, paternal F0 irradiated spermatogonia develop the capacity to transmit unstable cellular reprogramming (Vance et al., 2002) and biochemical responses (Baulch and Raabe, 2005) as far as F3 and F4 generations, respectively. Radiation-induced chromatin changes induced in early spermatogonia persist through spermatogenesis and produce heritable chromatin alterations in spermatozoa three generations later (Baulch et al., 2007), further emphasizing the potential for genetic or epigenetic dysregulation as a mechanism for the propagation of germline instabilities.

Paternal exposures to environmental toxins and therapeutic drugs also provide strong evidence for the transgenerational transmission of genomic instabilities. Transient gestational exposure to vinclozolin (antiandrogenic) or methoxychlor (estrogenic) induced decreased spermatogenic capacity and increased male infertility in the F1 generation. Altered reprogramming of the male germline, characterized by F1 phenotype, subsequently was observed through to the F4 generation (Anway et al., 2005). Alterations in the methylation patterns of male germline candidate genes/DNA sequences are passed through at least the paternal alleles and were found in the F2 and F3 germlines. Moreover, several of the identified imprinted-like genes/DNA sequences are related to epigenetic linked disease states (Chang et al., 2006). Generations F1 to F4 developed a consistently high incidence of various diseases and tissue abnormalities, supporting the role of epigenetic germline disruption in disease onset (Anway et al., 2006).

Etoposide, a topoisomerase II inhibitor commonly used in chemotherapy regimes, induced high frequencies of chromosomal aberrations in meiotic germ cells, specifically pachytene and preleptotene spermatocytes. Analysis of metaphase stage zygotes revealed that etoposide induced unstable structural aberrations and aneuploidies present in the spermatozoa are transmitted to the progeny (Marchetti et al., 2001). Direct evidence for heritable genomic damage beyond the first generation offspring following paternal chemotherapy is lacking;

however, these data clearly highlight the potential risks associated with fertilization via sperm carrying altered genetic and/or epigenetic material.

As demonstrated, paternal irradiation and genotoxic exposures have the capacity to induce DNA-based changes and genomic instabilities that are subtle enough to allow the completion of spermatogenesis and fertilization, thereby propagating potentially detrimental instabilities across multiple generations. Importantly, compromised genomic material may lead to developmental defects, increased sensitivity to exogenous exposures and cancer predisposition in the offspring (Baulch et al., 2007). Further characterization of the genetic and epigenetic mechanisms involved in transgenerational effects following paternal chemical exposures is necessary.

1. 8 DNA Damage Response Mechanisms

1.8.1 DNA Damage Recognition

DNA double strand breaks (DSB), generated from normal or pathological endogenous processes or exogenously by DNA-damaging agents, are among the most detrimental DNA lesions, compromising the genomic and structural fidelity of the cell. Unrepaired DSBs can lead to gene mutations, chromosomal translocations and aberrations, inducing genomic instability and increased susceptibility to diseases, including cancer (Khanna and Jackson, 2001; Bassing and Alt, 2004; Celeste et al., 2003a). Homologous recombination (HR) and non-homologous end joining (NHEJ) are two highly conserved pathways prominent in the repair of DSBs (Khanna and Jackson, 2001). Recently, post-translational chromatin modifications in response to DNA DSBs have been identified as an additional mechanism facilitating nucleosome mobilization and DNA repair (Loizou et al., 2006; Murr et al., 2006).

1.8.1.1 Phosphorylated Histone H2AX

Phosphorylation of the histone H2A variant, H2AX, is the most extensively characterized chromatin modification occurring in response to DNA DSBs. H2AX is critical for the maintenance of genomic integrity; H2AX deficient mice exhibit radiation sensitivity, growth retardation, chromosomal instability, DNA repair defects and male infertility (Celeste et al., 2002; Bassing et al., 2002). In response to DNA damage, phosphatidylinositol-3 kinase-like (PI3K) family of kinases, including ataxia telangiectasia mutated (ATM), ATM- and Rad3-related (ATR) and DNA dependent protein kinase (DNA-PK), rapidly phosphorylate the unique carboxy-terminal tail of H2AX at serine 139 along megabase-sized chromatin domains flanking each lesion site in the mammalian cell. Hundreds to thousands of H2AX molecules become phosphorylated per DSB, producing highly amplified nuclear micro-domains that are recognized reliably by anti-γ-H2AX antibodies (Figure 1.7). The 1:1 correspondence between γ-H2AX nuclear foci and DSB sites has allowed immunofluorescence visualization of foci to become a gold standard for DSB detection (Rogakou et al., 1999; Sedelnikova et al., 2002; Fernandez-Capetillo et al., 2004).

Although H2AX is dispensable for the initial recruitment of repair and signaling factors, it is essential for protein assembly and retention at sites of DNA damage (Celeste et al., 2003b; Bassing et al., 2002). Several components of the DNA damage response localize to foci in association with γ-H2AX, including Brca1, 53BP1, MDC1, Rad51 and the Mre-11-Rad50-Nbs1 (MRN) complex (Paull et al., 2000; Schultz et al., 2000; Stucki et al., 2005; Kang et al., 2005; Lukas et al., 2004; Stewart et al., 2003; Rappold et al., 2001). Accordingly, it is proposed that γ-H2AX may act as a catalyst for complex formation; more persistent breaks result in larger γ-H2AX foci and presumably accumulate a greater concentration of repair proteins (Pilch et al., 2003).

γ-H2AX plays an important role in spermatogenic chromatin remodeling, meiotic recombination (Mahadevaiah et al., 2001; Hamer et al., 2003) and sex chromosome condensation, synapsis and transcriptional inactivation (Fernandez-

Capetillo et al., 2003). Furthermore, DNA damage generated by cyclophosphamide or oxidative stress induced γ-H2AX in spermatocytes and spermatozoa, respectively, highlighting involvement in DNA damage detection and repair during male germ cell development (Aguilar-Mahecha et al., 2005; Li et al., 2006). The phosphorylation of H2AX has been directly linked to DNA repair via the recruitment of cohesion complexes; damage-recruited cohesin maintains chromatid proximity, facilitating post-replicative repair by HR (Unal et al., 2004; Alastalo et al., 1998; Strom et al., 2004).

An emerging function of H2AX phosphorylation is the recruitment of histone modifiers and chromatin remodelers, such as NuA4 (histone acetyl-transferase complex), Ino80 and Swr1 (ATP-dependent chromatin remodeling complex), to the vicinity of the DNA lesion, thereby altering chromatin structure and enhancing DNA accessibility to repair factors (Downs et al., 2004; van Attikum and Gasser, 2005b; van Attikum and Gasser, 2005a; van Attikum et al., 2004; Morrison et al., 2004; Murr et al., 2006). Together, these data reveal γ-H2AX as a crucial component of the DNA damage response, mediating changes in chromatin structure while promoting sustained protein assembly for DNA repair, in order to preserve genomic integrity and the faithful transmission of epigenetic information.

1.8.1.2 Poly(ADP)ribose polymerase (PARP-1)

Poly(ADP-ribosyl)ation of histones and other nuclear proteins is involved in many physiological and pathophysiological processes, including cellular signaling, transcription, cell cycle regulation and mitosis (Hassa et al., 2006; Kim et al., 2005). However, it has been most notably associated with the DNA damage response, playing important roles in DNA repair pathways (Malanga and Althaus, 2005), apoptosis and necrosis (Andrabi et al., 2006; Yu et al., 2006). Poly(ADP-ribose) polymerase-1 (PARP-1), an abundant 113 kDa nuclear protein, functions as a 'molecular nick sensor', binding with high affinity to DNA strand breaks and causing immediate stimulation of its catalytic activity by more than 500-fold (Simonin et al., 1993). Activated PARP-1 catalyzes the cleavage of

nicotinamide adenine dinucleotide (NAD+) to form chains of poly(ADP-ribose) polymers which are transferred to various acceptor proteins, thereby regulating DNA metabolism and altering chromatin structure to make damaged DNA more accessible to repair machinery following genotoxic stress (Figure 1.8) (Hassa et al., 2006; Realini and Althaus, 1992; Tulin and Spradling, 2003).

Gene disruption studies support the role of PARP-1 as a guardian of the genome. PARP-1-deficient mice are hypersensitive to the effects of genotoxic agents, are more prone to tumor development, have reduced repair capacity and display higher genomic instability (Masutani et al., 2000; de Murcia et al., 2003; Demurcia et al., 1997; Trucco et al., 1998). PARP-1 is primarily involved in single strand break repair (SSBR) and base excision repair (BER), where it mediates the recruitment of factors which include XRCC1, DNA ligase III, DNA polymerase β and condensin I (El Khamisy et al., 2003; Okano et al., 2003; Masson et al., 1998; Caldecott et al., 1996; Heale et al., 2006). PARP-1 involvement in nucleotide excision repair (NER)(Flohr et al., 2003) and double strand break repair (Wang et al., 2006), as well as in the regulation of alkylation-induced sister chromatid exchange formation (Meyer et al., 2000), has been reported.

Interestingly, in addition to the requirement of PARP-1 for DNA repair (Atorino et al., 2001; Di Meglio et al., 2004; Kopeika et al., 2004), poly(ADP-ribosyl)ation undergoes dynamic changes coincident with chromatin remodeling events during spermatogenesis (Meyer-Ficca et al., 2005; Maymon et al., 2006) and zygotic development (Imamura et al., 2004), highlighting the essential contribution of PARP-1 to both DNA repair and epigenetic modifications in germ cell development and embryogenesis.

1.8.2 DNA repair

1.8.2.1 DNA Repair during Spermatogenesis

Unscheduled DNA synthesis (UDS) is the most commonly used assay to indirectly measure DNA repair in spermatogenic cells; [^{3}H]-thymidine

incorporation in cells which are not in S phase reflects nucleotide excision repair. Examination of an extensive list of chemicals, UV irradiation and X-rays has determined that UDS occurs in spermatogonia, meiotic spermatocytes and early spermatids, however, late spermatids and mature spermatozoa were negative for UDS for all genotoxic agents tested (Sotomayor et al., 1978; Sotomayor and Sega, 2000).

Transcript profiling has established the presence and expression patterns of DNA repair enzymes during germ cell differentiation. DNA repair genes involved in NER, BER, HR repair and mismatch repair (MMR)(Richardson et al., 2000; Aguilar-Mahecha et al., 2001b) are differentially expressed during spermatogenesis. Protein analysis confirmed functional and efficient repair mechanisms for BER and NER in spermatogonia, spermatocytes and round spermatids; pachytene spermatocytes displayed the highest level of expression, while repair dramatically declined to negligible levels in post-meiotic cell types (Olsen et al., 2001; Jansen et al., 2001; Xu et al., 2005). Recently, using an *in vitro* plasmid recombination assay, it was determined that pachytene spermatocytes are also the most proficient germ cell stage at HRR (Srivastava and Raman, 2007). Therefore, surveillance mechanisms, such as γ-H2AX, which detect DNA double strand breaks in pachytene spermatocytes (Aguilar-Mahecha et al., 2005), may lead to damage resection via HR-mediated DSB repair. The restricted repair capacity of post-meiotic spermatids is likely due to their highly condensed chromatin state, which limits the access of repair machinery to DNA (van Loon et al., 1993); lack of UDS (Sotomayor and Sega, 2000) or degradation of DNA repair proteins may also contribute to the limited repair capacity during late spermatogenesis. Unrepaired DNA damage, accumulated in the mature spermatozoon from exposures during spermatogenesis, is, therefore, dependent on the maternally derived repair enzymes in the oocyte to solve the problem.

1.8.2.2 DNA Repair Pathways in the Zygote and Early Embryo

DNA damage arising prior to fertilization poses a significant risk to the developmental competence of the embryo; efficient repair in the early embryonic cleavage stages is of great importance to ensure genomic integrity of the next generation (Jaroudi and Sengupta, 2007). Early studies assessing the repair capacity of fertilized mouse oocytes demonstrated the occurrence of unscheduled DNA synthesis (or DNA repair replication) in both parental pronuclei in response to irradiation- or drug-induced damage of either the oocyte or spermatozoon (Brandriff and Pedersen, 1981). The utilization of DNA repair inhibitors (arabinofuranosyl cytosine (ara-C), 3-aminobenzamide (3AB) and caffeine) and dominant-lethal studies further supported the notion that DNA damage acquired in spermatozoa or oocytes can be reduced by a variety of repair processes in the egg cytoplasm after fertilization (Matsuda and Tobari, 1989; Genesca et al., 1992; Generoso et al., 1979).

The developmental programme of the newly fertilized oocyte is initially controlled by maternally derived proteins and transcripts (Schultz, 2002), suggesting that zygotic DNA damage resolution may be also dependent on DNA repair enzymes stored in the oocyte (Vinson and Hales, 2002; Gurtu et al., 2002). Recently, studies assessing the expression of mRNAs involved in DNA damage sensing, repair, cell cycle regulation and cell death have revealed dynamic temporal expression patterns during specific stages of preimplantation development (Zheng et al., 2005; Wells et al., 2005; Zeng et al., 2004; Jurisicova et al., 1998). *In vitro* embryo culture leads to dysregulation of many genes involved in various repair pathways as assessed at the blastocyst stage (Zheng et al., 2005). Further evidence for an early damage response in the embryo was revealed in preimplantation stage rat embryos following paternal cyclophosphamide exposure. Most significantly, as early as the zygote, dramatic increases in genes participating in nucleotide excision repair, mismatch repair and recombination repair were observed, while mRNA expression for base excision repair and other recombination repair transcripts were decreased (Harrouk et al., 2000a). Therefore, the early preimplantation embryo appears to

possess the components necessary to detect, respond to and potentially repair damage induced by exogenous stressors; however, very little information exists on the function and interaction of DNA damage sensing and repair proteins in the newly fertilized embryo.

1.9 Embryonic Fate of Paternally-Transmitted DNA Damage

Preconceptional exposure of male germ cells to irradiation or a range of mutagens produces genetic aberrations in the zygote that are predictive of ensuing reproductive outcomes. The finding that embryonic fate is determined by the end of G1 of the first cell cycle (Marchetti et al., 2004) suggests that, although cell cycle checkpoints seem to be inefficient in the initial stages of development (Handyside and Delhanty, 1997), DNA damage recognition and regulation in the zygote are essential steps controlling the life or death signal influencing postimplantation developmental success. This further highlights the importance of elucidating the early events that are activated to manage genetic and epigenetic aberrations of paternal origin (Marchetti et al., 2004).

1.10 Cyclophosphamide

1.10.1 Mechanisms of Action

Alkylating agents confer efficacy by acting in a cell cycle non-specific manner, although rapidly dividing cells are the most susceptible. Disruption of the double stranded DNA may occur at any time during the cell cycle, compromising normal cellular functions (Colvin, 1999).

As a prodrug, the nitrogen mustard alkylating agent cyclophosphamide must undergo metabolic activation by hepatic cytochrome P450 enzymes to form the main active metabolite, 4-hydroxycyclophosphamide (4-OHCPA). 4-OHCPA exists in equilibrium with its tautomer aldophosphamide, which is converted to acrolein and phosphoramide mustard (Figure 1.9). Phosphoramide mustard, the

ultimate alkylating agent, contains a bifunctional nitrogen mustard moiety that covalently binds to nucleophilic compounds such as the N-7 position on guanine (Anderson et al., 1995). Reaction of the second chloroethyl group in the nitrogen mustard with DNA or protein produces inter- and intrastrand DNA crosslinks or DNA-protein crosslinks, as well as DNA strand breaks, inhibits DNA synthesis and leads to cell death (Hengstler et al., 1997). Acrolein metabolites produce cytotoxicity via the induction of DNA single strand breaks (Crook et al., 1986) and play a major role in cyclophosphamide toxicity, causing side effects including hemorrhagic cystitis and cardiotoxicity (Fraiser et al., 1991; Kehrer and Biswal, 2000).

Cyclophosphamide is routinely used in combination with other chemotherapeutic agents for the treatment of various malignant diseases, including leukemia, lymphoma, neuroblastoma, retinoblastoma as well as lung, breast, ovarian, testicular and pediatric brain cancers. In addition, cyclophosphamide is used for the treatment of non-malignant disorders in cases in which autoimmune phenomena are indicated in the disease pathogenesis; examples for use as an immunosuppressive agent include systemic lupus erythematosis, rheumatoid disease and Wegener's granulomatosis (Colvin, 1999; Kenney et al., 2001).

Numerous disease dependent dosing schedules exist and adjustments must be made according to the toxicity profile and/or usage in drug combinations. In general, when used for adult and childhood malignancies, cyclophosphamide is given acutely in the dose range of 600 – 1000 mg/m^2 (1 - 5 mg/kg/day) every 3 to 4 weeks. Chronic oral administration, 100 – 200 mg (1.5 – 3.0 mg/kg) of cyclophosphamide daily, over the course of 60 – 90 days is used for patients requiring immunosuppression. Commonly, the dose limiting toxicity is myelosuppression in cases of malignant disease; alternatively, when immunosuppression is desirable, such as in the case of bone marrow transplantation, dose escalation is limited by cardiotoxicity (Moore, 1991). Other adverse effects of cyclophosphamide include gastrointestinal disturbances,

alopecia, hemorrhagic cystitis, secondary malignancies and amenorrhea or azospermia leading to infertility (Colvin, 1999).

1.10.2 Gonadotoxic Effects of Cyclophosphamide

Gonadal function is highly compromised in the male after cyclophosphamide treatment. Testicular biopsies show significant depletion of the testicular epithelium which is required for normal spermatogenesis and steroidogenesis. Therapeutic treatments for various malignant and non-malignant diseases, using cyclophosphamide as a single agent or in a combination regime, result in azoospermeria in 60% of patients; infertility may be reversed and appears to be dose-dependent (Meistrich et al., 1992b). Patients undergoing cancer treatment became azoospermic within 4 months from the commencement of therapy; recovery of spermatogenic function was observed to occur in 40% of men 5 years post-treatment, with cumulative drug dose being the most reliable predictor of regained fertility. The incidence of persistent gonadal dysfunction correlated with a cumulative dose exceeding 7.5 g/m^2 (or more than 300 mg/kg); patients receiving doses <7.5 g/m^2 recovered more than 70% of the time (Rivkees and Crawford, 1988; Meistrich et al., 1992b; Wetzels, 2004). A similar incidence of azoospermia, 58.8%, was observed following childhood exposure to cyclophosphamide, suggesting that male adult survivors of pediatric malignancies are equally at risk for long-term gonadal damage and infertility (Kenney et al., 2001). Despite the elevated incidence of oligozoospermia and azoospermia, there are case reports of paternity following high-dose cyclophosphamide exposure (Letendre and Moore, 1997; Sanders et al., 1996; Check et al., 2000).

Detailed assessment of the consequences of chronic cyclophosphamide administration has shown various time- and dose-dependent effects on male germ cells with almost no effect on the orderly process of spermatogenesis in rats (Trasler et al., 1985; Trasler et al., 1988; Anderson et al., 1995). Using the alkaline elution assay to determine total DNA damage, a significant increase in DNA single strand breaks and DNA-DNA crosslinks were observed in sperm

chromatin after 6 weeks, but not after 1 week, of cyclophosphamide exposure (Qiu et al., 1995a); DNA template function, as well as the *in vitro* nuclear elongation pattern, of spermatozoa were altered markedly compared to controls (Qiu et al., 1995b). Cyclophosphamide also interrupts the fidelity of mitotic and meiotic processes. Exposure of male mice damaged the synaptonemal complex, induced synaptic failure, altered centromeric DNA sequences (Backer et al., 1988; Allen et al., 1988) and has been reported to induce gene conversions and frameshift mutations (Schimenti et al., 1997), demonstrating a genotoxic effect during meiotic prophase. Interestingly, pre-meiotic cyclophosphamide exposure has been shown also to induce both structural and numerical chromosomal abnormalities in mouse secondary spermatocytes (Pacchierotti et al., 1983).

The capacity for developing male germ cells to respond to cyclophosphamide-induced alterations in genomic integrity was assessed using gene array technology. Post-treatment, numerous genes involved in DNA repair, oxidative stress defense and heat shock were differentially expressed during rat spermatogenesis. A single high dose treatment of cyclophosphamide (70 mg/kg) primarily increased gene expression in round spermatids (Aguilar-Mahecha et al., 2001a); conversely, chronic low dose treatment (6 mg/kg/day for up to 6 weeks) dramatically reduced gene expression in both pachytene spermatocytes and round spermatids (Aguilar-Mahecha et al., 2002). The discrepancy observed between acute and chronic treatment regimes suggests that a single high dose exposure induces mechanisms to repair the alkylation damage or eliminate the damaged cell, while the extent of damage accumulated during a chronic exposure compromises the cells ability to respond via damage resolution mechanisms. Accordingly, significantly increased apoptosis, detected by TUNEL assay, was most pronounced in spermatogonia and spermatocytes following an acute high dose cyclophosphamide exposure (Cai et al., 1997). Absence of apoptosis in repair deficient postmeiotic germ cells suggests an inability to activate an appropriate cell death response, enhancing genomic susceptibility to the damaging effects of cyclophosphamide, thus contributing to the heritable

defects observed during embryogenesis (Dulioust et al., 1989; Hales et al., 1992).

The germ cell phase specificity for cyclophosphamide-induced DNA damage was determined by comet assay. Although pre-meiotic germ cells are at considerable risk for error induction, overall DNA damage was greatest after germ cell exposure during mid-spermiogenesis (step 9-14 spermatids), reflecting specific susceptibility to cyclophosphamide alkylation during sperm chromatin remodeling (Codrington et al., 2004). Extensive examination of the effects of cyclophosphamide treatment on epididymal sperm chromatin structure confirmed the mid-spermiogenic spermatids to be the most sensitive germ cell phase (Codrington et al., 2007). The acridine orange DNA denaturation and chromomycin A3 assays demonstrated that cyclophosphamide exposure significantly increased denaturation susceptibility and induced alterations in chromatin packaging, respectively. Measurement of total thiol (monobromobimane fluorescence) and protamine contents (HUP1N protamine 1 antibody) further revealed reduced chromatin stability from round spermatid exposure, demonstrating that cyclophosphamide-induced chromatin alterations directly affect genetic integrity of the spermatozoal genome. Interestingly, 2D basic gel electrophoresis identified altered expression of proteins involved in various aspects of spermatid differentiation, sperm maturation and fertilization after cyclophosphamide exposure. Together, alterations in the sperm proteome, in conjunction with chromatin instability may interrupt the precise coordination of events post-fertilization leading to improper embryo development (Codrington et al., 2007).

1.10.3 Adverse Male-Mediated Effects on Embryogenesis

In man, efforts to demonstrate a link between fetal malformations, adverse developmental outcomes and preconceptional paternal exposures to chemotherapeutic drugs have lead to inconsistent and inconclusive reports (Gulati et al., 1986; Fried et al., 1987; Senturia et al., 1985). Rodent studies show unequivocally that preconceptional exposure of the father to

cyclophosphamide leads to adverse developmental effects in the offspring (Trasler et al., 1985; Trasler et al., 1986; Trasler et al., 1987; Auroux and Dulioust, 1985).

Male germ cells show differential susceptibility to cyclophosphamide exposure (Codrington et al., 2004); adverse developmental effects depend on the extent of damage and the spermatogenic phase of exposure (Figure 1.10) (Trasler et al., 1985; Trasler et al., 1986; Trasler et al., 1987; Anderson et al., 1995). Chronic low dose cyclophosphamide exposure for 2 weeks leads to increased postimplantation loss; maximal loss occurred following 4 weeks of treatment, correlating postimplantation loss with damage acquired during spermiogenesis. Behavioral testing demonstrated that post-meiotic germ cells were also the most sensitive to the induction of learning impairment in offspring sired by cyclophosphamide-exposed males (Fabricant et al., 1983; Auroux and Dulioust, 1985). A chronic 6 week exposure, initially targeting spermatocytes, significantly increased the incidence of preimplantation loss; treatment of male rats with cyclophosphamide for 7-9 weeks increased fetal malformation and growth retardation. The malformations observed when germ cells were first exposed as spermatogonia were primarily hydrocephaly, edema and micrognathia (Trasler et al., 1986); theses malformations, as well as open eyes, omphalocele, syndactyly, gigantism and dwarfism, persisted in the F2 generation (Hales et al., 1992). Incidentally, increased postimplantation loss, postnatal mortality and learning deficits were also evident in multiple generations following cyclophosphamide treatment of the F0 male (Hales et al., 1992; Auroux et al., 1990; Dulioust et al., 1989).

Chronic cyclophosphamide treatment induces specific and heritable male germ cell damage, compromising progeny outcome. Studies have been done to elucidate the complex mechanisms involved in male-mediated cyclophosphamide-induced embryotoxicity. Examination of cleavage stage embryos (gestational day 3 and 4) sired by cyclophosphamide-treated males revealed a reduction in cell number and an increased cell doubling time, to 16 hours as compared to 12 hours in corresponding controls (Austin et al., 1994).

Decreased [^{3}H]thymidine incorporation confirmed compromised DNA synthetic ability and proliferation rate. At the blastocyst stage, embryonic cell loss occurred gradually, in a cell lineage non-specific manner; both trophectoderm and inner cell mass cells were proportionally affected (Kelly et al., 1994). Interestingly, selective death of inner cell mass cells was observed in day 7 implantation sites, while the trophectoderm appeared morphologically normal following paternal cyclophosphamide exposure (Kelly et al., 1992). Random segregation of paternal DNA strands into both the inner cell mass and the trophectoderm (Ito et al., 1988) supports the observation of lineage non-specific effects; however, increased sensitivity of inner cell mass cells as cleavage stage development progresses highlights the requirement for proper regulation of paternal specific genes in the embryo proper. A reduction in embryonic cell number led to decreased cell-cell interactions and dysregulated expression of adhesion molecules (cadherins and connexin 43) and structural elements (β-actin, collagen and vimentin) disrupting the cellular communication in embryos sired by drug-treated males (Harrouk et al., 2000c). A continuous series of cell-cell interactions are imperative for the maintenance and proliferation of inner cell mass cells, successful embryogenesis and, thus, the production of live offspring (Suzuki et al., 1995).

Major events of the developmental program are dysregulated in preimplantation embryos fertilized by cyclophosphamide-exposed spermatozoa. The rate of sperm nuclear decondensation and male pronuclear formation is proportional to disulfide bond content (Perreault et al., 1987). Chronic cyclophosphamide treatment of male rats for 6 weeks altered spermatozoal decondensation patterns, decreased reducible sulfhydryl content in vitro (Qiu et al., 1995b) and led to advanced male pronuclear formation, assessed by the rat spermatozoa-hamster egg in vitro fertilization assay (Harrouk et al., 2000b). During the first cell cycle of the newly fertilized oocyte, the chromatin changes that occur in the maternal and paternal genomes determine the normal onset of gene expression. RNA synthesis ([^{32}P]UTP incorporation) in rat embryos fathered by cyclophosphamide-treated males was constant from the one- to

eight-cell stage, while total RNA synthesis peaked dramatically at the four-cell stage in embryos sired by control males. Paternal cyclophosphamide administration prior to conception also induced defects at the level of transcriptional machinery in the early cleavage stage embryo. Bromouridine triphosphate (BrUTP) incorporation and Sp1 transcription factor immunostaining were considerably increased and spread over both the nuclear and cytoplasmic compartments in 2-cell embryos sired by cyclophosphamide-exposed spermatozoa; immunofluorescence staining was restricted to the nucleus in embryos fathered by control males. Furthermore, chronic paternal exposure to cyclophosphamide altered the embryonic expression profile of numerous genes with defined roles in DNA repair, imprinting and growth as early as the one- and two-cell stages of development (Harrouk et al., 2000b; Harrouk et al., 2000a).

Fertilization by spermatozoa with cyclophosphamide-induced DNA damage may alter the coordinated events of pronuclear formation and disrupt the development of both the male and female pronuclei, subsequently leading to dysregulation of zygotic gene activation and aberrant regulation of the developmental clock (Harrouk et al., 2000b; Harrouk et al., 2000a).

1.11 Rationale for Thesis Studies

A decline in the reproductive capacity of current generations has provoked both scientific and public awareness of the potential impact of toxicant exposures on fertility. The incidence of infertility, specifically defined as the inability to conceive after a year of regular intercourse without contraception, has almost doubled over the last 20 years, affecting 15% of all couples (Runnebaum et al., 1997; Evers, 2002). Importantly, this indisputably represents an underestimation of the problem as this does not include couples who do not seek intervention. Clinical and epidemiological studies have noted a considerable increase in male reproductive problems, including genital abnormalities, low and probably declining semen quality and testicular cancer (Boisen et al., 2001). Consistent

with reported declines in sperm quality (Carlsen et al., 1992; Swan et al., 2000), male factor is responsible for 40% of cases of infertility worldwide.

The complex and highly proliferative nature of male germ cell production makes spermatogenesis a prime target for a diverse range of genotoxic agents that cause various types of genomic changes in the spermatozoa. Exposures to environmental, occupational and therapeutic toxicants have been shown to have detrimental effects on sperm quality (Jensen et al., 2006; Yang et al., 2006; Schrader et al., 2001). Furthermore, sperm with significant levels of nuclear damage retain the ability to fertilize; however, a significant risk of pregnancy loss and adverse developmental outcomes is imparted to the embryo (Marchetti et al., 1999). While it is becoming increasingly clear that paternal exposures contribute significantly to human infertility and adverse progeny outcomes, the underlying mechanisms are not well understood.

Cancer is a major health concern for men in the reproductive years; in fact, 1 in 71 men are diagnosed with a malignancy before 39 years of age (American Cancer Society, 2005). Early diagnosis and the advanced success of aggressive combination drug regimes have led to notable improvements in relative 5-year survival rates for all cancers; 1 in 30 men among the United States population is a cancer survivor. Remarkably, for children with diagnosed cancers, the combined 5-year survival rate has increased substantially to about 80% (Jemal et al., 2007). A growing number of young men achieve long-term survival following cancer therapy; therefore, heightened concern for the impact of altered germ cell quality, infertility and future fatherhood of healthy offspring is elicited.

To date, the debate continues over whether there is, in fact, an increased risk for pregnancy loss, birth defects, genetic syndromes or disease in the children of cancer survivors, regardless of the methods of conception, natural or assisted (Meistrich and Byrne, 2002; Green et al., 2003). The limitations of human epidemiological studies account for the lack of conclusive information; this is not reassuring given the indisputable evidence for adverse progeny outcomes following paternal drug exposures in animal models. Cyclophosphamide, an extensively studied anticancer agent, clearly impacts

germ cell quality in a stage specific manner and adversely affects progeny outcome, as evidenced by increased pre- and post-implantation loss, impaired embryonic development and/or birth defects (Trasler et al., 1985). Furthermore, these abnormalities, as well as behavioural deficits, are transmitted across multiple generations (Hales et al., 1992; Auroux et al., 1990), suggesting alterations in the epigenetic code in addition to genotoxic mutagenesis. A functionally intact paternal genome is essential for successful embryogenesis (Ward et al., 1999; Harrouk et al., 2000b); any interruption to sperm DNA integrity, chromatin structure and/or gene expression could negatively impact on progeny health and development. Rodent studies are invaluable tools to elucidate the mechanisms by which paternal exposures to genotoxic agents may affect male germ quality adversely and lead to detrimental effects during embryogenesis.

The purpose of this dissertation is to advance our current understanding of the mechanisms by which preconceptional paternal cyclophosphamide exposure alters male germ cell quality and the consequences on early post-fertilization events. Accordingly, the following hypothesis was proposed: Paternal cyclophosphamide exposure: (1) damages male germ cells in a phase-dependent manner that results in altered genomic integrity and spermatozoal function; and (2) causes germ cell damage that leads to altered chromatin remodeling and epigenomic instability during zygotic development.

To address the hypothesis, the following objectives were set forth:

(1) Determine the impact of chronic cyclophosphamide exposure on spermatozoal function by assessing alterations in sperm motility.
(2) Determine the aneugenic potential of chronic cyclophosphamide exposure by assessing the induction of numerical chromosomal abnormalities.
(3) Determine the consequences of altered sperm quality on chromatin remodeling and post-translational modifications on the zygotic chromatin.

(4) Determine the consequences of altered sperm quality on the ability of the zygotic pronuclei to detect DNA damage and initiate a DNA repair response.

The results of these studies have important implications for understanding the mechanisms by which paternal toxicant exposure alters the genome in a manner that adversely impacts successive generations. Furthermore, these findings provide valuable information in the context of the increasing use of assisted reproductive technologies in society.

FIGURES

Figure 1.1. Schematic representation of the stages of meiosis during spermatogenesis. Primary spermatocytes undergo reduction division, or meiosis I, to produce two secondary spermatocytes. During meiosis I, chromosomes pair, synapse, recombine and desynapse again, prior to cytokinesis. Meiosis II occurs rapidly, similar to mitotic divisions, producing four haploid spermatids. Adapted from Cobb and Handel (1998) Semin Cell Dev Biol 9:445-450.

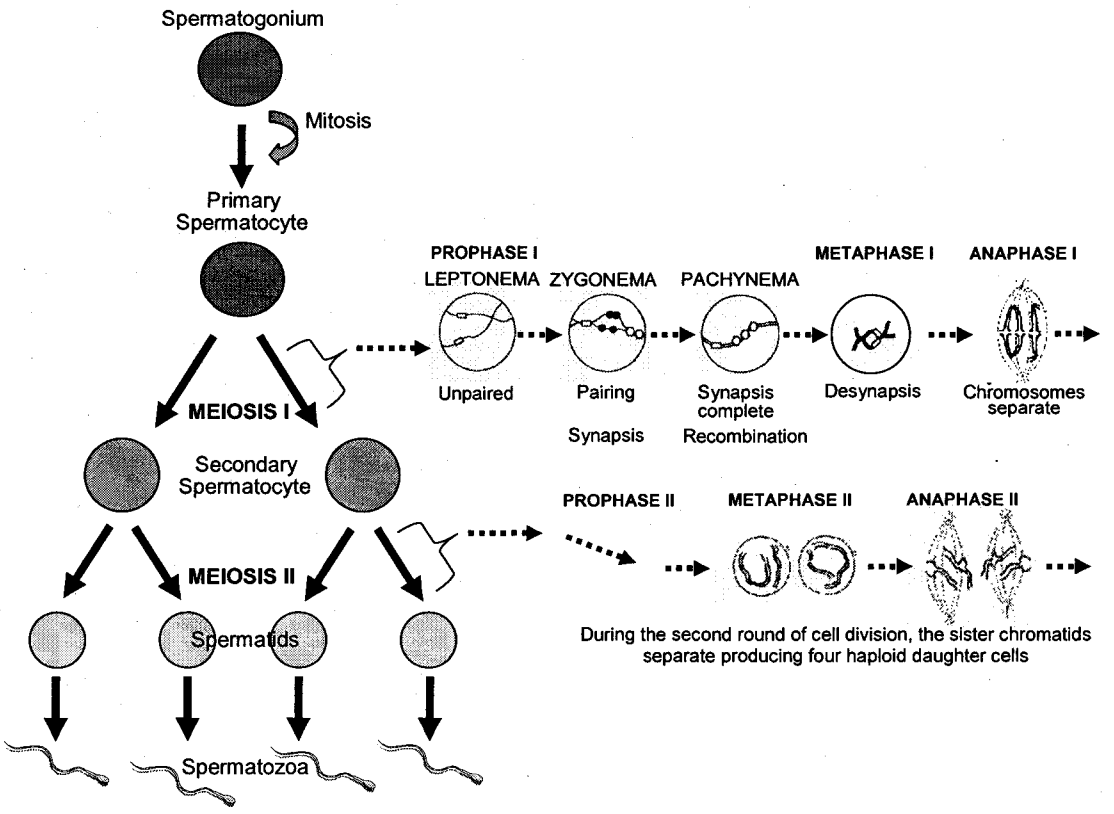


Figure 1.2. Epigenetic regulation during spermatogenesis. Histone modifications occur in male germ cell stage-specific waves associated with nucleoprotein exchange and subsequent chromatin condensation occurring in the late stages of spermiogenesis. During the proliferation phase through to early spermatocytes, histones are hyperacetylated and hypomethylated; during the meiotic phase and differentiation phase histones become hypoacetylated and hypermethylated. Histone H4 undergoes an additional wave of hyperacetylation in early elongating spermatids. DNA methylation is prominent from spermatogonia through the meiotic phase of spermatogenesis. Red bars represent hyper – acetylation, methylation; blue bars represent hypo – acetylation, methylation. Adapted from Kimmins and Sassone-Corsi (2005) Nature 434:583.

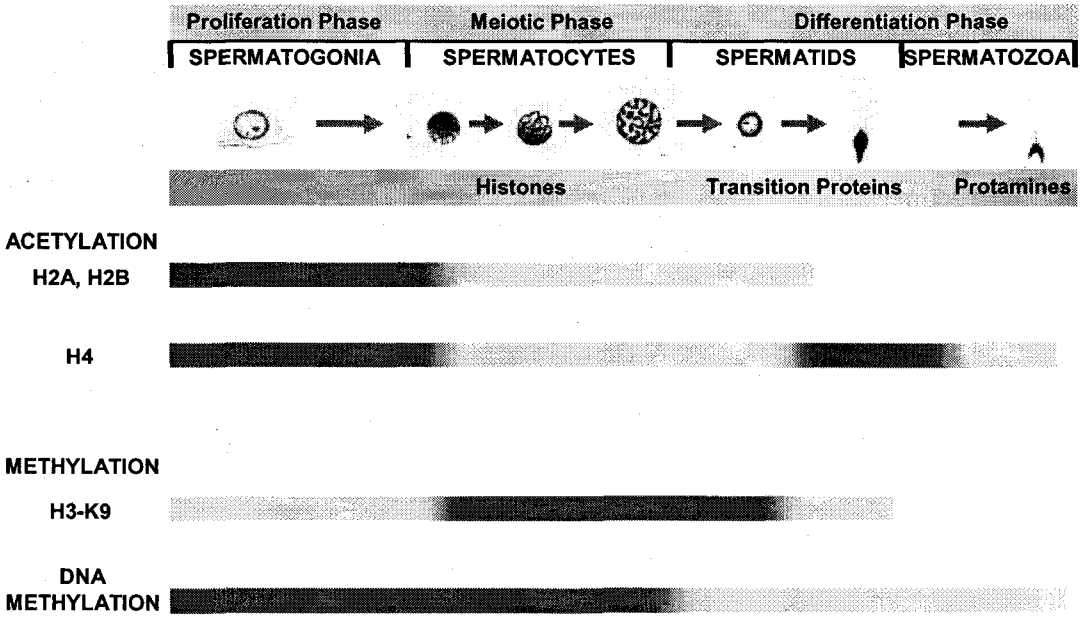


Figure 1.3. Schematic representation of a rat spermatozoon and axonemal cross-section. (A) The mammalian spermatozoon is composed of the connecting piece, the midpiece, the principle piece and the end piece. (B) Schematic cross-section through the midpiece segment demonstrating the mitochondrial sheath (MS), outer dense fibers (ODF), and the central pair of microtubule doublets (MD) surrounded by the nine outer microtubule doublets (OMD) of the axoneme (9 + 2 array). Associated with the OMD are radial spokes and dynein arms, which are implemental for flagellar motion. Adapted from Turner (2006) Reprod. Fert. Dev 18:25.

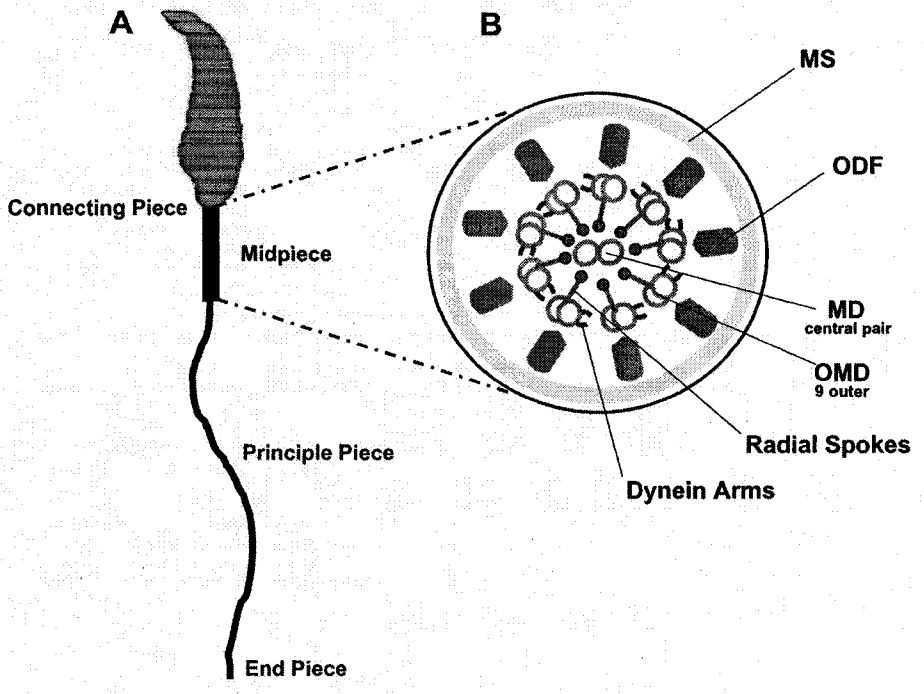


Figure 1.4. Paternal chromatin remodeling in the oocyte after fertilization. Immediately following fertilization, the nucleoprotein exchange that occurred during spermatogenesis is rapidly reversed. Sperm-derived protamines are removed by anaphase II and replaced by the maternal store of histones. Commencing at pronuclear formation and continuing throughout the zygotic cell cycle, male and female pronuclei progressively decondense, dramatically increasing chromatin areas. Adapted from McLay and Clarke (2003) Reproduction 125:625.

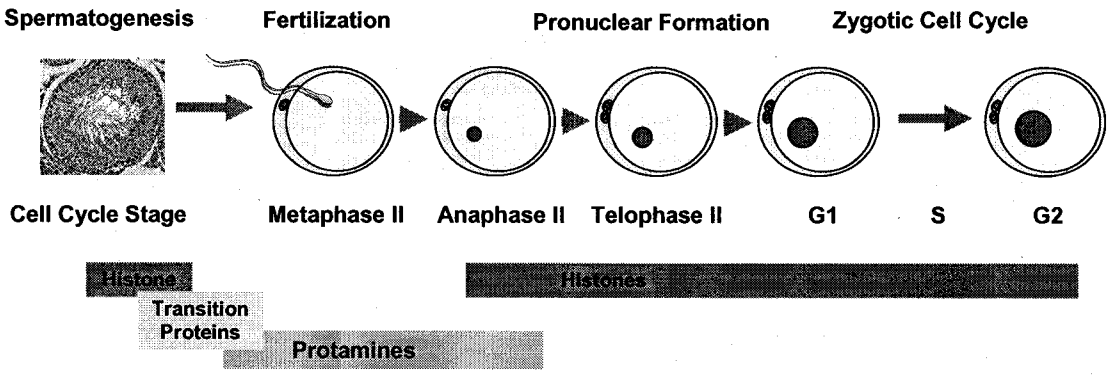


Figure 1.5. Stages of pronuclear development. Developmental progression of the zygote can be categorized into pronuclear stages as the male and female pronuclei migrate toward the center of the cell. At pronuclear stage (PN) 1, the pronuclei are small and are located at the periphery of the embryo; at PN2, pronuclei continue to decondense and begin to migrate; at PN3, male and female pronuclei are large in size and are more central; at PN4, further decondensed pronuclei are in close approximation to each other in the center of the embryo; and at PN5, parental pronuclei are large and centrally apposed. Adapted from Adenot et al., Development (1997) 124: 4615.

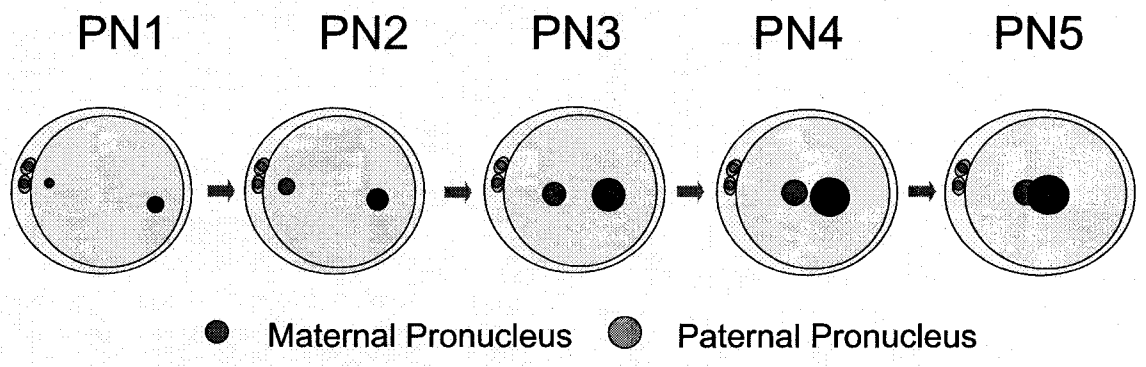


Figure 1.6. Germ-cell stage specific targeting for the assessment of adverse effects following toxicant exposure. The kinetics of spermatogenesis allows the determination of the precise susceptibility of various germ-cell phases throughout spermatogenesis. The number of weeks that males were exposed to a toxicant dictates the germ cells phases that were targeted. For example, 7 to 9 weeks of exposure targets all germs cells from spermatogonia through to mature spermatozoa; conversely, a 1 week exposure targets spermatozoa during epididymal transit.

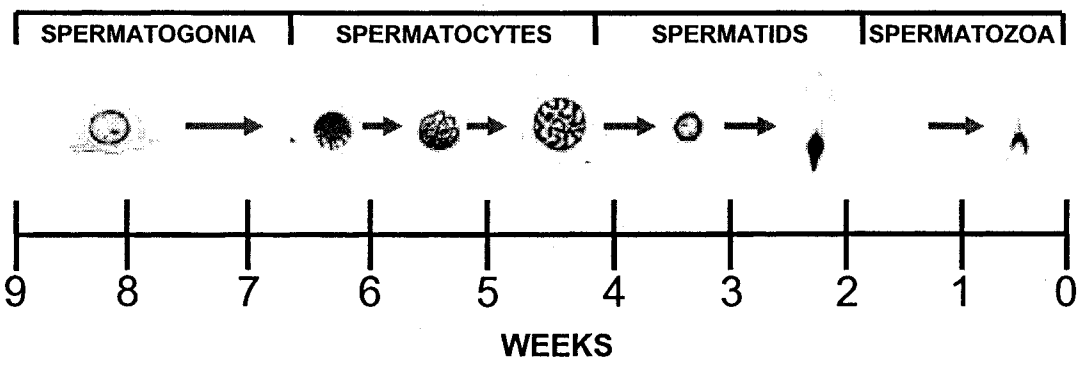


Figure 1.7. Phosphorylation of histone H2AX in response to DNA double strand breaks. The phosphorylation of H2AX by PI3K family members is proposed to be the first component of a DNA-damage specific histone code. Many molecules of phosphorylated H2AX are recruited to the DNA strand break and subsequently function to assemble and retain repair factors for lesion resolution.

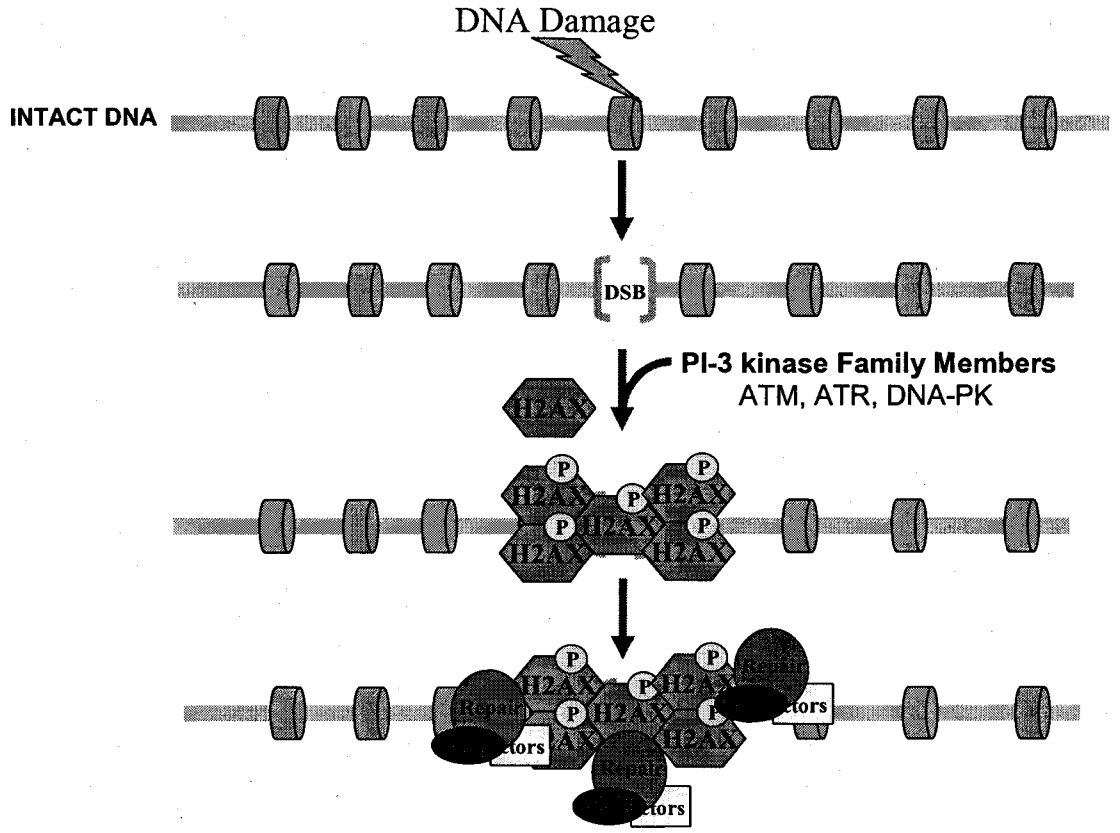


Figure 1.8. Poly(ADP-ribose) polymerase-1 acts as a molecular nick sensor for DNA single strand breaks. PARP-1 binds with high affinity to DNA single strand breaks; NAD+ is cleaved into ADP-ribose and nicotinamide producing chains of poly(ADP-ribose) (PAR), initiating the molecular switch for DNA repair or transcriptional block. Chromatin proteins are modified and dissociate from DNA; DNA is then accessible to DNA repair enzymes. Adapted from http://www.kudospharma.co.uk/r_d/parp.php.

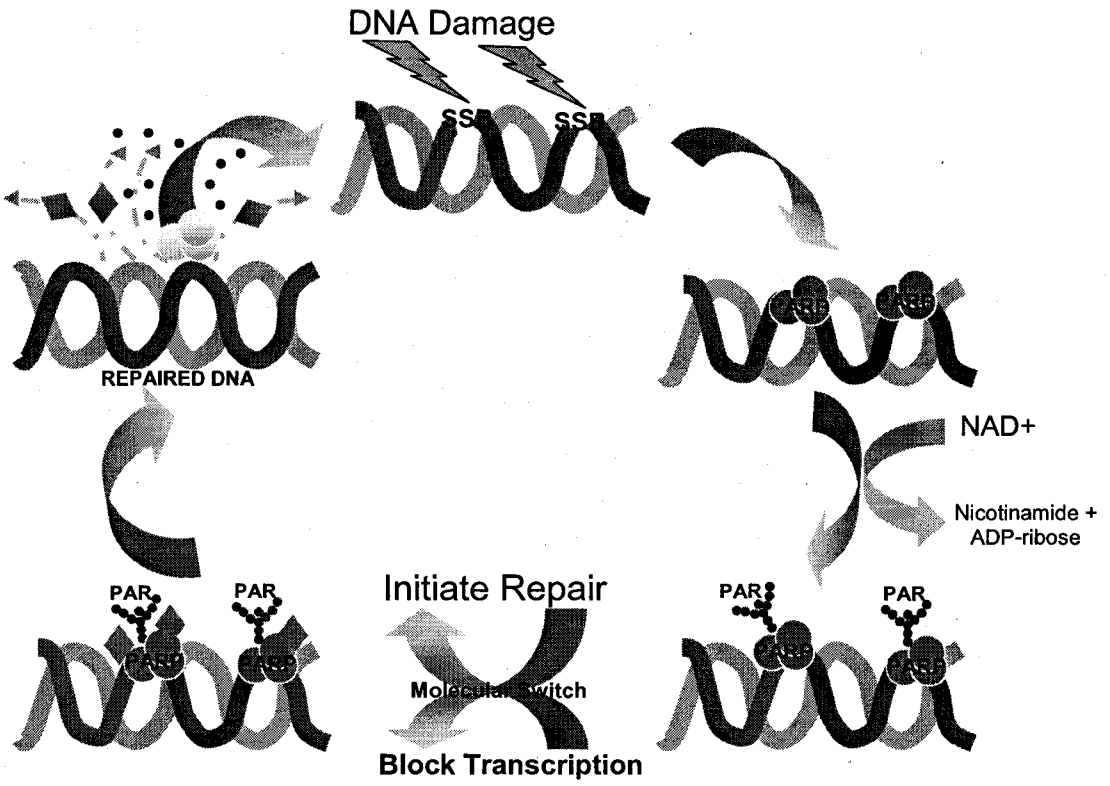


Figure 1.9. Cyclophosphamide metabolism. Cyclophosphamide is converted by cytochrome p450 enzymes to active metabolites; mainly, 4-OHCPA which is further converted through aldophosphamide to phosphoramide mustard and acrolein. Adapted from de Jonge et al. (2006) Brit J Cancer 94:1226-1230.

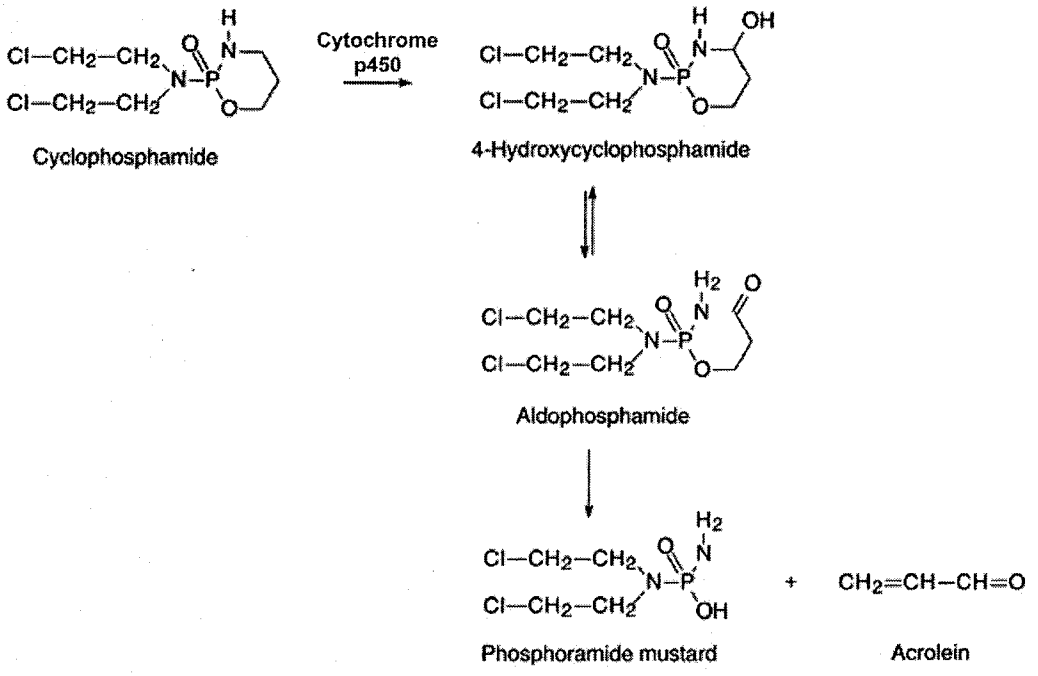
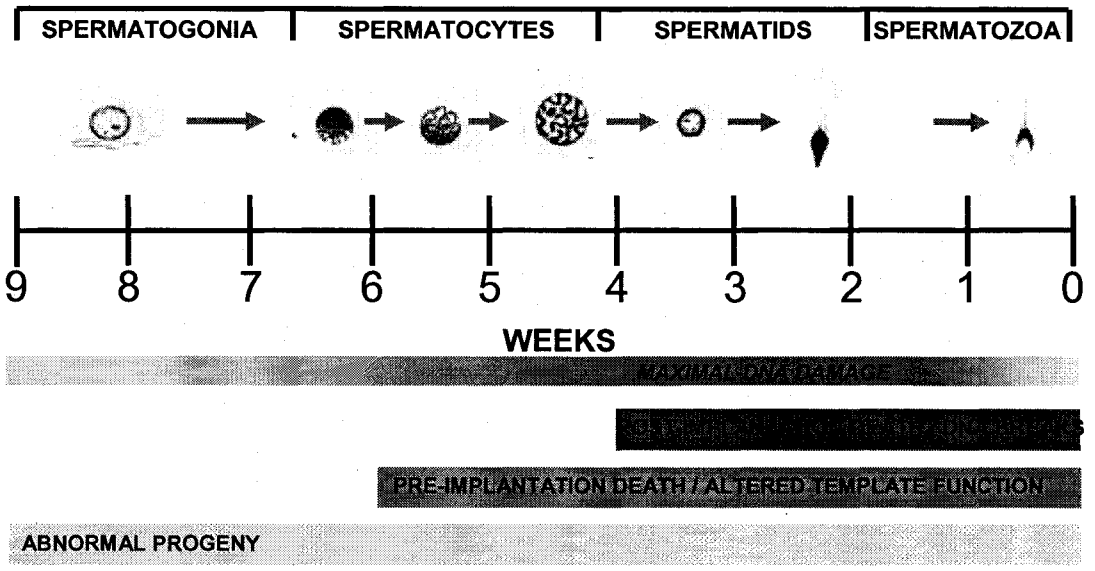


Figure 1.10. Male germ-cell phase-specific effects of chronic cyclophosphamide exposure. Maximal DNA damage is induced following 4 weeks of drug exposure, corresponding to increases in post-implantation embryo loss. Pre-implantation embryo loss results from 6 weeks of cyclophosphamide exposure; 9 weeks of treatment leads to malformed and growth retarded fetuses.



TABLES

Table 1.1. Male germ-cell stage susceptibility to mutagenicity induction following exposure to chemotherapeutic and environmental chemicals

Chemotherapeutic Chemicals	Stem Cells	Spermatogonia	Spermatocytes	Spermatids	Spermatozoa
Bleomycin	SLT	SLT			
Busulfan				DL / SLT	DL / SLT
Chlorambucil				DL / SLT / HT	DL / HT
Clormethine				DL / SLT	DL / HT
Cyclophosphamide				DL / SLT / HT	DL / SLT / HT
Diethyl sulfate				SLT / HT	SLT / HT
Dacarbazine				DL / SLT	DL / SLT
Ethylmethane sulfonate				DL / SLT	DL / SLT / HT
Ethyl nitrosourea	SLT	SLT		DL / HT	DL
Etoposide			DL / SLT / HT		
Isopropyl methanesulfonate			DL	DL / HT	DL / HT
Melphalan			DL / HT	DL / SLT / HT	DL
6-Mercaptopurine		DL			
Methylmethane sulfonate				DL / SLT / HT	DL - SLT
Methyl nitrosourea		SLT		DL / HT	
Mitomycin C			DL / HT	DL / HT	
Procarbazine	SLT	DL	DL	DL, HT	
Triethylene melamine				DL	DL / HT
Trophosphamide				DL / SLT / HT	DL / SLT / HT
x-rays		DL	DL	DL	DL - HT
Environmental Chemicals					
Acrylamide				DL / SLT / HT	DL / SLT
Benzopyrene					DL
1, 3-Butadiene				DL	DL / HT
Diepoxybutane				DL	DL
Ethylene Oxide				DL / HT	DL / HT
Glycidamide				DL	DL / HT

SLT, Specific Locus Test [a]

DL, Dominant Lethal [b]

HT, Heritable Translocation [b]

[a] Russell et al. (2004) Genetica 122:25-36

[b] Modified from Wyrobek et al. (2007) Environ. Mol. Mut. 48:71-95

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CONNECTING TEXT

Chronic cyclophosphamide exposure of male rats has been demonstrated to induce morphological abnormalities in the spermatozoal tail; however, the consequences on spermatozoal function were unknown. In chapter II, we employ computer assisted sperm analysis to quantitatively assess the effect of drug treatment on the kinematic parameters of spermatozoal motility.

CHAPTER II

Quantitative Evaluation of the Effect of Chronic Cyclophosphamide Exposure on Spermatozoal Movement Characteristics using Computer Assisted Sperm Analysis

ABSTRACT

Preconceptional paternal drug exposure leads to adverse progeny outcomes. Chronic treatment of male rats with the alkylating agent cyclophosphamide revealed a dose-dependent and time-specific increase in pre- and post-implantation loss, as well as malformed and growth retarded fetuses. Additionally, cyclophosphamide affected the structural integrity of the spermatozoal flagellum; cross-sectional analysis revealed a disruption in the normal association of the axonemal structure with the outer dense fibres. The overall mechanism of motion consists of flagellar bending to produce propulsive motions and entire sperm body movement with rigid body velocity. Therefore, of interest was whether cyclophosphamide exposed spermatozoa were capable of acquiring characteristically normal motility. Male rats were treated with either saline or cyclophosphamide (6 mg/kg/day) for 1, 3, 6 or 9 weeks; caput and cauda epididymal spermatozoa were analyzed using computer assisted sperm analysis (CASA) to quantitatively evaluate the various kinematic parameters describing sperm motion. Exposure for 1, 3 or 6 weeks, but not for 9 weeks, significantly altered beat cross frequency ($P < 0.05$), lateral head displacement ($P = 0.002$), straightness ($P = 0.003$) and linearity ($P = 0.023$) in a time-dependent manner, representing changes in spermatozoal swimming patterns following cyclophosphamide exposure. The altered function observed following 3 and 6 weeks of drug exposure, reflects the disruption of spermatozoal structural integrity acquired during spermiogenesis. The subtle change detected following 1 week of cyclophosphamide exposure demonstrates toxicant action on spermatozoa during epididymal transit. The lack of effect on sperm motion characteristics of a 9 week exposure suggests that the most damaged sperm may be eliminated or that spermatozoa may elicit a compensatory response to prolonged low dose drug exposure. Alterations in the kinematic parameters of sperm motility reflect subtle morphological changes in axonemal structure following chronic cyclophosphamide exposure. Toxicant disruption of midpiece assembly and/or signalling could lead to compromised transmission of the kinetic energy produced by the axoneme, and thus, altered spermatozoal movement.

INTRODUCTION

Spermatogenesis is a highly ordered process by which diploid spermatogonial stem cells undergo multiple mitotic divisions, meiotic reduction and post-meiotic modifications in order to produce mature haploid spermatozoa. The various stages of differentiation and biochemical and morphological restructuring make male germ cells highly vulnerable to damage resulting from toxic environmental and therapeutic exposures (Clermont, 1972; Boekelheide, 2005).

Cyclophosphamide, a widely used alkylating agent in cancer treatment and immunosuppression (Colvin, 1999), adversely affects developing male germ cells in a time and phase-dependent manner. Specifically, a one week treatment given to male Sprague-Dawley rats, targeting spermatozoa during epididymal transit, induced DNA single strand breaks; a chronic 6 week treatment induced a significant increase in DNA single strand breaks and DNA-DNA cross-links in sperm chromatin (Qiu et al., 1995a). In addition to the overall nuclear damage induced by chronic drug exposure, cyclophosphamide treatment also disrupts the normal close approximation of the 2 central to the 9 peripheral microtubule doublets in the mid-piece of the spermatozoal tail. A significantly increased percentage of sperm with tail defects was observed in the caput epididymidis after 1, 3, 6 or 9 weeks of cyclophosphamide exposure, signifying that the induction of aberrant tail morphology occurred prior to entry into the epididymis (Trasler et al., 1988). Together, the extent of DNA damage and the structural defects observed in the machinery responsible for generating flagellar motive force (Turner, 2003) in the spermatozoa of drug-treated males led us to question the consequences of chronic cyclophosphamide exposure on functional endpoints such as the acquisition of motility.

Sperm motility is essential to male fertility and, as such, has become an important component in the evaluation of reproductive toxicants (Slott et al., 1997; Kaneto et al., 1999a; Kaneto et al., 1999b; Henderson and Robaire, 2005) as well as in clinical cases of infertility (De Geyter et al., 1998; Shibahara et al., 2004). Complex mechanisms regulate mammalian sperm function, therefore,

subtle deregulation of flagellar structure or any one critical signaling pathway may disrupt normal movement (reviewed in (Turner, 2006). In fact, there are currently 21 knockout mouse models displaying specific flagellar anomalies, demonstrating that specific genes are involved in motility disorders (Escalier, 2006). This information further emphasizes the importance of function analysis in toxicology studies as subtle changes in motion parameters or impaired motility may signify underlying genetic damage and/or morphological defects. Early detection of genetic aberrations is imperative in the era of assisted reproductive technology, where impairments in the process of natural fertilization can be bypassed, potentially passing on clinical manifestations of infertility and additional health risks to future generations (Turner, 2006).

In the past, standard semen analysis used subjective techniques which were associated with inter-scorer and inter-laboratory variations. According to the World Health Organization (WHO) criteria, sperm motility has been graded routinely from a to d with respect to the extent of sperm motion; grade a, fast progressive, grade b, slow progressive, grade c, nonprogressive and grade d, immotile (WHO, 1999). Computer assisted sperm analysis (CASA) now provides an automated tool for quantitative assessment of multiple motility parameters to precisely describe the kinematics of sperm motion. Several movement characteristics, describing vigour, velocity and sperm swimming patterns, are measured based on well-defined thresholds (Boyers et al., 1989); as such, the CASA evaluation method serves as a predictive indicator for the fertilization potential of spermatozoa (Larsen et al., 2000).

CASA technology plays an important role in evaluating the modes and mechanisms of toxicant actions on the male reproductive system; variations in the defined motion parameters of epididymal spermatozoa are precise indicators of adverse effects on spermatogenesis. Monitoring changes in spermatozoal motion is a valuable indicator of toxicity in short-term studies and for low dose exposures as CASA is capable of detecting subtle alterations in function prior to the manifestation of harmful testicular effects and infertility (Perreault and Cancel, 2001). The objective of the present study was to investigate the effects

of chronic low dose cyclophosphamide treatment on epididymal sperm motion parameters to gain insight into the kinematic components of movement that may be affected by exposure to the DNA damaging agent, cyclophosphamide.

MATERIALS AND METHODS

Animal Treatments

Adult male Sprague-Dawley rats (body wt, 350-400 g) were obtained from Charles River Canada (St. Constant, QC, Canada) and housed at the McIntyre Animal Resources Centre, McGill University. Rats were kept on a 12-hr light / 12-hr dark photoperiod and received food and water ad libitum. After allowing one week for acclimatization, rats were randomly assigned to one of four treatment groups (n = 10 per group) and gavaged 6 days per week with either saline or 6 mg/kg/day cyclophosphamide (CAS 6055-19-2, Sigma Chemical Co., St. Louis, MO) for 1, 3, 6 or 9 weeks, respectively. The respective chronic treatment regimes were selected in order to target specific phases during spermatogenesis: cyclophosphamide treatment for 1 week targets germ cells undergoing epididymal spermatozoal maturation; drug administration for 3 weeks initially targets spermatids through to cauda epididymal spermatozoa; drug administration for 6 weeks ensures that pachytene spermatocytes are the first germ cells to be targeted through to cauda epididymal spermatozoa; cyclophosphamide treatment for 9 weeks ensures that spermatogonial germ cells are first exposed through all phases of spermatogenesis to mature spermatozoa (Fig. 1). One day subsequent to the completion of each treatment regime, animals were sacrificed by CO_2 inhalation and decapitation; epididymides were immediately removed and processed for sperm analysis as described below. All animal protocols were conducted in compliance with the Canadian Council on Animal Care guidelines as outlined in the Guide to the Care and Use of Experimental Animals.

Collection of Epididymal Spermatozoa and Motility Analysis

Procedures for spermatozoal motility assessment were done as previously described (Slott et al., 1991; Zubkova and Robaire, 2004; Henderson and Robaire, 2005). Briefly, spermatozoa from the distal caput and distal cauda epididymides were collected into a petri dish containing 3 ml of pre-warmed (37°C, pH 7.4) motility medium (Hank's balanced salt solution, sodium bicarbonate, HEPES, D-glucose and sodium pyruvate with soybean trypsin inhibitor and BSA added immediately prior to use) and were allowed to disperse for 5 minutes on a heating block at 37°C. Sperm concentrations were optimized (~1 x 10^5 spermatozoa/ml) by the addition of fresh medium to each sample; 20 μl of sample was then aliquoted onto an 80 μm 2X Cell Sperm Analysis Chamber slide for computer assisted sperm analysis (CASA) on the Hamilton-Thorne IVOS Motility Analyzer, version 12 (Hamilton-Thorne Research, Beverly, MA, USA). Five aliquots with approximately 200 sperm per aliquot were analyzed for each male ($n \geq 5$) in order to resolve the percentages of motile and progressively motile sperm as well as the following primary kinematic parameters determined by CASA: average path velocity (VAP), straight line velocity (VSL), curvilinear velocity (VCL); head motion parameters included the amplitude of lateral head displacement (ALH) and beat cross frequency (BCF); derived parameters measured included straightness (STR = VSL/VAP x 100) and linearity (LIN = VSL/VCL x 100) (Fig. 2). The HMT-IVOS system set-up values were experimentally optimized for rat sperm motility data acquisition using version 12 of the toxicology software; settings used for the current study are listed in Table 1.

Statistical Analysis

All CASA measurements were expressed as cumulative means calculated from the mean values obtained from the 5 aliquots for each male. Student's t-tests were used to compare motion differences between the control and drug-exposed spermatozoa ($P < 0.05$). Data are expressed as the mean ± SEM.

Statistical analyses were done by using the SIGMASTAT 3.0 software package (SPSS, Chicago, USA).

RESULTS

Motility Profiles during Spermatozoal Maturation

Swimming patterns of spermatozoa from the caput and distal cauda epididymides of control rats were compared in order to examine the changes in motion parameters coincident with spermatozoal maturation during epididymal transit. Spermatozoa released from the caput epididymidis presented with an erratic beating pattern, resulting in a circular swimming motion, whereas motile spermatozoa from the distal cauda epididymidis showed a significant increase ($P < 0.01$) in forward progressive movement (Fig 3*A*). Furthermore, various kinetic parameters serving as sensitive indicators of sperm maturation were changed significantly as spermatozoa acquired fertilizing ability during epididymal transit. Specifically, the overall vigor of the sperm motion, as demonstrated by significantly increased VAP ($P < 0.05$), VSL ($P < 0.001$) and VCL ($P < 0.05$) (Fig. 3*B, C, D*), was changed dramatically in spermatozoa from the caput to the cauda epididymides, reflecting the acquisition of coordinated and symmetrical flagellation patterns. The BCF was decreased significantly ($P < 0.01$) (Fig. 3*E*) in cauda epididymal spermatozoa, while the percent straightness (STR) was increased significantly ($P < 0.001$), providing further evidence of straight, rhythmic wave-like patterns of motion in the cauda epididymal spermatozoa analyzed under the current study conditions and operational settings.

Effect of Cyclophosphamide Treatment on Spermatozoal Motility

For all of the selected treatment regimes, cyclophosphamide-exposure had no effect on the percent motility, progressive motility or any of the motion parameters analyzed for spermatozoa obtained from the caput epididymidis (Table 2). Similarly, the overall motility, percent progressive motility and the velocity measures (VAP, VSL, VCL) of epididymal spermatozoa released from the distal cauda were not altered following cyclophosphamide exposure (Table

3). In contrast to observations obtained from caput spermatozoa, cauda epididymal spermatozoa taken from drug-treated males showed subtle changes in a time-specific manner compared to corresponding controls (Table 3). Following a 1-week cyclophosphamide treatment, BCF was increased significantly ($P = 0.015$); 3-weeks of treatment resulted in significantly increased BCF ($P = 0.048$) and STR ($P = 0.003$); 6 weeks of chronic CPA exposure resulted in a significantly decreased ALH ($P = 0.002$), while both STR and LIN were increased significantly ($P = 0.003$ and 0.023, respectively). Interestingly, no changes were detected following 9-weeks of chronic cyclophosphamide exposure compared to control rats.

DISCUSSION

Sperm motility assessment using CASA technology is a useful and sensitive measure of kinematic motion changes during spermatozoal maturation and in response to toxicant exposures. Various movement parameters are changed as spermatozoa transit through the epididymis. Structural maturation of spermatozoa, coincident with the epididymal segment-specific acquisition of function, is associated with an enhanced ability to swim progressively with a high degree of vigour to facilitate movement through the female reproductive tract and fertilization (Aitken et al., 1985; Mortimer et al., 1986). Maturational profiles of epididymal sperm have been established previously in the bull, ram, boar, primates and laboratory animals (Yeung and Cooper, 2002). Comparison of the kinematic profiles from rat caput and cauda epididymal spermatozoa confirmed the sensitivity of the CASA threshold settings to differentiate between immature erratic movement and the mature patterns of motility achieved prior to ejaculation. Accordingly, dramatic increases in forward progressive movement, vigour (VAP, VSL, VCL) and straightness were observed, while BCF was significantly decreased (Fig. 3). These changes accurately correspond to the expected pattern of coordinated and symmetrical flagellation, conferring a straight rhythmical wave-like motion in the mature spermatozoa (Soler et al., 1994). Chronic cyclophosphamide had no demonstrated effect on the motion

characteristics of caput epididymal sperm compared to untreated controls (Table 2).

Computer assisted sperm analysis is an important constituent of male reproductive toxicity evaluation; adverse effects on spermatogenesis may be rapidly reflected by aberrant function of mature epididymal spermatozoa (Perreault and Cancel, 2001). In fact, sperm motion analysis employing the CASA system has been particularly useful for detecting the toxicity of various chemicals with previously demonstrated testicular and/or epididymal effects (Slott et al., 1990; Slott et al., 1997; Kaneto et al., 1999a; Kaneto et al., 1999b; Kato et al., 2001; Kawaguchi et al., 2004). Following chronic cyclophosphamide administration for 1, 3, 6 or 9 weeks, the overall percent motility, forward progression and velocity of spermatozoa were not altered compared to spermatozoa obtained from untreated males (Table 3); a lack of effect of cyclophosphamide on motility was previously demonstrated by manual counting of motile sperm (Qiu et al., 1995b). Interestingly, the sophisticated quantitative measurement of multiple endpoints allowed the identification of toxicant-induced alterations in the motion pattern of treated spermatozoa previously undetectable by manual evaluation. Cyclophosphamide exposure induced time-specific alterations after 1, 3 and 6 weeks in BCF, ALH, STR and LIN, demonstrating the utility of the CASA system for the detection of subtle functional changes after toxicant exposure. Intriguingly, no changes in sperm motion characteristics were detected following 9 weeks of chronic cyclophosphamide treatment (Table 3). However, it is important to note that despite minimal effects on motility and fertility, 9 weeks of treatment produced a number of adverse effects on progeny outcome, including implantation loss, growth retarded and malformed fetuses (Trasler et al., 1985).

The respective cyclophosphamide treatment regimes (1, 3, 6 and 9 weeks) induced morphological abnormalities in the flagellar midpiece region of caput epididymal spermatozoa; the normal association of the axonemal structure with the outer dense fibers (ODF) was disrupted (Trasler et al., 1988). Outer dense fiber formation and the subsequent association with the microtubule

doublets is a multistep process, occurring from step 8 – 19 of spermiogenesis, first developing along the forming midpiece and gradually migrating distally along the principle piece (Irons and Clermont, 1982). Elaborate mechanisms regulating the synthesis, storage and transport of numerous proteins are required for the precise assembly of ODFs that are the most massive component of the spermatozoon (Oko, 1998; Kierszenbaum, 2001).

The cyclophosphamide induced midpiece defect was first detected in step 19 spermatids, reflecting a toxic effect during the haploid phase of germ cell development (Trasler et al., 1988). Drug induced alterations in the proteins and/or mechanisms responsible for the orderly development of the spermatozoal tail could, therefore, be accountable for the organizational abnormalities of the 9 + 2 axonemal structure. Furthermore, ODFs function to optimize energy conversion, playing an important role in the generation of movement; dynein-driven sliding of the outer doublet microtubules controls the flagellar waveform (Lindemann, 1996). Toxicant disruption of the motor components, in conjunction with structural defects of the flagellar midpiece, could ultimately interfere with the accurate transmission of the kinetic energy produced by the axoneme. The functional compromise observed after 3 and 6 weeks of cyclophosphamide exposure is, therefore, a reflection of the importance of the structural integrity of flagellar substructures formed during spermiogenesis. Alterations in the kinematic parameters describing spermatozoal swimming patterns, including BCF, ALH, STR and LIN, appear to be sensitive indicators of subtle morphological changes in midpiece structure. Moreover, the increased BCF and decreased ALH observed following 3 or 6 weeks of cyclophosphamide treatment, respectively, may be indicative of a compensatory mechanism requiring reduced energy to effectively confer a greater rate of tail oscillation (Kawaguchi et al., 2004).

Of note, following the completion of these studies, Higuchi and colleagues (Higuchi et al., 2001) demonstrated an overall lack of effect of short term, high dose cyclophosphamide treatment, 20 mg/kg/day for 1 week, on motility parameters using the CASA system. Critical issues concerning the timing of

drug exposure may directly influence the absence of detectable alterations in sperm motion parameters. Male rats were treated for 1 week and were subsequently sacrificed after a recovery period of 1 day, 3 weeks or 8 weeks following cyclophosphamide exposure. The mature spermatozoa that were assessed by CASA were, therefore, epididymal spermatozoa, round spermatids or spermatogonia, respectively, during the period of drug exposure. As such, the phase of elongating spermatids, during which key components of the spermatozoal flagellum develops, was not targeted by the respective drug treatment regimes. Furthermore, testicular and epididymal weights, and sperm counts were decreased significantly, suggesting that the administered dose of 20 mg/kg/day was toxic to developing germ cells; highly damaged sperm may have been eliminated.

The midpiece tail defect previously described was not observed in cauda epididymal spermatozoa following 1 week of cyclophosphamide exposure, reinforcing that the primary site of toxicant action occurred in the testis during late spermiogenesis (Trasler et al., 1988). Interestingly, BCF was significantly increased following 1 week of cyclophosphamide exposure, suggesting a secondary mode of toxicity occurring during epididymal transit that weakly influences the pattern of sperm motion. Higuchi and colleagues (Higuchi et al., 2001) also found significant differences in STR and BCF after 1 day and 3 week recovery, respectively; however, the observed changes were of small magnitude highlighting the importance of CASA set-up standardization for inter-experimental comparisons. The maturation of motility patterns during epididymal transit is a complex phenomenon regulated by a multitude of molecular and intracellular mechanisms (Turner, 2006). Alkylation by cyclophosphamide of proteins responsible for assembly and/or signaling, as well as the possible alteration of the epididymal intracellular environment, may lead to subtle changes in the pattern of movement executed by the spermatozoa. In the current study, quantitative assessment of sperm motility using the CASA system has demonstrated that structural alterations to the flagellum following cyclophosphamide treatment appear to be substantiated by subtle changes in the

spermatozoal swimming pattern. Parameters such as ALH and BCF, which describe the vigour of flagellar beating concomitantly with the frequency of cell rotation, are important indicators of IVF outcome (Barlow et al., 1991; Jeulin et al., 1996) and the capacity of human spermatozoa to penetrate cervical mucus and peri-oocyte envelopes (Aitken et al., 1985; Mortimer et al., 1986). Furthermore, changes in the pattern or shape of sperm motion, STR and LIN, have been positively correlated with IVF outcomes or pregnancy rates in some studies (Sukcharoen et al., 1995; Sukcharoen et al., 1998) and have emerged as sensitive markers for adverse effects that are below the level of detection of velocity parameters for specific chemicals (Kato et al., 2001). Therefore, our findings highlight the importance of CASA in establishing low level effects that, in the case of chronic cyclophosphamide exposure, may be biologically significant.

An extremely important observation is that the motion alterations detected by CASA are subtle enough that they do not interfere with fertility. Cyclophosphamide exposure has little effect on the general health of the male reproductive system (Trasler et al., 1985); however, it is well established that the treatment regimes tested result in nuclear damage (Qiu et al., 1995a) and detrimental effects on progeny outcome (Trasler et al., 1985; Trasler et al., 1987). Together, this raises concern for the transmission of structural and genetic damage to the offspring and highlights the importance of assessment of the quality of the genome in conjunction with sperm motility to evaluate the potential of a chemical for male mediated developmental toxicity.

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FIGURES

Figure 2.1. Schematic representation of male germ cell development with respect to differentiation phases during rat spermatogenesis. Timed administration of cyclophosphamide allows germ-cell phase specific targeting for the characterization of adverse effects during spermatogenesis. Oral drug administration for 1 week targets spermatozoa during epididymal transit, for 3 weeks initially targets spermatids and for 6 or 9 weeks initially targets pachytene spermatocytes or spermatogonia, respectively.

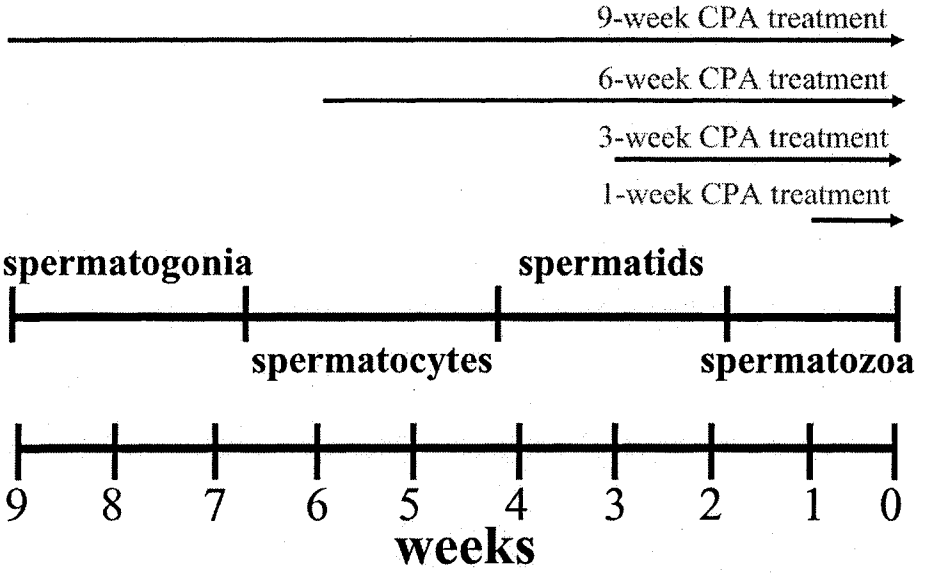


Figure 2.2. Schematic diagram of the kinematic parameters measured by CASA. VCL, curvilinear velocity, is the average velocity measured over the actual point-to-point track followed by the cell (μm / second); VSL, straight line velocity, is the average velocity measured in a straight line from the beginning to the end of each track (μm / second); VAP, path velocity, is the average velocity of the smoothed cell's pathway (μm / second); BCF, beat cross frequency, represents the frequency at which the spermatozoon's head crosses its average path (Hz); ALH, is the amplitude of lateral head displacement (μm). Additional parameters derived by CASA which are not represented schematically include: LIN, linearity, is the average value of the ratio VSL/VCL and measures the divergence of the cell track from a straight line; STR, straightness, is the average value of the ratio VSL/VAP and measures the divergence of the cell path from a straight line.

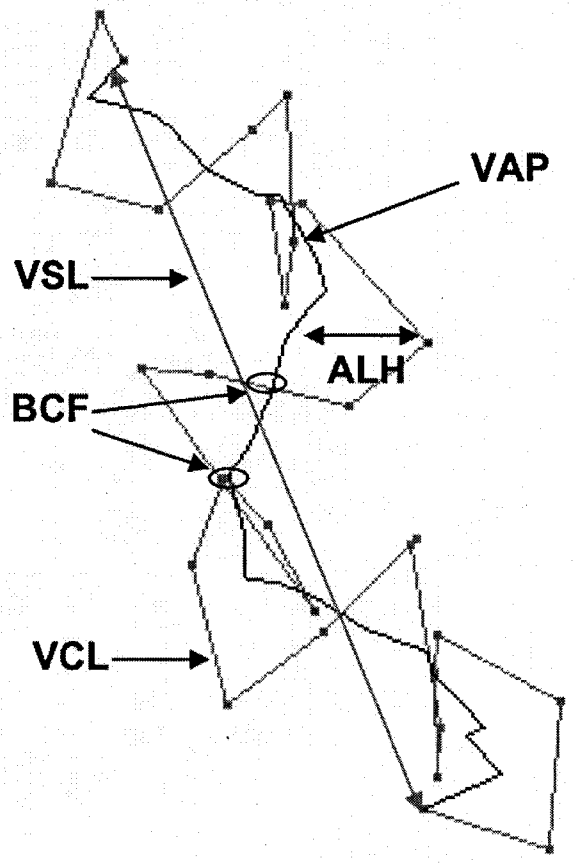
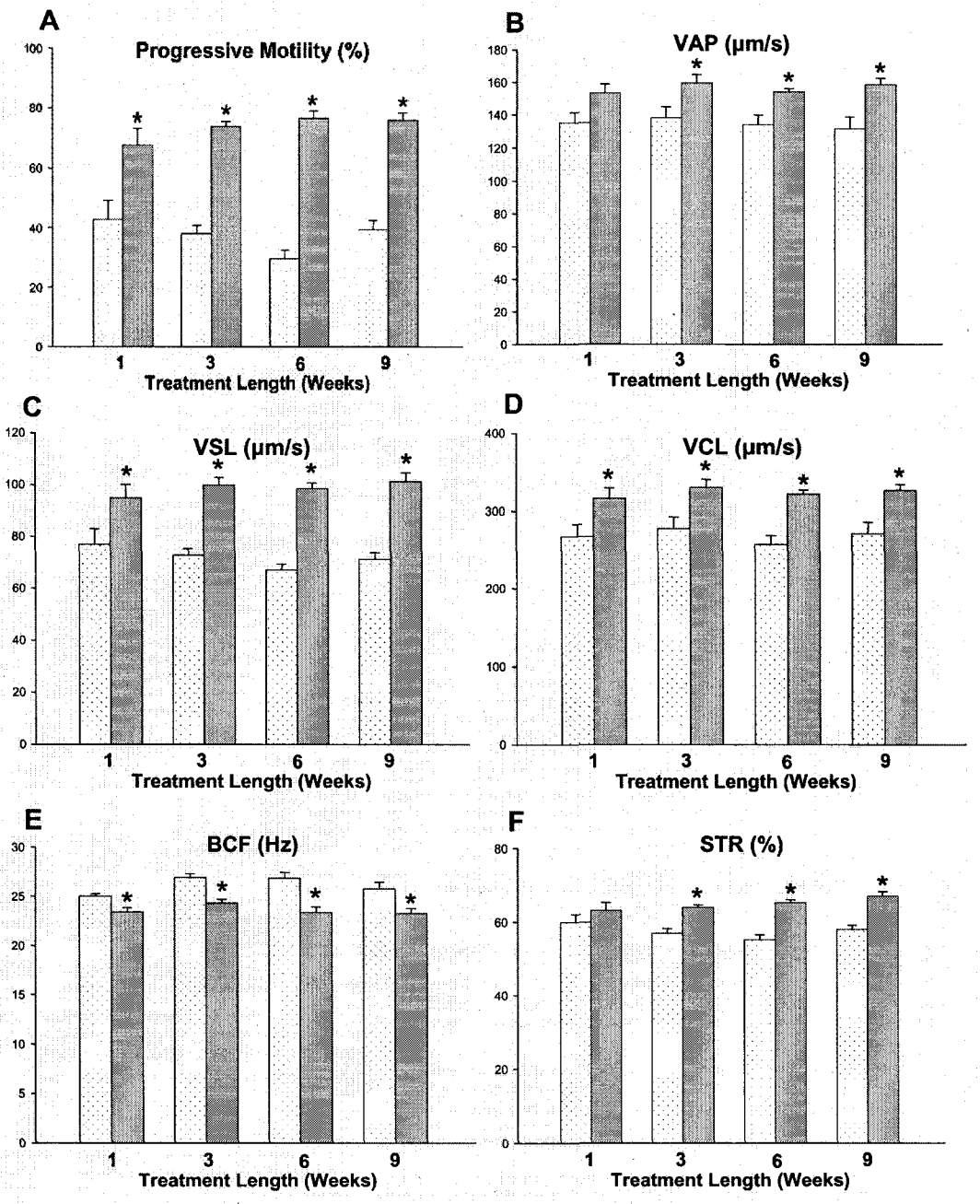


Figure 2.3. CASA motility profiles of caput and cauda epididymal spermatozoa from control males. The progressiveness, velocity and vigor of movement are dramatically altered during epididymal transit. Treatment length represents the number of weeks male rats were orally administered saline emphasizing reproducibility and consistency between control experiments. Speckled bars, caput epididymal spermatozoa; grey bars, cauda epididymal spermatozoa. *, $P < 0.05$.


A
Progressive Motility (%)
100
80
60
40
20
0
1
3
6
9
Treatment Length (Weeks)
B
VAP (µm/s)
180
160
140
120
100
80
60
40
20
0
1
3
6
9
Treatment Length (Weeks)
C
VSL (µm/s)
120
100
80
60
40
20
0
1
3
6
9
Treatment Length (Weeks)
D
VCL (µm/s)
400
300
200
100
0
1
3
6
9
Treatment Length (Weeks)
E
BCF (Hz)
30
25
20
15
10
5
0
1
3
6
9
Treatment Length (Weeks)
F
STR (%)
80
60
40
20
0
1
3
6
9
Treatment Length (Weeks)

TABLES

Table 2.1. Optimized HMT-IVOS system settings used for rat toxicology analysis.

Item	Set-up Values
Frames Acquired	30
Frame Rate	60 Hz
Minimum Contrast	80
Minimum Cell Size	7 Pixels
Minimum static Contrast	15
Straightness (STR), Threshold	40.0 %
VAP Cutoff	20.0 µm/s
Progressive Minimum VAP	50.0 µm/s
VSL Cutoff	30.0 µm/s
Cell Size	25 Pixels
Cell Intensity	80
Static Head Size	0.29 to 8.82
Static Head Intensity	0.18 to 1.80
Static Elongation	0 to 47
Slow Cells Motile	YES
Magnification	0.82
Video Frequency	60
Temperature, Set	37.0 °C
Chamber Depth	80.0 µm
Chamber Type	2X-Cel
Field Selection Mode	SELECT

Table 2.2. Motion paramaters of caput epididymal sperm determined by computer-assisted sperm analysis

	1 week		3 weeks		6 weeks		9 weeks	
	Saline (1ml/d)	CPA (6.0 mg/kg/d)	Saline (1ml/d)	CPA (6.0 mg/kg/d)	Saline (1ml/d)	CPA (6.0 mg/kg/d)	Saline (1ml/d)	CPA (6.0 mg/kg/d)
No. of Rats	6	6	6	6	5	5	6	7
Total sperm analyzed	5143	4134	5131	5128	4731	4360	6062	6936
Motility (%)	75.9 ± 1.7	72.0 ± 3.4	78.7 ± 1.9	78.7 ± 1.8	73.1 ± 2.9	71.0 ± 2.5	73.4 ± 3.7	69.5 ± 4.3
Progressive Motility (%)	42.7 ± 6.4	35.0 ± 3.6	37.9 ± 3.0	40.9 ± 1.7	29.6 ± 2.8	34.1 ± 3.9	39.3 ± 3.2	33.6 ± 3.6
Velocity Parameters								
VAP (um/sec)	135.0 ± 6.4	137.5 ± 8.6	138.0 ± 7.0	137.1 ± 3.7	134.0 ± 5.8	132.5 ± 4.1	131.5 ± 7.1	126.2 ± 4.2
VSL (um/sec)	76.9 ± 6.1	72.6 ± 3.4	72.7 ± 2.7	73.6 ± 1.6	67.1 ± 2.3	69.9 ± 2.3	71.2 ± 2.6	68.5 ± 1.7
VCL (um/sec)	267.0 ± 15.6	272.2 ± 18.9	277.4 ± 15.1	279.1 ± 6.3	256.5 ± 11.9	268.7 ± 9.8	270.4 ± 15.0	257.8 ± 8.2
Head Motion Parameters								
ALH (um)	19.4 ± 0.7	19.8 ± 1.0	19.7 ± 0.7	19.5 ± 0.3	19.9 ± 0.4	19.5 ± 0.4	19.2 ± 0.7	19.1 ± 0.4
BCF (Hz)	25.0 ± 0.3	26.0 ± 0.6	26.9 ± 0.4	25.9 ± 0.6	26.8 ± 0.6	26.0 ± 0.4	25.8 ± 0.6	25.9 ± 0.9
Dervied Parameters								
STR (%)	60.0 ± 2.0	56.0 ± 1.1	57.0 ± 1.2	57.0 ± 0.4	55.2 ± 1.3	56.7 ± 0.4	57.9 ± 1.2	57.9 ± 0.8
LIN (%)	31.1 ± 1.0	29.4 ± 0.8	29.3 ± 0.8	29.0 ± 0.5	29.2 ± 0.8	28.6 ± 0.6	29.0 ± 1.0	29.7 ± 0.6

No significant differences from saline control
Data represent the mean ± S.E.M.

Table 2.3. Motion paramaters of cauda epididymal sperm determined by computer-assisted sperm analysis

	1 week		3 weeks		6 weeks		9 weeks	
	Saline (1mL/d)	CPA (6.0 mg/kg/d)	Saline (1mL/d)	CPA (6.0 mg/kg/d)	Saline (1mL/d)	CPA (6.0 mg/kg/d)	Saline (1mL/d)	CPA (6.0 mg/kg/d)
No. of Rats	6	5	6	6	5	5	6	7
Total sperm analyzed	6576	4701	6172	5878	4517	4216	6668	7646
Motility (%)	84.4 ± 1.7	81.0 ± 3.8	87.5 ± 1.6	87.6 ± 1.7	88.1 ± 1.7	85.9 ± 2.0	87.9 ± 1.4	88.4 ± 1.2
Progressive Motility (%)	67.5 ± 5.3	70.0 ± 3.9	73.6 ± 1.8	74.3 ± 1.7	76.4 ± 2.6	76.6 ± 2.7	75.7 ± 2.5	78.9 ± 1.5
Velocity Parameters								
VAP (um/sec)	153.3 ± 5.7	147.3 ± 4.4	159.5 ± 5.2	157.4 ± 2.9	154.0 ± 2.1	147.5 ± 1.9	158.5 ± 3.8	150.9 ± 2.6
VSL (um/sec)	94.9 ± 5.2	97.2 ± 2.5	100.0 ± 2.9	105.3 ± 2.2	98.5 ± 2.3	99.9 ± 4.2	101.3 ± 3.3	100.2 ± 1.4
VCL (um/sec)	316.4 ± 13.4	324.1 ± 5.7	330.2 ± 10.8	341.6 ± 7.2	322.3 ± 4.8	307.8 ± 4.8	326.8 ± 7.1	315.6 ± 3.3
Head Motion Parameters								
ALH (um)	21.1 ± 0.7	20.2 ± 0.6	21.7 ± 0.7	20.9 ± 0.3	21.1 ± 0.3	**19.8 ± 0.1****	21.3 ± 0.3	20.6 ± 0.2
BCF (Hz)	23.4 ± 0.4	**25.2 ± 0.4***	24.3 ± 0.4	**25.7 ± 0.52***	23.3 ± 0.6	22.5 ± 0.2	23.3 ± 0.5	22.4 ± 0.4
Dervied Parameters								
STR (%)	63.1 ± 2.2	67.2 ± 0.9	63.9 ± 0.7	**67.9 ± 0.7****	65.3 ± 0.7	**69.2 ± 0.6****	67.0 ± 1.3	67.5 ± 0.8
LIN (%)	31.0 ± 0.7	30.8 ± 0.4	31.2 ± 1.0	31.5 ± 0.8	31.4 ± 0.4	**33.4 ± 0.6***	32.7 ± 0.7	32.4 ± 0.4

* Significantly different from saline control, $P < 0.05$

** Significantly different from saline control, $P < 0.01$

Data represent the mean ± S.E.M.

CONNECTING TEXT

The studies in chapter II demonstrate that chronic cyclophosphamide exposure, targeting various phases of spermatogenesis, does not lead to an overall compromise in spermatozoal motility. Therefore, of particular concern, is the ability of this alkylating agent to induce genomic defects during male germ cell development that may be transmitted to the progeny. In chapter III, by using fluorescent *in situ* hybridization of epididymal spermatozoa, we determine whether chronic drug treatment alters the normal chromosomal complement in mature spermatozoa, and establish germ cell phase susceptibility for the induction of chromosomal abnormalities during spermatogenesis.

CHAPTER III

Numerical Chromosomal Abnormalities in Rat Epididymal Spermatozoa following Chronic Cyclophosphamide Exposure

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ABSTRACT

Chronic low dose treatment of male rats with cyclophosphamide, a chemotherapeutic agent, is known to affect progeny outcome adversely in a dose and time-specific manner, resulting in increased pre- and post-implantation loss as well as malformations. There is concern for the genetic quality of the mature gametes exposed to cyclophosphamide during mitosis and meiosis. The goal of our study was to determine the effect of chronic cyclophosphamide treatment during spermatogenesis on the frequency of numerical chromosomal anomalies in epididymal spermatozoa. Male rats were treated with either saline or cyclophosphamide (6 mg/kg/day) for 6 or 9 weeks and cauda epididymal spermatozoa were collected. The rat sperm Y-4 FISH assay was used to assess the induction of spermatozoal disomy, nullisomy and diploidy involving chromosomes Y and 4. The overall frequency of numerically abnormal spermatozoa was elevated about 2-fold ($p<0.001$) after 9 weeks of cyclophosphamide treatment. Exposure for 9 weeks, but not for 6 weeks, significantly increased the frequency of spermatozoa with chromosome 4 disomy ($p<0.02$) and nullisomy ($p<0.05$), but disomy Y and diploidy were not significantly increased with treatment compared to corresponding controls. Independent of treatment, only 27% of aneuploid spermatozoa presented with morphological abnormalities, but all diploid spermatozoa were approximately twice the size of normal cells. Thus, cyclophosphamide disrupts meiotic events prior to pachynema during spermatogenesis, emphasizing the potential for adverse progeny outcomes following genotoxic damage.

INTRODUCTION

Aneuploidies are the most serious and frequent chromosomal defects in humans. Numerical chromosomal abnormalities are associated with congenital defects [1] and are critical in both the early initiation stages and the progression of a wide array of malignant tumors [2]. An abnormal chromosome complement can be transmitted to the progeny from either the mother or the father and can profoundly affect the viability, health and development of the embryo. Pregnancies involving numerical abnormalities for chromosomes 13, 18, 21, X and Y survive to term and account for approximately 0.3% of newborns that remain affected, making aneuploidy the leading known genetic cause of physical disabilities and mental retardation [3, 4].

In general, autosomal aneuploidies represent major maternal contributions, while the paternal contributions are reflected more frequently in the sex chromosomes. At birth, paternal meiotic error contributes to all XYY, 80% of cases of sex chromosome monosomy for Turner's syndrome (XO), and about one-half of Klinefelter syndrome (XXY) [3, 4]. Although the incidence and consequence of aneuploidy in humans has been well documented, the events and mechanisms that may lead to the induction of aneuploidy in germ cells remain poorly understood. Consequently, aneuploidy is an important genetic peril in human reproduction and a focus for research into genotoxicity and mechanisms of induction [3, 5].

Preconceptional paternal drug exposure is a source of detrimental effects on the genetic makeup and well being of the embryo and infant [6]. The recent availability of sperm fluorescence *in situ* hybridization (FISH) with chromosome-specific DNA probes makes it possible to analyze spermatozoal samples from exposed and unexposed individuals with the advantage of rapidly and accurately generating large data sets [7]. Using FISH, the frequency of numerical chromosome abnormalities in both normal [8, 9, 10] and infertile men [11, 12] and increased incidences of aneuploid spermatozoa among cigarette smokers

[13], following consumption of caffeine and alcohol [14], and after drug and pesticide exposure [15, 16, 17] have been established.

Animal studies offer the advantage that specific windows of toxicant exposure may be assessed in order to deduce the susceptibility of specific germ-cell stages to potential aneugens and to elucidate the mechanisms by which genotoxic damage occurs during spermatogenesis. Consequently, FISH has been extended and optimized for use in mice [18, 19], rats [20], and pigs [21]. The mouse epididymal sperm FISH assay for aneuploidy was used to examine the sensitivity of the assay in detecting aneuploidy and diploidy following treatment of male mice with a number of test chemicals (reviewed by Adler et al. [22]) and has provided evidence that the sperm FISH assay is able to bridge the gap between humans and rodents [23].

Chemotherapeutic agents were observed to be gonadotoxic in a dose-dependent and time-specific manner, rendering a proportion of cancer survivors with persistent oligozoospermia or azoospermia (reviewed by Thomson et al. [24]). Recent data demonstrating that aneuploid spermatozoa were as competent as normal spermatozoa for fertilization and progression to the first cell cycle of zygotic development [25, 26] underline the concern for the potential of adverse progeny outcome due to germ cell mutagenesis following natural conception. Paternal drug exposure leading to increased DNA damage, structural aberrations, and aneuploidy in the spermatozoa can be heritably transmitted, resulting in an increased incidence of offspring with chromosomal abnormalities [26]. Evidence that genetically damaged spermatozoa are capable of accomplishing fertilization naturally [25, 26] or after ICSI [27] has tremendous implications for cancer survivors, emphasizing the need to further assess the induction of genetic damage following exposure to commonly used chemical toxicants.

Cyclophosphamide, a bifunctional alkylating agent, is extensively used as an anticancer chemotherapeutic agent in childhood [24, 28] and adult malignancies [29], as well as an immunosuppressive agent for organ transplantation, systemic lupus erythematosus, glomerulonephritis, multiple

sclerosis, and other benign diseases [29]. Cyclophosphamide targets rapidly dividing cells, disrupting cell growth, mitotic activity, differentiation, and functions via alkylation of DNA at the N7 position of guanine [29, 30]. The cytotoxicity mediated by the formation of DNA-DNA cross-links, DNA-protein cross-links and single strand breaks is greater than for other drugs, however many damaged cells survive cyclophosphamide exposure [29, 30].

The complex, sequential stages of differentiation and chromosomal assortment make male germ cells highly vulnerable to chemicals that interact with DNA [31, 32]. A number of studies have confirmed that cyclophosphamide is a DNA damaging agent during spermatogenesis. Exposure of rat spermatocytes to cyclophosphamide resulted in synaptic failure, fragmentation of the synaptonemal complex and altered centromeric DNA sequences [33]. Cyclophosphamide also induced gene conversions and frameshift mutations in spermatocytes, indicating a genotoxic effect of the genome during meiotic prophase [34]. Furthermore, chronic paternal cyclophosphamide exposure revealed a dose-dependent and time-specific increase in pre- and post-implantation loss as well as impaired embryonic development and birth defects [35, 36, 37]. These adverse effects on progeny raise concerns regarding the effect of cyclophosphamide treatment on the genetic quality of the mature male gamete. Therefore, the goal of this study was to establish whether chronic cyclophosphamide treatment, given in a clinically relevant dose, results in chromosomal anomalies such as aneuploidy or diploidy in rat spermatozoa.

MATERIALS AND METHODS

Animal Treatments

Adult male Sprague-Dawley rats (350-400 g) were purchased from Charles River Canada (St. Constant, QC, Canada) and housed at the McIntyre Animal Resources Center, McGill University. Rats were maintained on a 14L:10D light cycle and received food and water ad libitum. One week after arrival, males were randomly assigned to one of two treatment groups of 10

animals each and gavaged with either saline or 6 mg kg^{-1} day^{-1} cyclophosphamide (CAS 6055-19-2, Sigma Chemical, St. Louis, MO) six times per week, for either 6 or 9 weeks. The selected treatment regime targets two separate phases of spermatogenesis: (1) cyclophosphamide administration for 6 weeks ensures that the cells to be exposed are pachytene spermatocytes through to cauda epididymal spermatozoa; (2) cyclophosphamide administration for 9 weeks first exposed spermatogonial germ cells throughout spermatogenesis to cauda epididymal spermatozoa. Thus, the selected treatment times allowed specific targeting of meiotic (6 weeks) and mitotic (9 weeks) germ cells, respectively (Fig. 1). Rats were killed one day subsequent to the completion of each treatment regime by CO_2 inhalation and decapitation. All animal protocols were conducted in accordance with the guidelines outlined in the Guide to the Care and Use of Experimental Animals, prepared by the Canadian Council on Animal Care.

Isolation of Epididymal Spermatozoa and Slide Preparation

Cauda epididymal sperm were isolated following the procedure described by Lowe et al. [18] with minor modifications. Briefly, the epididymides were removed surgically, trimmed free of fat and placed in 2.2% sodium citrate, pre-warmed to 37°C, to remove any excess debris. Each epididymis was clamped with a hemostat at the corpus-cauda junction and an incision through several tubule segments was made in the distal cauda epididymis using a #11 scalpel blade. The region of the incision was then gently rinsed in 0.6 ml of 2.2% sodium citrate at 37°C to allow the spermatozoa to swim out of the epididymis. The spermatozoal suspension was separated into five 100 µl aliquots, immediately frozen in liquid nitrogen and stored at -80°C until shipped to Lawrence Livermore National Laboratory (LLNL), Livermore, CA, on dry ice for subsequent slide preparation and FISH. Spermatozoal smears were prepared as previously described [20]. Each slide was evaluated using a phase contrast microscope to ensure the sample preparation and spermatozoal concentration in the smears

from the saline and cyclophosphamide treated rats were indistinguishable. The most uniform set of slides were selected for hybridization. All slides were coded prior to hybridization by an individual not involved in the analysis. Smears not used for hybridization were stored in nitrogen gas at -20°C until used.

Pretreatment and Fluorescent in situ hybridization of Spermatozoa

Smears of rat spermatozoa were each fixed in 3:1 methanol to acetic acid and air-dried prior to commencing pretreatment. To prepare the smears for rat FISH, spermatozoa were decondensed by incubating the slides in a Coplin jar for 30 min in 10mM dithiothreitol (DTT, Sigma Chemical, St. Louis) on ice allowing the maintenance of spermatozoal head and tail structure. Slides were briefly rinsed in ddH_2O and allowed to dry completely at room temperature before they were used for hybridization. The methods used for hybridizations were previously described by Lowe et al. [18, 20] and were followed with minor modifications, as follows. The spermatozoa on each slide were denatured in 70% formamide, 2X SSC, pH 7.0 at a temperature of 78°C for 2 min and dehydrated in an ice-cold alcohol series. The probe mixture, containing DNA probes specific for rat chromosome 4 (25S5) and Y (9.1ES8) as previously described [20, 38], was denatured at 78°C for 10 min. Denatured hybridization mix (10 μl) was immediately applied to the air-dried spermatozoal smears and incubated for 24 to 48 hours in a moist chamber at 37°C. Five posthybridization washes were carried out at 45°C for 5 min each: three times in 50% formamide, 2X SSC, pH 7.0, once in 2X SSC and once in PN buffer (0.1M NaH_2PO_4 / 0.1 M Na_2HPO_4, pH 8.0, 0.1% Nonidet P-40 (Sigma Chemical, St. Louis, MO). The posthybridization washes were completed by a 5 min incubation in PN buffer at room temperature. The probes for chromosome 4 and Y were visualized by fluorescein isothiocyanate (FITC)-avidin and rhodamine-anti-digoxigenin, respectively, using 30 μl of Biotin-Digoxigenin Dual Colour Detection Kit (Oncor, Gaithersburg, MD). Spermatozoal nuclei were counterstained with 0.01 μg/ml 4,6-diamidino-2-phenylindole (DAPI) in Vectasheild mounting medium (Vector Laboratories, Burlingame, CA).

Scoring of Hybridized Spermatozoal Smears

All hybridized slides were coded, recoded and decoded by an individual not involved in scoring. Descriptions of the spermatozoa scored were recorded using the CYTOscore© program developed at LLNL for utility with the human and rodent sperm FISH assays. At least 10 000 cells were scored for each of the 20 animals (five rats for each of the cyclophosphamide and saline groups treated for 6 and 9 weeks) for a total of 200 716 spermatozoa. Slides were independently scored using a fluorescent photo microscope equipped with a double band-pass filter for the simultaneous visualization of red (rhodamine) and green (FITC) hybridization signals, single fluorochrome filters for confirmation of individual colour domains (rhodamine: 41002C HQ:R; FITC: 41001 HQ:F, Chroma Technology Corp., Brattleboro, VT), as well as a filter for blue fluorescence (DAPI) used to identify spermatozoal nuclear counterstain. Phase-contrast was used to confirm the location of signals within the boundaries of the spermatozoal nucleus as well as to detect the presence of debris. Spermatozoa that were disrupted or overlapping were excluded from the analysis. The hybridization efficiency was > 99.9% across all slides and spermatozoa were equally decondensed such that no difference was observed between treated and control samples. Photographic images were captured using a RS Photometrics CoolSNAP fx camera and imported into CorelDRAW9®.

The spermatozoa were classified according to the specific combination of fluorescent signals observed in each nucleus as previously described [20]. Chromosomal anomalies were categorized as disomy 4 (Y-4-4, 0-4-4), disomy Y (Y-Y-4), nullisomy 4 (0-Y) or autodiploid (Y-Y-4-4) in cells where all of the previously established scoring criteria were also met [18].

Morphological characteristics of the chromosomally abnormal spermatozoa were recorded simultaneously with the scoring of chromosomal abnormalities on the hybridized slides. Each genotypically abnormal spermatozoon was scored as morphologically normal in the presence of a hook-shaped head equal in size to spermatozoa with normal fluorescent genotypes (Y-4, 0-4). Atypical forms were classified by head shape abnormalities such as

amorphous head, enlarged head or double head [39]. Differences in spermatozoal head size and shape were confirmed using phase-contrast and nuclei were measured with an ocular micrometer (100X objective).

Statistical Analysis

Chi Square analyses were employed to compare the frequencies of aneuploid and diploid spermatozoa observed from control animals with those treated chronically with cyclophosphamide ($p < 0.05$). Statistical analyses were done using SigmaStat 2.03 software package (SPSS Inc., Chicago, IL).

RESULTS

The ability of chronic cyclophosphamide treatment to induce aneuploidy in rat epididymal spermatozoa was evaluated using the dual-color rat Y-4 sperm FISH assay previously developed and optimized by Lowe et al. [20]. The number and color of fluorescent DNA probes specific for chromosome 4 (FITC) and chromosome Y (DIG) determined the phenotype of each spermatozoon. Cytogenetically normal and abnormal spermatozoa are illustrated in Fig. 2. Spermatozoa with a single green domain (0-4) or a single green and a single red domain (Y-4) presumably represent chromosomally normal cells (Fig.2A). Of the 200,716 spermatozoa analyzed, 49.69 % displayed a Y-4 phenotype while 50.14% of the cells carried the 0-4 phenotype; presumably, these were cells carrying an unlabeled X chromosome. Thus, the ratio of X-bearing spermatozoa to Y-bearing spermatozoa was approximately 1, as expected, for all animals analyzed (Table 1). Spermatozoa with other combinations of colored domains represent spermatozoa with numerically abnormal genotypes and were assigned to the categories of hyperhaploidy disomy (Figs.2B, C, D), nullisomy (Fig.2E), or autodiploidy (Fig. 2F).

The analyses of the aneugenic effect of cyclophosphamide treatment are presented in Table 1. Treatment with 6.0 mg kg^{-1} of cyclophosphamide for 6 weeks did not induce significant changes in the frequency of hyperhaploid spermatozoa for either chromosome 4 or chromosome Y; however, a 2.3-fold

increase in frequency was observed for one of the subcategories of disomy 4 (Y-4-4), but not the other or nullisomy 4. The incidence of diploid spermatozoa, which resulted from errors in meiosis II, was elevated significantly following 6 weeks of treatment compared with the corresponding control value. In contrast, 9 weeks of cyclophosphamide treatment induced a significant increase in the frequency of spermatozoa with disomy 4 (Y-4-4), as well as the aggregate frequency of spermatozoa carrying chromosome 4 or Y disomies. Although the frequency of disomy Y and of disomy 4 of the 0-4-4 type increased by 2-fold and 1.5-fold, respectively, these values were not significantly different than corresponding control values. Nullisomy 4 was significantly greater following 9 weeks of cyclophosphamide treatment than in the concurrent controls, however, this was not different from that observed after 6 weeks of treatment. The incidence of Y-Y-4-4 autodiploid sperm did not differ significantly between treated and control rats. Nevertheless, the overall frequency of numerically abnormal spermatozoa, including all aneuploid and diploid cells, was higher in treated (range 4 to 14) than in control (range 14 to 19) by about 2-fold ($p < 0.001$) following 9 weeks of cyclophosphamide treatment.

In a previous study, the frequency of chromosome 4 disomy was significantly higher than that of Y-chromosomal disomy [20]. This was confirmed by our study (Table 1). The average frequency of chromosome 4 disomy after 6 or 9 weeks of cyclophosphamide treatment was significantly greater than Y-chromosomal disomy ($p < 0.001$). A similar difference ($p < 0.001$) was observed when comparing the frequencies of disomy 4 and Y in both of the control groups. Thus, independent of treatment, disomy 4 was significantly more frequent than disomy Y.

As a result of the rapid advancement of assisted reproductive technologies that require the selection of germ cells that appear most suitable for fertilization, it is of interest to determine the proportion of numerically abnormal cells that are identifiable based on phenotypical anomalies, namely size and shape (Table 2). Independent of treatment, the overall number of chromosomally abnormal spermatozoa detected on the basis of phenotype defects was 64 cells

out of a total of 205 chromosomally abnormal cells. Aneuploid spermatozoa that were large in size (Fig. 3A,B) occurred about 11-fold more frequently than those cells that presented with shape irregularities. The proportion of spermatozoa large in size with simultaneous shape abnormalities (Fig. 3C) presented at an occurrence that was about 5-fold greater than that for cells with shape defects alone, but was about 2.5-fold less that those cells that were only large in size. The percentage of aneuploid cells detected based on atypical appearance was not significantly increased following cyclophosphamide treatment when compared to the corresponding saline values for 6 and 9 weeks of treatment (Table 2). Overall, these results highlight the fact that only a small proportion of aneuploid cells can be identified as a consequence of morphological irregularities, emphasizing the high potential for selection of numerically abnormal spermatozoa for assisted reproduction. Diploid cells (Fig.2F), in contrast, were observed to be approximately two times the size of normal cells in all cases, suggesting that diploidy can be selected against based on size and hence DNA content of the cell.

DISCUSSION

Chronic treatment of male rats with cyclophosphamide prior to mating results in male-mediated fetal loss and teratogenicity [35, 37, 40]. Evidence of chromosomal anomalies, obtained by karyotyping the somatic tissue of malformed offspring fathered by cyclophosphamide-exposed males, suggested a link between the genetic complement and the health of individual progeny [41]. In the current study we used fluorescence *in situ* hybridization specific for rat chromosomes Y and 4 in epididymal spermatozoa to measure the chromosome complement directly in the spermatozoa of cyclophosphamide-treated rats. The present data demonstrate that chronic cyclophosphamide treatment, initially exposing either spermatogonia or pachytene spermatocytes (Fig. 1), induced a germ cell stage-specific increase in the frequency of numerical chromosomal abnormalities. Cyclophosphamide primarily induced chromosomal aneuploidy in

germ cells prior to meiosis, as demonstrated by the increased frequency of aneuploid spermatozoa following 9 weeks of treatment.

Aneugenic effect of chronic cyclophosphamide exposure on male germ cells

The administration of a single dose of cyclophosphamide to male mice, exposing male germ cell stages from spermatogonia to metaphase I [42], or at a time coincident with premeiotic S-phase [33], resulted in a significant increase in metaphase II hyperhaploidy [33, 42]. Although the observed increases in aneuploidy in cyclophosphamide-damaged spermatocytes were minimal [33], a statistically significant correlation of metaphase II hyperhaploidy was found with pairing anomalies that may lead to disruptions in the meiotic exchange process and result in a predisposition to chromosome malsegregation [33, 42].

To determine the stage-specificity of the induction of non-disjunction events, as well as to identify the potential genetic risk of the ensuing mature gametes, rats were treated chronically with one of two cyclophosphamide regimes of differing exposure lengths (see Materials & Methods; Fig. 1). That aneuploidy was increased when germ cells were first targeted as spermatogonia, but not as pachytene spermatocytes (Table 1), is consistent with the hypothesis that: (1) there is a critical window prior to pachynema during which the alkylation by cyclophosphamide interferes with molecular events that are responsible for ensuring faithful chromosome recombination and segregation during meiosis; and (2) germ cells with numerical chromosome defects can develop to mature spermatozoa, thereby introducing the risk of delivering a defective genome to the developing embryo.

Germ cells at different stages have differential responses to genetic damage [43, 44]. Although spermatogonia are quite well protected against bifunctional nitrogen mustards, these drugs are highly mutagenic to successive pre- and post-meiotic germ cells [45, 46]. Together, previous cytogenetic observations [33, 42] and the current induction of aneuploidy in pre-meiotic-exposed germ cells suggest that cyclophosphamide acts primarily on preleptotene and zygotene/pachytene stages by interfering with different genes

and regulatory proteins functioning in synaptonemal complex formation, crossing-over and chiasmata formation [47, 48], affecting the fidelity of subsequent processes of disjunction.

Damaged germ-cells may persist as a result of a decrease in apoptosis [49, 50]; the dose of cyclophosphamide selected for the present study may not have induced sufficient DNA damage to trigger a significant degree of apoptosis in premeiotic and meiotic cells. Alternatively, repeated low dose drug exposure may alter the expression of genes necessary to trigger an apoptotic response [49, 50] or overwhelm protective mechanisms such as DNA-repair [41]. The maintenance of genomic integrity following toxicant exposure largely depends upon the capacity of the cells to repair damaged DNA. Cyclophosphamide exposure markedly repressed the expression of genes involved in the nucleotide excision repair, base excision repair and mismatch repair pathways, as well as cell cycle kinases involved in the meiotic checkpoint after chronic treatment [51], perhaps increasing susceptibility to genetic damage.

Sex chromosomes generally appear to be particularly susceptible to non-disjunction in male meiosis resulting from failed recombination in the pseudoautosomal region of the XY bivalent [52]. Our study demonstrated that the frequency of spermatozoa with disomy Y was significantly less than those with disomy 4 across all groups, confirming the finding of the previous rat aneuploidy baseline study [20]. Chromosome Y disomy is an error that occurs uniquely in the paternal second meiotic division and might be expected to occur less frequently than autosomal errors that result from errors in either of the meiotic divisions [20]. As neither disomy Y nor the incidence of Y-Y-4-4 autodiploidy was significantly elevated following cyclophosphamide treatment, cyclophosphamide may induce aneuploidy primarily through errors at meiosis I. As such, an increase in the frequency of X-Y-4 would also be predicted. The development of an X-probe would permit us to further examine this issue by revealing the incidence of X-Y-4-4 diploidy due to failure of meiosis I, as well as permitting further definition of abnormal genotypes involving the X-chromosome.

Aneuploidy in phenotypically abnormal spermatozoa

The incidence of aneuploidy in human spermatozoa is not significantly different between spermatozoa with a normal versus an aberrant head morphology [53, 54]. Conversely, certain morphological abnormalities reflect increased cytogenetic abnormalities in immature germ cells and spermatozoa [55]. In the present study, aneuploidy was associated with phenotypic spermatozoal defects in approximately 27% of the cells. Although there is no clear correlation between aneuploidy and morphology, a large spermatozoal head may be reliably associated with a diploid chromosome complement. Yurov et al. [56] found that the majority of human spermatozoa with a large head were diploid, whereas normal sized heads were generally haploid. This was demonstrated also in rats using dual color FISH [57]. Consequently, it was suggested that the majority of spermatozoa hyperhaploid for chromosome 4, with Y-4-4 and 0-4-4 fluorescent phenotypes, that were large in size correspond to diploid cells carrying (X)-Y-4-4 or (X-X)-4-4 genotypes, respectively [57]. We can speculate that a proportion of the observed aneuploidy is due to diploidy from errors in meiosis I. Most strikingly, a very low proportion of genetically abnormal spermatozoa may be recognized on the basis of phenotype, highlighting the need for genetic and molecular analysis of spermatozoa from males attempting artificial insemination.

Sperm FISH measures quantitatively the level of aneuploidy induction following toxicant exposure, however, the precise mechanism of induction is not fully addressed. The presence of genotoxic damage in rat epididymal spermatozoa, together with the observation that genetically abnormal spermatozoa are capable of fertilization and zygotic development [26], raise concern for a higher risk of adverse male-mediated effects on progeny following chemotherapy with cyclophosphamide.

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FIGURES

Figure 3.1. Schematic presentation of male germ cell differentiation during rat spermatogenesis. From timing the period of cyclophosphamide exposure, germ cell phase-dependent susceptibility can be characterized. Drug administration by gavage for 6 weeks initially targets pachytene spermatocytes, while a 9 week treatment initially targets spermatogonia. For each treatment regime, rats were treated chronically (6 mg/kg/day) and cauda epididymal spermatozoa were analyzed by rat sperm Y-4 FISH. (This figure was originally published in Barton et al. (2003) Biol. Reprod. *69(4):1150-1157*).

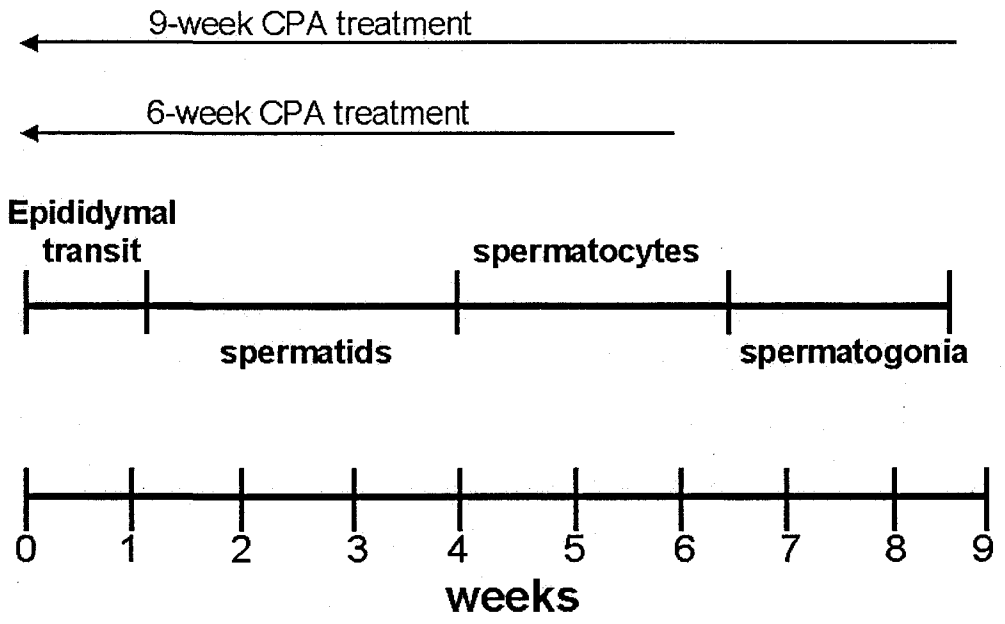

Figure 3.2. Microscope images of rat epididymal spermatozoa labeled using Y-4 sperm FISH. The nuclei (blue) of each spermatozoa is stained with DAPI, chromosome 4 probe is stained with FITC (green) and the chromosome Y probe with DIG (red). (A) Y-4 normal haploid spermatozoa. (B) A presumably X carrying disomy 4 spermatozoa (0-4-4). (C) A Y carrying disomy 4 spermatozoa (Y-4-4). (D) Disomy Y spermatozoa. (E) A nullisomy 4 spermatozoa (0-Y). (F) A meiosis II autodiploid spermatozoa (Y-Y-4-4). Spermatozoa were imaged at 1,000x magnification. (This figure was originally published in Barton et al. (2003) Biol. Reprod. *69(4):1150-1157*).

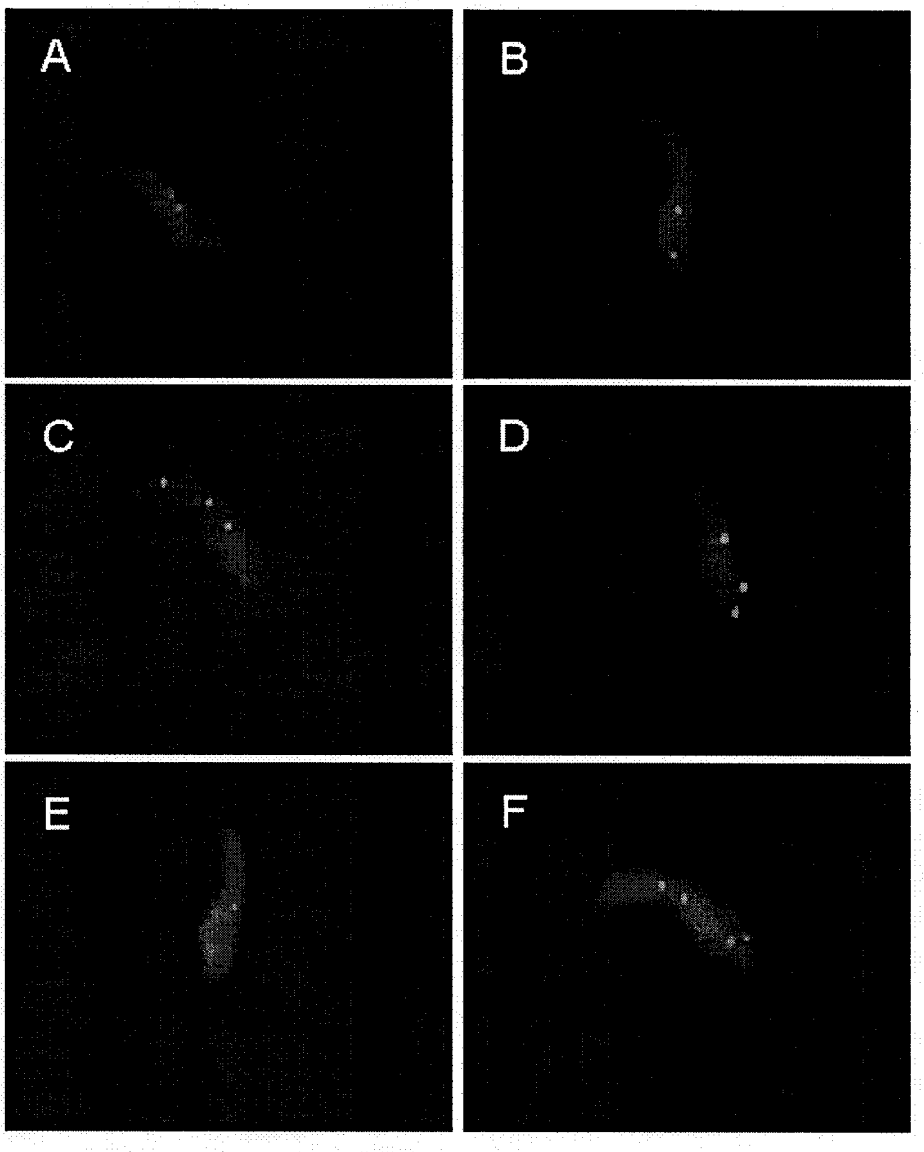
A
B
C
D
E
F

Figure 3.3. Microscope images of abnormal rat epididymal spermatozoa illustrating morphological abnormalities of size and shape. (A) Abnormal disomy 4 (Y-4-4) spermatozoa (left) and normal haploid spermatozoa (right). (B) Disomy 4 (Y-4-4) spermatozoa large in size with normal shape. (C) Disomy 4 (Y-4-4) spermatozoa large in size with abnormal shape. Spermatozoa were imaged at 1,000x magnification. (This figure was originally published in Barton et al. (2003) Biol. Reprod. *69(4):1150-1157*).

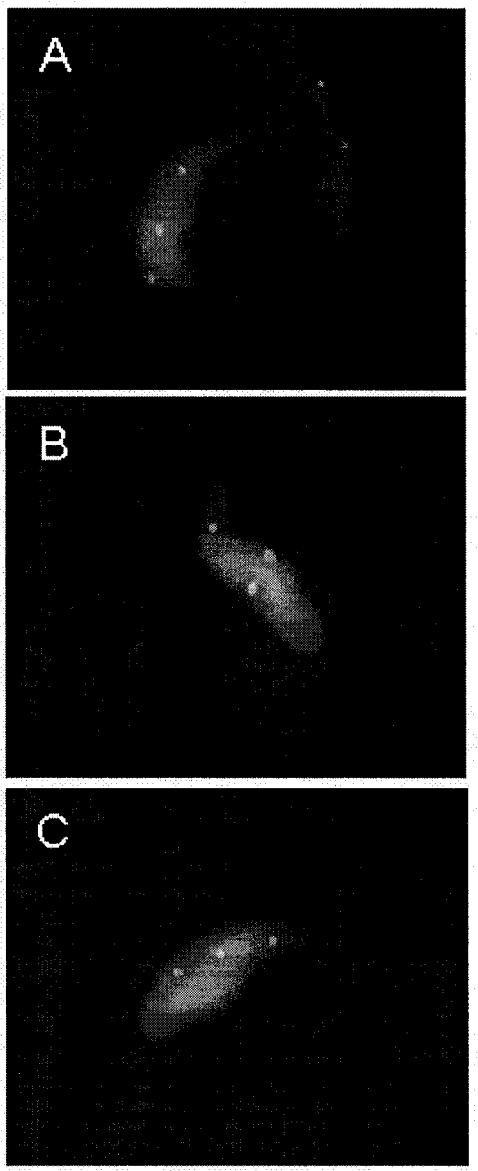
A
B
C

Table 3.1. Frequencies of aneuploid and diploid epididymal sperm following chronic cyclophosphamide Treatment (This table was originally published in Barton et al. (2003) Biol. Reprod. *69(4):1150-1157*)

	Saline control 6 Weeks (1mL/d)	CPA 6 weeks (6.0 mg/kg/d)	Saline control 9 Weeks (1mL/d)	CPA 9 Weeks (6.0 mg/kg/d)
No. of rats	5	5	5	5
Total cells scored	50,260	50,129	50,151	50,176
Normal, haploid				
Y-4	25,094	25,013	24,843	24,797
0-4	25,111	25,052	25,236	25,244
Sex ratio (Y-4/0-4)	0.999	0.998	0.98	0.98
Anomalies[a]				
Disomy Y				
Y-Y-4	0.4	0.6	1.2	2.6
Disomy 4				
Y-4-4	1.4	3.2	4	7.6[b]
0-4-4	3	2	2	3
Total Disomy 4	4.4	5.2	6	10.6[b]
Sum of Disomy Y and 4	4.8	5.8	7.2	13.2[b]
Nullisomy 4				
Y-0	1.8	2.2	0.6	2.2[c]
Meiosis II Diploidy				
Y-Y-4-4	0	0.8[c]	0.6	1
Total frequency of abnormal sperm	6.6	9.4	8.4	16.6[d]

[a] Frequency per 10,000 sperm

[b] Significantly different from corresponding saline control, $P < 0.05$ (Chi-Square test)

[c] Significantly different from 9 week saline control, $P < 0.02$ (Chi-Square Test)

[d] Significantly different from 9 week saline control, $P < 0.001$ (Chi-Square test)

Table 3.2. Morphological analysis of aneuploid spermatozoa (This table was originally published in Barton et al. (2003) Biol. Reprod. *69(4):1150-1157*)

	Saline control 6 weeks (1mL/d)	CPA 6 weeks (6.0 mg/kg/d)	Saline control 9 weeks (1mL/d)	CPA 9 weeks (6.0 mg/kg/d)	TOTALS
No. of rats	5	5	5	5	20
Total cells scored	50,260	50,129	50,151	50,176	200,716
No. chromosomally abnormal spermatozoa	33	47	42	83	205
Aneuploid Spermatozoa					
Total No. of disomy & nullisomy cells	33	43	39	78	193
Cells with large size	1	12	7	15	35
Cells with abnormal shape	2	1	0	0	3
Cells with abnormal size & shape	2	3	1	8	14
No. of aneuploid cells with abnormal phenotype	5	16	8	23	52
Percent of aneuploid cells with abnormal phenotype	15.1	37.2	20.5	27.7	26.9
Diploid Spermatozoa					
No. of Y-Y-4-4 cells	0	4	3	5	12
No. of diploid cells with abnormal size	0	4	3	5	12
Percent of diploid cells with abnormal size	0	100	100	100	100

CONNECTING TEXT

The objective of the work described in chapter III was to elucidate the induction of chromosomal aberrations, or genetic defects, in spermatozoa following toxicant exposure. We established that aneuploidy was significantly increased only when pre-meiotic germ cells were exposed. Thus, we propose that genetic aberrations are not sufficient to account for the spectrum of adverse developmental consequences observed following chronic paternal cyclophosphamide treatment. Forming a layer on top of the nucleotide sequence, or genetic code, is an epigenetic code which undergoes dramatic alterations during germ cell development and early post-fertilization, until becoming more stable in differentiated cells. Modifications in epigenomic programming and/or embryonic reprogramming via paternal cyclophosphamide exposure could also contribute to embryo loss, developmental deficits and heritable disease. In chapter IV, we assess the competency of the one- and two-cell stage embryo to accurately undergo epigenetic reprogramming following fertilization with drug-exposed spermatozoa; specifically, we assess changes in the dynamic regulation of histone acetylation and DNA methylation.

CHAPTER IV

Epigenetic programming in the preimplantation rat embryo is disrupted by chronic paternal cyclophosphamide exposure

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ABSTRACT

Preconceptional paternal exposure to cyclophosphamide, a widely used anticancer agent, leads to increases in embryo loss, malformations [Trasler, J. M., Hales, B. F. & Robaire, B. (1985) *Nature* 316, 144-146] and behavioral deficits in offspring; these abnormalities are transmissible to subsequent generations [Hales, B. F., Crosman, K. & Robaire, B. (1992) *Teratology* 45, 671-678, Auroux, M., Dulioust, E., Selva, J. & Rince, P. (1990) *Mutat. Res.* 229, 189-200]. Little information exists on the mechanisms underlying this male-mediated developmental toxicity. We assessed the impact of paternal cyclophosphamide exposure on the dynamic regulation of histone H4 acetylation at lysine 5 (H4-K5) and DNA methylation (5-MeC) in preimplantation rat embryos. Zygotes sired by drug-treated males displayed advanced developmental progression, increased pronuclear areas, and disruption of the epigenetic programming of both parental genomes. Early post-fertilization zygotic pronuclei were hyperacetylated; by mid-zygotic development, male pronuclei were dramatically hypomethylated while female pronuclei were hypermethylated. Micronuclei were substantially elevated and H4-K5 localization to the nuclear periphery was disrupted in 2-cell embryos fertilized by cyclophosphamide-exposed spermatozoa. This finding demonstrates that paternal exposure to this drug induces aberrant epigenetic programming in early embryos. We hypothesize that disturbances in epigenetic programming contribute to heritable instabilities later in development, emphasizing the importance of epigenetic risk assessment after chemotherapy.

INTRODUCTION

Long-term survival rates after childhood and reproductive-aged cancers are rising dramatically, eliciting concern for the health of offspring conceived after cytotoxic therapy (1). Of great concern is the ability for germ-line instabilities to be manifested in the surviving offspring and, thus, be propagated across multiple generations. Paternally transmitted chromosomal aberrations are present in the mouse zygote following preconceptional exposure to six mutagens; the extent of abnormalities was suggested to predict the fate and developmental competence of the embryo (2, 3). The first evidence of persistently elevated mutation rates in second generation progeny is following paternal exposure to irradiation in mice (4). A plausible mechanism by which toxicant perturbation of DNA may lead to heritable alterations in the genome is by epigenetic modifications. The impact of aberrant epigenetic reprogramming in cloning on embryonic development has recently gained much attention (5, 6). We report the first experimental evidence of epigenetic deregulation in the naturally fertilized zygote following paternal exposure to a drug.

Cyclophosphamide, an extensively used chemotherapeutic (1, 7) and immunosuppressive agent, targets rapidly dividing cells, alkylating DNA at the N7 position of guanine (7). Spermatogenesis is characterized by complex, sequential stages of differentiation, chromosomal assortment and chromatin remodeling (8), rendering male germ cells highly susceptible to genotoxic agents. The bifunctional alkylating agent, cyclophosphamide, induces DNA-DNA cross-links, DNA-protein cross-links, and single-strand breaks (7), altering the unique structural organization of the paternal genome during chromatin remodeling (9). Sperm chromatin remodeling is essential for imposing predetermined epigenetic programs and involves a wave of hyperacetylation, replacement of histones by sperm-specific protamines (10), nuclear condensation and spermatid remethylation (11), producing a transcriptionally inert spermatozoal genome. After fertilization, the paternal chromosomes decondense and undergo extensive remodeling in association with dramatic epigenetic changes that are occurring

asymmetrically in both parental genomes to establish coordinated parent-specific programs ensuring accurate embryogenesis (12-14).

Epigenetic disturbances that occur soon after fertilization may be particularly detrimental as somatic cells and germ-line cells will be involved prior to lineage specification. The inheritance of epigenetic defects may lead to developmental and neurological abnormalities, aberrant disease phenotypes and subtle changes in gene expression resulting in elevated cancer predisposition in the offspring and subsequent generations (reviewed in (15, 16)). We propose that the DNA damaging effects of paternal cyclophosphamide exposure disrupt the epigenome of male germ cells during this vulnerable state of chromatin remodeling in a manner that is transmissible to the embryo, as well as future offspring.

MATERIALS AND METHODS

Drug treatment, *in vivo* rat embryo production and collection. Adult male (body wt, 350-400 g) and virgin female (body wt, 225-250 g) Sprague-Dawley rats were purchased from Charles River Canada (St. Constant, Quebec) and housed at the Animal Resources Centre, McIntyre Medical Building, McGill University. Animals received food and water ad libitum and were maintained on a 12L:12D photoperiod. Following one week of acclimatization, male rats were randomly assigned into one of two treatment groups, each consisting of 10 animals, and gavaged with either saline or 6 mg/kg/day cyclophosphamide six times per week for 4-5 weeks (17-20). The selected treatment regime ensures the targeting of the fundamental process of sperm chromatin organization and packaging during spermiogenesis (9).

On the fifth week of treatment, each male was mated overnight with two control virgin females in proestrus. At 900 hr the following morning, designated as day 0 of gestation, pregnancies were confirmed; sperm-positive females were sacrificed at 1300 hr on day 0 or at 1100 hr on day 1 in order to collect 1-cell stage and 2-cell stage embryos, respectively. Oviducts and proximal uteri were isolated, 1-cell stage embryos were released into warm (37°C) 1% hyaluronidase

(Sigma) for cumulus cell dissociation; 2-cell embryos were flushed from the oviducts of pregnant dams with warm (37 °C) M2 culture medium (Sigma). Embryos were prepared for immunofluorescent staining as described below. All animal protocols were conducted in compliance with the guidelines outlined in the Guide to the Care and Use of Experimental Animals, prepared by the Canadian Council on Animal Care.

Immunofluorescence. All embryo manipulations were done at room temperature unless otherwise stated. Embryos sired by saline and cyclophosphamide-exposed males were stained in parallel. Preimplantation embryos were washed in 1X phosphate-buffered saline (PBS), pH 7.4 (Mg^{2+}- and Ca^{2+}-free) containing 1 mg/ml polyvinylpyrrolidone (PVP). Zonae pellucidae were removed by rinsing embryos in a drop of Acid Tyrode's solution. Methods used for indirect immunofluorescence were described previously by Santos et al. (5, 13). Embryos were fixed in 4% paraformaldehyde in PBS for 15 min, washed in 0.05% Tween-20 in PBS for 5 min, permeabilized for 30 min in a solution of 0.2% Triton X-100 in PBS, re-washed in 0.05% Tween-20 in PBS for 5 min and blocked overnight at 4 °C in 1% BSA, 0.05% Tween-20 in PBS. Immunofluorescence staining for methylation patterns required additional treatment of all samples with 4 N HCL, 0.01% Triton X-100 for 20 min followed by a 15 min neutralization of cells with 100 mM Tris-HCL, pH 8.5 subsequent to permeabilization.

Embryos were incubated for 1 hr in rabbit polyclonal anti-histone H4 acetylated at lysine 5 (1:500 dilution; Abcam Inc.) to recognize the preferentially acetylated isoform of histone H4 (14). They were washed vigorously in a series of fresh blocking solution (1x10 min, 1x30 min, 1x10 min) and incubated for 1 hr in Alexa Fluor® 488 goat anti-rabbit IgG secondary antibody (1:200 dilution; Molecular Probes); blocking solution washes were repeated. DNA was stained with 10 μg/ml 4',6-diamidino-2-phenylindole (DAPI) for 30 min, embryos were washed in 0.05% Tween-20 in PBS for 10 min and mounted in 3 μl of

VectaSheild antibleaching mounting medium (Vector Laboratories, Inc) on premarked slides.

DNA methylation was visualized with a mouse monoclonal anti-5-methylcytosine antibody (1:25 dilution) (5, 12, 13). Embryos were incubated for 1 hr with the primary antibody, washed repeatedly in blocking solution, and incubated for 1 hr with anti-mouse fluorescein conjugated-IgG secondary antibody (1:40 dilution; Amersham Biosciences). DNA was stained with 2 μg/ml propidium iodide (Molecular Probes) for 15 min and embryos were mounted. Control experiments included the incubation of 1-cell and 2-cell stage embryos in secondary antibody only to confirm nuclear staining specificity. Additionally, the second polar body serves as an internal staining control for 5-MeC as it does not undergo demethylation during preimplantation development.

Confocal Microscopy. Fluorescence was visualized using a Zeiss LSM 510 Axiovert 100M confocal microscope equipped with a Plan-Apochromat x63/1.4 oil DIC objective. The optimal conditions for LSM fluorescence imaging for each primary antibody were determined experimentally; identical brightness and contrast settings were used when collecting original images from both 1-cell and 2-cell stage embryos. All embryos were scanned at a speed of 7 with an optical slice of 0.7 μm, zoom factor equal to one and a pinhole setting of 96 μm. Sixteen scans of each optical section were compiled and averaged by the computer software to give the final image that was 1024 X 1024 pixels in size. All embryos stained with anti-histone H4 acetylated at lysine 5 or anti-5-methylcytosine, were imaged with a detector gain setting of 775 or 992, respectively. Images were collected and transferred into monochrome 8-bit TIFF files for quantitative analysis.

Quantitative Analysis. Quantitative analysis was done on single optical images of all embryos with the microcomputer imaging device MCID® version 7.0 Imaging Research Inc. software. All threshold settings for intensity and saturation were maintained constant across all experimental groups.

Approximately 100 embryos were examined for each preimplantation cleavage stage and for each treatment. The pronuclear and nuclear areas were measured as the total grained area contained within the region of interest, consisting of chromatin regions with the exclusion of the early embryonic precursor nucleoli. Fluorescence intensity was expressed as the mean integrated value of all pixels contained within the sample outline as measured on a brightness scale of 0 to 1. Zygotes were qualitatively categorized into five pronuclear stages (PN1-PN5) based on the interpronuclear distance of the paternal and maternal genomes with respect to each other in the cytoplasm as well as pronuclear morphology for further qualitative comparisons and statistical analysis. Morphological analysis consisted of embryo classification according to the appearance of nucleoli within the paternal pronucleus: 1 large nucleolus; 1 large nucleolus with few smaller nucleoli; or multiple small nucleoli, corresponding to PN1-2, PN3 and PN4-5 respectively, confirming the pronuclear staging obtained from interpronuclear distance (13, 14).

Statistical Analysis. Student's t-tests or Mann-Whitney Rank Sum Tests were used to compare the progression of zygotic development, total grain areas and fluorescence intensity of parental pronuclei and 2-cell stage nuclei of embryos from control animals with those that were fertilized with spermatozoa chronically exposed to cyclophosphamide ($P < 0.05$). Chi-square analyses were employed to compare the proportion of 2-cell stage embryos with H4-K5 peripheral staining and micronuclei sired by saline or cyclophosphamide males ($P < 0.05$). Error bars represent the mean $\pm$ s.e.m. Statistical analyses were done using SigmaStat 2.03 software package (SPSS, Inc., Chicago, IL).

RESULTS

Paternal Cyclophosphamide Exposure Disrupts Zygotic Development. The transmission of DNA damage incurred in the male genome during four weeks of cyclophosphamide administration (20) significantly disrupted the rate of zygotic development following *in vivo* fertilization (Fig. 1). The proportion of zygotes

sired by males that were chronically exposed to cyclophosphamide was significantly decreased (P = 0.029) very early post-fertilization at pronuclear stages 1 and 2 (PN1 and 2), while at PN3 there was an increasing trend, compared to corresponding controls. At PN4, the proportion of zygotes fertilized by cyclophosphamide-exposed spermatozoa was significantly increased (p = 0.039) compared to controls. Comparable numbers of zygotes sired by control and cyclophosphamide males were observed by PN5.

We further investigated pronuclear integrity in zygotes sired by cyclophosphamide-treated males by assessing the chromatin areas of parental pronuclei (Fig. 2). Immediately following fertilization, PN1 and 2, pronuclear grain areas of embryos sired by cyclophosphamide-treated males were not different from controls. Interestingly, the areas of both paternal and maternal pronuclei were significantly enlarged at PN3 and 4 ($p < 0.01$), maintaining increased chromatin dispersion at PN5, compared to controls. Thus, fertilization by spermatozoa chronically exposed to a toxicant perturbed chromatin structure in both parental genomes, namely excessive decompaction, and led to aberrant developmental progression in the zygote.

Aberrant Reprogramming in Parental Genomes following Paternal Cyclophosphamide Exposure. For the first time, we have demonstrated that paternal exposure to a chemotherapeutic agent prior to conception disrupts the temporal patterns of both H4-K5 (Fig. 3) and 5-MeC immunostaining (Fig. 4) in the rat zygote, indicating that genotoxic effects with an epigenetic basis are manifested very early post-fertilization.

In the rat and mouse zygote (14), paternal chromatin outcompeted the maternal genome for the pool of acetylated histone H4 during PN2 (G1) of the first cell cycle (Fig. 3*Aa*), while levels of pronuclear staining in both parental genomes became comparable by PN3 and 4 (S-phase)(Fig. 3 *Ab* and *Ac*), and remained constant for the duration of PN5 (G2)(Fig. 3*Ad*). Conversely, in zygotes fertilized by cyclophosphamide-exposed spermatozoa, both male and female pronuclei displayed enhanced levels of H4-K5 staining as early as PN2

(Fig. 3*Ae*); fluorescence continued to increase dramatically becoming more dense and intense at PN3 (Fig. 3*Af*) compared to corresponding controls. As zygotes progressed to PN4 and 5 (Fig. 3 *Ag* and *Ah*), the pronuclei remained highly acetylated, similar to those observed in saline embryos (Fig. 3 *Ac* and *Ad*). Quantitative analysis of H4-K5 fluorescence intensity confirmed that male and female pronuclei in embryos sired by cyclophosphamide-treated males were significantly hyperacetylated beginning in G1 ($p < 0.05$) and lasting into S-phase ($p < 0.001$), corresponding to PN2 and 3, respectively. In the later stages of zygotic development, the extent of H4 acetylation was not different between drug-exposed and control embryos (Fig. 3*B*).

DNA methylation reprogramming, an epigenetic modification that is crucial for competent embryogenesis (5), was also markedly different in zygotes sired by cyclophosphamide-treated fathers (Fig. 4). Immediately after fertilization, both haploid pronuclei were equally methylated in zygotes from saline (Fig. 4*Aa*) and cyclophosphamide-treated males (Fig. 4*Ae*). In controls, the male pronuclei underwent a gradual process of active genome-wide demethylation while the female pronuclei remained hypermethylated with respect to the paternal genome (Fig. 4*Ab - d*). At PN5 (Fig. 4*Ad*), the male pronuclei were significantly ($p < 0.001$) undermethylated compared to the female pronuclei. In contrast, the male pronuclei in zygotes fertilized by drug-exposed spermatozoa were dramatically hypomethylated at PN3 (Fig. 4*Af*) and remained globally undermethylated until the parental genomes were in close apposition at PN5 (Fig. 4*Ae - h*).

Intriguingly, quantitative analysis of 5-MeC fluorescence intensity established that at PN3 (Fig. 4*B*) both parental pronuclei from cyclophosphamide-exposed spermatozoa were significantly affected ($p < 0.002$). The fluorescence intensities of male pronuclei were decreased while those of the female pronuclei were aberrantly increased compared to corresponding controls. Male pronuclei from cyclophosphamide-treated fathers were consistently and significantly demethylated compared to controls through PN5 (Fig. 4*B*). These data emphasize the role of sperm chromatin composition in encoding parent-specific programs during preimplantation development (12).

Transmission of Aberrant Programming after the First Embryonic Cleavage. Genetic and epigenetic damage persisted to the 2-cell stage (Fig. 5). Chronic cyclophosphamide treatment resulted in a marked induction in the proportion of 2-cell stage embryos with micronuclei (Fig. 5 *Ab* and *Ad*) compared to controls (Fig. 5 *Aa* and *Ac*); 63% versus 4% (Fig. 5*B;* $p < 0.001$), respectively. Interestingly, embryonic micronuclei were marked by opposing epigenetic states; they were acetylated (Fig. 5 *Ab*) and unmethylated (Fig. 5 *Ad).* Similar chromatin abnormalities were not present in control embryos (Fig. 5 *Aa* and *Ac*).

Preconceptional paternal drug treatment also disrupted the spatial localization of histone H4 acetylation in 2-cell embryos with and without micronuclei (Fig. 5 *Aa* and *Ab*). Acetylation of H4-K5 was enhanced at the nuclear periphery of embryos sired by control males (Fig. 5*Aa*); however, this spatially restricted staining pattern was often obliterated in embryos fertilized by drug-exposed spermatozoa (Fig. 5*Ab*). Analysis of the subset of embryos without micronuclei revealed that significantly fewer embryos sired by cyclophosphamide-exposed males portrayed the sequestration of H4-K5 immunofluorescence staining at the nuclear periphery (Fig. 5*C*; $p = 0.004$). In contrast, the pattern of 5-MeC staining was not spatially altered and immunofluorescence intensity was unchanged (data not shown), in 2-cell stage embryos without micronuclei sired by cyclophosphamide-treated males (Fig. 5A*d*), compared to the corresponding controls (Fig. 5*Ac*).

DISCUSSION

The delivery of a sperm nucleus with highly organized genetic material is required for competent participation of the paternal genome in early post-fertilization development (21); subtle structural disturbances in sperm DNA are sufficient to impede normal embryogenesis (22). Rat spermatozoa from males chronically exposed to cyclophosphamide have normal fertility (17) but altered nuclear decondensation patterns *in vitro*, decreased sulfhydryl content (23) and advanced male pronuclear formation in hamster oocytes (19). In the current study, the proportion of embryos sired by cyclophosphamide-treated males that

were at PN1 and 2 was significantly reduced; significantly more zygotes had advanced to PN4, compared to controls. Alkylation of sperm DNA and/or nuclear proteins such as protamines may loosen the chromatin structure and reduce nuclear compaction, thereby affecting the nuclear organization of mature spermatozoa. Accelerated pronuclear formation was also observed after inhibition of poly(ADP-ribosyl)ation, a covalent modification involved in epigenetic remodeling, following *in vitro* fertilization of mouse oocytes (24).

Nuclear decondensation rate prior to pronuclear formation is directly related to disulphide bond content (25). Distorted chromatin packaging may lead to an unusually permissive conformation after fertilization, allowing inopportune access of proteins with chromatin modifying potential. Intriguingly, fertilization by cyclophosphamide-exposed spermatozoa increased chromatin decompaction in both parental pronuclei (Fig. 2), emphasizing the unique interaction of two sets of chromosomes coexisting as apparently separate entities in the zygote (26). Destabilization of maternal chromatin architecture in response to the introduction of a damaged paternal genome suggests that common regulatory factors controlling differential gene activity between parental chromosomes may be affected.

Histone acetylation and DNA methylation constitute intricate regulatory mechanisms that play essential roles in DNA packaging and programming of epigenetic information during preimplantation development (6, 27). Our finding that preconceptional paternal exposure to a chemotherapeutic agent perturbs both H4-K5 (Fig. 3) and 5-MeC immunostaining (Fig. 4) in the rat zygote suggests that many fundamental cellular processes may be dysregulated.

Zygotes sired by drug-treated males were hyperacetylated very early post-fertilization (Fig. 3). The possible consequences of hyperacetylation, as early as G1 of the 1-cell stage, are numerous since histone modifications are involved in the regulation of replication, transcription and cell cycle progression (28). Histone acetylation is also required for DNA repair and genomic integrity (29), suggesting that immediately post-fertilization the zygote initiates a heightened

damage response attempting to repair genomic perturbations incurred during spermiogenesis, when germ cells themselves are unable to repair DNA.

Core histone acetylation is intimately associated with transcriptionally competent chromatin and, as predicted by the histone code hypothesis (28), signals for the recruitment of the specific protein complexes required to regulate zygotic gene expression. Studies in which histone deacetylase activity is inhibited demonstrate that chromatin-modifying proteins are active as early as G1 in the zygote and that accurate modulation is critical for preimplantation development (14). In bovine, the onset of DNA replication in both pronuclei is directly regulated by the paternal genome; moreover, the first zygotic S-phase plays a pivotal role in chromatin template organization for subsequent transcriptional activities (30). The histone H4 hyperacetylation induced as a result of paternal cyclophosphamide exposure may permit atypical relief of the repressed chromatin state and provide a window of opportunity for promiscuous binding and assembly of transcriptional complexes, leading to inappropriate patterns of gene expression. Histone acetylation has been implicated in cell memory (14); therefore, the adverse consequences of abnormal patterns of histone H4 acetylation may be transmitted from one cell generation to the next, as well as to future progeny, thereby fitting the definition of an "epigenetic code"(31).

DNA methylation, an epigenetic mark associated with transcriptional repression and genomic stability (27), is also significantly altered in the male and female pronuclei of zygotes fathered by cyclophosphamide-exposed males. The patterns of DNA methylation established during early embryogenesis serve as a global repression mechanism essential to regulate genomic structure and preserve stable epigenetic chromosomal states (27). Deficient reprogramming of DNA methylation in cloned bovine embryos is highly correlated with diminished developmental potential (6), while inappropriate gene repression has been linked to numerous human diseases (reviewed in (16)). Thus, the greatly altered zygotic DNA methylation pattern observed following paternal cyclophosphamide

treatment may contribute to the serious neurological deficits and developmental failure transmitted to three successive generations (18, 32).

The presence of a unique phenomenon, zygotic pronuclear cross talk, was revealed in embryos fertilized by spermatozoa chronically exposed to cyclophosphamide. Interestingly, the maternal pronucleus was transiently destabilized, specifically, hyperacetylated (Fig. 3) and hypermethylated (Fig. 4), as a result of damage to the spermatozoal genome. Paternal and maternal genomes have distinct roles post-fertilization. The damage-free maternal genome has the ability to initiate a p53-dependent checkpoint in response to DNA damage delivered by irradiated spermatozoa (33). We provide the first evidence that fertilization by spermatozoa carrying drug-induced DNA damage leads to aberrant epigenetic reprogramming of both the paternal and maternal genomes. Deregulation of parental pronuclear methylation, as well as nuclear hypermethylation after the first cleavage division, may be responsible for the altered expression of imprinted genes (19) and contribute substantially to impaired post-implantation development.

Micronuclei in 2-cell stage embryos are a sensitive measure of the clastogenic effects of paternal drug exposure (34). The genetic damage accumulated during the most sensitive phase of spermatogenesis (9) cannot be fully repaired in the zygote and continues to be transmitted through successive cleavage stages. Previous studies (17) have shown that the current cyclophosphamide regimen does not affect preimplantation embryo survival but results in 80% postimplantation loss, comparable with the proportion of embryos presenting with micronuclei. The two-cell embryos without micronuclei had aberrant epigenetic markings, suggesting that there may be programming abnormalities in the absence of extensive chromosomal damage. A unique observation is that embryonic micronuclei are marked by opposing epigenetic states; specifically, acetylated (Fig. 5*Ab*) and unmethylated (Fig. 5*Ad*), suggesting a permissive chromatin conformation. Hypomethylation after exposure to 5-azacytidine is implicated in chromatin decondensation and micronucleus formation (35).

Peripheral enrichment of H4-K5 is transiently restricted to the 2-cell embryo, correlating with a major burst of transcription during the onset of zygotic gene activation (36). The spatial localization of histone H4 acetylation is disrupted following paternal cyclophosphamide treatment, suggesting a dysregulation of cell cycle progression at the 2-cell stage compared to control embryos. It has been proposed that a replication-dependent mechanism moves chromatin from replication sites to the nuclear periphery; histones are further acetylated, gaining a permissive groundstate for transcription factor binding, thus, establishing a gene expression pattern compatible with continued development (37). Defects at the level of the transcriptional machinery and alteration of the gene activation program are evident in 2-cell embryos sired by cyclophosphamide-treated males (19), supporting the hypothesis that the chromatin conformation necessary to temporarily localize acetylated histone H4 is disturbed.

Our results indicate that cyclophosphamide-induced DNA damage in the paternal genome relays defective messages as early as the first round of DNA replication in the zygote, manifesting profound epigenetic changes in chromatin structure and function in a manner subtle enough to maintain fertility, thus, perpetuating long-term genetic instabilities.

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FIGURES

Figure 4.1. The progression of zygotic development is disrupted following chronic paternal cyclophosphamide exposure. Zygotes fertilized by drug-exposed spermatozoa displayed advanced progression to pronuclear stage 4. The number of embryos analyzed at each pronuclear stage was as follows. Saline, n = 49, 96, 40, 15 for PN1 & 2, 3, 4, 5, respectively. Cyclophosphamide, n = 19, 115, 68, 14 for PN1 & 2, 3, 4, 5, respectively. Open bars, saline sired zygotes; filled bars, cyclophosphamide sired zygotes; * $P < 0.05$.

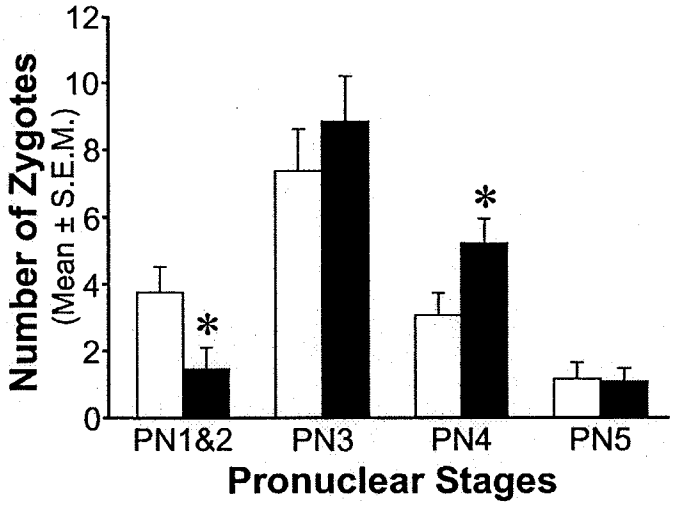


Figure 4.2. Paternal and maternal pronuclei of zygotes from cyclophosphamide-treated males are dramatically increased in size. Zygotic chromatin was counterstained with propidium iodide to determine pronuclear area. The number of embryos analyzed at each pronuclear stage was as follows. Saline, n = 29, 47, 19, 5 for PN1 & 2, 3, 4, 5, respectively. Cyclophosphamide, n = 12, 51, 38, 5 for PN1 & 2, 3, 4, 5, respectively. Cross-hatched bars, saline male pronucleus; black bars, cyclophosphamide male pronucleus; open bars, saline female pronucleus; gray bars, cyclophosphamide female pronucleus. * $P < 0.01$.

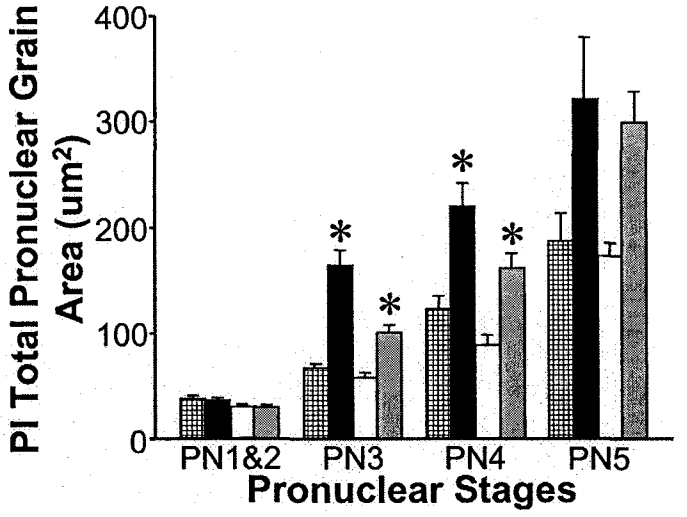


Figure 4.3. Histone H4 hyperacetylation in rat zygotes fertilized by cyclophosphamide-exposed spermatozoa. (*A*) Embryos were stained by indirect immunofluorescence using an antibody to histone H4 acetylated at lysine 5 (green); DNA was counterstained with DAPI (blue). Acetylated histones assembled preferentially onto the paternal genome (a) became similar in both parental genomes (*b* and *c*), and remained constant for the duration of PN5 (*d*). In contrast, both parental genomes of zygotes sired by cyclophosphamide-treated males displayed increased H4-K5 staining soon after fertilization (*e* and *f*), returning to control levels later in development (*g* and *h*). (Scale bar represents 20 μm.) (*B*) Quantitative analysis of H4-K5 fluorescence intensity. Paternal and maternal pronuclear histone H4 is significantly hyperacetylated in zygotes sired by cyclophosphamide-treated rats from PN1 through PN3. The number of embryos analyzed at each pronuclear stage was as follows. Saline, n = 20, 49, 21, 10 for PN1 & 2, 3, 4, 5, respectively. Cyclophosphamide, n = 7, 64, 30, 9 for PN1 & 2, 3, 4, 5, respectively. M, male pronucleus; F, female pronucleus, PB, polar body; cross-hatched bars, saline male pronucleus; black bars, cyclophosphamide male pronucleus; white bars, saline female pronucleus; gray bars, cyclophosphamide female pronucleus. * $P < 0.05$, ** $P < 0.001$.

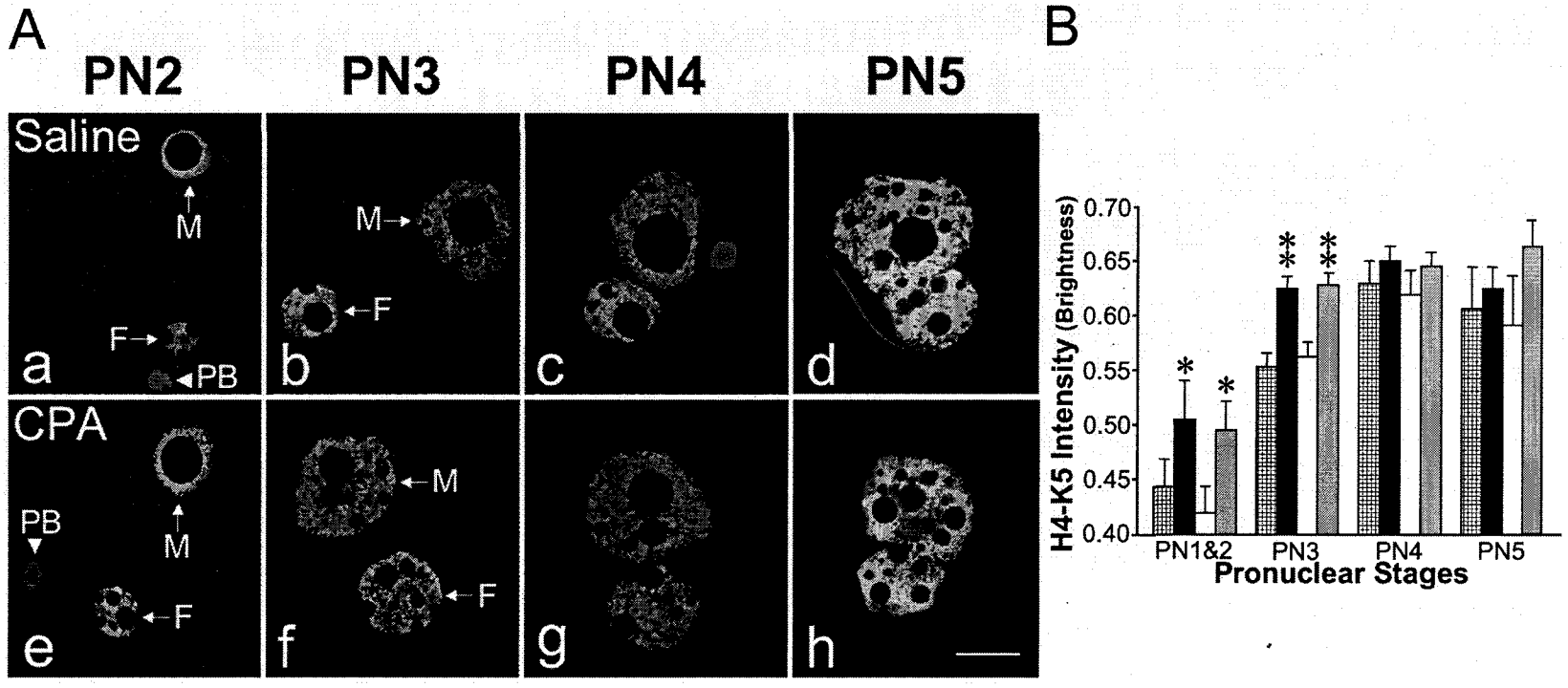

A
PN2
PN3
PN4
PN5
Saline
M
F
PB
a
b
c
d
CPA
PB
M
F
e
f
g
h
B
H4-K5 Intensity (Brightness)
0.70
0.65
0.60
0.55
0.50
0.45
0.40
PN1&2
PN3
PN4
PN5
Pronuclear Stages

Figure 4.4. Abnormal DNA methylation patterns in rat zygotes fertilized by cyclophosphamide-exposed spermatozoa. (*A*) Embryos were stained by indirect immunofluorescence using an antibody to 5-methylcytosine (green); DNA was counterstained with propidium iodide (red). Paternal pronuclei are dramatically hypomethylated in zygotes fertilized by drug-exposed spermatozoa (*e* – *h*), compared to controls (*a* – *d*) (Scale Bar represents 20 μm.) (*B*) Quantitative analysis of 5-MeC fluorescence intensity. In addition to the highly significant hypomethylation of the damaged paternal genome, maternal pronuclear 5-MeC immunofluorescence was significantly increased at PN3 in zygotes sired by cyclophosphamide-treated rats. The number of embryos analyzed at each pronuclear stage was as follows. Saline, n = 29, 47, 19, 5 for PN1 & 2, 3, 4, 5, respectively. Cyclophosphamide, n = 12, 51, 38, 5 for PN1 & 2, 3, 4, 5, respectively. M, male pronucleus; F, female pronucleus, PB, polar body; cross-hatched bars, saline male pronucleus; black bars, cyclophosphamide male pronucleus; white bars, saline female pronucleus; gray bars, cyclophosphamide female pronucleus. * $P < 0.05$, ** $P < 0.002$; §, hypomethylation of paternal compared to maternal pronuclei, $P < 0.001$.

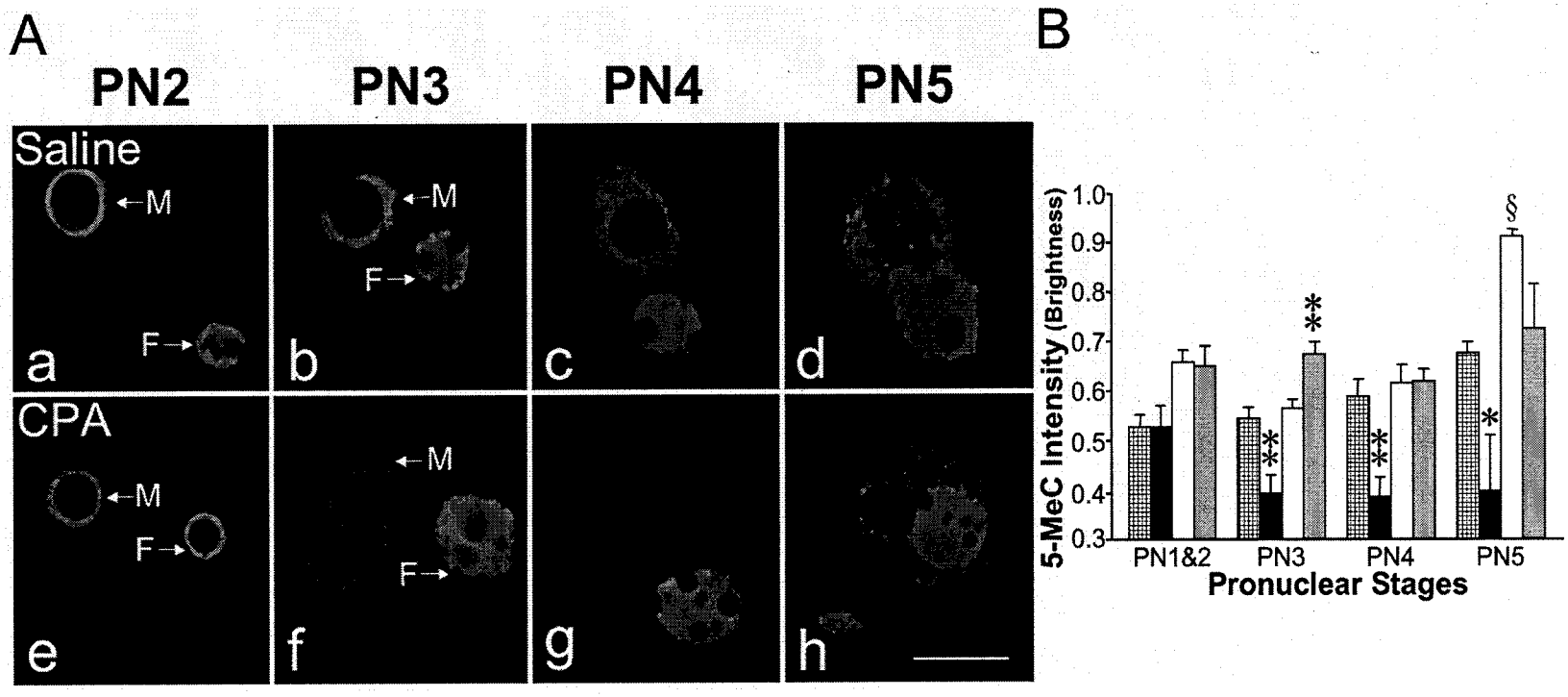
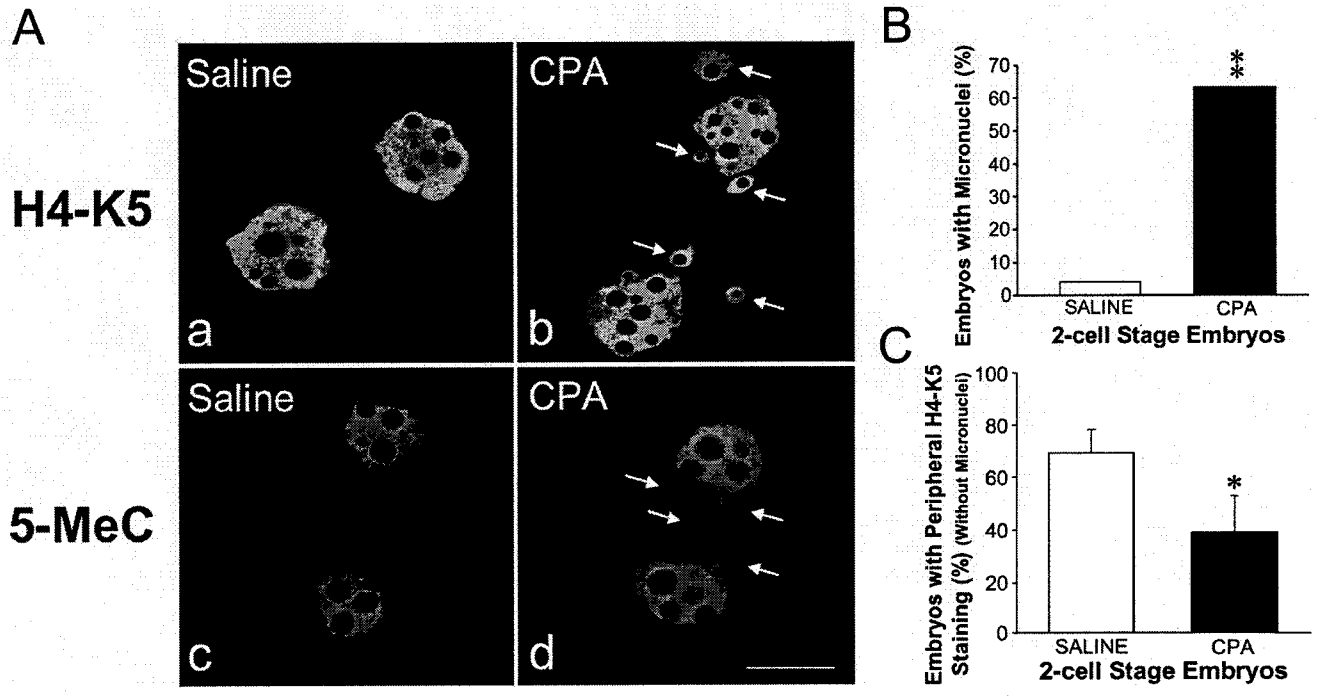

A
PN2
PN3
PN4
PN5
Saline
CPA
M
F
a
b
c
d
e
f
g
h
B
5-MeC Intensity (Brightness)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
PN1&2
PN3
PN4
PN5
Pronuclear Stages
*
**
§

Figure 4.5. Chromatin integrity and the localization of histone H4 acetylation at lysine 5 are disrupted in 2-cell stage embryos sired by cyclophosphamide-exposed males. (*A*) Embryos were stained by indirect immunofluorescence using an antibody either to H4-K5 (*a, b*) or to 5-MeC (*c, d*) (green); DNA was counterstained with DAPI (blue) or propidium iodide (red), respectively. Acetylation of histone H4-K5 was enhanced at the nuclear periphery of embryos sired by control males (*a*), however was widespread in embryos fertilized by cyclophosphamide-exposed spermatozoa (*b*). 2-cell stage embryos fertilized by cyclophosphamide-exposed spermatozoa contain micronuclei that are acetylated (*b*) and unmethylated (*d*). (Scale bar represents 20 mm.) (*B*) The proportion of embryos with micronuclei is dramatically increased following chronic paternal cyclophosphamide exposure compared to controls. (*C*) Paternal cyclophosphamide exposure significantly decreased the proportion of embryos with a spatially restricted H4-K5 staining pattern in the 2-cell stage subset without micronuclei. Saline: n = 88, cyclophosphamide: n = 26. Open bars, saline sired embryos; filled bars, cyclophosphamide sired embryos; arrows indicate micronuclei. * $P = 0.004$, ** $P < 0.001$.


A
H4-K5
5-MeC
Saline
CPA
Saline
CPA
a
b
c
d
B
Embryos with Micronuclei (%)
70
60
50
40
30
20
10
0
**
SALINE
CPA
2-cell Stage Embryos
C
Embryos with Peripheral H4-K5 Staining (%) (Without Micronuclei)
100
80
60
40
20
0
*
SALINE
CPA
2-cell Stage Embryos

CONNECTING TEXT

As established in the preceding chapter, preconceptional paternal cyclophosphamide exposure dysregulates epigenetic reprogramming in both parental pronuclei in the zygote. Normal embryogenesis requires an intact paternal genome; this is achieved by the coordinated integration of DNA damage recognition and repair responses that may be elicited during early embryo development to ensure genomic stability. In chapter V, we elucidate two pathways by which the zygote detects and responds to DNA damage introduced into the embryo via the paternal genome.

CHAPTER V

Epigenetic regulation of DNA damage recognition in the rat zygote following chronic paternal cyclophosphamide exposure

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ABSTRACT

The detrimental effects of preconceptional paternal exposure to the alkylating anticancer agent, cyclophosphamide, include aberrant epigenetic programming, dysregulated zygotic gene activation and transgenerational heritable abnormalities in offspring. The adverse developmental consequences of genomic instabilities transmitted via the spermatozoon emphasize the need to elucidate the mechanisms by which the early embryo recognizes DNA damage in the paternal genome. Little information exists on DNA damage signaling in the zygote. We assessed the impact of paternal cyclophosphamide exposure on histone H2AX phosphorylation (γH2AX) and poly(ADP-ribose) polymerase-1(PARP-1) to determine the capacity in the rat zygote to recognize genomic damage and initiate a response to DNA lesions. An amplified biphasic γH2AX response was triggered in the paternal pronucleus in zygotes sired by drug-treated males; the maternal genome was not affected. PARP-1 immunoreactivity was substantially elevated in both parental genomes, coincident with the second phase of γH2AX induction in embryos sired by cyclophosphamide-exposed spermatozoa. Thus, paternal exposure to a DNA damaging agent rapidly activates signals implemental for DNA damage recognition in the zygote. Inefficient repair of DNA lesions may lead to persistent alterations of the histone code, resulting in heritable instabilities. We propose that the response of the early embryo to disturbances in spermatozoal genomic integrity plays a vital role in determining its outcome.

INTRODUCTION

An intact paternal genome is crucial for successful embryogenesis; subtle alterations in sperm nuclear organization disrupt genomic and epigenetic regulation during development (Barton *et al.,* 2005; Harrouk *et al.,* 2000b; Ward *et al.,* 2000). Preconceptional exposure of male rodents to chemotoxicants or irradiation leads to the transmission of chromosomal aberrations (Marchetti *et al.*, 2004), germ-line instabilities (Barber *et al.,* 2002; Barber *et al.,* 2006) and a variety of abnormalities (Auroux *et al.,* 1990; Hales *et al.,* 1992) in the offspring that may persist for multiple generations.

Cyclophosphamide, a cytotoxic alkylating agent used to treat various cancers and autoimmune diseases, targets rapidly dividing cells, inducing DNA cross-links and DNA single strand breaks (Colvin, 1999). Spermatogenesis is a complex, sequentially ordered process, rendering male germ cells particularly susceptible to genotoxic agents. Chronic preconceptional exposure of males to cyclophosphamide generates genetic instabilities in spermatozoa (Codrington *et al.,* 2004) that are introduced into the oocyte at fertilization (Harrouk *et al.,* 2000a).

Sophisticated mechanisms to recognize and repair DNA damage have been described extensively in eukaryotic cells (Nordstrand *et al.*, 2007). In contrast, male germ cells lack an effective repair system to eliminate lesions incurred during spermiogenesis and spermatozoal maturation (Codrington *et al.,* 2004; Spermon *et al.,* 2006); disruptions in the integrity of sperm DNA must be resolved early post-fertilization to ensure faithful transmission of genetic information throughout embryogenesis. Following fertilization with mutagen exposed spermatozoa, the egg has basic DNA repair capacity (Generoso *et al.,* 1979). Additionally, mRNA analyses provide evidence for the expression of numerous repair genes during early development (Harrouk *et al.,* 2000a; Zheng *et al.*, 2005); however, there is very limited knowledge of the mechanisms implemented by the mammalian zygote in response to paternally transmitted DNA damage.

Histone phosphorylation and poly(ADP-ribosyl)ation are well characterized posttranslational modifications implemental in the maintenance of genomic stability (Celeste *et al.,* 2002; Masutani *et al.,* 2000). Histone H2AX is rapidly phosphorylated at sites of DNA double strand breaks; phosphorylated H2AX (γH2AX) subsequently recruits numerous essential repair proteins to the vicinity of the DNA lesions (Paull *et al.,* 2000). In addition to a prominent role in the genotoxic stress response in mid-spermatogenic germ cells (Aguilar-Mahecha *et al.,* 2005), γH2AX foci are observed coincident with major chromatin remodeling events in the testis (Hamer *et al.,* 2003), coalesce in the condensing X-Y body of spermatocytes, conferring transcriptional inactivation (Fernandez-Capetillo *et al.,* 2003) and have female-specific roles in chromosomal dynamics of the oocyte (Roig *et al.,* 2004). Collectively, these sites emphasize the importance of H2AX phosphorylation as a histone posttranslational modification implemental in the epigenetic control of chromatin modifications and cellular functions. Interestingly, γH2AX signaling has been observed immediately after gamete fusion in the mouse zygote (Derijck *et al.*, 2006).

Poly(ADP-ribosyl)ation of nuclear proteins plays a fundamental role in the detection of DNA strand breaks, the recruitment of repair factors to the lesion site, and the epigenetic regulation of chromatin structure (Schreiber *et al.,* 2006). Poly(ADP-ribose) polymerase 1 (PARP-1) catalyzes poly(ADP-ribosyl)ation, binds with high affinity to DNA strand breaks, and is an integral mediator of DNA base excision repair and single strand break repair (de Murcia *et al.,* 2003). Although poly(ADP-ribosyl)ation has been linked to epigenetic chromatin remodeling events in the preimplantation embryo (Imamura *et al.,* 2004), the kinetics of PARP-1 activation in response to male-mediated genotoxic stress have not been reported. We propose that the DNA damage incurred in the paternal genome due to preconceptional cyclophosphamide exposure initiates early signaling pathways for the detection of DNA strand breaks in the zygote.

MATERIALS AND METHODS

Drug Regime, *in Vivo* Rat Embryo Generation and Collection. Sprague-Dawley rats were purchased from Charles River Canada (St. Constant, Quebec) and housed at the Animal Resources Centre, McIntyre Medical Building, McGill University. Animal housing conditions, treatments and embryo protocols were done following the procedures previously described (Barton *et al.,* 2005). Briefly, adult male rats (body weight, 350-400 g) were gavaged with saline or 6 mg/kg per day of cyclophosphamide (CAS 6055-19-2; Sigma Chemical Co., St. Louis, MO, USA) 6 times per week for 4-5 weeks (Harrouk *et al.,* 2000a; Harrouk *et al.,* 2000b). The selected treatment regime ensures that the embryos are sired by spermatozoa that were initially exposed during the highly sensitive phase of spermiogenesis, encompassing sperm chromatin organization and packaging (Codrington *et al.,* 2004). Early in the fifth week of treatment, each male was mated overnight with two virgin females (body weight, 225-250 g) in proestrus; pregnancies were confirmed the following morning, designated gestation day 0. Sperm-positive females were euthanized at 1300 hours; oviducts were isolated and cumulus-oocyte-complexes released into warm (37°C) 1% hyaluronidase (Sigma Chemical Co., St. Louis, MO, USA) for cumulus cell dissociation. Zygotes were washed in 1X PBS, pH 7.4 (Mg^{2+} and Ca^{2+} free), containing 1 mg/ml polyvinylpyrrolildone; zonae pellucidae were removed subsequently by briefly rinsing embryos in a drop of Acid Tyrode's solution. Embryos were prepared for immunofluorescence staining as described below. All animal protocols were conducted following the guidelines outlined in the Guide to the Care and Use of Experimental Animals, prepared by the Canadian Council on Animal Care.

Immunocytochemistry. Rat zygotes, sired by saline or cyclophosphamide-treated males, were manipulated in parallel using previously described indirect immunofluorescence techniques with minor modifications (Barton *et al.,* 2005). All embryo incubations were done at room temperature unless otherwise indicated; all washes were done in 0.05% Tween 20 in PBS for 5 min. Zygotes

were fixed in 4% paraformaldehyde for 15 min, washed, permeabilized in PBS containing 0.2% Triton X-100 for 30 min, rewashed thoroughly, and blocked overnight at 4°C. Embryos to be used for γH2AX detection were blocked in a solution of 10% goat serum (Vector Laboratories, Burlington, ON, Canada), 2% BSA and 0.05% Tween 20 in PBS; goat serum was omitted from the blocking solution for embryos to be processed for PARP-1 immunostaining. Embryos were incubated in primary antibody solution for 1 h, washed vigorously in a series of fresh blocking solution (1 X 10 min, 1 X 30 min and 1 X 10 min) and incubated for 1 h in secondary antibody; washes in blocking solution were repeated. Incubation with primary and secondary antibodies was done at 37°C or at room temperature for fluorescent detection of γH2AX or PARP-1, respectively. DNA was stained with 10 μg/ml DAPI for 30 min, embryos were washed and mounted in 3 μl of Vectashield antibleaching mounting medium (Vector Laboratories, Burlington, ON, Canda) on premarked slides.

Antibodies. Primary antibodies were mouse monoclonal IgG1 anti-γH2AX (phosho-Serine-139), clone JBW301 (1:500 dilution; catalog number 05-636, Upstate Biotechnology, Charlottesville, VA, USA) and mouse monoclonal IgG1 anti-Poly(ADP-Ribose) Polymerase-1, clone C-2-10 (1:200 dilution; catalog number AM30, Calbiochem, San Diego, CA, USA). Secondary antibodies used for immunofluorescence detection were sheep anti-mouse fluorescein conjugated-IgG (1:200 dilution; catalog number N1031, Amersham Pharmacia Biosciences, Baie d'Urfe, QC, Canada) and horse anti-mouse Texas Red IgG (1:200 dilution; catalog number TI-2000, Vector Laboratories, Burlington, ON, Canada), respectively. Nuclear staining specificity was confirmed by secondary antibody only incubations. The second polar body was found to be a useful internal staining control for γH2AX as it is highly stained during zygotic development.

Confocal Microscopy. Optical z-sections of individual embryos were recorded using a Zeiss LSM 510 Axiovert 100M confocal microscope equipped with a

Plan-Apochromat X63/1.4 oil DIC objective. Prior to extensive imaging, optimal conditions for laser scanning confocal microscopy were established experimentally; z-stacks were captured using identical parameter settings for all embryos. Fluorescein, Texas Red and DAPI fluorochromes were excited by 488 nm, 543 nm and 730 nm (2-photon) lasers, respectively; multitracking was used to allow sequential imaging of individual fluorescence emissions avoiding crosstalk between channels. Pinhole diameters were set at 0.84 and 1.00 Airy units, resulting in individual optical slices of 0.6 μm or 0.8 μm for γH2AX and PARP-1, respectively. Images were scanned at a speed of 6 (pixel time of 3.20 μsec) with an average of 2 scans per optical section, in 1024 X 1024 pixels for an optimal resolution of 0.14 x 0.14 μm pixel size. Each data stack was acquired at a zoom factor equal to one using detector gain settings of 1150 and 1050 for γH2AX and PARP-1, respectively. Photobleaching of fluorescent signals was avoided by scanning a single embryo within a specified boundary one time only. Digital images were collected and transferred into appropriate file formats for respective analytical imaging software. In accordance with our previous classification criteria (Barton *et al.*, 2005), zygotes were categorized qualitatively into five pronuclear stages (PN) based on interpronuclear positioning of parental genomes and morphological assessment of the paternal pronucleus.

Quantification of γH2AX. Image stacks generated by laser scanning confocal microscopy were imported into IMARIS® (Bitplane AG, Zurich, Switzerland), an automated imaging software for detailed processing of multi-dimensional images. γH2AX staining in male and female pronuclei was observed to be qualitatively different; the 3D reconstruction of each data set was cropped and parental pronuclei processed separately. γH2AX foci were quantified independently using IMARIS Spots and Isosurface measurement modules which provide a detailed comparative analysis of each distinct 3D focus. Prior to automated focus detection, image stacks were smoothed using a Gaussian filter; optimal threshold limits were set to eliminate insignificant background objects. Using the spots module, foci within the selected detection range (minimum diameter, 0.200 μm;

maximum diameter, 5.00 μm; threshold, 25) were modeled as spherical structures, each belonging to a spatial position along the x, y, and z-axis; the calculated sum number of foci per pronucleus was used for statistical analysis. γH2AX focal volume was assessed using the Isosurface module that creates a computer-generated representation of specified real volume objects within a data set; a threshold limit of 65 was used. Precise measurements and detailed statistical data derived from each object were exported into Excel-readable files for further categorization and statistical analysis.

Quantification of PARP-1. Single optical images representing characteristic staining patterns for each of the maternal and paternal pronuclei were imported as monochrome eight-bit TIFF files for quantitative analysis using MCID 7.0 imaging software (Imaging Research, St. Catherine's, ON, Canada). Optimal threshold limits for intensity and saturation were reserved across treatments. The density of PARP-1 immunofluorescence was measured as proportional grain area, defined as grain area/scan area. For each zygote, the perimeter of the male and female pronucleus was manually outlined to determine distinct scanning areas used for grain ratio calculations. Circles with a calibrated dimension of 20 μm X 20 μm, generating a scan area of 313.959 μm^2, were placed adjacent to each parental pronucleus to obtain background measures. Proportional grain area per pronucleus was adjusted to account for background immunofluorescence in all cases.

Statistical Analysis.

Student's t-tests or Mann-Whitney rank sum tests were used to compare the γH2AX parameters and PARP-1 proportional grain areas of zygotic pronuclei from control animals with those that were sired by males chronically exposed to cyclophosphamide ($P < 0.05$). Error bars represent the mean ± S.E.M. SIGMASTAT 2.03 software package (SPSS, Chicago, IL) was used for all statistical analyses.

RESULTS

Characterization of γH2AX immunoreactivity post-fertilization in the absence of genotoxic stress. Nucleosomal architecture and chromatin remodeling of the parental genomes are markedly different in the newly fertilized oocyte (Mclay and Clarke, 2003). Early post-fertilization (PN1 and PN2), the maternal chromatin was extensively stained with dense γH2AX fluorescence. The paternal genome displayed interconnecting amorphous clusters of various sizes throughout the chromatin at PN1; progression to PN2 resulted in the reorganization of γH2AX staining into multiple uniform punctate foci (Fig. 1a and b). At both pronuclear stages, γH2AX volume within the maternal chromatin was significantly greater than observed for the paternal genome (PN1, $P = < 0.001$; PN2, $P = 0.022$) (Fig. 2A). Remarkably, at PN3, the numbers of γH2AX foci were dramatically reduced in both parental pronuclei (Fig. 1c) in zygotes fertilized by control males; male and female pronuclei remained consistently depleted of foci through PN4 and 5 (Fig. 1d and e). γH2AX appears to play a prominent role in the complex process of chromatin remodeling of parental genomes immediately after fertilization, as well as having a possible role in the silencing of the maternal genome during G1 of the zygotic cell cycle.

Biphasic γH2AX Response in the Male Genome after Paternal Cyclophosphamide Exposure. After fertilization, at PN1, the male pronucleus of embryos sired by cyclophosphamide-exposed fathers displayed enhanced levels of γH2AX fluorescence (Fig. 1f); at PN2, staining was markedly reduced to small foci, comparable to those observed in zygotes fertilized by spermatozoa from control males (Fig. 1g). Interestingly, at PN3, γH2AX fluorescence re-emerged in the male pronucleus of zygotes fertilized by cyclophosphamide-exposed spermatozoa and continued to increase dramatically as zygotic development progressed through PN4 and 5 (Fig. 1h – j). Quantitative analysis confirmed the biphasic response of the paternal genome to DNA damage. The volume of γH2AX foci within the male pronuclei of embryos sired by cyclophosphamide-treated fathers was significantly increased at PN1 (P =

0.013), corresponding to phase 1, but it was not different from controls at PN2. This was followed by a progressive elevation through S-phase (PN3 and 4) into G2 (PN5). This second phase represents a selective response of the zygote to paternal transmission of DNA damage (Fig. 2A). The maternal genome of zygotes fertilized by cyclophosphamide exposed spermatozoa was not significantly different from controls (Fig. 1f – e and 2A). In conjunction with observations from embryos fertilized by control males, this demonstrates an initial requirement for H2AX phosphorylation regardless of the presence of paternal genomic damage and highlights the absence of a γH2AX response in the intact maternal pronucleus of zygotes sired by cyclophosphamide-treated males.

Number and Pronuclear Distribution of γH2AX Foci in Zygotes Fertilized by Cyclophosphamide-Exposed Spermatozoa. Quantitative analysis of spots further revealed the dramatic induction of γH2AX foci after paternal cyclophosphamide treatment. The number of γH2AX foci was significantly higher in paternal pronuclei of zygotes fertilized by cyclophosphamide exposed spermatozoa at PN1 ($P = 0.009$) and increased dramatically at PN3 ($P = <0.001$), PN4 ($P = <0.001$) and PN5 ($P = 0.018$), compared to corresponding controls. In maternal pronuclei of embryos sired by cyclophosphamide-exposed males, the numbers of γH2AX foci were consistently low throughout zygotic development, similar to both parental genomes in controls (Fig. 2B).

Two distinct γH2AX focal populations have been described in normal and irradiated mammalian cells (McManus and Hendzel, 2005). To determine whether paternally mediated DNA damage induced distinguishable populations of γH2AX foci in the zygote, we characterized the volume distribution of γH2AX foci in embryos sired by cyclophosphamide-treated and control males (Fig. 3). In parental pronuclei of zygotes fertilized by control males, the majority of γH2AX foci were classified as category 1 or 2, with focal volumes between 0.2 – 0.99 μm^3 or 1.0 – 4.99 μm^3, respectively. Interestingly, at PN1, male pronuclei in embryos fertilized by cyclophosphamide-treated spermatozoa displayed

significantly more foci in volume categories 1 (P = 0.014) and 3 (5.0 – 9.99 μm^3; P = 0.001); at PN3 the numbers of γH2AX foci in categories 2 (P = <0.001) and 3 (P = 0.002) were elevated, while at PN4, categories 1 (P=0.004), 2 (P = 0.001) and 3 (P = 0.016) were all increased significantly compared to control embryos at corresponding pronuclear stages. Preconceptional paternal cyclophosphamide treatment had no effect on the kinetics of γH2AX focal volumes in maternal pronuclei. These data demonstrate that DNA damage imparted to the embryo via the spermatozoon causes an increase in the population of larger γH2AX foci in addition to the accumulation of smaller foci.

To further characterize the γH2AX damage response in the zygote, we qualitatively assessed 3D focal distribution within the male and female pronuclei. As indicated above, the female chromatin at PN1 and 2 was highly stained by γH2AX (see Movie 1 and 2, supporting information); at PN3, 4 and 5, female pronuclear staining was dramatically reduced to a single focus located at the perimeter, occasionally accompanied by sparse small foci situated randomly throughout the chromatin (see Movie 3 and 4, supporting information). At PN2, γH2AX foci were dispersed evenly throughout the male pronuclei of zygotes sired by control and cyclophosphamide exposed fathers. However, in contrast to the small number of foci distributed intermittently throughout the paternal pronucleus of controls from PN3 through 5 (Movie 3), zygotes sired by cyclophosphamide-exposed males displayed γH2AX clusters concentrated in the perinucleolar regions as well as throughout the pronucleus (Movie 4). Therefore, γH2AX foci are distributed throughout the entire male pronucleus following paternal cyclophosphamide treatment.

Paternal Cyclophosphamide Exposure Activates Poly(ADP-ribosyl)ation during Zygotic Development. We investigated PARP-1 induction in the zygote following chronic paternal cyclophosphamide exposure to determine the competence of the early embryo to activate signaling networks with known involvement in the repair of DNA lesions. Immediately after fertilization, both haploid pronuclei displayed minimal PARP-1 immunofluorescence staining in

embryos sired by saline and cyclophosphamide-exposed males (Fig. 4a and e). In controls, male and female pronuclei maintained baseline levels of staining from PN3 through PN5 (Fig. 4b - d). Intriguingly, in zygotes sired by cyclophosphamide-treated fathers, PARP-1 immunofluorescence was dramatically elevated at PN3 in both parental genomes and remained intense through zygotic development, coincident with the second phase of γH2AX foci induction (Fig. 4f - h). Furthermore, a large proportion of male and female pronuclei in zygotes at PN3 to 5 displayed dense PARP-1 staining in perinucleolar regions (Fig. 4f - h), compared to controls. Quantitative analysis confirmed that PARP-1 proportional grain areas of male and female pronuclei were significantly increased at PN3 ($P = < 0.001$) and 4 ($P \leq 0.005$) and maintained a hyperactivated state at PN5, compared to controls (Fig. 5). Thus, fertilization by spermatozoa chronically exposed to a genotoxic agent rapidly triggered DNA damage response pathways in both parental genomes, further emphasizing the importance of zygotic pronuclear cross talk in the maintenance of genomic stability.

DISCUSSION

The phosphorylation of H2AX has specialized physiological functions beyond DNA damage detection and repair in the zygote. Prior to fertilization, the metaphase II chromosomes of the dormant oocyte are extensively labeled with γH2AX (Derijck *et al.,* 2006). Fertilization initiates a multi-stage process of chromatin remodeling of both gametes; oocyte chromatin with meiotic features is transformed to strictly mitotic chromatin and highly compacted sperm chromatin is decondensed to produce a transcriptionally competent paternal genome (Mclay and Clarke, 2003). Interestingly, DNA-damage independent γH2AX foci have been described in mitotic cells (McManus and Hendzel, 2005), confirming a biological role for γH2AX in meiotic and mitotic chromosomes. During G1 (PN1-2) of the first cell cycle, inactive maternal chromatin, that is poorly accessible to transcription factors (Spinaci, M. *et al.,* 2004), was highly stained (Fig. 1, Derijck *et al.,* 2006); paternal chromatin undergoing protamine-histone exchange and

elaborate remodeling displayed large amorphous clusters in PN1 that were rapidly reduced to punctate foci in PN2. Intriguingly, γH2AX was remarkably depleted in both pronuclei at S-phase (Fig. 1), corresponding to the onset of replication and transcription in the 1-cell embryo; DNA synthesis in the male pronucleus precedes that in the female (Ferreira and CarmoFonseca, 1997). We propose that H2AX phosphorylation is involved in the control of chromatin structural reorganization, ensuring proper temporal execution of early zygotic events.

Exogenous genotoxic insults induce phosphorylation of H2AX over megabases of chromatin, tethering each double strand break (Kruhlak *et al.*, 2006). Alterations in spermatozoal genomic integrity, acquired during 4 weeks of preconceptional exposure to cyclophosphamide, were detected effectively in the early embryo. Intriguingly, γH2AX emerged in two distinct phases in the paternal genome of zygotes sired by cyclophosphamide-treated males, each corresponding to important regulatory processes of the first zygotic cell cycle. The initial spike of γH2AX (Fig. 2A) occurred in the recondensing male pronucleus (PN1) during nucleoprotein exchange (Mclay and Clarke, 2003). The replacement of protamines by histones is a phosphorylation sensitive process, emphasizing a dual role for γH2AX in chromatin remodeling and DNA damage recognition in the early male pronucleus. Zygotes sired by cyclophosphamide-exposed males were hyperacetylated at histone H4 as early as G1 of the first cell cycle (Barton *et al.*, 2005). Histone acetylation regulates H2AX phosphorylation (Park *et al.*, 2003) which is required for the recruitment of additional histone acetyl-transferase complexes to sites of DNA double strand breaks (Murr *et al.*, 2006). Alkylation of sperm DNA and nuclear proteins by cyclophosphamide may distort nuclear compaction, thereby generating an abnormally permissive chromatin conformation, permitting enhanced accessibility to chromatin modifying proteins and DNA repair factors. Immediately after fertilization, histone hyperacetylation and H2AX phosphorylation may cooperate to facilitate chromatin reconfiguration at lesion sites in zygotes with paternal DNA damage, thereby triggering a DNA damage histone code that mediates events

implemental for DNA damage resolution beginning in S-phase (Murr *et al.,* 2006; Park *et al.,* 2003).

DNA double strand breaks initiate local chromatin expansion independent of γH2AX. However, consistent with our previous observation of increased chromatin dispersion from PN3 through 5 in zygotes fertilized by cyclophosphamide-exposed spermatozoa (Barton *et al.,* 2005), γH2AX may be required for sustained chromatin relaxation, thus augmenting accessibility at DNA damage sites (Kruhlak *et al.,* 2006) in the male pronucleus during the second γH2AX induction phase (Fig. 2A). Cyclophosphamide-induced hyperacetylation and hypomethylation of the male pronucleus further supports the existence of a highly permissive chromatin state (Barton *et al.,* 2005). γH2AX is dispensable for the initial recruitment of signaling and DNA repair proteins; however, repair factor concentration, assembly and stabilization at double strand break sites is dependent on H2AX phosphorylation (Celeste *et al.,* 2003). The striking increase in γH2AX focal volume and numbers (Fig. 2A and 2B) in PN3 to 5 in response to paternal cyclophosphamide exposure may function to recruit and maintain the localization of numerous repair factors to the vicinity of the damage, assisting in the processing of the DNA lesions. The accumulation of a higher concentration of repair proteins may further affect chromatin structure and alleviate transcriptional repression during the repair process.

Large-scale genomic repositioning does not occur as a result of DNA double strand breaks; therefore, an increasing focal size has been suggested to correlate with γH2AX spreading over a large chromatin domain, providing additional binding sites for DNA damage response proteins (Kruhlak *et al.,* 2006). Zygotes fertilized by spermatozoa chronically exposed to cyclophosphamide displayed an increased number of larger foci, beginning in S-phase through G2 (Fig. 3). These late-arising larger foci may represent complex lesion repair and/or the persistence of residual unrepaired DNA damage (Hamada *et al.*, 2006). Interestingly, γH2AX foci were clustered throughout the male pronucleus following preconceptional paternal drug exposure, lending further support to the

proposition that DNA damage can be recognized and repaired in any region of the chromatin (Bewersdorf *et al.*, 2006).

Constitutive poly(ADP-ribosyl)ation is required for competent developmental progression of the preimplantation embryo and is proposed to act as a facilitator of chromatin remodeling in conjunction with other epigenetic modifications; specifically, poly(ADP-ribosyl)ation antagonizes DNA methylation (de Murcia *et al.,* 2003; Imamura *et al.,* 2004). In response to genotoxic stress, PARP-1 catalytic activity may increase 500-fold on binding to DNA strand breaks, playing a key role in the temporal and spatial organization of their repair (Schreiber *et al.,* 2006). PARP-1 is essential for recovery from DNA damage by alkylating agents (Demurcia *et al.*, 1997); inhibition of PARP-1 in oocytes impacts negatively on embryo survival (Imamura *et al.,* 2004). PARP-1 staining in zygotes sired by cyclophosphamide-exposed males increased dramatically from PN3 through 5 (Fig. 4), corresponding to the second surge of progressive γH2AX induction. PARP-1 associates with γH2AX following irradiation (Du *et al.*, 2006), suggesting the cooperation of distinct posttranslational modifications in the activation of DNA damage response pathways that are required for strand break resolution in the early embryo. Accordingly, in response to paternally transmitted DNA damage, pronuclear PARP-1 activation may act as a catalyst for chromatin relaxation (Realini and Althaus, 1992) in early S-phase, permit enhanced cofactor access and subsequently recruit single strand break and base excision repair proteins to the site of chromosomal damage (Schreiber *et al.,* 2006).

Epigenetic programming that is crucial for embryogenesis is disrupted in both parental pronuclei following paternal cyclophosphamide exposure (Barton *et al.,* 2005). Intriguingly, the fascinating phenomenon of pronuclear cross talk was also evident in the epigenetic control of DNA-damage signaling; PARP-1 was hyperactivated in the maternal, as well as the paternal pronucleus, in response to interruptions in spermatozoal genomic integrity (Fig. 5). The damage-free maternal genome has the ability to activate a p53-dependent S-phase DNA damage checkpoint upon fertilization by irradiated sperm; DNA synthesis was suppressed (Shimura *et al.*, 2002). The spatial localization of PARP-1

immunofluorescence in parental pronuclei of zygotes sired by drug-treated males reflects the perinucleolar and intranuclear S-phase replication patterns of the first cell cycle (Ferreira and CarmoFonseca, 1997), indicating the potential involvement of analogous chromatin regions and/or proteins during DNA repair. Thus, we provide evidence that spermatozoal genomic instabilities destabilize the maternal genome, necessitating a collaborative activation of DNA damage pathways in both maternal and paternal pronuclei. The widespread drug-induced damage imparted to the embryo via an altered paternal genome, if left unrepaired, is likely to be detrimental to the development and survival of the embryo postimplantation.

Thus, cyclophosphamide-induced DNA-damage in the spermatozoal genome initiates distinct yet inter-related mechanisms for the detection and signaling of male-mediated genomic lesions. The enhanced presence of γH2AX and PARP-1 at G2 in embryos sired by males exposed to a genotoxicant indicates that the resolution of paternally mediated DNA damage is incomplete prior to the first cleavage division; sophisticated mechanisms for the complete resection of DNA lesions are required in later cleavage stages to ensure the faithful transmission of genomic sequence information. Inefficient repair and/or aberrant epigenetic programming of damaged sites may lead to persistent modifications of the histone code, leading to heritable mutations, chromosomal aberrations and diseases such as cancer in the offspring and subsequent generations.

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FIGURES

Figure 5.1. Histone H2AX phosphorylation in rat zygotes fertilized by cyclophosphamide-exposed spermatozoa. Embryos were labeled by indirect immunofluorescence using an antibody to γH2AX (Ser 139) (green); DNA was counterstained with DAPI (blue). Paternal pronuclei display dramatic γH2AX induction in zygotes fertilized by drug-exposed spermatozoa (f – j) compared to controls (a – e). M, male pronucleus; F, female pronucleus; PB, polar body. (Scale Bar: 20 μm.)

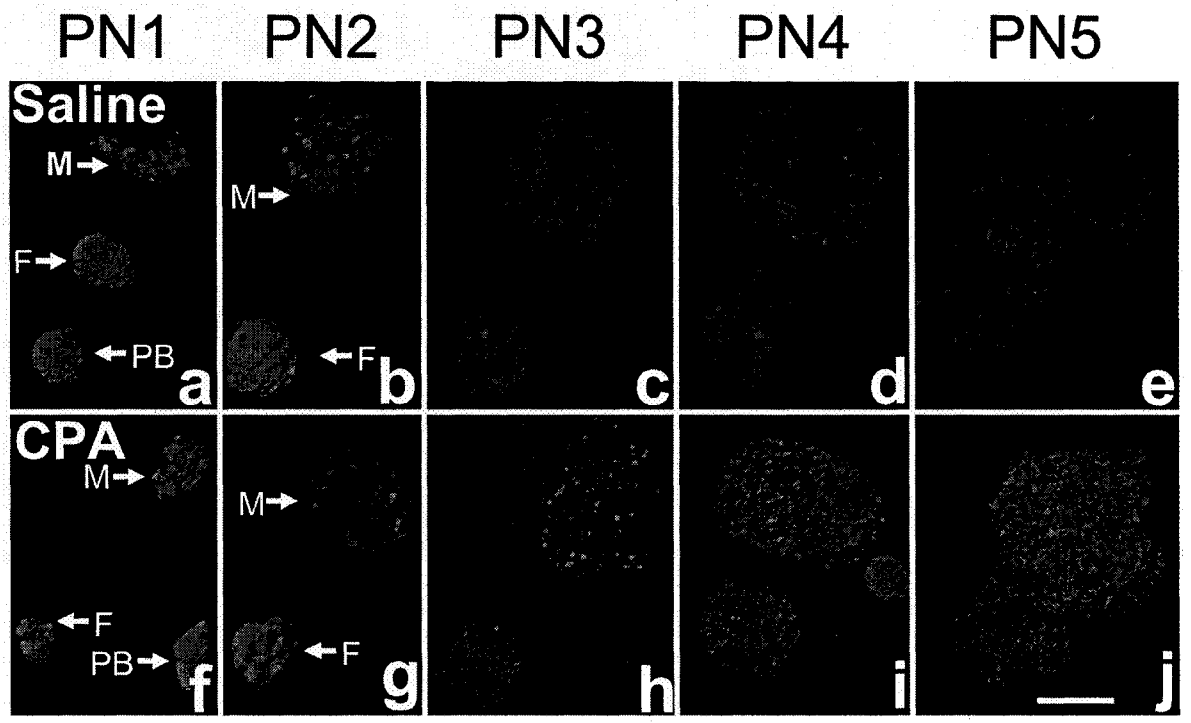


Figure 5. 2. (A) The focal volumes of γH2AX were significantly increased in a biphasic pattern in paternal pronuclei of zygotes sired by males chronically exposed to cyclophosphamide. (B) The numbers of γH2AX foci were significantly elevated in paternal pronuclei of zygotes fertilized by cyclophosphamide-exposed males. The number of embryos analyzed at each pronuclear stage was as follows: saline, n = 5, 11, 37, 28, 5 for PN1, 2, 3, 4, 5, respectively; cyclophosphamide, n = 7, 7, 43, 23, 7 for PN1, 2, 3, 4, 5, respectively. Crosshatched bars, saline male pronucleus; black bars, cyclophosphamide male pronucleus; white bars saline female pronucleus; gray bars, cyclophosphamide female pronucleus. *, P < 0.02, **, P < 0.001.

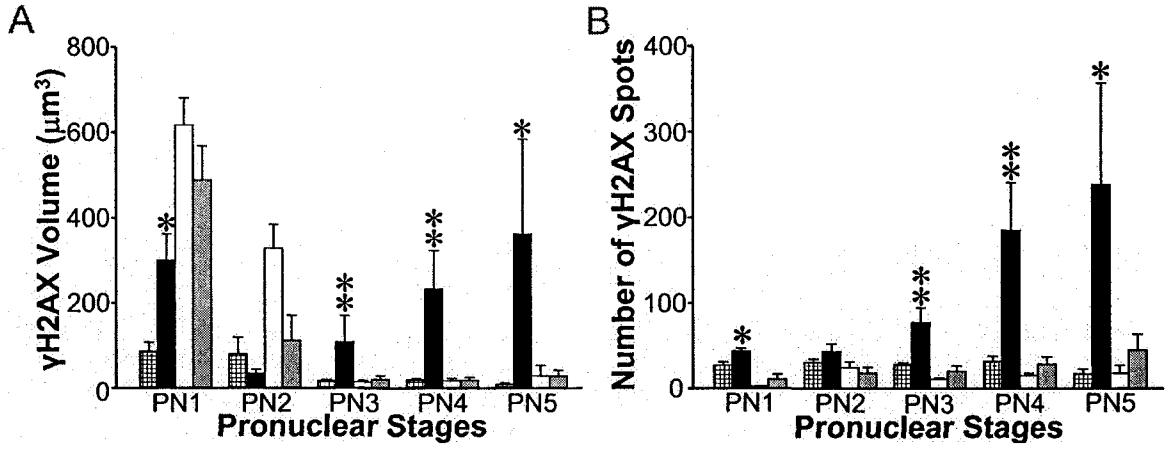


Figure 5.3. The focal volume categories 1 and 2 of γH2AX account for the majority of foci induction in parental pronuclei of zygotes fertilized by saline or cyclophosphamide-exposed males; populations of larger γH2AX foci were increased following paternal cyclophosphamide exposure. Stacked bar graph demonstrates the proportional contribution of each focal volume size category. Gray bars, category 1, 0.2 – 0.99 μm^3; speckled bars, category 2, 1.0 – 4.99 μm^3; white bars, category 3, 5.0 – 9.99 μm^3; black bars, category 4, >10.0 μm^3.

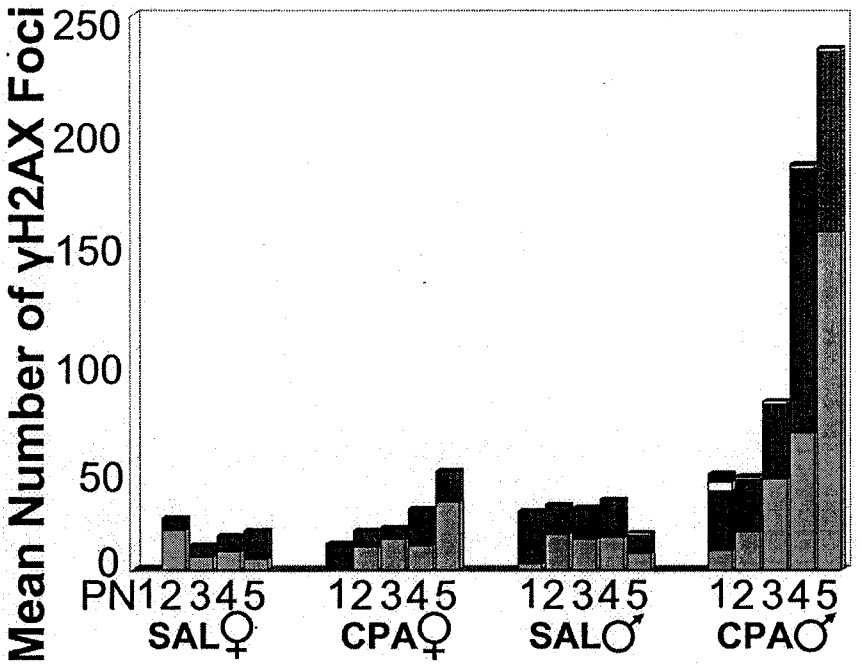


Figure 5.4. PARP-1 activation is initiated in S-phase in paternal and maternal pronuclei following cyclophosphamide-induced DNA damage of the spermatozoal genome. Embryos were labeled by indirect immunofluorescence using an antibody to poly(ADP-ribose) polymerase-1 (red); DNA was counterstained with DAPI (blue). Both parental pronuclei display prominent PARP-1 immunofluorescence in zygotes fertilized by drug-exposed spermatozoa (f – h) compared to controls (b – d). M, male pronucleus; F, female pronucleus; PB, polar body. (Scale Bar: 20 μm.)

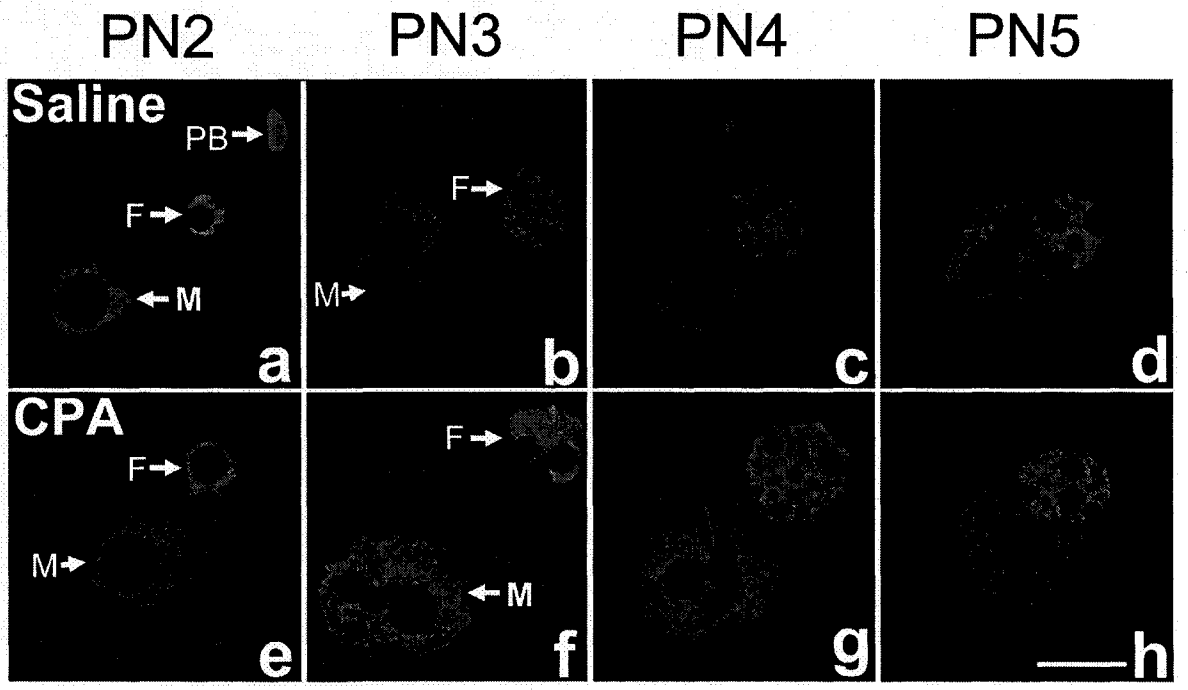
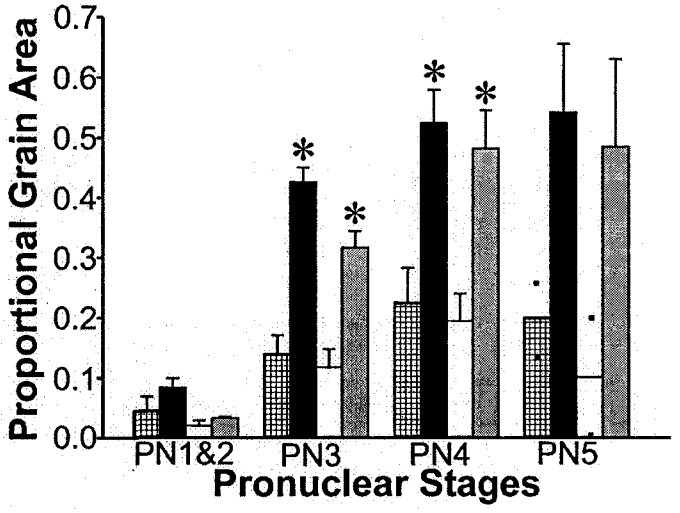


Figure 5. 5. Quantitative analysis of PARP-1 proportional grain area. Maternal and paternal pronuclear PARP-1 staining was significantly increased at PN3 and 4, and remained hyperactivated at PN5 in zygotes sired by cyclophosphamide-treated rats. The number of embryos analyzed at each pronuclear stage was as follows: saline, n = 10, 16, 11, 2 for PN1 & 2, 3, 4, 5, respectively; cyclophosphamide, n = 3, 28, 11, 3 for PN1 & 2, 3, 4, 5, respectively. Crosshatched bars, saline male pronucleus; black bars, cyclophosphamide male pronucleus; white bars saline female pronucleus; gray bars, cyclophosphamide female pronucleus. *, $P < 0.01$.



CHAPTER VI

DISCUSSION

DISCUSSION

The increasing awareness that the altered integrity of the genome of spermatozoa significantly contributes to infertility and progeny outcome has prompted the development of increasingly stringent approaches to assess semen quality. The World Health Organization (WHO) semen analysis parameters include measures of sperm concentration, motility and morphology (WHO, 1999); however, subtle sperm defects and/or genomic disturbances are undetectable via standard assessment criteria. Various techniques beyond the scope of WHO guidelines are available to investigate spermatozoal DNA integrity, chromatin structure and chromosomal abnormalities (Agarwal and Allamaneni, 2005). It is envisioned that detailed assessment of DNA damage will provide enhanced reliability for predicting pregnancy outcome; this is particularly important in the era of assisted reproductive technologies (Morris et al., 2002; Tomsu et al., 2002; Seli et al., 2004).

Evidence from animal studies (Marchetti et al., 1999) and the zona-free hamster penetration test using human spermatozoa (Ahmadi and Ng, 1999) clearly indicate that extensive DNA damage does not limit fertilization capacity; therefore, defects in the genomic material may be passed on to the offspring, compromising embryogenesis and, potentially, postnatal child health and development. Cyclophosphamide is an excellent model of a therapeutic exposure with demonstrated action as a male-mediated developmental toxicant; pregnancy loss and developmental defects occur in a manner that is dependent on whether pre- or post-meiotic germ cells were first exposed to the drug (Trasler et al., 1987). Of particular interest was the discovery that the effects induced by preconceptional paternal cyclophosphamide exposure persisted for multiple generations (Hales et al., 1992). The array of adverse effects following paternal cyclophosphamide exposure suggests that defects arise at different levels within the spermatozoal genome; genetic alterations and disturbances of the epigenome may both contribute to heritable instabilities. The studies in this thesis have assessed the impact of drug exposure on spermatozoal function, genetic integrity and the fascinating consequences of altered spermatozoal

genomic integrity on epigenetic reprogramming and the initiation of DNA damage responses in the zygote. In this final chapter, the significance of this work will be discussed and the implications and relevance in the field of reproductive medicine will be further highlighted.

6.1 Spermatozoal Quality: Numerical Chromosomal Aberrations

Cyclophosphamide exposure in male rats induces single strand breaks and cross-links in spermatozoal DNA; the template function of spermatozoal nuclei was altered also (Qiu et al., 1995). If fact, using the comet assay, it was established that DNA damage accumulation is greatest following spermiogenic exposure (Codrington et al., 2004). The results using CASA clearly demonstrate that in the presence of nuclear damage, spermatozoa retain their overall functional capacity. Although, in several instances, toxicant exposure leads to altered spermatozoal concentration and disrupted motility, exposure to the current alkylating agent provides a clear example in which basic quality measures are unaffected. The finding that cyclophosphamide exposed spermatozoa are generally motile, in spite of the documented nuclear damage, further underscores the potential limitations of standard functional assessment if not accompanied by more extensive chromatin evaluation.

An important function of the spermatozoon is the delivery of an intact paternal genome to the oocyte. Therefore, if the spermatozoon is functionally capable of transmitting the paternal DNA following toxicant exposure, there is great interest in evaluating the genetic factors that may influence the reproductive outcome. Fluorescence *in situ* hybridization (FISH) in spermatozoa is a robust test that extends beyond the characterization of general chromatin integrity and allows for molecular cytogenetic analysis of large numbers of spermatozoa per individual. Rat sperm FISH analysis, employing DNA probes for chromosomes Y and 4, detected a significant increase in the frequency of chromosome 4 disomy and nullisomy following 9 weeks of chronic cyclophosphamide administration, for the first time illustrating the aneugenic

potential of this alkylating agent in a chromosome specific manner in rat spermatozoa.

The technological state of the rat sperm FISH assay is currently restricted to two probes. Extending the present findings over the genome, it is proposed that a marked elevation in the overall incidence of aneuploidy induction would be observed following drug exposure. Differences in the susceptibility of different chromosomes to non-disjunction and aneuploidy induction have been obtained from preimplantation embryos; the most common aneuploidies were for chromosomes 22, 16, 21 and 15 (Munne et al., 2004). Correspondingly, a prevalence of trisomy 22 and an increased frequency of numerical anomalies for chromosomes 21, 16 and gonosomes have been demonstrated in human sperm; evidence suggests a similar aneuploidy rate for remaining chromosomes (Spriggs et al., 1996; Shi and Martin, 2000; Egozcue et al., 1997; Guttenbach et al., 1997). Chromosomal features that may confer increased susceptibility to meiotic errors of disjunction include shorter length, centromere position, pericentromeric and repetitive sequences, recombination patterns and general chromatin characteristics (Warburton and Kinney, 1996).

Human chromosomes can be classified into seven groups, A to G, based on length and centromere position to assess aneuploidy distribution among chromosomes. Of particular interest, aneuploidies in groups A and B, which consist of chromosomes 1, 2, 3 and 4, 5, respectively, were less frequent than expected (Pellestor et al., 2002). Accordingly, rat chromosome 4, being a comparatively large chromosome (Hamta et al., 2006) may also be less susceptible to aneuploidy induction following genotoxicant exposure. Multicolour FISH using probes for chromosomes X, Y and 8 offers further evidence for toxicant induced dose dependent differential susceptibility of chromosomal aberrations in male mouse germ cells following exposure to the widely used acetylcholineesterase inhibitor, trichlorofon (Sun et al., 2000). Whether germ cell toxicants act preferentially on chromosomes that display an inherent susceptibility to non-disjunction is unknown. The development of DNA probes specific for additional rat chromosomes would indisputably increase the power of

identifying aneugenic potential of various exposures. The ability to identify all sex chromosome anomalies would be clinically relevant in assessing paternal contributions to aneuploidy; production of a probe specific for the X chromosome would provide valuable information. Furthermore, an enhanced ability to simultaneously assess spermatozoal numerical abnormalities and structural chromosomal aberrations in the rat, as has been established for human spermatozoa (Sloter et al., 2000), would improve the detection of transmissible genetic damage following paternal exposures.

Spermatogenic phase-specific cyclophosphamide administration established that pre-meiotic germ cells were most susceptible to aneuploidy induction; initially targeting spermatogonia, but not pachytene spermatocytes, increased the incidence of numerical chromosomal anomalies in epididymal spermatozoa. The precise molecular mechanisms responsible for cyclophosphamide induced aneuploidy remain elusive; however, the expression of several genes with important roles in meiosis is altered by chronic treatment (Aguilar-Mahecha et al., 2002). The ability of cyclophosphamide to alkylate proteins suggests that chromosomal structures and functions critical for proper synaptonemal complex formation and recombination may be damaged. Identification of disturbances in regulatory proteins that are involved in synapsis (SCP1, SCP2, SCP3), recombination (RAD50-MRE11-NBS1, RAD51, DMC1, MLH1) and DNA repair during meiosis (RPA, BRCA1, BRCA2) (Cohen and Pollard, 2001) will help to elucidate the impact of chronic cyclophosphamide exposure on the molecular events required for accurate meiotic progression.

Although numerical chromosomal abnormalities in spermatozoa were significantly increased compared to controls following cyclophosphamide exposure, it is expected that genetic aberrations alone, are not responsible for the various developmental deficits observed following chronic paternal cyclophosphamide treatment. Thus, we proposed that epigenetic alterations may also contribute to the adverse developmental outcomes. A clever analogy by Gosden and Feinberg describes epigenetic information as a penciled in code

surrounding the primary DNA sequence; any 'smudge' in the pencil may alter normal gene activity (Gosden and Feinberg, 2007). Modifications of the epigenome via paternal cyclophosphamide exposure could also lead to incompetent embryogenesis and heritable disease.

6.2 Importance of Paternal Contributions for Developmental Success: Sperm is more than just a shuttle for DNA.

The genome undergoes major chromatin reorganization, transitions in epigenetic information, and paternal-specific marking of imprinted genes to produce a highly unique heterogeneous sperm nucleus which forms the basis of male-specific epigenetic inheritance (Rousseaux et al., 2005). The manner by which exogenous insults may disturb critical events during male germ cell development, thus, leading to aberrant paternal epigenetic information to be transmitted to the offspring is largely unknown. Results from the experiments presented in this thesis clearly demonstrate that preconceptional paternal cyclophosphamide exposure affects important chromatin components during spermiogenesis that are required for the establishment and/or regulation of accurate epigenetic events post fertilization. To our knowledge, this is the first demonstration that paternal exposure to any drug dysregulates epigenetic programming in preimplantation embryos.

6.2.1 Paternal Transmission of Epigenomic Marks

It is generally accepted that during spermiogenesis, the nucleosome-based chromatin structure is transformed into the sperm-specific chromatin structure by the removal of most of the histones from the genome and subsequent replacement by protamines. How then is it plausible that histone modifications can be transmitted via the male germ line to the offspring? Evidence from human and mouse indicates that nucleohistone domains remain in mature spermatozoa (Gatewood et al., 1987; Pittoggi et al., 1999); human sperm nuclei maintain about 15% of the histone complement, while in mouse

sperm a lesser percentage of the genomic DNA remains associated with histones (Balhorn et al., 1987; Kourmouli et al., 2004). Intriguingly, a recent study demonstrated that nucleosomes containing specific acetylated histone modifications are in fact retained in the chromocenter of elongating spermatids; remarkably, these epigenetic marks were found to be transmitted by the spermatozoon to the zygote (van der Heijden et al., 2006).

A wave of hyperacetylation in elongating spermatids is associated with the histone-to-protamine exchange (Hazzouri et al., 2000); acetylation weakens the DNA-nucleosome and nucleosome-nucleosome interactions facilitating a more open chromatin state. Therefore, it is postulated, that if hyperacetylation promotes nucleosome disassembly, reduced acetylation levels and a more stable chromatin structure would encourage nucleosome retention during spermiogenesis (van der Heijden et al., 2006).

The crosslinking capacity of cyclophosphamide may result in the aberrant joining of DNA interstrands and/or DNA to proteins, inducing atypical conformational changes of proteins associated with particular interactions; consequently, the wave of acetylation may be altered. Alternatively, as it was noted that hypoacetylation alone is insufficient for nucleosome retention in elongating spermatids (van der Heijden et al., 2006), the chromosomal organization and stability may be altered by DNA strand breaks, alkylation and crosslinking, preventing proper removal of nucleosomes. Notably, using 2D basic gel electrophoresis, histones were also identified in rat epididymal spermatozoa, suggesting the presence of residual nuclear proteins. Further experiments are necessary to distinguish nuclear histones from histones of the perinuclear theca (Codrington et al., 2007). This finding leads me to speculate that selected histone modifications induced as a result of toxicant exposure may be directly transmitted in the paternal genome to the oocyte in rats, as has been demonstrated in mice. Nucleosome retention in spermatozoa and direct transmission of altered epigenetic marks to the offspring following cyclophosphamide exposure remains to be elucidated.

Paternal chromatin remodeling is complete soon after fertilization and nucleosomal chromatin is reestablished for further participation in zygotic reprogramming. Altered histone marks could, therefore, immediately affect the coordinated integration of the paternal contribution with maternal processing. Intriguingly, parental pronuclei were hyperacetylated as early as G1 in zygotes fertilized by cyclophosphamide exposed spermatozoa compared to controls. The finding that histone H4 acetylation at lysines 8 and 12, but not at lysines 5 and 16, was paternally transmitted (van der Heijden et al., 2006) implies that not all modifications are directly inherited; however, as our results demonstrate, the state of the paternal genome is directly related to the precision of epigenetic programming.

Cyclophosphamide exposure during the critical events of spermiogenesis may interfere with the dynamic acquisition of epigenetic marks by a number of non-mutually exclusive mechanisms: (1) for each modification there exists a cohort of enzymes responsible for depositing or removing the correct mark; drug induced alterations in the enzymes and/or their respective encoding genes would compromise the epigenetic code, (2) modifications of proteins that recognize and bind to epigenetic marks would promote aberrant regulation, (3) genetic damage inducing vast structural change in the spermatozoal genome could affect the ability of epigenetic factors to accurately interact with the DNA, (4) genetic damage accumulated during spermatogenesis may promote dysregulation of epigenetic reprogramming post fertilization via altered susceptibility to maternal factors and subsequent post-translational modifications of histone tails (Spotswood and Turner, 2002). Evidence is lacking in support of any one particular mechanism as it is extremely difficult to discern the genetic-epigenetic interplay in the male-mediated developmental toxicity of the current drug.

Cyclophosphamide altered male germ cell expression of various genes involved in DNA repair and post-translational modifications (Aguilar-Mahecha et al., 2002). Furthermore, DNA damage incurred in the male genome following chronic drug exposure is transmitted to the zygote and is also associated with altered expression profiles of DNA repair genes during early preimplantation

development (Harrouk et al., 2000a). Together, these studies strongly favor the existence of an intricate relationship between the accumulated genetic damage in the paternal genome and the observed epigenetic dysregulation. As the principle epigenetic regulatory mechanism for the establishment of gene expression patterns is through chromatin alterations and remodeling (Spotswood and Turner, 2002), the study of histone-to-protamine exchange and the status of epigenetic modifications (acetylation, methylation and phosphorylation) during spermiogenesis following cyclophosphamide exposure would be useful to elucidate the direct contribution of paternal histone modifications to altered zygotic regulation.

Another level of the epigenetic code comprises DNA methylation. The establishment of DNA methylation patterns in the embryo has been proposed to be the key component for setting up the structural profile of the genome (Hashimshony et al., 2003). Interestingly, DNA methylation levels and histone modifications are functionally interrelated; disruptions in histone modifications or histone-DNA interactions dramatically alter DNA methylation (Li, 2002). In fact, in the paternal genome of the zygote, DNA methylation is significantly altered by trichostatin A (TSA) (an inhibitor of histone deacetylase), highlighting the contribution of acetylation at specific histone residues to methylation patterns in the zygote.

Accordingly, male pronuclei in zygotes fertilized by spermatozoa exposed to cyclophosphamide prior to conception were dramatically hypomethylated, while female pronuclei were hypermethylated compared to controls, by mid-zygotic development succeeding the hyperacetylated state observed in G1. Embryos treated *in vitro* with TSA are also hyperacetylated compared to controls. Transient perturbation of acetylation patterns did not impede preimplantation development; early cleavage stage embryos were suggested to compensate for temporary deviations in acetylation levels. Conversely, prolonged culture of cleavage stage embryos in the presence of TSA blocked development at the one- or two-cell stage (Adenot et al., 1997). Thus, it can be postulated, that *in vitro* culture in TSA of zygotes fertilized by cyclophosphamide-treated males

would exacerbate the excessively active chromatin state of the paternal pronucleus, and induce further activation in the maternal genome. Together, this would lead to an overall precocious alleviation of transcriptional repression, further disrupting the gene expression profile that is required for competent development; early preimplantation embryo loss would likely ensue. The importance of the transcriptionally repressive state during preimplantation development has been demonstrated by Ma and colleagues; the two- to four-cell cleavage is inhibited by continuous TSA induced histone hyperacetylation (Ma et al., 2001). The findings of our studies reinforce the idea that disruptions to the stability of the paternal chromatin influence developmental programming through interactions with various epigenetic components of the genome.

Promptly following fertilization, active demethylation of the male pronucleus establishes parental asymmetry in the zygote. This has traditionally been thought to be required for the erasure of gametic epigenetic information such that *de novo* embryo specific patterns may be reset. The need for paternal specific active demethylation has been proposed as a mechanism for the general alleviation of repression of paternal alleles for the onset of zygotic transcription (Santos and Dean, 2004). The importance for accurate DNA methylation status has been demonstrated most conclusively by the lack of developmental success in embryo cloning; abnormal DNA methylation patterns contribute to poor quality embryos (Yang et al., 2007; Shi et al., 2004; Bourc'his et al., 2001; Santos et al., 2003; Kang et al., 2001).

Most interestingly, the paternal genome in zygotes obtained from round spermatid injection (ROSI) was highly remethylated prior to the first mitosis, indicating that spermiogenesis is crucial for acquisition of the ability to regulate DNA methylation in the paternal genome. Discordant chromatin architectures between immature and mature germ cell genomes are suspected to produce the clear difference in DNA methylation of the paternal zygotic pronucleus. Specifically, histone modifications are implicated in the maintenance of DNA methylation patterns in zygotes fertilized by mature spermatozoa (Kishigami et al., 2006). Accordingly, Santos and colleagues proposed a model suggesting

that the differential modifications in parental pronuclei are regulated by the presence or absence of specific histone deacetylases and histone methyltransferases and specifically, their substrate availability (Santos et al., 2005). In further support of this model, DNA methyltransferase expression was also defective in cloned mouse embryos (Chung et al., 2003). As such, cyclophosphamide exposure during spermiogenesis may alter nuclear conformation, which upon fertilization, is aberrantly permissive to chromatin regulating complexes, conferring premature and pronounced signals for atypical pronuclear acetylation and DNA methylation. Coordinated timing of events in relation to the zygotic clock is essential for successful development; precocious modulation of chromatin may severely impinge on the competence of cellular differentiation and the setting of heritable epigenetic information.

Expanding knowledge of epigenetic programming during gametogenesis and early embryogenesis has revealed that not all epigenetic marks are erased between generations; paternal imprint marks are resistant to genome demethylation in the early preimplantation embryo. DNA methylation, histone modifications and chromatin packaging are thought to be the key epigenetic mediators of imprinted gene expression. Therefore, observed changes in DNA methylation, histone acetylation and perturbed chromatin structure following paternal cyclophosphamide exposure imply the possibility of alterations at the level of paternally inherited alleles, specifically, modifications of paternal imprints.

6.2.2 Genomic Imprinting

The establishment of paternal-specific methylation imprints during spermatogenesis predominantly occurs from spermatogonial stem cells through to meiotically dividing spermatocytes (Davis et al., 1999; Kerjean et al., 2000; Trasler, 2006). Methylated imprint control regions are marked by various combinations of histone modifications during spermatogenesis that may also be vulnerable to disruption by genotoxic agents leading to aberrant genomic imprints. These epigenetic marks are likely to be important for chromatin assembly during spermiogenesis and may influence post fertilization events

(Delaval et al., 2007). Drug-induced errors in imprint expression in the male germ line could lead to activation of the normally silent allele, due to paternal imprint relaxation, or to repression of the normally active allele, from failure to appropriately mark the paternal allele for expression (Trasler and Doerksen, 1999). Limited information is available relating the direct effect of paternal exposures to disruptions in genomic imprinting.

The derivation of diploid embryos containing same sex genomes, specifically, gynogenetic embryos (two female genomes) and androgenetic embryos (two male genomes), elegantly demonstrated the significant contribution of the paternal genome for normal development. Unbalanced imprinted gene expression in gynogenetic embryos leads to deficient placental development but relatively normal embryonic development. Conversely, placentation is normal in androgenetic embryos but development of the embryo proper is abnormal (Barton et al., 1984; Mcgrath and Solter, 1984; Surani et al., 1984). These findings clearly demonstrate that paternally linked epigenetic information is transmitted to the embryo and is dynamically involved in normal development, albeit with an emphasis on healthy placentation.

Interestingly, chronic paternal cyclophosphamide exposure has lineage-specific lethal effects on inner cell mass (ICM) cells as opposed to effects on extraembryonic tissues (Kelly et al., 1992). Therefore, the paternal genome is unmistakably crucial for proper development of both the ICM and trophectoderm (TE) cell lineages. That the ICM and TE cells are asymmetrically methylated by the blastocyst stage suggests that gene expression in the differentiated lineages is differentially susceptible to the level of DNA methylation; ICM cells are hypermethylated in comparison to the hypomethylated cells of the TE (Dean et al., 2005). The hypomethylated state of the male pronucleus in zygotes following paternal cyclophosphamide exposure may interfere with the ability of the ICM cells to acquire the hypermethylated state necessary for continued development. Trophectoderm cells, that give rise to the placenta and extraembryonic tissues, remain hypomethylated and may not be influenced by disrupted patterns of DNA methylation. In fact, control by differentially methylated regions, that typically are

essential for expression or repression of imprinted genes, are not required for allelic marking of several imprinted genes in the placenta (Lewis et al., 2004). Therefore, disruptions in the methylation status of the paternal genome may result in a greater degree of adverse effects on the embryo proper due to abnormal epigenetic marks on specific genes. As such, it is possible that methylation marks at imprinted loci are abnormal. Worth noting is the fact that primordial germ cells, derived from ICM cells, in turn differentiate into gametes (Ginsburg et al., 1990); abnormal epigenetic programming beginning in ICM cells may lead to heritable changes in future generations. Dysregulated expression of candidate imprinted genes required for growth and development (Harrouk et al., 2000b) provides further support for the role of altered parental imprints in the adverse male-mediated effects of cyclophosphamide.

The inability to appropriately modify histones in clusters of imprinted genes on the paternal allele, due to structural alterations and disrupted genomic integrity, could interfere with acquisition of lineage specific monoallelic expression. Any disturbance in imprinting processes and transmission can lead to abnormal embryogenesis and disease; loss of imprinting control has important causative roles in several developmental disorders which display a broad spectrum of phenotypic anomalies. The altered epigenetic reprogramming observed in zygotes fertilized by cyclophosphamide-exposed spermatozoa is predicted to confer long term consequences on development. One prominent and probable mechanism that needs further investigation is paternal imprinting.

6.3 Embryonic Response to Spermatozoal Damage: Repair or Abort?

The question of the ability of the early embryo to detect and repair DNA damage, whether imparted by the spermatozoa or acquired during the early cleavage stages, is attracting tremendous interest recently. More than 25 years ago, it was shown that the zygote was capable of unscheduled DNA synthesis (Brandriff and Pedersen, 1981); however, the exact mechanisms available to detect and implement DNA repair are largely unknown. Our studies enhance the

current understanding of the zygotic DNA damage response to paternally transmitted damage; γ-H2AX and PARP-1 are dramatically elevated in stage specific patterns during developmental progression of the 1-cell stage embryo, highlighting the importance of post-translational modifications in the DNA damage response.

In vitro fertilization using irradiated mouse spermatozoa, has since verified that an H2AX phosphorylation response is elicited promptly in the zygote following paternal transmission of DNA damage (Derijck et al., 2006). Intriguingly, *in utero* irradiation of preimplantation mouse embryos provides a divergent perception of the early embryos competence to mount a response to genomic damage. Despite the faint presence of phosphorylated ATM and DNA-PKcs, γ-H2AX foci were absent in one- and two-cell stage embryos; however, γ-H2AX signal was detectable following cleavage to the four-cell stage, morula and blastocyst (Yukawa et al., 2007; Adiga et al., 2007a). Rather than the conclusion that early embryos are deficient in cell cycle checkpoint control and DNA damage detection and repair, as put forth by the corresponding authors, these studies, in collaboration with our results, implicate the importance of the maternal genome in regulating the early damage response.

The first embryonic cleavages are dependent on the accumulation of RNA and proteins in the cytosol of the oocyte; the mouse zygotic genome is not activated until the late 2-cell stage (Schier, 2007). Included in this pool are numerous mRNAs that encode for proteins involved in the sensing and repair of both SSBs and DSBs to provide a defense against genomic instability (Zheng et al., 2005). Preimplantation mouse embryos are highly radiosensitive (Jacquet, 2004). Thus, the lack of DNA damage response may be due to a severe impairment in the latent supply of proteins required to execute various repair pathways. Fittingly, γ-H2AX appears after zygotic genome activation when transcription of the zygotic genome begins replacing the degraded maternal transcripts; thus, the ability to respond to genomic alterations is re-established. Therefore, paternally transmitted DNA damage can be immediately detected by maternal supplies of effector proteins. In the absence of whole embryo damage,

the maternal genome successfully communicates with the spermatozoal genome to elicit a functional response.

Paternal cyclophosphamide exposure triggered a biphasic γ-H2AX response corresponding to early G1, and S-phase into G2, respectively. In addition to the proposed role of H2AX phosphorylation in chromatin remodeling of the paternal genome during G1 in zygotes fertilized by drug exposed and control spermatozoa, γ-H2AX appears to initiate an early signaling cascade for DNA DSB repair. DSBs are repaired by homologous recombination (HR) and non-homologous end joining (NHEJ). NHEJ functions primarily in G1/early S-phase, while HR is restricted to late S-phase/G2; these pathways may coexist and act sequentially depending on the complexity and severity of the damage (Hassa and Hottiger, 2005). γ-H2AX colocalizes with several factors involved in NHEJ (Mre11/Rad50/Nbs1 (MRN) complex), HR (Brca1, Rad51), DNA damage induced cell cycle checkpoints (53Bp1, Mdc1) and has more recently been shown to be involved in the regulation of chromatin structure (Paull et al., 2000; Celeste et al., 2002; Ward et al., 2003; Lukas et al., 2004). That we observed two phases of γ-H2AX focus induction suggests that multiple pathways are involved in the processing of DNA damage in the spermatozoal genome.

Interestingly, PARP-1 hyperactivation also occurred in early S-phase through G2, emphasizing the involvement of additional pathways, particularly SSB repair and BER, in the zygotic DNA damage response. Our observations clearly demonstrate the involvement of post-translational modifications in early embryonic damage recognition and repair processes. The distinct modifications that serve as initial damage markers to activate subsequent DNA damage signaling cofactors and chromatin modifiers, thereby regulating the recruitment of repair machineries, remain to be elucidated. Evolving knowledge of the histone modifications involved in DNA-damage pathways, in collaboration with the generation of gene-targeted mice, will advance our understanding of the intricate pathways that determine the success of DNA repair in the early embryo (Hassa and Hottiger, 2005; Nordstrand et al., 2007).

In the case of severe damage, the simultaneous activation of many DNA repair pathways (NER, BER and DSB repair) could trigger major chromatin modifications leading to precocious activation of zygotic gene function which may overwhelm the embryo's resources, pushing the embryo into survival conflict (Baumann et al., 2007). PARP-1 has been proposed to play a role in the regulation of the switch between cell survival and cell death in the presence of DNA damage. Engagement of the apoptotic pathway in response to genotoxicity occurs downstream of p53 activation (Huber et al., 2004). Mouse zygotes fertilized by irradiated sperm respond to aberrant genomic integrity via a p53-dependent S-phase damage checkpoint (Shimura et al., 2002), suggesting the possibility that PARP-1 activation in the zygote may be another level of protection for the mammalian genome. Fascinatingly, it is proposed that the developmental programme of the embryo is already established at the one-cell stage (Hardy et al., 2001; Marchetti et al., 2004). Thus, PARP-1 may also play a role in marking the embryo for continued development or failure by cell death (apoptosis) when the damage is irreparable. Remarkably, apoptosis is not initiated until the blastocyst stage and occurs only in ICM cells (Fatehi et al., 2006; Adiga et al., 2007a; Adiga et al., 2007b), indicating that the apoptotic pathway is not functionally competent until after the morula stage.

Although the overall success of complete repair of DNA lesions in the zygote appears to be limited, the early preimplantation cleavage stages seem to function as a time of genome surveillance and a phase during which DNA damage is detected, with the goal of lesion resolution, prior to the blastocyst stage when elimination of deleterious cells begins. Micronuclei were present in 2-cell rat embryos following fertilization with cyclophosphamide exposed spermatozoa, indicating persistent genetic damage which may in fact activate apoptosis in later stages. The presence of micronuclei was involved in p21 activation at the blastocyst stage prior to the onset of apoptosis in mouse embryos (Adiga et al., 2007b). Thus, the observed ICM specific cell death in embryos sired by cyclophosphamide exposed males (Kelly et al., 1992) may, in

part, be due to the hierarchical activation of DNA damage response pathways stemming from the zygotic programme.

As the knowledge of DNA repair in the early embryo is in its infancy, many captivating questions remain: Is the preimplantation embryo capable of efficient DNA repair? Can specific types of lesions be completely resolved? Do pathway limitations exist? Is the preimplantation period truly a time of assessment that stipulates the decision of life or death that is ultimately executed post implantation? What mechanisms are involved? Is there a discrepancy between genetic and epigenetic instability? What are the kinetics of the damage response with respect to damage severity? Could it be that a large amount of chromosomal damage elicits apoptosis, while epigenetic damage, that is insufficient to largely disrupt development, confers the probability of heritable instabilities that survive post implantation? Are damage sensors for genetic damage more sophisticated while sensors for epigenetic alterations may not be as strict due to the natural complexity associated with epigenetic regulation and inheritance?

A thorough assessment of DNA repair proteins in conjunction with histone modifications, levels of DNA damage that persist between cleavage stages, and apoptosis induction during preimplantation development, would begin to shed light on the mechanisms regulating and/or determining the outcome of embryos fertilized by damaged spermatozoa.

6.4 Emergence of Zygotic Pronuclear Cross Talk: A Unique Phenomenon

The intriguing phenomenon of pronuclear cross talk emerged as a key finding in conjunction with alterations in epigenetic reprogramming and the initiation of DNA damage recognition and repair signals following fertilization with cyclophosphamide-exposed spermatozoa. Alterations in the maternal genome, as a consequence of preconceptional paternal drug exposure, illustrate that information pertaining to the integrity of the paternal genome can flow between the pronuclei before physical union occurs. Pronuclear cross talk, therefore, must be mediated across the cytoplasm.

Changes in chromatin structure in the male pronucleus, in response to paternal genomic instabilities, may provide premature opportunities for the binding of modified histones acquired from the oocyte cytoplasm. Signals of the advanced chromatin state of the male pronucleus may epigenetically stimulate the female pronucleus to rapidly progress to an equivalent developmental state to avoid detrimental discrepancies in pronuclear asymmetry. Accordingly, the pronuclear areas of both maternal and paternal pronuclei were significantly larger in zygotes sired by cyclophosphamide treated males. The availability of maternal supplies of epigenetically modified histones in the zygotic cytoplasm, together with the rate of change in concentration, may play an important role in the coordinated dysregulation of both parental genomes. Subsequently, chromatin modifications sustaining an aberrantly permissive condition in parental genomes may initiate abrupt activation of additional control mechanisms involved in DNA damage response pathways; ATM activation may be a result of damage induced alterations in higher chromatin structure (Tse et al., 2007).

Interestingly, lack of pronuclear cross talk was shown following the assessment of γ-H2AX induction and localization, demonstrating the specificity of this marker for the detection of DNA double strand breaks in the drug-exposed paternal genome; the maternal genome was devoid of evident DNA damage. Conversely, PARP-1 was hyperactivated in the male and female pronucleus following paternal cyclophosphamide treatment, indicating that, in fact, signals downstream of the PI3K transducer kinases, ATM and ATR, can be transmitted across the cytoplasm, eliciting a DNA damage response in the undamaged female pronucleus. The p53-dependent pronuclear cross talk that occurs in response to fertilization with irradiated spermatozoa is the only other example to date illustrating this phenomenon (Shimura et al., 2002). Interestingly, PARP-1 positively regulates the p53 response to DNA damage (Wieler et al., 2003) and may act in concert to activate signaling pathways implemental in cell cycle control and DNA repair. Thus, the wealth of knowledge that exists on p53 regulation in response to genotoxic stress provides exciting insight for the

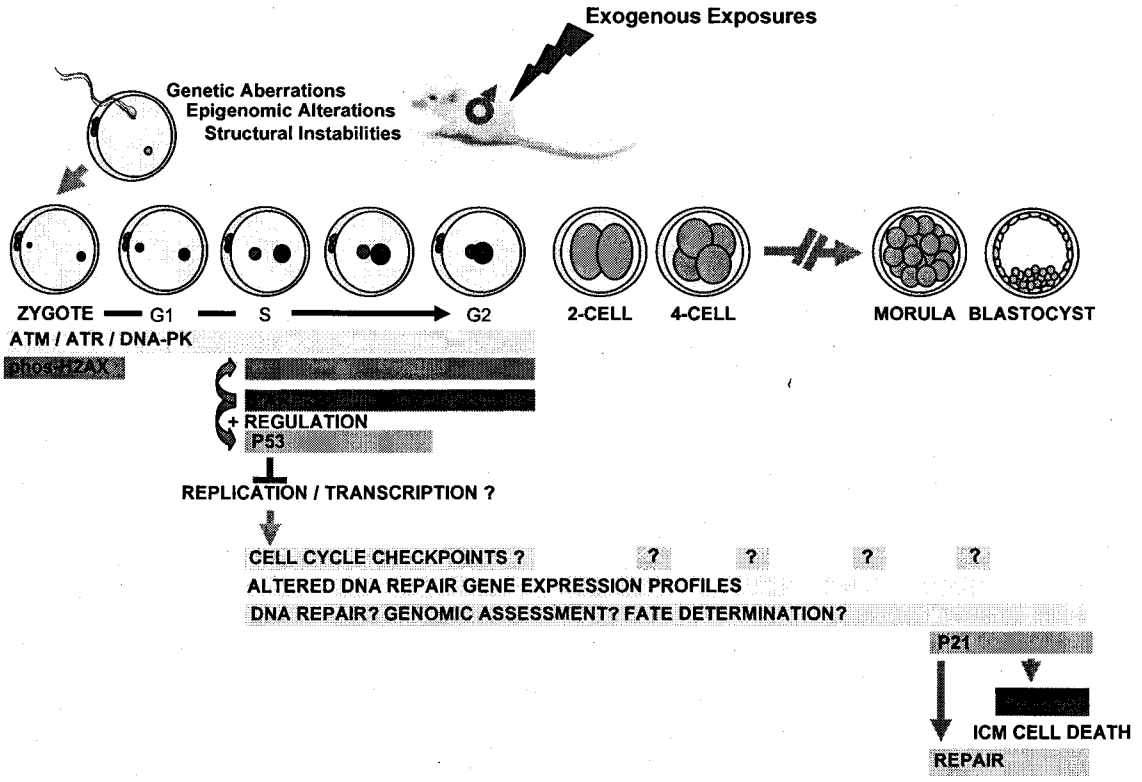
postulated mechanisms regulating the DNA damage dependent pronuclear cross talk highlighted in this thesis.

Maternal and paternal pronuclear apposition in the zygote is a critical event that establishes appropriate spatial association to facilitate genomic union. Migration of parental pronuclei to the center of the zygote is dependent on the formation of cytoplasmic microtubules (Schatten et al., 1985; Navara et al., 1994; Van Blerkom et al., 1995) which extend between male and female pronuclear surfaces. Microtubules are also required for the concentration of membrane material and motor proteins (dynein, dynactin) around maternal and paternal chromatin. Pronuclear movement occurs during S-phase (Payne et al., 2003); coincidently, p53 (Shimura et al., 2002) and PARP-1 pronuclear cross talk is also initiated in S-phase, suggesting a link for the establishment of cytoskeleton filament systems between parental genomes and pronuclear communication. Genotoxic exposures initiate signaling pathways that activate cytoplasmic factors that are involved in cell cycle arrest, DNA repair and/ or apoptosis; consequently, proteins must localize and accumulate in the nucleus to further activate downstream targets. The shuttling between the cytoplasm and the nucleus may be regulated in part by epigenetic modification of specific proteins, stress activated signals and/or cellular structures that direct navigation toward the nucleus (O'Brate and Giannakakou, 2003). Intriguingly, p53 interacts with microtubules for cytoplasmic trafficking and nuclear accumulation; movement from the cytoplasm to the nucleus is guided by dynein (Giannakakou et al., 2000; Giannakakou et al., 2002). Therefore, the dynamic regulatory role of microtubule scaffolds in many cellular functions of mitosis appears to extend to intracellular trafficking and possibly reflects a means of achieving communication between the paternal and maternal genomes in the zygote. Elucidation of the interactions between additional components of stress response pathways, cell cycle checkpoint proteins and the cytoskeleton will be an exciting avenue for future research.

These studies demonstrate that sensors (γ-H2AX) and effectors (PARP-1) are functional in the zygote and effectively communicate the DNA damage signal

across the cytoplasm to the undamaged maternal genome. To date, the earliest responses to paternal DNA damage have been assessed and establish that a dynamic interaction exists between developing genomes; however, an extensive investigation of key downstream cell cycle engines, regulators of DNA repair and replication is needed. It may be that the zygote is limited to the extent of the response that can be functionally elicited; that p21 does not function until the morula/blastocyst stage provides the first evidence for this hypothesis (Adiga et al., 2007b). In collaboration with the findings of others, I propose the following hierarchical model for damage sensing and repair during preimplantation development; many of the areas in this scheme require elucidation (Figure. 6.1).

Figure 6.1. Diagram of the known and proposed events involved in DNA damage sensing and repair during preimplantation development. Events supported by experimental evidence are shown in colour; events requiring elucidation are shown in grey.



6.5 Implications of the Findings in Reproductive Medicine

Although genetic anomalies play a significant role in paternally mediated syndromes, I will focus on the evolving field of epigenetics in which invigorating findings highlight the significant contribution to all aspects of gamete development, embryogenesis, ageing and disease.

6.5.1 Impact of Assisted Reproductive Technologies

In vitro fertilization (IVF) is typically used to circumvent problems of oocyte accessibility for spermatozoa; spermatozoal function must be uninhibited for successful fertilization to occur *in vitro.* As such, damage to the paternal genome as well as altered spermatozoal function may impede successful fertilization using this technology; the associated risk of birth defects in offspring appears to be small (Van Voorhis, 2006). On the other hand, intracytoplasmic sperm injection (ICSI) is a more invasive form of assisted reproduction which is commonly used for treatment of male factor infertility characterized by spermatozoal abnormalities in morphology, concentration, motility and genomic integrity (Tesarik and Mendoza, 2007). ICSI relies on the direct injection of an ejaculated or epididymal spermatozoa (Devroey et al., 1995b), or immature testicular spermatids (Devroey et al., 1995a; Chan et al., 2001), into an oocyte to achieve a pregnancy, thus bypassing the natural barriers of fertilization. This procedure is not selective against abnormal sperm (Harari et al., 1995; Rybouchkin et al., 1997). Toxicant exposure may disrupt germ cell quality in numerous ways, including abnormal morphology, altered motility, aberrant genetic integrity and/or chromatin packaging, as well as more subtle epigenetic errors or altered gene expression. To ensure healthy offspring, genetic material must be passed unaffected to the egg. The use of assisted reproductive techniques such as ICSI may increase the risk of transmitting genetic and/or epigenetic damage to the child. In fact, it has been suggested recently that infants born following assisted reproductive technologies are at an increased risk for congenital birth defects (Hansen et al., 2005) and epigenetic and imprinting

syndromes (De Rycke et al., 2002; Maher, 2005; Paoloni-Giacobino, 2007); cytotoxic therapy may exacerbate the problem.

Spermatozoa with genetic abnormalities may be selected unknowingly for ICSI; only a small proportion of aneuploid spermatozoa were morphologically abnormal following cyclophosphamide exposure. Fortunately, preimplantation genetic diagnosis (PGD) using FISH aims to detect the presence of chromosomal aberrations to avoid the transfer of embryos containing genetic disease (De Rycke et al., 2002). To improve the selection of better quality embryos for transfer, prolonged embryo culture has been introduced (Gardner and Lane, 1998). This raises additional concerns as *in vitro* culture of mouse embryos affects development, behaviour and imprinting in offspring (Doherty et al., 2000; Ecker et al., 2004). Accordingly, recent attention has focused on the introduction of epigenetic errors to the embryo that may be inherent in the spermatozoa of infertile men, or induced as a consequence of cryopreservation, procedural manipulations and culture of gametes and embryos (Sakai et al., 2005). Discrepancies between epigenetic regulation, the timing of imprinting and the embryonic clock may cause major developmental disturbances, resulting in obvious adverse phenotypic characteristics or mortality; subtle defects, influencing gene expression, thereby creating a predisposition to diseases such as cancer, may be unnoticed.

Preconceptional paternal cyclophosphamide exposure leads to advanced developmental progression, aberrant pronuclear decondensation, altered DNA methylation and histone acetylation, as well as initiation of DNA damage recognition pathways in the zygote, indicating that epigenetic defects can be detected very soon after fertilization with damaged spermatozoa. There are currently no markers or standard indications to predict the probability of epigenetic disease in human offspring generated by assisted reproductive technologies. Furthermore, the first ICSI children are still too young to know the long-term consequences of potential genomic and/or epigenomic alterations on their reproductive status and predisposition to disease (Van Voorhis, 2006). The studies presented in this thesis clearly demonstrate that an intact paternal

genome is absolutely essential for competent genetic and epigenetic progression during early embryogenesis and implicates the importance of focusing on the identification of biological markers which could be used, in conjunction with PGD, to recognize epigenetic or imprinting disorders prior to embryo transfer.

6.5.2 Epigenetic Transmission of Heritable Disease

Developmental phenotype is dependent on genotype and the unique epigenotype that is now known to be heavily influenced by various endogenous and exogenous exposures. Epigenetic alterations due to environmental exposures are progressively being highlighted as early molecular events involved in cancer etiology. Interestingly, cancer cells are characterized by an overall imbalance in DNA methylation patterns and chromatin packaging; global hypomethylation, and thus, an aberrantly permissive chromatin conformation, is evident in tumor cells (Weidman et al., 2007; Brock et al., 2007). Furthermore, epigenetic alterations during development are also involved in psychosocial and behavioural disorders in later life. Intriguingly, methylation status has been implicated in the etiology of schizophrenia, fragile X syndrome, fetal alcohol syndrome, and depression (Crews and McLachlan, 2006). Thus, it is becoming increasingly evident that epigenetic programming during embryogenesis is critical for determining the physical, mental and behavioural health of the individual.

Experiments recently published by Skinner and colleagues, indicate that embryonic or early postnatal exposure to the environmental toxicants, vinclozolin or methoxyclor, may also promote various disease states that are transmissible to subsequent generations. Importantly, the adult-onset defects were only transmitted through the male germline. Transgenerational disease phenotypes, presenting in an age-dependent manner, included, increased frequencies of tumors, prostate disease, kidney disease, immune abnormalities and severe male factor infertility, which continued to emerge through the third generation (Anway et al., 2005; Anway et al., 2006b; Anway et al., 2006a). An altered DNA methylation pattern in the germ cells of the second and third generation males was suggested to be the source of disease propagation (Anway et al., 2005;

Anway et al., 2006a). Although the doses used in these studies were higher than environmentally relevant exposures, the observation that maternal xenobiotic exposures may interfere with epigenetic programming in the germline remains an important finding (Dolinoy et al., 2007).

The transgenerational penetrance of epigenetic modifications, specifically through inefficient erasure of marks during gametogenesis and incomplete epigenetic reprogramming, has been clearly shown for the murine metastable epialleles, A^{vy} (viable yellow agouti) and $Axin^{Fu}$ (axin fused). The *Agouti* gene (A^{vy}) dictates coat colour; CpG methylation status of the A^{vy} IAP is correlated with coat colour which varies from yellow (unmethylated) to brown (methylated) in wild-type mice (Morgan et al., 1999). Interestingly, unmethylated IAP sequence in the Agouti region also leads to increased obesity and tumorigenesis (Miltenberger et al., 1997); the variable phenotypes associated with dramatic variation in methylation are transmitted to the offspring via the maternal germ line (Waterland and Jirtle, 2003). $Axin^{Fu}$ IAP methylation is inversely related to the severity of the kinked tail phenotype; less methylation results in more severe tail kinks. Supporting the concept of transgenerational epigenetic inheritance, both $Axin^{Fu}$ parents produce offspring with tail kinks. Furthermore, as evidenced by the corresponding methylation status of mature spermatozoa and somatic tissues, $Axin^{Fu}$ does not appear to undergo epigenetic reprogramming during gametogenesis (Rakyan et al., 2003).

The induction of epigenetic defects, including DNA methylation changes, in the paternal germline following toxicant exposure has not been extensively investigated. That histone acetylation and DNA methylation patterns are dysregulated in the zygote following paternal cyclophosphamide exposure provides the first link that preconceptional male germline exposure to therapeutic drugs can directly influence the establishment of epigenetic programmes during early embryogenesis. These findings have substantial implications for epigenetic manifestations in the paternal genome following therapeutic exposures that could relay adverse heritable developmental and health outcomes to subsequent generations. This is substantiated by the observation that paternal

cyclophosphamide exposure has a significant impact on the fertility and survival of second generation progeny (Hales et al., 1992). The studies presented here underscore the need for more extensive analysis to determine the mechanisms by which drug exposures may alter the spermatozoal epigenome.

6.5.3 Creation of Artificial Gametes: An Intricate Template to 'Reproduce'

The field of reproductive medicine has made remarkable advances in the options available to combat male infertility, giving the opportunity for parenthood to individuals who would otherwise not be able to naturally conceive due to unknown or therapeutically induced infertility. At the extreme end of the reproductive technologies is the generation of gametes from embryonic stem cells (ESCs) or stem cells induced from fibroblasts (Wernig et al., 2007; Okita et al., 2007) for use with ICSI. Although the prospect of creating germ cells for infertility treatment is exciting, it is not without a substantial amount of risk. Studies thus far have demonstrated that ESCs are capable of developing into haploid spermatogenic cells, albeit with abnormal morphology, that may produce normal-looking preimplantation embryos. Unfortunately, development into live offspring was not highly successful and most mice produced from *in vitro* generated gametes displayed a high rate of developmental abnormalities, indicative of deficits in epigenetic programming (Nayernia et al., 2006). This highlights, once again, the absolute requirement for unaltered genetic and epigenetic programming during the intricate sequential phases of spermatogenesis for the production of healthy offspring.

Our findings demonstrating that genotoxic exposure during a critical window of spermatogenesis leads to epigenetic alterations in the zygote, in the absence of functional deficits, raise particular concerns for the use of gametes generated *in vitro*. In particular, the complete extent of epigenetic programming and the establishment of chromatin signatures that occur during spermatogenesis are currently unknown. Furthermore, as we begin to gain a better understanding of the transgenerational basis of epigenetic modification, it is plausible that the use of gametes produced from ESCs could increase the

propagation of heritable disorders, diseases and increase the prevalence of infertility in future generations. There is much to be gained from experimentation with *in vitro* development of gametes; however, competent duplication of the intricate programming required for the successful production of healthy offspring requires intense scrutiny (Lucifero and Reik, 2006; Whittaker, 2007).

Collectively, the studies in this thesis demonstrate that spermatozoa carrying significant amounts of genomic damage are functional; furthermore, disturbances in DNA integrity are transmitted to the zygote, necessitating the initiation of DNA repair early in embryonic development. Intriguingly, that preconceptional paternal cyclophosphamide exposure altered epigenetic reprogramming in the zygote inspires great interest in resolving the epigenetic mechanisms provoking the transgenerational effects following exposure to therapeutic toxicants.

ORIGINAL CONTRIBUTIONS

1. Computer assisted sperm analysis demonstrated that chronic cyclophosphamide treatment subtly alters spermatozoal motion characteristics. Thus, spermatozoa carrying nuclear and structural damage are motile, and are capable of transmitting defects to the progeny.

2. Chronic cyclophosphamide exposure increased the frequency of chromosome 4 disomy and nullisomy in epididymal spermatozoa that were initially targeted as spermatogonia. By using spermatogenic phase-specific drug exposures, it was demonstrated that cyclophosphamide induced aneuploidy by disrupting critical events that occur prior to pachynema during spermatogenesis.

3. Independent of drug exposure, only a limited percentage of aneuploid spermatozoa presented with morphological abnormalities; this raises concern for the selection of chromosomally abnormal spermatozoa for assisted reproductive technologies. Conversely, all diploid spermatozoa were approximately twice the size of normal cells.

4. Chronic preconceptional paternal cyclophosphamide exposure alters zygotic development and pronuclear chromatin structure. Zygotes sired by drug-treated males had advanced rates of development and increased pronuclear areas in both paternal and maternal genomes.

5. Epigenetic reprogramming was altered in zygotes fertilized by cyclophosphamide treated males, evidenced by altered levels of histone acetylation and DNA methylation in both parental genomes. This finding was the first demonstration that paternal exposure to any drug induced aberrant epigenetic programming in early embryos.

6. Genetic and epigenetic damage persists to the 2-cell embryo stage. Micronuclei were substantially elevated and the spatial localization of acetylated histone H4 was altered in embryos fertilized by cyclophosphamide-treated spermatozoa. Epigenetic damage occurred independently of dramatic chromosomal damage; 2-cell embryos without micronuclei also had altered epigenetic markings.

7. Paternal exposure to cyclophosphamide rapidly activates signals for DNA damage detection in the zygote. A biphasic phosphorylated H2AX response was induced in the paternal pronucleus of zygotes sired by drug-treated males.

8. Poly (ADP-ribose) polymerase-1 was substantially elevated, coincident with the second phase of γ-H2AX induction, in the paternal and maternal pronucleus of zygotes fertilized by cyclophosphamide-exposed spermatozoa. The response of the early embryo to paternally derived genetic and epigenetic disturbances plays an important role in determining developmental outcome.

9. The studies assessing reprogramming and the DNA damage response in the zygote highlighted the emergence of the unique phenomenon of pronuclear cross talk. The maternal genome was disrupted as a result of spermatozoal genome instabilities introduced into the zygote following paternal drug exposure. This exciting phenomenon was evidenced by altered acetylation, methylation and enhanced PARP-1 in the maternal genome, as well as in the damaged paternal genome.

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