EFFECTS OF CHEMOTHERAPEUTIC AGENTS FOR TESTICULAR CANCER ON MALE RAT REPRODUCTIVE ORGANS AND SPERMATOZOAL NUMBERS, MOTILITY, AND MORPHOLOGY

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Abstract

Testicular cancer (TC) is the most common cancer affecting men of reproductive age; however treatment with bleomycin, etoposide, and cis-platin (BEP) results in extremely high cure rates. The post-treatment quality of life of TC patients is therefore a major concern. The goal of this study was to determine the effects of BEP on sperm count, motility, and morphology in an animal model. Male Sprague Dawley rats were treated with BEP for 9 weeks. Rats were killed, and the numbers, motility, and morphology of the spermatozoa from the epididymides were analyzed. After BEP treatment, sperm counts decreased by almost 10-fold when compared to control ($11.9x10^7$ versus $1.65x10^7$ sperm per epididymis). The percent of spermatozoa that were motile was > 30% lower in the treated group compared to control group. Morphological defects increased significantly in both the midpiece and principal piece of the flagella. These results indicate that BEP treatment has significant effects on spermatogenesis in the rat model.

Résumé

Le cancer du testicule fait partie des cancers les plus fréquents chez l'homme en âge de se reproduire. Le traitement combiné à la bléomycine, l'ectoposide et la cis-platine (BEP) permet d'obtenir d'excellents résultats. Toutefois, les conséquences sur la qualité de vie de ces patients après le traitement reste une question majeure. Le but de cette étude a été de déterminer les effets du traitement BEP sur le nombre, la motilité et la morphologie des spermatozoïdes dans un modèle animal. Après le traitement de rats Sprague Dawley mâles pendant 9 semaines, le nombre, la motilité et la mobilité des spermatozoïdes prélevés au niveau de l'épididyme ont été analysés. Le traitement induit une diminution significative du nombre de spermatozoïdes d'un facteur 10 ($11,9.10^7$ *vs* $1,65.10^7$ spermatozoïdes par épididyme). D'autre part, le pourcentage de spermatozoïdes motiles est diminué de 30% après le traitement par rapport au groupe témoin. Enfin, nous avons observés une augmentation significative des défauts morphologiques au niveau de la pièce intermédiaire et du flagelle des spermatozoïdes. L'ensemble de ces résultats suggère que le traitement BEP a des effets délétères sur la spermatogenèse chez le rat.

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Preface

Format of the Thesis

This thesis comprises a paper which is included in the form in which it will be submitted for publication. It is a manuscript based thesis in compliance with section 1.C. of the "Guidelines for Thesis Preparation", Faculty of Graduate Studies and Research, McGill University. These guidelines state that "Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly-duplicated text (not the reprints) of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" with respect to font size, line spacing and margin sizes and must be bound together as an integral part of the thesis. (Reprints of published papers can be included in the appendices at the end of the thesis). The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges preceding and following each manuscript are mandatory. The thesis must conform to all other requirements of the "Guidelines for Thesis Preparation" in addition to the manuscripts."

The Introduction, Chapter 1, contains an introduction to sperm morphology and motility in the rat model, and to the chemotherapeutic regimen used to treat testicular cancer. The introduction ends with the rationale for and the hypothesis of the study presented in the thesis. Chapter 2 includes a paper that will be

submitted for publication. All experiments and analyses were performed by the candidate, with the exception that Ludovic Marcon aided in administering the drugs to and killing the animals, and Johanne Ouelette aided with the electron microscopy. Chapter 3 includes a general discussion of the results.

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CHAPTER 1

INTRODUCTION

Introduction

1. Male Reproduction in the Rat Model

1.1. Sperm Production

1.1.1 Spermatogenesis

Spermatogenesis is the continuous process that takes place within the seminiferous tubules of the testes by which diploid spermatogonial stem cells mature into haploid elongated spermatozoa (1). Closest to the basement membrane of the seminiferous tubules are the spermatogonial stem cells, which divide by mitosis to populate the tubules (2). These stem cells then differentiate, becoming primary spermatocytes, undergoing the first meiotic division to become secondary spermatocytes, and finally undergoing the second meiotic division to become haploid spermatids (1). Spermatids, initially round, undergo intense cellular remodeling; they grow a flagellum, replace nuclear histones with protamines, and shed the majority of their cytoplasm to become spermatozoa (3;4). These spermatozoa are then shed into the lumen of the seminiferous tubules where they are transported to the epididymis to undergo maturation (5).

1.1.2 Sperm Maturation in the Epididymis

Upon being released into the lumen of the seminiferous tubules, spermatozoa are transported to the distal region of the tubules, into the rete testes, the efferent ducts, and finally into the epididymis. The epididymis consists of a single highly convoluted tubule that can be divided into four

segments based on morphology and function: the initial segment, the caput, the corpus, and the cauda. Passage through the epididymis takes approximately one week, during which time the sperm mature (6;7). As the sperm pass through the epididymis, they undergo various biochemical changes, shed their remaining cytoplasm, gain the ability to be progressively motile, and the ability to fertilize (6;8-11). When maturation is complete, spermatozoa are stored in the cauda epididymidis until ejaculation occurs.

1.2. Sperm Morphology and Motility

1.2.1 Sperm Morphology

A spermatozoan is made up of several distinct structures, including the head and the flagellum. The head contains the nucleus, which consists of highly condensed chromatin (12). The head can be divided into 3 segments: the acrosomal region, the equatorial segment, and the postacrosomal region (3;13). The surface molecules of the acrosome are involved in the recognition of the zona pellucida protein (ZP3) of the oocyte (14). The equatorial segment is a cytoskeletal element that is maintained until it is incorporated into the oocyte (15;16). The flagellum generates sperm motility, and thus is important for fertilizing ability.

1.2.1.1 Overall Structure of the Flagellum

The flagellum of a rat sperm can be divided into four major parts: the connecting piece, the midpiece, the principal piece, and the endpiece (3). The

connecting piece attaches the flagellum to the nucleus. The midpiece begins at the connecting piece, and terminates approximately one-fourth of the way down the flagellum, where the principal piece begins. The principal piece makes up approximately two-thirds of the length of the flagellum. The short remaining piece of the flagellum is known as the endpiece. The overall structure of a spermatozoan, with emphasis on the ultrastructure of the flagellum, is shown in figure 1.

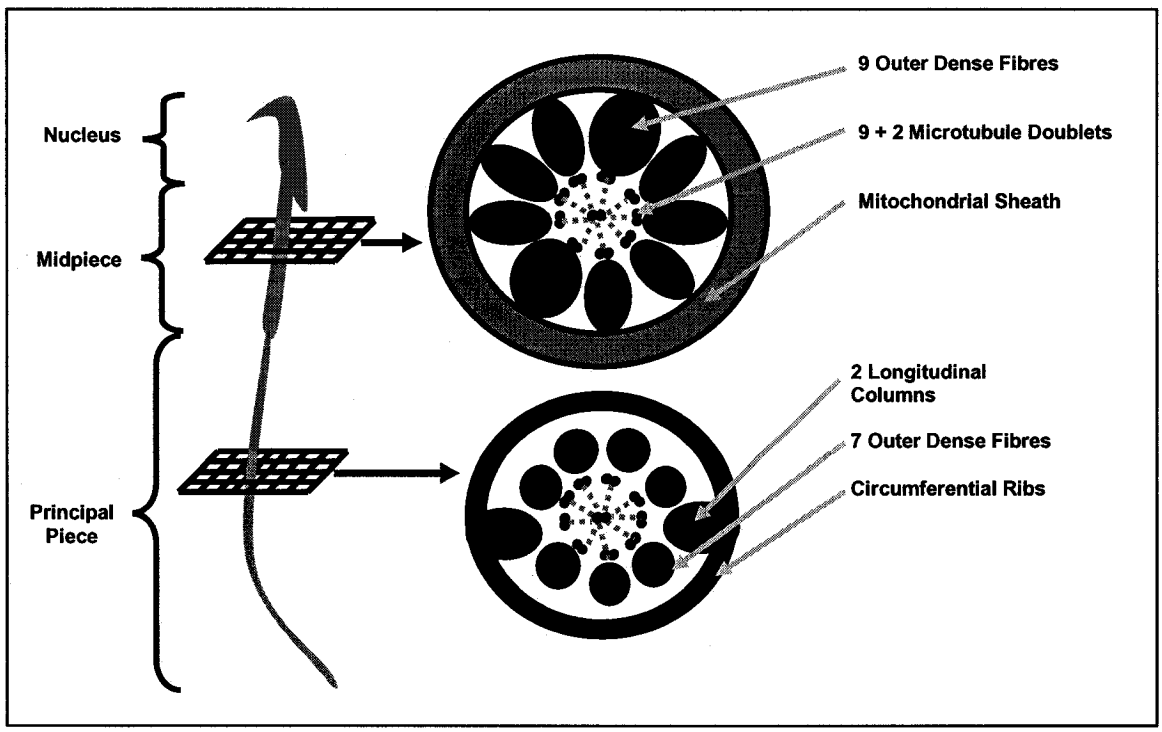

Figure 1. Structure of a spermatozoan. The nucleus, midpiece, and principal piece are shown on the left, with cross-sections of the midpiece and principal piece on the right.

Running the entire length of the flagellum is the axoneme, which is responsible for generating the motor force required for motility (17;18). The axoneme is a cytoskeletal structure that consists of a ring of 9 pairs of microtubule doublets, each of which projects a radial spoke to a central doublet pair (3;18). Inner and outer dynein arms project from each doublet and are

responsible for generating motive force via activation of a dynein ATPase which causes sliding of adjacent outer microtubule pairs (19).

1.2.1.2. Structure and Function of the Midpiece

The midpiece is characterized by the presence of 9 outer dense fibres (ODFs) that surround the axoneme, each of which is associated with a microtubule doublet. It has long been suggested that the role of the ODFs is to provide structural support and elasticity to the flagellum (3;20). This hypothesis is supported by recent findings that the proteins that make up the ODFs consist of keratin-like intermediate filament proteins (17;21;22).

Surrounding the ODFs is a ring of mitochondria known as the mitochondrial sheath. Although motility is generated throughout the entire length of the flagellum, mitochondria are located only in the mitochondrial sheath of the midpiece. Several proteins have been found that are unique to the mitochondria of spermatozoa, and are not found in somatic cells. These include mouse spergen-1 (23), OXCT2a and 2b, which are rate determining enzymes in ketosis (24), as well as a sperm specific hexokinase (25).

1.2.1.3. Structure and Function of the Principal Piece

The characteristic feature of the principal piece is the replacement of ODFs 3 and 8 with the two longitudinal columns of the fibrous sheath (FS). It is thought that the fibrous sheath is involved in providing the stiff support for the flagellum that helps generate a planar beat (3;17;26). The fibrous sheath of

mouse sperm consists of 6 major proteins and many more minor proteins that are apparent on silver stained 2D gels (27;28). In addition, recent findings (29;30) indicate that proteins involved in motility and metabolism are found in the FS. This suggests that there may be a role of the FS in the generation of motility (17). Another postulated function of the FS is the protection of sperm from oxidative stress. This is based on the finding of glutathione-S-transferase in the FS (31;32). The two columns of the fibrous sheath are connected by circumferential ribs that provide support for the columns.

1.2.1.4. Structure and Function of the Endpiece

The end piece of the flagellum consists solely of the axoneme surrounded by the plasma membrane. The components of the axoneme terminate successively toward the end of the end-piece: the dynein arms disappear first, followed by the central pair of microtubules, and finally one of each pair of microtubule doublets (33). At the distal portion, the endpiece consists solely of 9 microtubule singlets.

1.2.1.5. Effects of Drugs on Sperm Morphology

Several drugs as well as physiological states have been shown to have a deleterious effect on rat sperm morphology. Cyclophosphamide, an alkylating agent, has been shown to disrupt the close association of the axoneme and the outer dense fibres (34). Cyclosporine A, an immunosuppressant, has been shown to induce several defects in sperm morphology, including compact

aggregation of numerous flagella in a single membrane, abnormal arrangement of mitochondrial or fibrous sheath with outer dense fibers, and fusion of flagella with a single intervening mitochondrial layer (35). In addition, aged rats have been shown to have an increase in a wide variety of flagellar defects, including an abnormal spatial arrangement of outer dense fibers, modifications in the number of ODFs and axonemal components, and more than one axonemal structure within one membrane (36).

1.2.1.6 Consequences of Altered Morphology

Proper flagellar structure is necessary for sperm motility, and in turn, fertility. This is demonstrated by the fact that many genetic mutations leading to altered flagellar structure result in infertility. For example, patients with Kartagener's syndrome, an autosomal recessive disease characterized by immotile ciliated cells due to a lack of dynein arms in the axoneme, are infertile (37). In the mouse, gene disruption studies have revealed several genes associated with flagellar structure that are important for fertility. For example, Clark et al. (38) recently discovered two novel genes located on chromosome 11 which, when knocked out, result in infertility. Further investigation revealed that the sperm axonemes in these mice had altered numbers of microtubules. In addition, mice with a disrupted AKAP4 gene were infertile due to failure to develop a fibrous sheath, and development of shortened flagella (39). These examples demonstrate the requirement of intact flagellar structure for fertility.

1.2.2 Sperm Motility

1.2.2.1 Requirement for Sperm Motility

The motion of spermatozoa can be divided into two classes: activated motion, and hyperactivated motion. The focus of the experiments outlined below will be on the former. Activated motion is seen in freshly ejaculated sperm (40), and its role is thought to be to drive the sperm through the female reproductive tract to the oviducts (41). In contrast, hyperactivated motility is involved in penetration of the oocyte (41). Activated motility is essential for natural fertilization, as immotile sperm are unable to pass through the uterotubal junction and reach the site of the oocyte (42).

1.2.2.2 Acquisition of Motility in the Epididymis

As sperm exit the testis and enter the epididymis, they are immotile. They gain the ability to swim during their passage through the epididymis (11). Jeulin *et al.* (43) showed that immotile sperm in the initial segment of the epididymis have rigid flagella, and acquisition of sperm motility occurs abruptly in the proximal caput epididymidis with different patterns of flagellar movement: vibrating, motile in place, motile with a static curvature of the midpiece resulting in a spinning motion or a circular path, and forward progressive movement with regular rotation of the head. The pattern of spermatozoal movement becomes homogeneous in the distal cauda epididymidis where the whole spermatozoal population swims linearly. The progressive acquisition of motility requires extensive interaction with the contents of the epididymal lumen (44-47).

1.2.2.3 Measurement of Sperm Motility

The motion of a large number of spermatozoa can be analyzed in a short period of time with the use of a computer-assisted sperm analysis (CASA) system. Using the CASA system, microscopic images of spermatozoa are detected by video technology. A computer captures each video image, records the location of the sperm head in each video frame, and reconstructs the sperm path by connecting these images (48;49). The system then generates values which provide information about the velocity, vigour, and pattern of the sperm's motion. The parameters of sperm motion provided by the CASA system have been shown in several studies to be correlated with the ability to fertilize both *in vitro (50;51)* and *in vivo* (52-54). Furthermore, CASA parameters have been shown to correlate with a high percentage of DNA damage as measured by the Sperm Chromatin Structure Assay (55).

The parameters that describe the velocity of the sperm are the average path velocity (VAP), curvilinear velocity (VCL), and the straight line velocity (VSL). The velocity of the average path uses the distance of the general trajectory of the spermatozoa. The curvilinear velocity is calculated using a two-dimensional projection of the actual three-dimensional velocity. The distance is the sum of the distances along the trajectory. The straight line velocity uses the distance between the first and last points of the trajectory (49).

Two additional parameters, the linearity (LIN) and the straightness (STR), use velocity ratios to further describe the motion of the spermatozoa. The

linearity is the ratio of the VSL to the VCL. If the trajectory of the spermatozoan is circular, the LIN will be low because the net distance traveled by the spermatozoa will be small compared to the total distance covered. The straightness is the ratio of the VSL to the VAP. Again, a more circular path would have a low STR, however the STR would be higher than the LIN because the curvilinear velocity is higher than the average path velocity (49;56).

The final two measures provided by the system are the amplitude of lateral head displacement (ALH) and the beat cross frequency (BCF). The ALH gives the average distance between the peak and trough of the sperm's average path. The calculation of the BCF is based on the fact that each peak or trough in the average path is the result of a new flagellar beat. The BCF is therefore the number of times the curvilinear path crosses the average path per second (49).

1.2.2.4 Effects of Drugs on Sperm Motility

Several drugs have been shown to alter sperm motility, both by decreasing the percent of motile sperm, and by altering specific parameters of sperm motion. For example, rats treated with PNU157706, a dual 5α-Reductase inhibitor, experienced a decrease in both percent motile and percent progressively motile sperm (57). Rats treated with alpha-chlorohydrin showed a decrease in the percent motile sperm, VAP, VCL, VSL, LIN, and STR, and an increase in BCF and ALH (58). In addition, certain chlorinated biphenyls lead to a decrease in percent motile sperm, VCL, VAP, VSL, ALH, and BCF (59).

2. Testicular Cancer

Testicular cancer can be divided into two classes: germ-cell, and non germ-cell cancer. Germ-cell tumours account for most (90-95%) cases of testicular cancer (60), and occur predominantly in young men (61). Germ cell tumours are further sub-divided into two groups based on histology: seminomatous, which retain their germ-cell lineage, and non-seminomatous, which can resemble any tissue type. Both types of germ-cell cancers are preceded by a common preinvasive lesion, known as carcinoma in situ (CIS) (62). One of the leading hypotheses on the origins of germ-cell cancer suggests that germ cells become neoplastic in fetal life when they are germ cell precursors, or gonocytes, and their development into invasive tumours depends on factors such as the presence of gonadotrophins or testicular steroids (63). Further support has been lent to this hypothesis by findings that the gene expression profile in CIS cells has a high degree of overlap with embryonic stem cells (64).

2.1. Epidemiology of Testicular Cancer

Testicular cancer is the most common cancer affecting men of reproductive age, and the incidence has been rising worldwide for several years (65;66). In Canada, men born between 1959 and 1968 were twice as likely to suffer from testicular cancer as men born between 1904 and 1913 (67). Despite this increasing incidence, the cure rate is quite high; several reports have shown the overall cure rate to be over 90% (68;69). Furthermore, when patients are

organized into prognostic groups, the 5-year survival rate is approximately 96% for those with favourable factors, such as minimal pulmonary metastasis (70).

Several reports suggest that the rise in the incidence of testicular cancer is due to environmental factors. More specifically, Sharpe and Skakkebaek (71) suggest that testicular cancer is one symptom of a multi-symptom disease called Testicular Dysgenesis Syndrome, the rise in incidence of which is due to increased estrogen exposure *in utero*. It has also been found that markers of high estrogen in the mother during gestation (age and parity) (72), as well as men whose mothers were on oral contraceptives within 12 months of conception (73), are associated with increased testicular cancer risk. While several studies have suggested links between estrogen exposure *in utero* and risk of testicular cancer, this hypothesis is still very controversial. Other reported risk factors include a history of cryptorchidism (74), low birthweight (73;75), bleeding or threatened miscarriage, and maternal smoking (76).

2.2 Treatment of Testicular Cancer

2.2.1. Regimen

The first step in the treatment of testicular cancer is orchidectomy of the cancerous testis. For patients with metastatic non-seminomatous tumours, or patients with stage I disease (disease confined to the testicle) and presence of vascular invasion in the orchidectomy sample, treatment with bleomycin, etoposide, and cis-platin (BEP) has become the regimen of choice (77). The

standard treatment includes 20 mg/m^2/day of cis-platin on days 1-5 of each week, 100 mg/m^2/day etoposide on days 1-5 of each week, and 30 units/week bleomycin. Patients who are considered to have favourable factors receive 9 weeks of treatment, while those that are poor-risk may receive 12 weeks (78).

2.2.2 Bleomycin

2.2.2.1 Structure and Mechanism of Action

Bleomycin (figure 2) is a widely used anti-tumour antibiotic that was originally isolated from the culture medium of *Streptomyces verticullus* (79;80). It is given systemically, and is used to treat testicular cancer, lymphomas, and head and neck cancers. It acts by inflicting DNA lesions through a free-radical driven process (81). There are many types of DNA lesions that can be produced, and the extent of formation of the lesions depends on the redox status of a given cell type (82-84). In addition to attacking DNA, bleomycin has also been shown to cleave mRNA, tRNA, and rRNA (85-88); however, RNA is not believed to be the main target of bleomycin because the extent of RNA cleavage is much less than that of DNA.

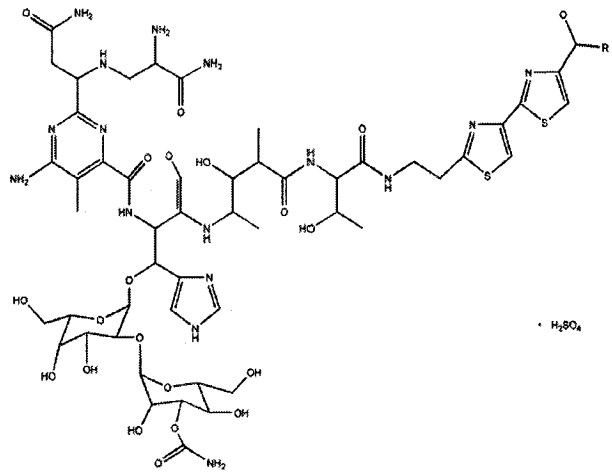

Figure 2. Molecular structure of bleomycin

2.2.2.2. Effects on the Male Reproductive System

Few studies have investigated the effects of bleomycin alone on the male reproductive system. In the mouse model, it has been shown that bleomycin can induce reciprocal translocations in spermatogonia (89;90). In addition, it has been shown using the specific locus test that bleomycin is mutagenic in spermatogonia (91). In humans, *in vitro* treatment of mature male germ cells with bleomycin resulted in an increase in structural chromosomal aberrations, including breaks, fragments, exchanges, and gaps (92).

2.2.3. Etoposide

2.2.3.1. Structure and Mechanism of Action

Etoposide (figure 3) is a derivative of a natural compound found in the roots of the Mayapple plant. It is mainly used to treat testicular cancer, as well as small cell lung carcinomas. It is generally given intravenously, and approximately one third of administered etoposide is excreted in the urine (93). It is a member

of a class of drugs referred to as topoisomerase II inhibitors. DNA topoisomerase II is a nuclear enzyme that relieves torsional stress that occurs during DNA replication by making transient DNA strand breaks (94;95). Topoisomerase II is essential for DNA replication and transcription, as well as chromosomal segregation and DNA recombination. Etoposide interferes with topoisomerase II by increasing the concentration of covalent DNA cleavage complexes. This action turns the topoisomerase into a toxin that causes DNA strand breaks.

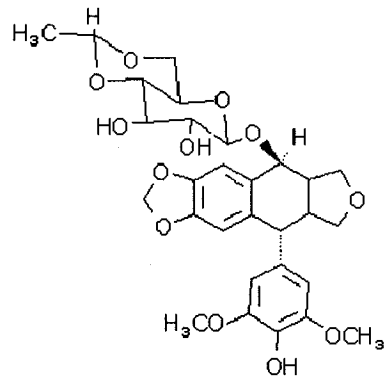

Figure 3. Molecular structure of etoposide

2.2.3.2 Effects on the Male Reproductive System

As is the case with bleomycin, relatively few studies have investigated the effects of etoposide alone on the male reproductive system. It has been shown by several groups, however, that the main target of etoposide during spermatogenesis is meiotic cells. In the mouse model, Russell *et al.* showed that etoposide significantly affected crossing over by inducing local decreases in recombination, a distal shift in locations of crossing-over, and an overall decrease in double crossovers (96). In addition, etoposide treatment led to

increased aneuploidy in spermatozoa, resulting in significant increases in hyperhaploid sperm, as well as diploid sperm (97). In the rat, etoposide was shown to be a potent inducer of micronuclei in early spermatids that had been exposed at different stages of meiotic prophase (98). In addition to affecting meiotic cells, etoposide is a potent inducer of apoptosis in all types of spermatogonia as well as spermatocytes (99). Only one study to date has investigated the effects of etoposide treatment in the male on progeny outcome: in matings that sampled treated pachytene and preleptotene spermatocytes, the resulting progeny showed significant increases in the percentages of zygotic metaphases with structural aberrations. The aberrations included acentric fragments and deletions, which are expected to result in embryonic lethality. This study also showed an increase in aneuploidy in both spermatocytes and zygotes (100).

2.2.4. Cis-Platin

2.2.4.1. Structure and Mechanism of Action

Cis-Platin (cis-diamminedichloroplatinum(II)) (figure 4) is a member of the family of platinum anti-cancer complexes. It is administered to patients intravenously, and is used to treat patients with solid tumours of the ovary, head, neck and testes. Upon entering the cell, it is hydrolyzed and passes through the nuclear membrane. Several studies have shown that the main target of cis-platin is DNA (101-103). The major products of cis-platin-DNA binding are intra-strand DNA cross-links involving adjacent guanidines or a guanidine adjacent to an

adenine (104). Cis-platin is thought to exhibit its cytotoxic effects by inhibiting both replication (105) and transcription (106;107) of DNA.

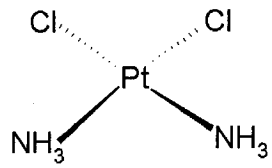

Figure 4. Molecular structure of cis-platin

2.2.4.2 Effects on the Male Reproductive System

Cis-platin has multiple effects on almost every part of the male reproductive system, including the testes, epididymides, sperm cells, as well as the endocrine system and progeny outcome.

Several studies have shown that sub-chronic treatment of adult male rats with cis-platin results in a decrease in serum testosterone (108). Chronically, cis-platin induces a decrease in serum and intratesticular testosterone as well as serum luteinizing hormone, without any effect on follicle stimulating hormone (109).

In the testes, acute treatment with cis-platin has been shown to kill rapidly dividing spermatogonia, resulting in a decreased number of spermatids post-treatment (110). It also causes an increase in germ cell apoptosis both 5 and 10 days post-treatment (111). Chronically, it has resulted in more pronounced reductions in spermatogonia, as well as an increase in morphologically abnormal spermatids (112). The tight junctions of Sertoli cells, as well as their secretory

function, have been shown to be compromised following chronic treatment with cis-platin (113).

In addition to the effects on the testes, cis-platin also causes a reduction in weight of the epididymis, ventral prostate, and seminal vesicles (114).

In the epididymis, acute treatment with cis-platin has been shown to cause a reduction in sperm count and sperm motility (115); however, no increase in sperm aneuploidy was observed (116). In a sub-chronic treatment, sperm counts, as well as sperm motility were decreased (117).

The progeny outcome of males treated with cis-platin is also significantly affected. In acute treatments in which males were mated one week after a single intraperitoneal injection, cis-platin resulted in an increase in pre-implantation loss, as well as a decrease in fetal weight (118). This implies an effect on epididymal sperm maturation. In a 9 week treatment, in which males were mated after each week of treatment, cisplatin resulted in increased pre- and post-implantation loss, a decrease in sex-ratio of the offspring, as well as an increase in malformed and growth-retarded fetuses (119).

2.3. Effects of BEP Treatment on Reproductive Function in Humans

Several studies have shown deleterious effects of BEP treatment for testicular cancer on male reproductive function in humans. De Santis *et al.* (120) summarized five studies that evaluated fertility after treatment of testicular cancer with BEP. It was concluded that after 4 courses of therapy, spermatogenesis and testicular exocrine function were altered. More specifically, after treatment

with BEP, there was a decrease in sperm count and sperm motility, and an increase in morphologically abnormal sperm (121). In most patients, however, the sperm count gradually improved after completion of chemotherapy (122), and an 80% recovery rate for spermatogenesis can be expected after 5 years (123).

Studies that have examined aneuploidy in sperm from men treated with BEP have produced conflicting results. While De Mas *et al.* (124) found an increase in aneuploidy for chromosomes 16, 18, and XY, Thomas *et al.* (125) found no change in aneuploidy for chromosomes X, Y 13, 18, and 21, and Martin *et al.* (126) actually found a decrease in aneuploidy for chromosome 1 after BEP treatment. Using the Sperm Chromatin Structure Assay, it has been determined that there is no increase in DNA fragmentation after either <2 or >2 cycles of BEP (127). However, one case report showed high levels of DNA denaturation as the sole semen abnormality in a patient after BEP treatment for testicular cancer (128).

Reports on progeny outcome in males treated with chemotherapy suggest that there is not an increased risk of congenital malformations (129;130); however, these studies are scarce, and do not have the power to detect an increased risk of less than 3-5 fold (131).

2.4. Value of Rat Model

While the reports of altered reproductive function in human males after BEP treatment provide valuable information, it is impossible to conclude from them that BEP is the cause of the malfunction. Semen quality of testicular cancer patients is already decreased at diagnosis, with significantly lowered sperm concentrations, lowered sperm counts, alterations in endocrine function (132), as well as decreased motility and morphology (133). In addition, the semen quality decreases further after orchidectomy, with a highly significant decrease in sperm concentration following removal of the cancerous testis (134). Due to these confounding factors, it is impossible to determine whether the altered reproductive function seen after completion of treatment is due to the chemotherapy, the orchidectomy, the cancer itself, or some combination of the three. Furthermore, most of the human studies lack properly matched control groups, and the patients vary in their duration of treatment and time between completion of treatment and analysis.

The use of a rat model provides the opportunity to elucidate the role of BEP treatment in the alteration of male reproductive function after diagnoses of, and treatment for, testicular cancer. Using the rat, it is possible to administer clinically relevant doses of BEP in a regimen that mimics that given to humans, but in the absence of testicular cancer or any surgery. In addition, the dose, duration of treatment, and time between treatment and analysis can be standardized. This model allows us to remove much of the variation inherent to the human studies, and further determine the role of BEP in impairing male reproductive function.

3. Effects of BEP Treatment on Male Reproduction in the Rat Model

While several studies have examined male reproductive function in human testicular cancer patients after BEP treatment, and several studies have investigated the effects of cis-platin, bleomycin, and etoposide individually in the rat model, no studies to date have examined the effects of BEP treatment in the rat model. To the best of our knowledge, this is the first study of its kind.

4. Hypothesis of the Current Study

Studies on human testicular cancer patients indicate that during the course of diagnoses and treatment for testicular cancer, sperm count and sperm quality decrease. While these studies lead us to speculate that the effects on the male reproductive system are due to BEP treatment, it is impossible to draw a conclusion due to confounding factors such as the presence of cancer, treatment with orchidectomy, and variability in doses and time between completion of treatment and data collection.

We hypothesize that treatment of healthy male rats with BEP alone, in the absence of cancer and orchidectomy, and in a dose and regimen that mimic the human clinical setting, will result in a decrease in sperm quality. More specifically, we hypothesize that BEP treatment will result in a decrease in epididymal sperm count and sperm motility, as well as an increase in morphologically abnormal sperm. We aimed to quantify these effects, and compare them to the known effects of each of the three drugs individually.

To test this hypothesis, healthy male rats were treated with BEP for 9 weeks. At the end of the treatment, the rats were killed and the number of spermatozoa in the caput-corpus epididymidis was counted using a hemocytometer. CASA analysis was used to assess the motility of cauda epididymal spermatozoa. Finally, electron microscopy was used to evaluate ultrastructural changes in the midpiece and the principal piece sections of the flagella of the spermatozoa.

CHAPTER 2

EFFECTS OF CHEMOTHERAPEUTIC AGENTS FOR TESTICULAR CANCER ON MALE RAT REPRODUCTIVE ORGANS AND SPERMATOZOAL NUMBERS, MOTILITY, AND MORPHOLOGY

ABSTRACT

Testicular cancer is the most common cancer affecting men of reproductive age. Advances in treatment of the disease, which includes the co-administration of bleomycin, etoposide, and cis-platin (BEP), have brought the cure rate to over 90%. This high cure rate, coupled with the young age of the patients, makes elucidation of the impact of the treatment on fertility and reproductive function increasingly important. The goal of this study was to determine the effects of BEP, in doses analogous to those given to humans, on spermatozoal count, motility, and morphology in an animal model. Male Sprague Dawley rats were treated daily with BEP for 3 cycles of 3 weeks, for a total of 9 weeks. After 9 weeks, rats were killed and spermatozoa from the epididymis were counted and analyzed for motility and morphology parameters. BEP treatment resulted in decreases in testicular and epididymal weights of 52% and 28%, respectively, when compared to control. Decreased tissue weights were accompanied by a decrease in spermatozoal count of nearly 90% ($11.9x10^7$ spermatozoa per caput epididymidis in control versus $1.65x10^7$ in BEP-treated). The percent motile spermatozoa in the treated rats was greater than 30% lower than that of control. Morphological defects increased by more than 2-fold in the midpiece, and by more than 6-fold in the principal piece of the flagella. The adverse effects of BEP treatment on spermatozoal numbers, motility and morphology may contribute to infertility.

Introduction

Testicular cancer is the most common cancer affecting men of reproductive age. While the incidence of testicular cancer has been rising steadily for several decades (135), mortality due to the disease has been declining (136). Advances in the treatment of the disease, which include the administration of bleomycin, etoposide, and cis-platin (BEP), have brought the 5-year survival rate to over 90% for those patients who are considered to be good-risk (137). This high cure rate makes the post-treatment quality of life of testicular cancer patients a concern, and due to the young age of the patients, consideration of the impact of the treatment on fertility and reproductive function has become increasingly important.

After treatment for testicular cancer, patients experience a decrease in spermatozoal count and motility, as well as an increase in morphologically abnormal spermatozoa (138). Spermatogenesis recovers in most men after 5 years (139). However, reports on the chromatin quality of the surviving spermatozoa are conflicting: one study found an increase in aneuploidy (124), another found no change (125), and yet another found a decrease in aneuploidy (126). The sperm chromatin structure assay revealed no increase in DNA fragmentation (127); however, one case report described a man whose sole semen abnormality after treatment with BEP was an increase in DNA denaturation (128).

While studies of the effects of BEP on spermatogenesis in human testicular cancer patients provide valuable information, their data are confounded

by the fact that the subjects have a diseased testis and have undergone orchidectomy. It has been shown that the semen quality of testicular cancer patients is already decreased at diagnosis (132;133). The semen quality further decreases following orchidectomy, even before the initiation of chemotherapy (134). Furthermore, the patients in these studies have received various numbers of cycles of BEP, and the time from treatment to semen collection is variable. It is thus extremely difficult to conclude whether the effects on fertility are due to chemotherapy, orchidectomy, or the cancer itself.

We hypothesized that even in the absence of testicular cancer and orchidectomy, the chemotherapeutic regimen used to treat testicular cancer is deleterious to spermatozoal production, morphology, and motility. We tested this hypothesis in the rat model.

Materials & Methods

Chemicals

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted.

Animals and Treatment Protocol

Adult male Sprague Dawley rats (300-350 g) were purchased from Charles River Canada (St. Constant, QC, Canada) and housed under controlled light conditions (14L:10D) in the Animal Resources Centre of McGill University. Animals were provided with food and water *ad libitum*, and were randomly

divided into 2 groups of 8 rats each. The rats from the control group were gavaged on days 1-5 of each week with 1 ml of 7:3 saline (Roche, Laval, QC): DMSO (Fischer Scientific, Fair Lawn, NJ). On day 2 of each week, control rats were given 1 ml of saline by IP injection. The animals from the drug-treated group were gavaged on days 1-5 of each week with 3.0 mg/kg cis-platinum (LKT Laboratories, St. Paul, MN) and 15.0 mg/kg etoposide (LKT Laboratories, St. Paul, MN) dissolved in 7:3 saline:DMSO. On day 2 of each week, they were given an IP injection of 1.5 mg/kg bleomycin (LKT Laboratories, St. Paul, MN) dissolved in saline. These doses were chosen based on the standard dose given to humans (140), adjusted for surface-area according to the formula: f x mg/kg = mg/m^2, where f=6.0 for the rat (141). The treatment was continued for 9 consecutive weeks. At the end of the 9 weeks, animals were anaesthetized and the ventral prostate, seminal vesicles, left testis, and left epididymis were removed, trimmed of fat, and weighed. The epididymides were sectioned into caput-corpus and cauda segments. The caput-corpus segments were frozen in liquid nitrogen for the determination of spermatozoal counts; spermatozoa from the cauda epididymidis were used for motility and morphology analyses, as described below. All animal studies were conducted in accordance with the principles and procedures outlined in the Guide to the Care and Use of Experimental Animals prepared by the Canadian Council on Animal Care (McGill protocol #4687).

Spermatozoal Counts

The previously frozen caput-corpus epididymides were homogenized in 5 ml of 0.9% saline, 0.1% merthiolate and 0.05% Triton X-100 (VWR International, Mississauga, ON), for 2 intervals of 15 seconds separated by a 30 second interval. Heads of spermatozoa were counted using a haemocytometer to assess the absolute number of sperm per caput-corpus epididymidis (142).

Spermatozoal Motility

Spermatozoa from the cauda epididymidis were used immediately for computer assisted sperm analysis (CASA), as previously described (143), with the exception that the medium used was as follows: Hanks' Balanced Salt Solution (Gibco Invitrogen Co., Grand Island NY), supplemented with 4.2 mg/ml Hepes, 0.35 mg/ml sodium bicarbonate, 2.0 mg/ml bovine serum albumin, 0.9 mg/ml D-Glucose, and 0.025 mg/ml soybean trypsin inhibitor, pH 7.3-7.4, 37°C (144). Briefly, the epididymis was trimmed free of fat, rinsed in medium, clamped at the corpus-cauda junction, and severed at the corpus side of the clamp. Several tubules of the distal cauda were pierced with a #11 scalpel, and the cauda was transferred to a Petri dish containing 10 ml of medium, allowing spermatozoa to disperse into the medium. The tissue was removed, and the spermatozoa were left to disperse for several minutes. An aliquot of 10 μl of the spermatozoa-containing medium was transferred to a pre-warmed 80 μm deep glass cannula for CASA analysis using the HTM-IVOS system (Hamilton-Thorne Research, Beverly, MA) and version 12 of the Toxicology software. For each animal, 4 slides, each with two 80 μm deep chambers, were analysed. At least

100 spermatozoa/slide were analyzed. The following parameters were determined: percent of motile spermatozoa, percent of progressively motile spermatozoa, curvilinear velocity (VCL), straight line velocity (VSL), average-path velocity (VAP), amplitude of lateral head displacement (ALH), beat cross frequency (BCF), linearity (LIN=VSL/VCL x 100), and straightness (STR=VSL/VAP x 100).

Spermatozoal Morphology

The cauda epididymidis was minced into 7 ml of phosphate-buffered saline (PBS) (Roche, Laval, QC). The suspension was filtered, washed several times with PBS, fixed for 1 hour in 1% glutaraldehyde (Mecalab LTD, Montreal, QC) in PBS, and washed again with PBS. The resulting pellet was suspended in 1% agarose (Gibco Invitrogen Co., Grand Island NY), and embedded for electron microscope analysis as follows. The samples were washed three times in 0.1 M sodium cacodylate buffer containing 3% sucrose, pH 7.4, post-fixed in 1% osmium tetroxide and 1.5% potassium ferrocyanide, and embedded in epoxy resin. Sperm ultrastructure was then analyzed on the electron microscope (Philips 410 Electron Microscope, Eindhoven, Netherlands). At least 100 midpiece cross-sections and 100 principal piece cross-sections per sample were photographed. The number of midpiece cross-sections and principal piece cross-sections with any abnormality, as well as the number with a cytoplasmic droplet, were recorded.

Statistical Analysis

All data was analysed using the Student's *t*-test. Data are presented as the mean ± SEM. The level of significance was considered as $P<0.05$.

Results

Animal and Tissue Weights

During the treatment, one rat from the BEP group became sick and was killed. The remaining BEP-treated rats gained less weight than the control rats, although the body weights of the BEP-treated rats did increase over the course of the treatment (Figure 5). We observed a dramatic effect on the weights of the testes and epididymides in the BEP-treated rats. There was a decrease in testis weight of approximately 50%, and a decrease in epididymal weight of approximately 30% in the BEP-treated rats when compared to controls. There was no significant effect on seminal vesicle or ventral prostate weights (Figure 6).

Spermatozoal Numbers, Motility, and Morphology

We observed a striking effect on the numbers of spermatozoa in the BEP-treated rats. The total number of spermatozoa per caput-corpus epididymidis in the BEP-treated rats was reduced by more than 90% when compared to control rats (Figure 7).

A number of the motility characteristics of the BEP-treated spermatozoa were significantly altered (Figure 8). The overall percent of motile spermatozoa was approximately 30% lower in the BEP-treated rats when compared to control.

While the range of values for percent motile spermatozoa was relatively small among the control group (67.8 – 77.3 %), the values for the drug-treated rats varied greatly; one rat had 7.5% motile spermatozoa, while another had 71.0 % motile spermatozoa. The percent of progressively motile spermatozoa was unchanged in the BEP-treated group. All three of the velocity parameters (VAP, VCL, VSL) were significantly decreased in the BEP-treated animals; both the VAP and the VCL decreased by approximately 10%, and the VCL by approximately 13%. In addition, the parameters reflecting beat characteristics were altered in the drug-treated rats; the ALH was approximately 20% lower, and the BCF approximately 10% higher when compared to control. The parameters that reflect the straightness (STR and LIN) with which the spermatozoa swim were not significantly altered in the drug-treated rats.

We observed an increase in the percent of spermatozoa with morphological abnormalities in the midpiece of the flagella after BEP treatment (figure 9). The midpiece of a control spermatozoon consists of 9 microtubule doublet pairs surrounding one central doublet. Each of the 9 peripheral doublet pairs is associated with one outer dense fibre. Surrounding the outer dense fibres is the mitochondrial sheath (figure 10a). In the control group, the only abnormalities seen were an abnormal spatial arrangement of outer dense fibres, and the presence of two or more flagellar sections within one membrane. Some of the abnormalities observed in the midpiece cross-sections of spermatozoa from BEP-treated rats (figure 10) were the same as the two abnormalities seen in the control group. Interestingly, other abnormalities were found in spermatozoa

from the BEP-treated animals that were not observed in the control group. These included an absence of the mitochondrial sheath, an abnormal number of outer dense fibres (either too many or too few), hemilateral absence of the axoneme and the outer dense fibres, a malformed mitochondrial sheath, the presence of some normal outer dense fibres and some that appear small and malformed, as well as sections that were malformed and had a combination of the abnormalities listed above in addition to excess cytoplasm that contained outer dense fibres and debris (figure 10). There was no increase in the percent of sperm with a cytoplasmic droplet in the BEP group.

In addition to defects in the midpiece, an increase in the percent of spermatozoa with abnormalities in the principal piece of the flagella was observed (figure 9). The principal piece of a sperm of a control rat (figure 11a) consists of the same 9 + 2 arrangement of microtubules as the midpiece; however, outer dense fibres 3 and 8 are replaced by the 2 longitudinal columns of the fibrous sheath. Surrounding the outer dense fibres are the circumferential ribs. In this region of the flagella the abnormalities seen in the BEP group were also seen in the control group, but at significantly higher numbers compared to the control group (figure 9); these included a hemilateral absence of the outer dense fibres, with or without an intact axoneme, and a missing outer dense fibre (figure 11).

Discussion

Although patients treated with BEP for testicular cancer experience significant reproductive problems, no studies to date have confirmed that these symptoms are directly the result of the chemotherapy. The use of an animal model allows us to administer BEP in a dose and regimen that is clinically relevant, without the presence of testicular cancer or orchidectomy. This provides the opportunity to elucidate the role of BEP in decreasing semen quality.

In the current study, male rats were treated continuously for 9 weeks with BEP. The BEP-treated animals experienced a substantial decrease in the weights of both the testes and epididymides. The reduction in spermatozoal numbers to approximately 10% of control values is most likely the cause of the decreased organ weights (145). We found that 71% of the BEP treated rats had a spermatozoal count that was decreased by >90% when compared to control. Relatively few studies have examined the impact of BEP treatment on spermatozoal numbers in men immediately after the completion of chemotherapy. The spermatozoal concentration in men who have undergone BEP gradually improves after completion of treatment (122), and thus it is difficult to compare the response in humans with the current study.

In a study by Stephenson et al (146), 57% of human patients treated with BEP had less than 50% motile spermatozoa. In the rat, motility of less than 70% is considered abnormal (147). In the current study, 86% of drug treated rats had

spermatozoal motility below 70%. Again, this discrepancy is most likely due to the recovery time in humans. In addition to the percent motility, the VAP, VSL, VCL, BCF, and ALH were significantly altered in the BEP treated rats. Of these parameters, VSL, VCL, and BCF have been shown to be correlated with fertility (148). This may explain why fertility remains a problem even in patients who are not oligospermic (149).

No studies to date have examined the morphology of spermatozoa from human testicular cancer patients at the electron microscope level. We observed an increase in the incidence of morphologically abnormal spermatozoa, as well as a larger variety of abnormalities, in BEP treated rats. Interestingly several of the midpiece abnormalities we observed uniquely in BEP treated rats have been reported after other drug treatments, such as cyclosporine A (35) or triptolide, a diterpene triepoxide isolated from a Chinese plant (150). These abnormalities, however, are extremely rare in control rats (36). The flagellum is formed during spermiogenesis in the testis, suggesting that the increase in abnormalities in BEP treated rats probably reflects a defect in spermiogenesis, rather than epididymal maturation. Nevertheless, a number of the flagellar defects found in selenium-deficient rats were detected only after the spermatozoa left the caput epididymidis (151), suggesting that the defects we observed may originate either during spermiogenesis in the testis or spermatozoal maturation in the epididymis.

To the best of our knowledge, this is the first study involving the administration of bleomycin, etoposide, and cis-platin concurrently in the rat model. Each of these drugs, however, has been given individually to rats

(96;119;152). Of the three drugs, only the effects of cis-platin on spermatozoal numbers, motility, and morphology have been examined (119). After 9 weeks of daily intra-peritoneal treatment with 0.5mg/kg cisplatin, rats experienced a decrease in spermatozoal count of approximately 60%, and a decrease in motility of approximately 50%. The effect on motility is much larger than we observed in our study; however, the effect on spermatozoal numbers is much smaller. The larger effect on motility with cis-platin alone may be a result of the route of administration, as it is interesting to note that the percent of sperm that were motile in the control rats in the study (56%) is well below what is considered normal (153). The larger effect on spermatozoal count in our study, however, is likely to be due to an additive effect of the three drugs, and underscores the value of using a combination regimen in the rat model.

In the rat, a decrease in sperm production of > 90% is required to impair fertility (154). Furthermore, changes in motility and morphology can also impair fertility. This suggests that the BEP treated rats may be infertile; however, further studies are required to determine the effects of BEP treatment on fertilization and progeny outcome.

These results clearly show that BEP treatment has a deleterious affect on spermatozoal quality in the male rat, resulting in a decrease in spermatozoal numbers and motility, as well as an increase in morphologically abnormal spermatozoa. This suggests that the chemotherapeutic agents, rather than cancer or orchidectomy, may be the cause of decreased spermatozoal quality in patients who have been treated for testicular cancer.

Figure 5. Body weight changes over the course of the 9-week treatment expressed as weight on the last day of treatment divided by weight on the first day of treatment (Control n=8, BEP n=7). The BEP-treated rats did not gain as much weight as the control rats. *P<0.01

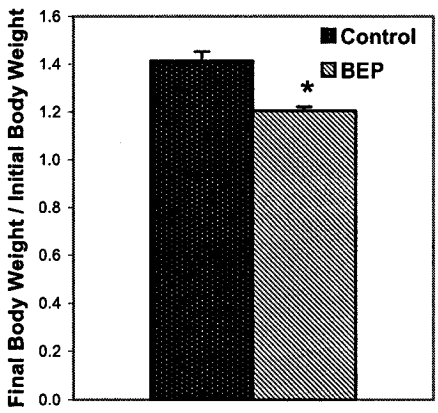

Figure 6. Weights of reproductive organs after 9 weeks of treatment with vehicle or BEP (Control n=8, BEP n=7). There was a significant decrease in the weights of the left testes and epididymides of the BEP-treated animals, while the weights of the left and right seminal vesicles and ventral prostate remained unchanged. ** P<0.001

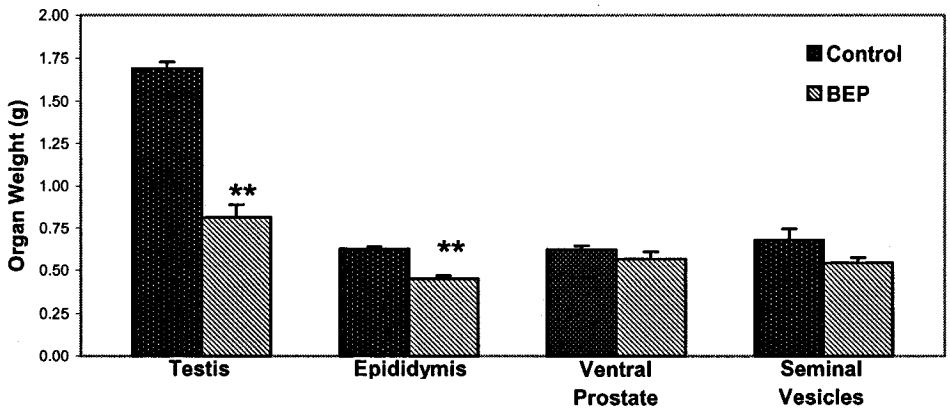

Figure 7. Spermatozoal counts in the caput-corpus epididymidis after 9 weeks of treatment with vehicle or BEP (Control n=8, BEP n=7). Spermatozoal heads were counted with a hemocytometer to determine the reserves of spermatozoa in the caput-corpus epididymidis. BEP-treated rats had a reduction in spermatozoal count of approximately 10-fold. **P<0.001

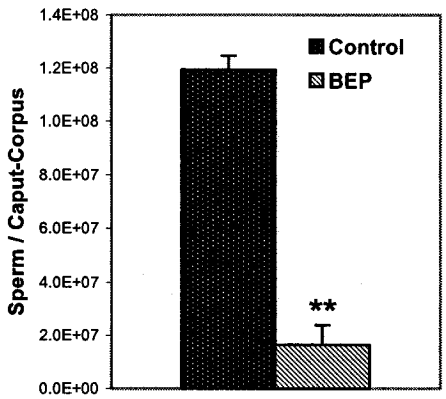

Figure 8. Effects of BEP treatment on percent of motile spermatozoa, percent progressively motile spermatozoa, as well as the motility characteristics of spermatozoa obtained from the distal cauda epididymidis of control or BEP treated rats (Control n=8, BEP n=7) (black bars = control; grey bars = BEP). VCL=curvilinear velocity, VSL=straight line velocity, VAP=average path velocity, ALH=amplitude of lateral head displacement, BCF=beat cross frequency, STR=straightness, and LIN=linearity. *P<0.01; **P<0.05

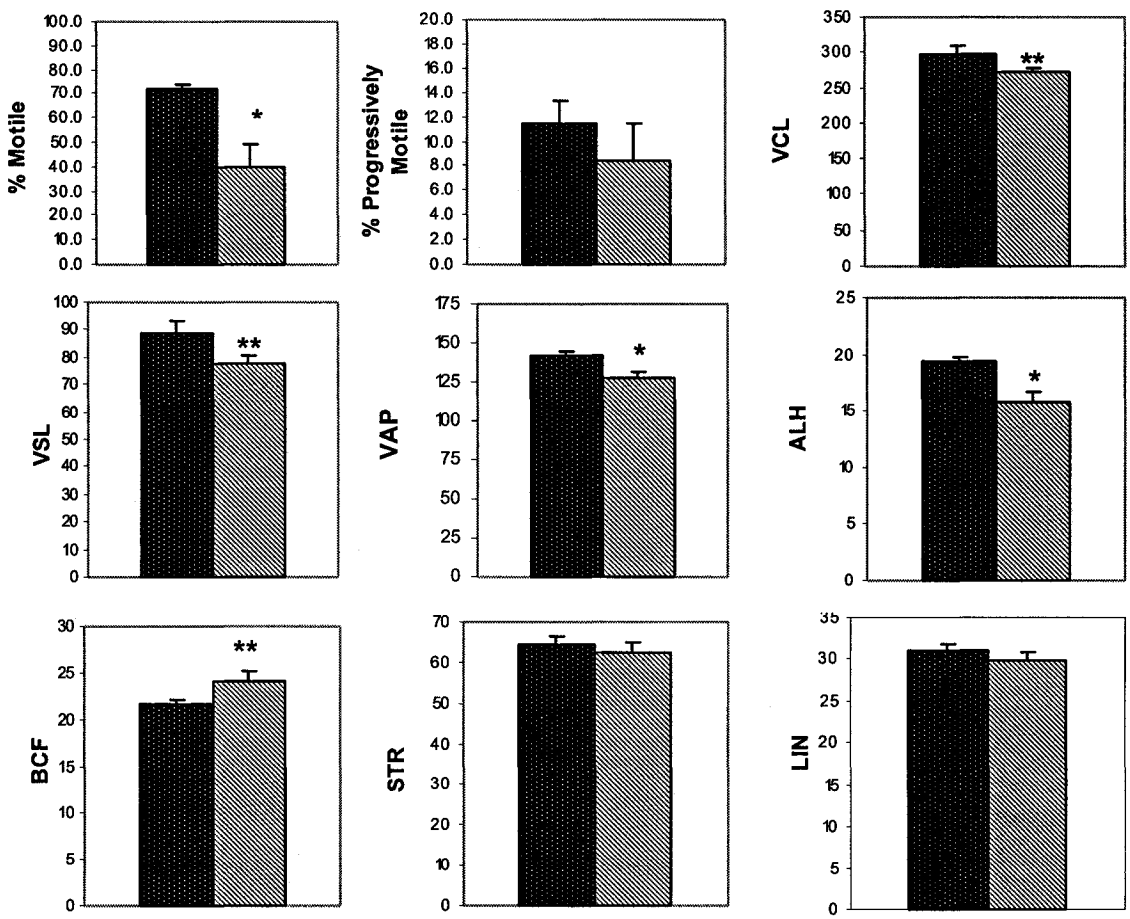

Figure 9. Percent abnormalities in both the midpiece and principal piece of spermatozoa from control or BEP-treated rats (control n=4, BEP n=4). At least 100 midpiece and principal piece cross-sections were counted per rat, and the numbers of sections with any abnormality were recorded. The BEP-treated animals experienced an increase in the percent of both midpiece and principal piece cross sections with abnormalities. *P<0.05

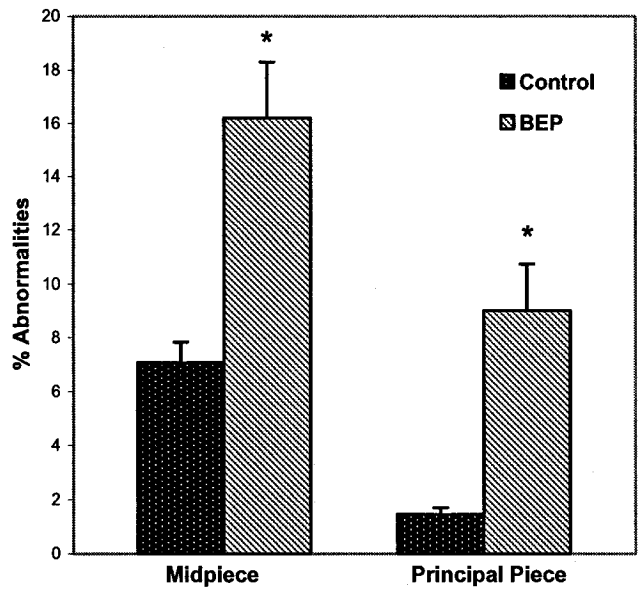

Figure 10. Morphology of the midpiece of control spermatozoa, displaying the 9+2 arrangement of microtubules (M), the 9 outer dense fibres (ODF), and the mitochondrial sheath (Mt) (A), as well as spermatozoa from rats treated with BEP (B-I). Abnormalities include an abnormal spatial arrangement of outer dense fibres (B), the presence of two flagellar sections within one membrane (C), absence of a mitochondrial sheath (D), an abnormal number of outer dense fibres (E), hemilateral absence of the outer dense fibres and axoneme (F), a malformed mitochondrial sheath (G), malformed outer dense fibres (H), as well as a malformed midpiece (I). Scale bars represent 100μm.

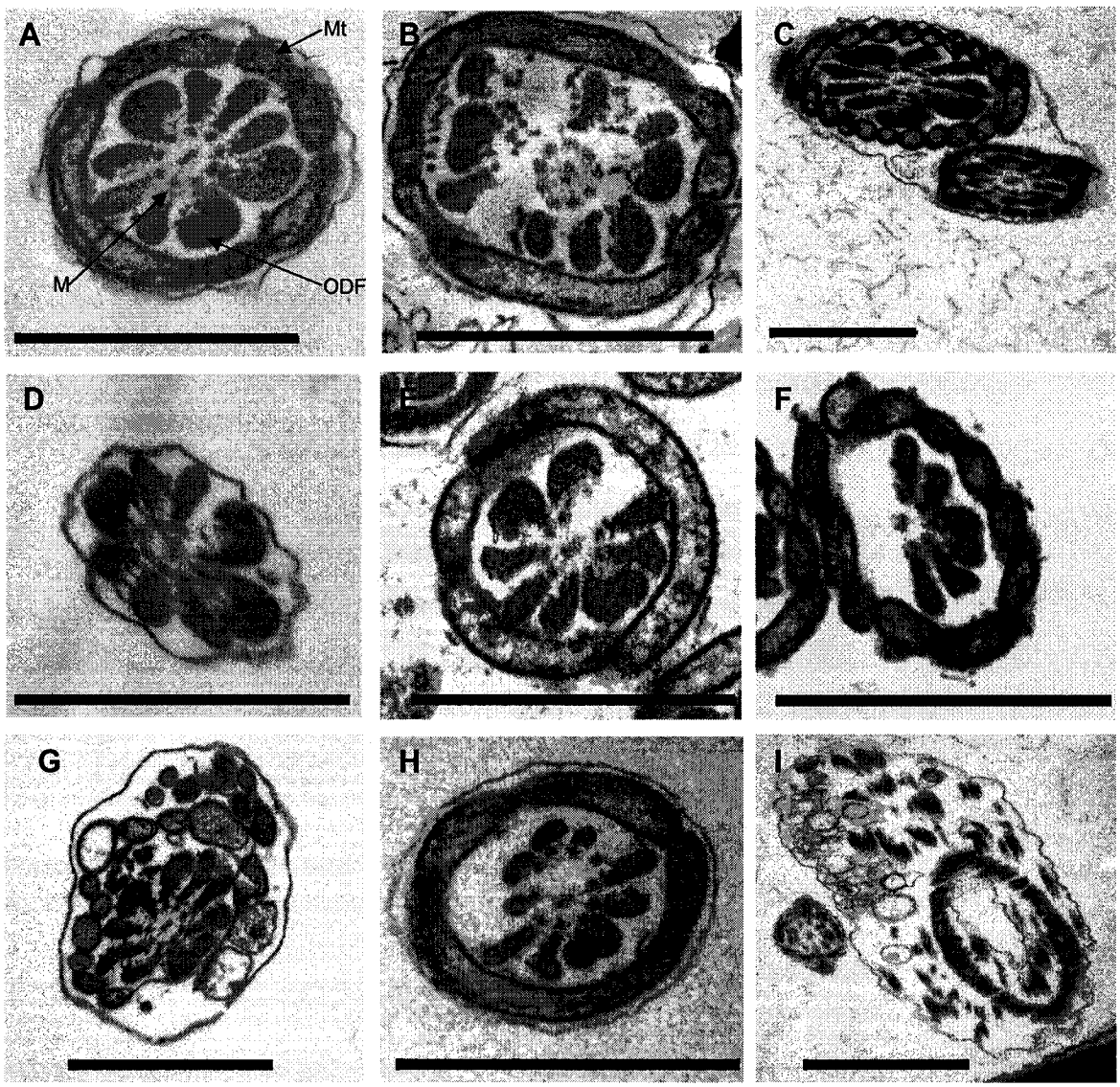
A
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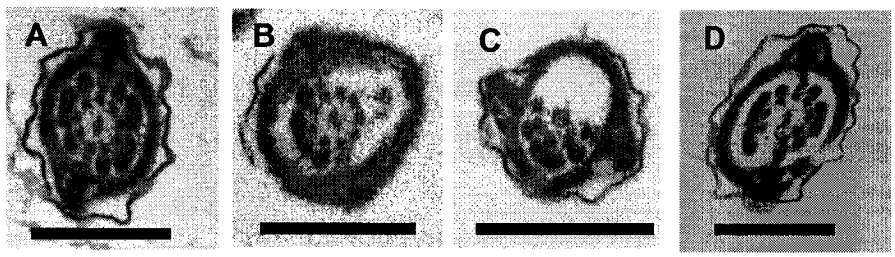
Figure 11. Morphology of the principal piece of a control spermatozoon (A), as well as spermatozoa from rats treated with BEP (B-D). Abnormalities include the hemilateral absence of the outer dense fibres, with (B) or without (C) hemilateral absence of the axoneme, as well as an abnormal number of outer dense fibres (D). Scale bars represent 50 µm.



CHAPTER 3

DISCUSSION & CONCLUSIONS

The incidence of testicular cancer has risen significantly in recent years; however, the cure rate of the disease is now reported to be over 90% (135;136). Because the disease affects mainly men in their reproductive years, the effects of the treatment on reproductive function have become progressively more important. Studies examining human tissues have provided valuable information regarding the effects of testicular cancer and its treatment on male reproductive function (146); however, they are confounded by several factors. These include evidence that sperm quality is already decreased at diagnosis, and further decreases after orchidectomy (132-134). Due to these confounding factors in human studies, we aimed to elucidate the role of combination chemotherapy in the rat model. This model allows us to use healthy "subjects", and eliminate the effects of the cancer itself, or any surgeries. It also allows for the standardization of dose and treatment regimen.

Human testicular cancer patients are treated with bleomycin, etoposide, and cis-platin (BEP) for cycles of three weeks. Patients that are considered good risk receive three cycles of treatment, while poor-risk patients may receive four cycles. In the current study, we treated rats with BEP for three cycles of three weeks. Humans receive each of the three drugs intravenously. Due to inherent difficulties with chronically treating rats intravenously, we designed a treatment regimen whereby etoposide and cis-platin were given by gavage, and bleomycin was given by an intraperitoneal injection.

Upon completion of treatment, rats were killed, and their reproductive organs were weighed. Spermatozoa from the cauda epididymidis were analyzed

for motility parameters using a computer assisted sperm analysis (CASA) program, as well as for morphology using electron microscopy. Spermatozoa from the caput-corpus epididymidis were counted using a hemocytometer to quantify epididymal sperm reserves.

We determined that with BEP treatment, the weights of the testis and epididymis decreased dramatically, suggesting a defect in sperm production. The seminal vesicles and ventral prostate weights remained unchanged, indicating that there were no anti-androgenic effects (155). The caput-corpus epididymal spermatozoal reserves were reduced by approximately 10-fold. CASA analysis showed a reduction in total sperm motility by approximately 30%, without a change in progressive motility. Additionally, the VAP, VSL, VCL, ALH, and BCF were significantly altered, suggesting that swimming is sluggish and less vigourous. Electron microscope analysis revealed a wide variety of malformations; specifically, an increase in the number of mid-piece and principal piece cross-sections that were malformed was observed. There was no increase in the number of cytoplasmic droplets in the BEP treated animals. All of the defects in the midpiece morphology that we observed have been previously reported, but are extremely rare in control animals (35;36). Because of the effects in the morphology, it is tempting to speculate that there is a defect in spermiogenesis, although a defect in epididymal maturation cannot be ruled out without repeating these experiments after a 1 week treatment, or analyzing sperm from the caput epididymidis. Obtaining cauda epididymal spermatozoa after a 1 week treatment would isolate sperm that were exposed to the drugs

only during epididymal passage. Additionally, obtaining caput epididymal spermatozoa after a 9 week treatment would isolate sperm that had not yet passed through the epididymis, and thus any defects seen would have to have been formed in the testes. Although an epididymal defect may seem unlikely, Olson *et al* (151) found that rats maintained on a selenium deficient diet sequentially developed flagellar defects throughout epididymal maturation. These defects occurred via an extrusion of the outer dense fibers from the ends of the mitochondrial sheath. Thus, it is possible that these defects can occur after spermiogenesis. It is interesting to note that in the selenium-deficiency study, when an ODF was missing, it was always ODF # 4, and when several were missing, #4 was always among the missing fibers. This is the same pattern we observed in the sperm of the BEP-treated rats. This suggests that there may be a similar mechanism involved in causing the morphological defects observed in selenium-deficient rats and BEP treated rats. To the best of our knowledge, there is no evidence of selenium deficiency in humans after treatment with BEP.

It has been shown that a reduction in sperm count by at least 90% is required to impair fertility in the male rat (154). The BEP treated rats had a reduction in epididymal sperm reserves of approximately this amount. In addition, several of the motility parameters have been shown to be correlated with fertility (148). This suggests that the BEP treated rats may have reduced fertility. Our laboratory has recently performed mating studies to determine whether BEP treatment affects male fertility and progeny outcome (unpublished observations). Surprisingly, when females that were mated to BEP-treated rats

were killed on gestation day 21 and the fetuses examined, there was no decrease in litter size when compared to control, and all females that were sperm positive after mating became pregnant. This suggests that a reduction in sperm count of much greater than 90% is required to impair fertility. In addition, we did not observe any fetal abnormalities or birth defects in the progeny of the BEP-treated males. This is unexpected especially in light of studies that have shown that cis-platin or etoposide alone can result in abnormal progeny outcome (100;118;119).

Further studies performed in our laboratory have shown that although there are no visible defects on gestation day 21, when the mothers were allowed to give birth and nurse the pups, there was a significant decrease in the number of pups surviving after birth. This suggests that although there were no gross abnormalities, the pups may have metabolic, behavioral, or respiratory problems. It is therefore likely that in addition to the morphological and motility defects shown here, the spermatozoa have suffered damage to their DNA that is being passed on to the next generation. This hypothesis is further supported by the fact that abnormal sperm morphology is correlated with high levels of DNA damage (156). In addition, all three drugs alone have been shown to cause DNA damage in rat spermatozoa: Bleomycin can induce reciprocal translocations (157) and is mutagenic to spermatogonia (152); etoposide can induce aneuploidy (97) and micronuclei (98); and cis-platin alone has in fact already been shown to affect progeny outcome (119). This hypothesis that paternal BEP-treatment can cause DNA damage can be tested using several assays available in our laboratory,

such as the comet Assay, Sperm Chromatin Structure Assay, or fluorescence in situ hybridization

In summary, we have shown that BEP treatment results in a decreased sperm count, as well as defective sperm motility and morphology in the male rat. These results are independent of the presence of testicular cancer or any surgery. Follow-up studies have indicated that there may be genetic damage that is being passed on to the progeny; however this needs to be confirmed with further studies.

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