Investigating the effects of chemotherapeutic agents for testicular cancer on the male reproductive system, progeny outcome and spermatogonial stem cells in the rat

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ABSTRACT

Testicular cancer (TC) is the most common type of cancer affecting men between the ages of 15 and 35 years old. The standard chemotherapy for testicular cancer is a combination of bleomycin, etoposide and cisplatin (called BEP regimen) that results in cure rates over 90%. In order to determine whether the BEP regimen induces adverse effects on the male reproductive system and progeny outcomes, in chapter 2, male Sprague-Dawley rats were treated with a combination of bleomycin, etoposide and cisplatin for 9 consecutive weeks. BEP treatment resulted in decreased testis and epididymal weights, and sperm counts, but despite the dramatic effects on spermatogenesis, paternal exposure to BEP did not affect fertility, nor were there adverse effects on litter size, pre-and post-implantation losses, fetal weight and death rate or sex ratio observed among progeny sired by treated males; however, increased mortality was observed in pups during the perinatal phase. In chapter 3, we evaluated the reversibility of a subchronic BEP treatment on the male reproductive system and progeny outcome. Male rats were treated with a 9 week subchronic BEP regimen; progeny outcome parameters were determined every 3 weeks for a total of 9 weeks during recovery. Subchronic BEP caused transient defects on spermatogenesis; however, a prolonged increase in pre-implantation loss was observed up to 9 weeks after completion of the treatment, suggesting that spermatogonial stem cells may be affected. Thus, in chapter 4, the impact

of BEP chemotherapy on spermatogonial stem cells (SSCs) was investigated. Testicular cell suspension from BEP-treated GCS-EGFP transgenic rats were transplanted into busulfan-treated recipient nude mice. The number and length of colonies-derived from transplanted stem cells was assessed as a function of stem cell activity after exposure to BEP. Finally, in chapter 5, we assessed the *in vitro* effects of bleomycin, etoposide and cisplatin alone and in combination on cultured rat stem/progenitor spermatogonia. These results indicated that BEP has deleterious effects on male reproductive functions including SSCs and progeny outcomes.

RESUME

Le cancer testiculaire (ou cancer du testicule) est le type de cancer le plus commun chez les hommes âgés de 15 à 35 ans. La chimiothérapie de première ligne pour le cancer du testicule est la combinaison de bléomycine, étoposide et cisplatine (appelé protocole BEP) qui permet un taux de guérison de plus de 90%. Afin de déterminer si le protocole BEP induit des effets néfastes sur le système reproducteur mâle ainsi que sur la progéniture, dans le second chapitre, nous avons traités des rats mâles (Sprague-Dawley) avec une combinaison de bléomycine, étoposide et cisplatine pendant 9 semaines consécutives. Le protocole BEP entraîna la diminution du poids des testicules et des épididymes, du nombre de spermatozoïdes, mais en dépit des effets spectaculaires sur la spermatogénèse, l'exposition du père au protocole BEP n'affecta pas la fertilité et aucun effet ne fut observé sur la taille de la portée, les pertes aux stades pré- et post- implantatoires, le poids et la mortalité fœtale ou encore sur le ratio mâle/femelle des fœtus issus de pères traités. Cependant, une augmentation de mortalité peu de temps après la naissance fut observée. Dans le chapitre 3, nous avons évalué la réversibilité des effets du protocole BEP sub-chronique sur le système reproducteur mâle et la progéniture. Des rats mâles furent traités avec un protocole BEP sub-chronique de 9 semaines ; les paramètres de la progéniture furent déterminés toutes les 3 semaines pour un total de 9 semaines pendant la période de rétablissement. Le protocole BEP sub-

chronique entraîna des effets transitoires sur la spermatogénèse, cependant une augmentation prolongée des pertes pré-implantatoires fut observé jusqu'à 9 semaines après la fin du traitement, suggérant que les cellules souches germinales puissent être affectées. Ainsi, dans le chapitre 4, l'impact de la chimiothérapie BEP sur les cellules souches germinales (CSGs) fut étudié. Des suspensions de cellules testiculaires provenant de rats transgéniques GCS-EGFP traités avec le protocole BEP furent transplantées dans des testicules récipiendaires traités au busulfan de souris immunodéficientes. Le nombre ainsi que la longueur des colonies provenant des cellules souches transplantées fut déterminé pour évaluer la capacité des cellules souches germinales exposées au BEP à rétablir la spermatogénèse. Enfin, dans le chapitre 5, nous avons évalué les effets in vitro de la bléomycine, de l'étoposide et du cisplatine seul ou en combinaison sur des cultures de spermatogonies. Ces résultats indiquent que le protocole BEP produit des effets néfastes sur les fonctions reproductives incluant les cellules souches germinales et la progéniture.

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LIST OF ABBREVIATIONS

As	A single
Ap	A paired
Aal	A aligned
ANOVA	Analysis of Variance
BSA	Bovine serum albumin
BEP	**B**leomycin-**E**toposide-cis**P**latin
DMEM	Dulbecco`s modified eagle`s medium
DMSO	Dimethyl sulfoxide
EGFP	Enhanced green fluorescent protein
FGF2	Fibroblast growth factor 2
GDNF	Glial-cell line derived neurotrophic factor
GFRA1	GDNF family receptor alpha 1
MEMα	Minimum eagle`s medium alpha
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
RT	Room temperature
SD	Standard deviation
SEM	Standard error of the mean
SSC	Spermatogonial stem cell
TC	Testicular cancer
TGCT	Testicular germ cell tumor
TUNEL	Terminal deoxynucleotidyl transferase-mediated digoxigenin-dUTP nick end labeling
ZBTB16	Zinc finger and BTB domain containing 16

PREFACE

Format of the thesis

This thesis is comprised of six chapters. Chapter 1 is a general introduction that includesan overview of the male reproductive system and SSC transplantation and culture as well as a literature review on testicular cancer, BEP chemotherapy and previous work on cisplatin, etoposide and bleomycin. Chapter 2 to 5 are data chapters that are presented as the duplicated text of published papers or papers submitted for publication in respect with the “Guidelines for Thesis Preparation” . Chapter 2 and 3 were published in Journal of Andrology (J Androl. 2006 Mar-Apr;27(2):189-200 and J Androl. 2008 Jul-Aug;29(4):408-17). Chapter 4 was submitted for publication to Journal of Andrology, 2010. Chapter 5 was published in Biology of Reproduction 2010 Aug 1;83(2):228-37. The copyright agreements of the respective publishers, Journal of Andrology by the American Society of Andrology and Biology of Reproduction by the Society for the Study of Reproduction, permit the inclusion of these manuscripts in this thesis. Chapter 6 contains a discussion of all the results of this thesis and potential future studies. It also includes a List of Original Publications. The appendix contains the ethics certificates for work on animal subjects and the copyright notices of the Journal of Andrology and Biology of Reproduction.

Contribution of authors

All of the experiments described in this thesis were performed by the candidate with the exceptions of the sperm motility and morphology analyses, and postnatal death analysis, in chapter 2, which were done by A.M Bieber and the spermatogonial transplantations in chapters 4 and 5, which were carried out by X. Zhang.

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Chapter 1

Introduction

1. General introduction

It is well documented that many chemotherapeutic agents used in clinic are toxic to the male reproductive system, in particular to the gonads [1-3]. These drugs may induce temporary or permanent infertility. Therefore, understanding the normal physiology of the male reproductive tract is essential for the characterization of the adverse effects of chemotherapeutic drugs.

1.1 Anatomy of the male reproductive system

The male reproductive system consists of the paired testes, the system of excurrent ducts that store and transport spermatozoa to the exterior (epididymides, vas deferens, efferent ducts, urethra), the accessory sex glands that empty into these ducts (seminal vesicles, prostate, bulbourethal glands), and the penis. This entire set of organs acts together to produce and maintain functional mature spermatozoa that are destined to be delivered to the female reproductive tract.

1.2 The mammalian testis - an overview

The testis (plural: testes) is the male gonad. In mammals, the testes are paired oval-shaped organs that lie inside the scrotum, suspended by the spermatic cords. The scrotum helps to maintain a testicular temperature 2-3 degrees lower than the body temperature that is essential for proper testicular function [4]. Each individual testis is covered by a dense fibrous layer of connective tissue called the tunica albuginea that maintains its content (Figure 1.1 A). The testes have two primary

functions: they are responsible for spermatogenesis, the process by which spermatozoa are produced and steroidogenesis with synthesis and release of male sex hormones or androgens. Therefore, they are components of both the reproductive system and the endocrine system [5].

1.2.1 Organization of the testis

The testis is itself composed of two discrete compartments: the interstitium or interstitial space and the seminiferous tubules. These two compartments are not only physically divided but also functionally distinct from each other, with spermatogenesis arising in the seminiferous epithelium and androgen synthesis in the Leydig cells of the interstitium (Figure 1.1 B). The interstitial compartment consists of loose connective tissue that contains blood and lymphatic vessels, nerves as well as connective tissue-associated cells (non endocrine cells) such as macrophages, fibroblasts, mast cells and lymphocytes. The prominent cell type of the interstitial compartment is the Leydig cell. Leydig cells are large polyhedral cells that are often found in small clusters around blood vessels and are responsible for the production and secretion of the sex hormones, i.e, primarily testosterone, but also other steroids such as estradiol. Thus, Leydig cells are the principal source of androgens in the males, the most important of which is testosterone. Testosterone is well-known to be involved in the expression of the male phenotype including masculinisation of the brain and sexual behaviour, the differentiation of the male sex

organs and secondary sex characteristics. More importantly, testosterone is crucial for the initiation and maintenance of spermatogenesis. The absence of testosterone results in the arrest of spermatogenesis and loss of fertility [6]. Thus, testosterone is essential for male fertility.

1.2.2 Structure of the seminiferous epithelium

The testis consists of long and highly convoluted seminiferous tubules that are connected at both ends to the rete testis (Figure 1.1 A). Such tubule structure increases the surface area of the seminiferous epithelium and consequently the number of spermatozoa produced. The seminiferous tubules, that make up approximately 90% of the weight of each testis, are the functional units of the testis in which spermatogenesis takes place. In the testis, seminiferous tubules are surrounded by a single layer of contractile peritubular myoid cells (Figure 1.1 B). Through their contractility, peritubular myoid cells are involved in the transport of spermatozoa and tubular fluid out of the testis to the efferent duct [7]. In addition, peritubular myoid cells secrete extracellular matrix components (including laminin, type I and IV collagens, fibronectins and proteoglycans) and growth factors that are part of the basement membrane. In mammals, the seminiferous epithelium of the adult testis is comprised of two distinct cell types: the germ cells at diverse stages of development and the supporting Sertoli cells, whose functions will be discussed later. Germ cells include spermatogonia, primary and secondary spermatocytes,

spermatids and spermatozoa. In the center of each tubule is a fluid-filled lumen containing spermatozoa.

1.2.3 The Sertoli cell

In the adult testis, the seminiferous epithelium consists of only one somatic cell type, the Sertoli cells and the developing germ cells. Sertoli cells are fully differentiated, non-dividing cells (therefore non-renewable) of a single type. Histologically, Sertoli cells are large columnar cells having cytoplasm spans from the basal lamina of the tubule wall to the adluminal space or lumen [8, 9]. In addition, adjacent Sertoli cells are connected by tight junctions herein forming the blood-testis barrier (BTB) which divides the seminiferous epithelium into two functional compartments: the basal compartment that contains spermatogonia and early spermatocytes (which are accessible to the vascular system) and the adluminal compartment that contains late spermatocytes, spermatids and spermatozoa (Figure 1.1 C, arrow head). This tight barrier acts as a diffusion barrier to maintain an optimal environment within the seminiferous tubules and help to prevent the diffusion of potential toxic substances from the blood and immune cells within the tubules [10, 11]. Sertoli cells provide various nutrients and structural support to germ cells throughout their development (providing nutrients and secrete numerous proteins such as inhibin, activin, androgen binding protein and growth factors). Sertoli cells are indispensable for the development and differentiation of spermatogenic cells; they provide the structural framework over which germ cells will proliferate and differentiate

thereby contributing to the generation of a microenvironment within the seminiferous epithelium. They orchestrate growth factor signalling so as to ensure spermatogonial stem cell development toward germ cell differentiation thereby maintaining quantitatively normal sperm production. They also facilitate germ cell movement towards the lumen and release of spermatozoa. Because Sertoli cells are a fixed population of non proliferating cells, the total number of Sertoli cells defines the sperm production capacity of an adult testis depending of the species. Within the seminiferous tubules, germ cells are organized in a defined architecture dictated by Sertoli cells which varies with stages of the seminiferous epithelium cycle. Therefore, the Sertoli cell population is of utmost importance for the male reproductive capacity.

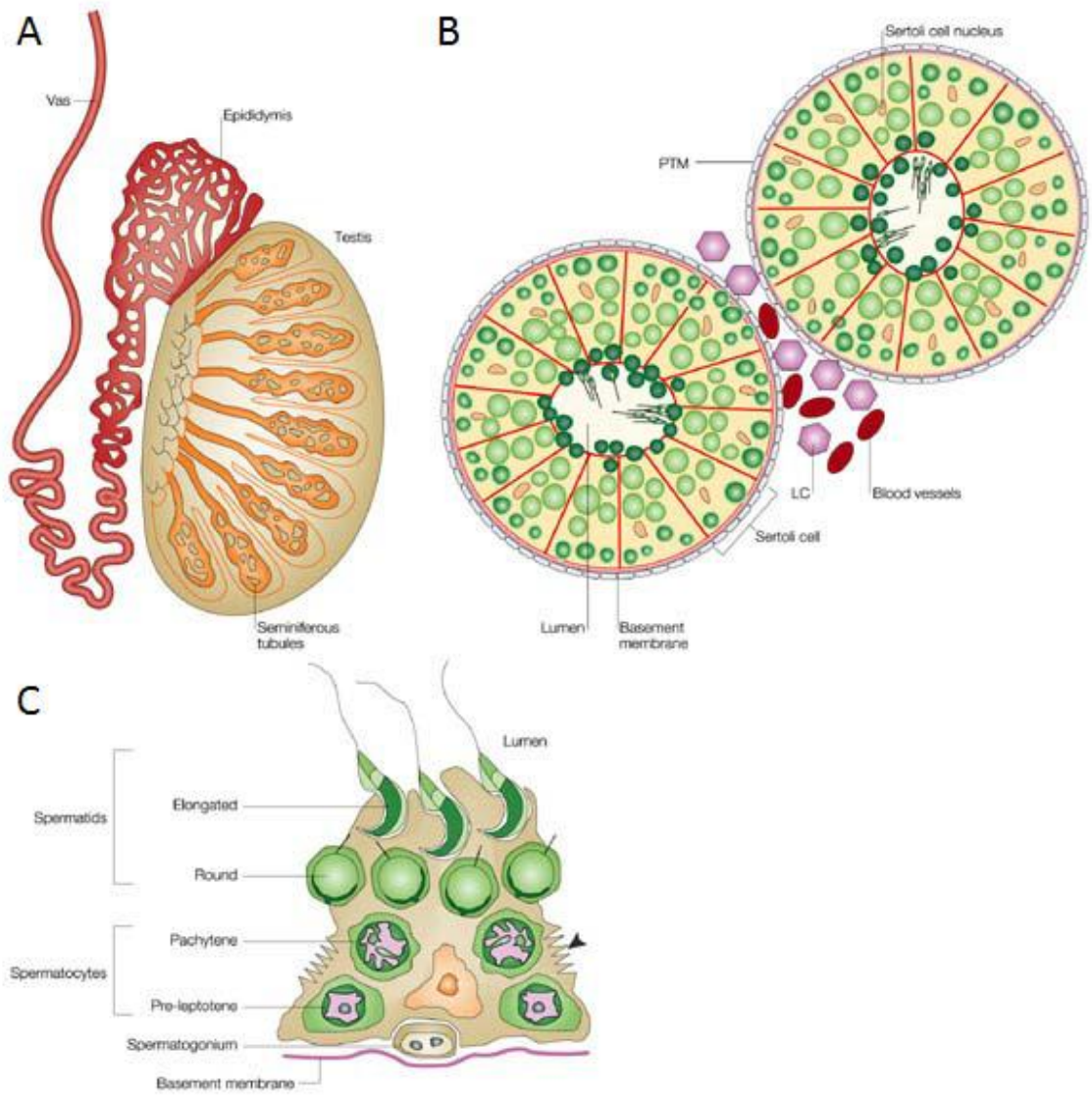

Figure 1.1 Organization of the mammalian testis.

A) A schematic representation of a longitudinal cross-section through an adult testis and subsequent vas deferens and epididymis. **B)** A schematic representation of a cross-section through seminiferous tubules within the basal membrane and separated by the interstitium. The interstitium contains the Leydig cells (LC) and blood vessels. Inside the seminiferous

tubules, the developing germ cells (green) at different stages of maturation are in close association with Sertoli cells (red). Mature spermatozoa migrate towards the lumen of the tubules. Each seminiferous tubule is surrounded by a layer of peritubular myoid cells (PTM). **C)** Sertoli cells lining the basement membrane are closely associated with developing germ cells throughout spermatogenesis. Early germ cells reside on the basement membrane while more differentiated germ cells move towards the lumen of the tubule. Modified from Cooke and Saunders [12].

1.3 Spermatogenesis

In mammals, spermatogenesis is one of the most productive self-renewing systems in the body, with millions of spermatozoa produced per gram of testis each day [13]. Spermatogenesis is a dynamic, yet highly organized biological process involving germ cell proliferation and differentiation that continuously produces male haploid spermatozoa from diploid spermatogonial stem cells. It is a complex sequence of events that can be subdivided into three main phases: the mitotic phase in which diploid spermatogonia proliferate and differentiate, the meiotic phase of spermatocytes in which haploid gametes are produced, and the post-meiotic phase called spermiogenesis in which the morphological changes of spermatids to spermatozoa occur. Spermatogenesis is primarily driven by the gonadotropic hormones luteinizing hormone (LH)/testosterone and follicle-stimulating hormone (FSH).

1.3.1 Spermatogonial proliferation phase

During the proliferation phase, the most immature spermatogonia that reside on the basement membrane of seminiferous tubules proliferate through a series of mitotic divisions and give rise to differentiated spermatogonia. In rodents, such as rats and mice, four populations of spermatogonia have been previously described: undifferentiated type A spermatogonia (A single (A_s), Apaired (A_{pr}) and Aaligned (A_{al})); differentiated type A spermatogonia (A_1, A_2, A_3, A_4); intermediate (In) spermatogonia; and type B spermatogonia (B) [14, 15]. These spermatogonia can be distinguished at the light microscopy level by the presence and amount of heterochromatin they contain in their nuclei. Type A spermatogonia have little heterochromatin, whereas type B have the most. Spermatogenesis begins with the differentiation of immature diploid spermatogonia to committed spermatogonia. Although it is not currently possible to identify unequivocally spermatogonial stem cells (SSCs), it is generally accepted that A-single spermatogonia (A_s) are the stem cells of spermatogenesis. A_s spermatogonia either renew themselves to maintain the pool of stem cells or differentiate into progenitor daughter cells termed Apaired (A_{pr}) as they are joined by an intercellular bridge resulting from incomplete cytokinesis [16, 17]. These cytoplasmic bridges promote the synchronous development of germ cells. The A_{pr} spermatogonia further divide to form chains of aligned spermatogonia (A_{al} 4, 8, 16 and occasionally 32) that from then on, remain connected by intracellular

bridges. In whole-mount preparations, A_s, A_{pr} and A_{al} spermatogonia can be distinguished according to their topographical arrangement along the basement membrane of seminiferous tubules. Clones of A_{al} spermatogonia then morphologically transform into differentiatied spermatogonia, termed A_1 through A_4. The differentiated A_4 spermatogonia further mature into intermediate and finally type B spermatogonia. In the rat, spermatogonia undergo a total of six mitotic amplifying divisions. This mitotic phase is important as it gives rise to large cohorts of differentiated spermatogonia and ensures that a large number of spermatozoa will be produced. In the rat spermatogenesis, the mitotic phase lasts for 12.9 days [18].

1.3.2 Meiotic phase

The meiotic phase begins when type B spermatogonia divide mitotically into two preleptotene spermatocytes. During the lengthy meiotic prophase, leptotene spermatocytes continue to differentiate as they form leptotene, zygotene, pachytene and diplotene spermatocytes. DNA replication occurs at this stage, as well as chromosomal pairing and genetic recombination. Diplotene spermatocytes in turn undergo the first meiotic division producing secondary spermatocytes .These secondary spermatocytes then undergo a second round of meiotic division to form haploid cells called round spermatids. Preleptotene and leptotene initially reside in the basal compartment of the seminiferous epithelium but as they

develop, leptotene spermatocytes pass through the tight junctions formed by the Sertoli cells to enter the adluminal compartment.

1.3.3 Spermiogenesis and spermiation

Spermiogenesis is the haploid phase of male germ cell development, and occurs prior to their release from the seminiferous epithelium. During this phase, round haploid spermatids undergo a series of extensive biochemical and morphological changes that result in the formation of elongated spermatids. These changes include condensation of the chromatin characterized by the exchange of histones to protamine, change in the shape of the nucleus, formation of the acrosome which derives from the Golgi apparatus, development of the flagellum (tail) and shedding of the germ cell cytoplasm. Excess cytoplasm is removed from the mature elongated spermatids in the form of residual bodies that are eventually phagocytosed by the Sertoli cells. In the rat, spermiogenesis is divided into 19 steps based on the development of acrosome and changes in nuclear morphology.

Spermiation represents the final step of spermatogenesis, and is the process by which spermatozoa are released from the seminiferous epithelium into the lumen of the tubule. In the rat, the total length of spermatogenesis ranges between 49-52 days depending on the strains. The spermatogonial phase lasts approximately 15 days, the meiotic phase lasts over 20 days, and spermiogenesis takes up another 20 days [19].

After formation in the seminiferous tubules, the spermatozoa are transported to the rete testes, and from there, through the efferent ducts to the epididymides where they gain the potential for motility and acquire the ability to fertilize an egg. The epididymis is a single-yet highly convoluted tubule. It is composed of five distinct segments: the initial segment, the intermediate zone, a head (caput), a body (corpus) and a tail (cauda). The functions of the epididymis include storage, protection, transport and maturation of spermatozoa [20].

Figure 1.2 Male germ cell development. Modified from Krawetz SA et al, 2009 [21].

1.3.4 Cycles of spermatogenesis

Spermatogenesis is a highly synchronized process that allows the germ cell development within the seminiferous tubules. At the histological level, cross sections of seminiferous tubules present with precise cell associations that are characteristic of specific stages of the spermatogenic process. Each pattern of cellular associations present in the seminiferous epithelium cycle is called a stage. These stages are designated by roman numbers. In the rat, fourteen different stages have been described (I-XIV) [13].

1.4 The spermatogonial stem cell

In the body, adult somatic stem cells have been described as rare populations of cells present in a variety of tissues such as skin, hair follicles, intestine, blood and bone marrow, mammary gland, and testis [22-23]. Although adult stem cells make up for only a small percentage of all cells found in a mature organ, these cells can regenerate a tissue after injury or disease and therefore play a critical role in tissue homeostasis. Adult stem cells can be broadly defined by their potential for indefinite self-renewal and their ability to produce lineage-committed progenitors which eventually differentiate into a particular cell type.

Male fertility requires the continuous production of large number of mature spermatozoa, and spermatogenesis is the process by which these cells are constantly produced. In mammals, spermatogenesis proceeds throughout the reproductive lifetime of an organism, supported by spermatogonial stem cells (SSCs), the male germ-line stem cells [24-26]. Like other adult stem cells, SSCs are unspecialized cells that are capable of self-renewing and differentiating into progenitor daughter cells. However, SSCs are the only stem cells of an organism responsible for the transmission of genetic information to the next generation. Following cytotoxic insults, spermatogonial stem cells are of special interest since these cells are the only germ cell type that can regenerate spermatogenesis, due to their self-renewal ability and their key role as

precursor cells to spermatozoa that are destined to transmit genetic information to the offspring [3].

Interaction of SSCs with their microenvironment is a critical process in maintaining normal spermatogenesis.In general, the stem cell microenvironment, also termed “niche”, provides a complex molecular milieu that regulates the self-renewal and differentiation activities of stem cells. Within the testis, the spermatogonial stem cell niche can be comprised of extracellular matrix molecules, cell-bound or soluble factors and multiple cellular components including Sertoli, Leydig and peritubular myoid cells and potentially other germ cells (differentiated progenitor cells may secrete factors that may induce or not cell self-renewal or differentiation) [106, 107]. In addition to these components, Yoshida and colleagues showed that type-A undifferentiated spermatogonia (As, Apr and Aal) were preferentially localized close to the vasculature system (blood vessels) and interstitial space around the seminiferous tubules [108]. To date, all the components of the so-called SSC niche that regulate SSC self-renewal and differentiation are still incompletely defined.

1.5 Spermatogonial stem cell transplantation

Although the development and function of spermatogenic germ cells have been studied extensively, the task of identifying and characterizing spermatogonial stem cells among others germ cells has proven inherently difficult mainly because of the scarcity of the SSCs and due to the absence of specific markers. Functional evidence for the

presence of stem cells within the testis germ cells was first demonstrated in rodents by the use of a transplantation experiment. In 1994, Brinster and colleagues developed a SSC transplantation assay by which restoration of spermatogenesis in infertile recipient testes was achieved [27, 28]. This assay not only validated the presence of SSCs among other germ cells but also allowed the investigation of their functional characteristics. This technique implies that a donor testis cell suspension is microinjected into the seminiferous tubules of an infertile recipient testis, usually through the efferent ducts and rete testis (Figure 1.3). SSCs present in the injected cell suspension colonize the seminiferous epithelium and regenerate complete spermatogenesis in the form of spermatogenic colonies. Under clonal conditions, each colony of spermatogenesis originates from a single SSC. Thus, by simply counting the numbers of donor-derived colonies, the number of SSCs can be determined. At present, the SSC transplantation technique is the only assay that allows the quantifiable measure of spermatogonial stem cells activity. Importantly, this assay also demonstrated that the restoration of spermatogenesis can be fully achieved and produce functionally competent spermatozoa that can generate normal offspring [29-31].

Interestingly, the technique of spermatogonial stem cell transplantation can also be applied between donor-germ cells and recipient testes from different species and is referred to as xenogeneic transplantation. In 1996, Brinster and al., demonstrated that rat

spermatogonial stem cells can colonize and restore complete spermatogenesis in infertile immunodeficient mouse testes [32]. They also showed that rat spermatogenesis was supported by mouse somatic Sertoli cells and that the timing of the rat spermatogenic cycle (≈ 52 days) remained unchanged in the mouse testis suggesting that the Sertoli cells do not dictate the rate of germ cell development [33]. Thus, SSC transplantation assay provides a versatile and robust experimental model for evaluating stem cell activity even through interspecies assays.

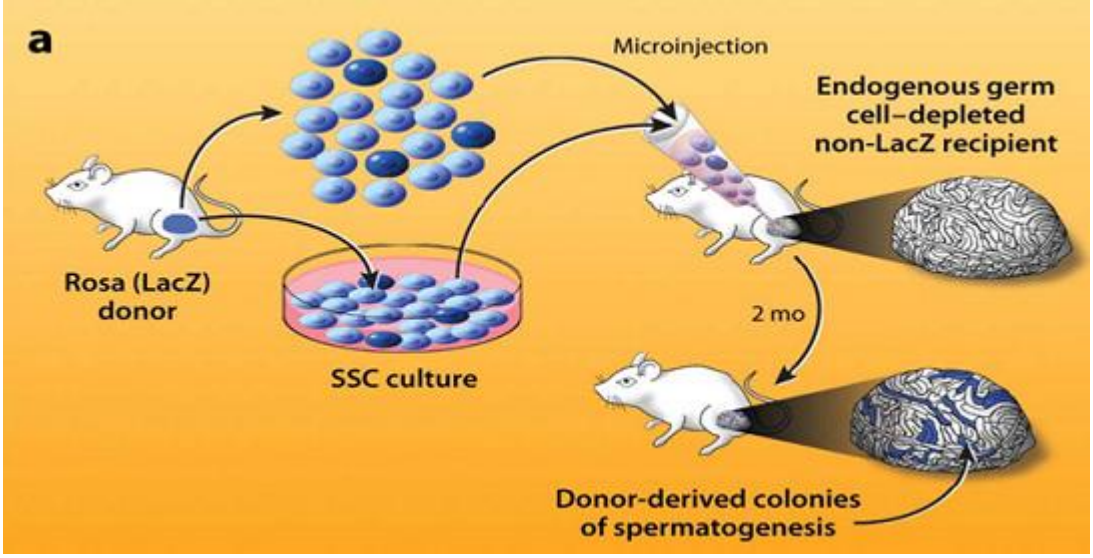

Figure 1.3 Spermatogonial stem cell transplantation assay. A germ cell suspension from freshly digested testis tissue or a germ cell in cultures both carrying a marker transgene (i.e. LacZ or GFP) can be used for transplantation. The cell suspension is microinjected into the seminiferous tubules of a recipient testis. After 2-3 months, donor-derived colonies of spermatogenesis can be detected in recipient testes. Only donor SSCs can colonize and regenerate colonies of spermatogenesis with transgene

expression. Each colony derives from one single SSC under clonal conditions. From Oatley JM and Brinster RL., 2008 [31].

1.6 In vitro spermatogonial stem cell culture

In recent years, a few culture systems with slight variations in protocol have been developed for the maintenance and expansion of rodent SSCs over long periods of time [34-36]. In 1998, Nagano et al., reported for the first time that mouse spermatogonial stem cells could be maintained in culture for as long as four months while retaining their ability to regenerate spermatogenesis and mature spermatozoa after transplantation [37]. In 2004, Kubota et al., were then able to demonstrate the successful in vitro generation and maintenance of mouse spermatogonial germ cell lines for up to six months [38]. A year later, Ryu et al., reported the development of a long-term culture system for rat SSCs by optimizing the mouse culture conditions [39]. In these studies, three fundamental aspects of SSC culture were defined. First, testis germ cells have to be enriched for SSCs using specific cell-surface markers in order to decrease the number of contaminating somatic cells that interfere with SSC maintenance in vitro. Second, the testis germ cells enriched for SSCs have to be cultured on mitotically inactivated STO feeder cells. Third, germ cells have to be maintained in a chemically defined serum-free medium since the presence of serum is detrimental to SSC proliferation in vitro. In addition, the absence of serum allowed for the identification of growth factors specifically required for SSC self-renewal such as GDNF and

FGF2 [38-41]. Therefore based on these observations, mouse and rat testis germ cells enriched for SSCs can be typically cultured on a layer of mitotically arrested STO feeder cells in a serum-free medium supplemented with GDNF and FGF2. Under these conditions, rodent SSCs form three-dimensional cell aggregates termed clusters. Each cluster presumably contains cells capable of generating new clusters upon passage allowing the net expansion of the cluster culture. This ability to form and sustain cluster growth over several passages has been attributed to the self-renewal activity of SSCs. Moreover, the presence of functional stem cells within these clusters was further demonstrated using transplantation experiments even after prolonged culture or cryopreservation [38, 39]. The establishment of culture systems that support self-renewal and proliferation of SSCs in vitro have greatly facilitated the study and understanding of SSC biology. For example, the growth factor GDNF has been described as essential for the self-renewal and proliferation of SSCs in vitro [41, 42]. However, SSC cultures have not yet been considered as potential in vitro models for SSCs toxicity.

1.7 Animal model

1.7.1 Rat as an animal model

Over the years, the laboratory rat has enormously contributed to all disciplines of biomedical research including toxicology and pharmacology. At present, the rat is a well-established animal model for physiological studies and because it is generally considered more relevant to humans

than mice, for analysis of diseases such as cancer, diabetes, cardiovascular and neurological diseases [43].

Rats have been used extensively in experimental toxicology investigations and nonclinical drug safety evaluation studies. In particular, they have proven to be a suitable model for reproductive toxicology studies. This is due in part because rats have a high fecundity and are easy to breed; their gestational period is relatively short (21-23 days) and results in large litter size (3-18 pups). In addition, rats have a low incidence of birth defects such as spontaneous abortions and fetal malformations. They also have been an informative animal model to investigate male reproductive toxicity since rat spermatogenesis and the different components of the male reproductive system have been comprehensively studied and described in details in the literature. Furthermore, the tissue sampling and ease of surgical techniques make it a popular model for this type of studies. The rat has become the de facto model for the majority of mating studies in rodents [44].

Previous studies have shown that the adult rat testis contained a higher number of SSCs than the mouse testis [45]. Therefore, rats also represent an excellent model for investigating spermatogenesis restoration and stem cell activity. In this thesis, male and female Sprague-Dawley rats were obtained from a commercial animal breeder (Charles River Canada Inc., St. Constant, Québec, Canada).

1.7.2 GCS-EGFP transgenic rats

For donor-derived colonies of spermatogenesis to be clearly and easily identified following transplantation, donor germ cells must express a marker transgene such as Lac-Z or GFP (green fluorescent protein). In this thesis, we used a specific strain of transgenic rats that express enhanced green fluorescent protein (EGFP) exclusively in both the male and female germ cell lineages. These rats were generated by microinjecting a ROSA-EGFP transgene into the pronucleus of Sprague-Dawley eggs [46, 47]. Of the four lines of ROSA-EGFP transgenic rats initially produced, one demonstrated EGFP expression restricted to the germ cells, and was designated as the Germ Cell Specific –EGFP (GCS-EGFP) line. The GCS-EGFP (homozygous *SD-Tg(Gt[ROSA]26Sor-EGFP)2–4Reh*) transgenic rats were obtained from Robert .E Hammer (University of Texas Southwestern Medical Center) and maintained as a breeding colony at the Animal research Centre (ARC, McGill University). Adult male GCS-EGFP transgenic rats were used to generate donor testis cell suspension in our transplantation experiments. Furthermore, the use of this rat strain has allowed us to rapidly separate postnatal undifferentiated spermatogonia based on their EGFP expression from contaminating testis somatic cells using fluorescence-activated cell sorting (FACS). Thus, the use of GCS-EGFP rats has greatly facilitated the establishment of rat SSC cultures (as described in Chapter 5).

1.8 Testicular cancer

Testicular cancer (TC) is a relatively rare form of cancer, but it still represents the most frequently diagnosed malignant solid tumors in young men between the ages of 15 and 35 years [48-51]. The incidence of testicular cancer has doubled in industrialized countries over the past 40 years [52]. Fortunately, during the same period, considerable progress in the management of the disease has been made. The introduction of cisplatin-based combination chemotherapy as standard care has dramatically improved the prognosis for patients with metastatic germ cell cancer; with a five-year disease-free survival rate over 90%, testicular cancer is now considered a highly curable form of cancer [51, 53]. At present, the standard treatment for testicular cancer combines the administration of bleomycin, etoposide and cisplatin (called BEP regimen) for three cycles. Given that the majority of testicular cancer patients become long-term survivors, the effects of BEP treatment on fertility and progeny outcome have become a matter of great concern for young testicular cancer patients as many of them have not started a family at the time of diagnosis. Several studies have reported decreased fertility in patients diagnosed with testicular cancer even prior to treatment [54-56]. However, due to the limited number of studies, it is still unknown to what extent the BEP treatment affects reproductive functions and progeny outcome of testicular cancer survivors in the long-term.

1.8.1 Incidence and etiology

Testicular cancer is unique in its epidemiological profile compared to other type of cancers as it occurs at the onset of the patient's reproductive life, between the ages of 15 and 35. Furthermore, its incidence varies according to the geographic area and ethnic background. The risk of testicular cancer is the highest among white men (Caucasians) of Northern Europe and US who are 5 times more likely to develop TC than black men, and about 3 times than Hispanics and Asians, suggesting the influence of genetic background in testicular cancer [57]. For unknown reasons, the overall incidence of testicular cancer has been continuously rising in developed/western countries over the past 30-40 years [52]. Although the precise etiology and pathogenesis of testicular cancer remain elusive, it is now well established that cryptorchidism (undescended testis) and male infertility are both risk factors associated with developing testicular cancer [58-60]. In addition, recent data indicate that other testicular disorders including contralateral testicular cancer, history of familial TC, and testicular atrophy might also be predisposing factors for testicular cancer. The testicular dysgenesis syndrome (TDS) hypothesis was first introduced by Skakkebaek et al, in 2001 and postulates that testicular cancer is linked to other male reproductive disorders such as cryptorchidism, hypospadias, and impaired spermatogenesis that all result from disturbed prenatal testicular development due to deleterious environmental factors [61].

1.8.2 Epidemiology – Testicular cancer current statistic in North America (United States and Canada)

Testicular cancer continues to be the most common form of cancer in men between the ages of 15 and 35. In the United States, the American Cancer Society estimates that about 8400 new cases of testicular cancer will be diagnosed in 2009, with an associated risk of dying of about 1 in 5,000 (380 related deaths) (American Cancer Society: Cancer Facts and Figures 2009. Atlanta, Ga: American Cancer Society, 2009). According to the current statistics of the Canadian Cancer Society, it is estimated that the number of domestic new cases of testicular cancer in 2009 would total approximately 900, with 30 of these expected to die (*Canadian Cancer Statistics 2009*, www.cancer.ca). With advances in the management of testicular cancer, an increasing number of testicular cancer patients have become long-term survivors. As a consequence, post-treatment quality of life has become an important issue for testicular cancer survivors.

1.8.3 Testis cancer histology

Ninety-five percent of all testicular cancers are of germ-cell origin, and termed germ cell tumors (GCTs). Germ cell tumors are believed to arise from the neoplastic transformation of primordial germ cells/gonocytes during embryogenesis in utero [62-64]. For clinical purposes, testicular cancers have been broadly divided into two main histologic groups, seminomas and nonseminomas, each comprising approximately 50% of cases. Seminomas have a better prognosis than nonseminomas and are

manageable with orchidectomy and surveillance with or without radiation therapy or chemotherapy. Nonseminomas have several histological subtypes which consist of embryonal carcinoma, yolk sac tumor, chroriocarcinoma, and teratoma, and are generally less sensitive to radiation, and frequently require both surgery and chemotherapy [62]. The diagnosis of testicular cancer is based on the histopathological evaluation of testis specimen from testicular biopsies and is confirmed with serum tumor marker levels. Patients are assigned to one of the three prognosis groups (good-, intermediate-, and poor-prognosis group) as defined by the International Germ cell Cancer Collaborative Group (IGCCG) based on the histopathology of testis specimen (seminomas or nonseminomas), primary site of the disease, the presence or not of metastases, and the levels of serum tumor markers [65, 66]. The stage of the disease determines the treatment strategy in patients with TC.

1.8.4 Treatment of testicular cancer: BEP chemotherapy

A major breakthrough in the chemotherapeutic treatment for testicular cancer was the introduction of cisplatin in the 1970s. In a series of randomized clinical trials, Einhorn and colleagues, at Indiana University, pioneered the development of multi-agent chemotherapy which for the first time included cisplatin in patients with advanced disease [67, 68]. Since the 1980s, bleomycin, etoposide and cisplatin (BEP) chemotherapy has become the gold standard first-line treatment for patients with metastatic germ cell cancer. In clinic, the BEP chemotherapy uses a combination of

bleomycin 30 mg per week on days 2, 8, and 15, etoposide 100 mg/m^2 per day on days 1 - 5 (500 mg/m^2 per cycle), and cisplatin at 20 mg/m^2 per day on days 1 - 5 (total 100 mg/m^2 per cycle), and is usually given for three to four cycles of 21 days each [64]. To date, BEP regimen is still the most effective systemic chemotherapy for metastatic germ cell cancer, since no other multi-agent combination has yet proven superior. BEP regimen will secure long-term disease-free survival in up to 90 % of testicular cancer patients within the good prognosis group. Most patients diagnosed as having testicular cancer who achieve a complete response after the first-line BEP chemotherapy are cured and relapses occur in less than 10% of patients. Moreover, patients in the intermediate and poor prognosis groups requires 4 cycles of BEP chemotherapy.

1.8.5 Effects of cancer treatments on spermatogenesis and fertility

Many anticancer drugs, particularly alkylating agents, have deleterious effects on testicular function. As mentioned previously, bleomycin, etoposide and cisplatin are the three anticancer drugs currently used in combination for the treatment of metastatic testicular cancer. These drugs are not tumor cells specific but rather target the more rapidly dividing cells indiscriminately. Since the seminiferous epithelium is highly proliferative, disruption of spermatogenesis is a common side effect of BEP treatment. The effects of BEP chemotherapy on testicular function have been well documented in testicular cancer patients and primarily depend on the cumulative doses of drugs received and the fertility

pretreatment status [69-71]. BEP chemotherapy induces impaired spermatogenesis characterized by reduced sperm concentration, reduced motility and increased abnormal sperm morphology. The vast majority of these patients become azoospermic shortly after initiation of BEP chemotherapy. This decrease in fertility is often transient, and recovery of spermatogenesis is seen in about 50% and 80% of patients after two and five years, respectively [72]. However, the risk of permanent azoospermia after BEP chemotherapy still exists and is directly related to the total cumulative dose of cisplatin; with high dose-treatment in which cumulative doses of cisplatin exceed 400 mg/m^2, irreversible impairment of spermatogenesis is usually observed [72-74]. While recovery of testicular function following BEP treatment is difficult to predict in time, recovery of a normal sperm count is more likely in patients with normal sperm count prior to starting treatment. Thus, the impact of BEP regimen on fertility is a major concern for testicular cancer survivors because of their young age at diagnosis, high cure rates, and long-life expectancy following treatment.

1.8.6 Impact of BEP on offspring

Given that the majority of testicular cancer patients survive the disease, the long-term effects of BEP chemotherapy are of major concern to patients particularly with respect to their fertility and ability to father normal and healthy offspring. BEP combination chemotherapy may induce azoospermia; fortunately, this is only transient and the majority of testicular cancer survivors will recover their fertility after treatment

completion [72]. However, the sperm quality may be reduced compared to what it was prior to treatment. The ultimate assessment of testicular function for testicular cancer survivors is the achievement of fatherhood. Although numerous studies have investigated the consequences of BEP chemotherapy on testicular functions, there is limited information available on the pregnancy outcomes in patients treated for testicular cancer. In 2004, Huyghe et al., send a questionnaire on reproductive events that occur before and after treatment to 451 testicular cancer patients. They reported that 110 out of the 164 testicular cancer patients who wanted children succeeded to achieve pregnancy after treatment completion, compared with 208 patients out of 228 before treatment. Thus, fertility rates were 30% lower in testicular cancer patients after completion of BEP chemotherapy. Therefore, most testicular cancer survivors appear to succeed in obtaining naturally conceived pregnancies, but it seems more difficult to father a child for these men compared to the normal population [75].

To date, no increase in the rate of adverse pregnancy outcomes such as congenital malformations or genetic diseases has been documented in the offspring of testicular cancer survivors. But these studies lack the sensitivity and statistical power to detect small effects. Thus, there is now a strong tendency to counsel patients and offer them sperm banking prior to starting BEP chemotherapy.

1.9 Cisplatin, etoposide and bleomycin

1.9.1 Cisplatin

Cisplatin, (cis-diamminedichloroplatinum(II); CDDP), is an inorganic platinum compound considered as one of the most potent anticancer agents available for chemotherapy. The anticancer activity of cisplatin was accidentally discovered in the 1960s [76]. Today, cisplatin is commonly used in clinic to treat a wide variety of human cancers including breast, ovarian, testis, lung, head and neck bladder, lymphoma and melanoma. Since its approval by the FDA (Food and Drug Administration) in 1979, cisplatin has been used with most success in the treatment of testicular germ cell tumors (TGCTs). Interestingly, although cisplatin displays clinical activity against a wide variety of solid tumors, testicular germ cell tumors appeared to be exquisitely sensitive to cisplatin. However, despite its remarkable efficacy in the treatment of testicular tumors, the clinical application of cisplatin in other types of cancer is often limited by several harmful side effects such as neurotoxcity, and nephrotoxicity and by the occurrence of both intrinsic and acquired resistance of tumor cells [77]. Over the years, the critical role of cisplatin in the context of multi-agent chemotherapy has been amply demonstrated in clinical studies that showed a direct relation between treatment outcome and dose concentration of cisplatin [78, 79]. In the treatment of metastatic germ cell cancer, cisplatin was originally combined with vinblastine and bleomycin, later vinblastine was replaced by etoposide [80].

At present, it is widely accepted that DNA is the primary biological target of cisplatin. The mechanism of action underlying the anticancer activity of cisplatin is not yet fully understood, but it mostly relies on the formation of DNA adducts, predominantly intrastrand cross-link adducts. Once formed, these DNA adducts are thought to mediate cytotoxicity by blocking transcription, inhibiting DNA replication, activating various signaling pathways including DNA-repair, cell cycle arrest and ultimately by inducing programmed cell death, or apoptosis [81, 82].

1.9.2 Effects of cisplatin on the male reproductive system

The effects of cisplatin on the male reproductive system have been widely reported. Several studies have demonstrated the gonadotoxicity of cisplatin alone in animal models such as rats and mice. Cisplatin treatment induced a progressive but irreversible depletion of spermatogenic cells [83-86]. The effects of cisplatin paternal treatment on progeny outcome and offspring were investigated following both acute and chronic exposure. After a single i.p injection of cisplatin, a significant increase in pre-implatantion loss and a decrease in fetal weight were observed [87]. Seethalakshmi and colleagues showed that chronic cisplatin treatment for 9 consecutive weeks of adult male rats decreased reproductive organ weights, sperm counts and sperm motility. Results of mating studies showed that, although fertility was not affected, significant increases in pre- and post-implantation loss rates in fetuses sired after cisplatin paternal exposure was observed. In addition, a decrease in male to female

sex-ratio as well as malformed and growth-retarded fetuses were found among fetuses of the offspring of cisplatin-treated males [88]. Taken together, these results indicate that cisplatin as a single agent has deleterious effects on both male reproductive system and progeny outcome.

1.9.3 Etoposide

Etoposide [VP-16], a semi-synthetic derivative of epipodophyllotoxin, is a potent antineoplastic agent widely used in the treatment of lymphomas, leukemia, and many solid tumors such as testicular and small lung cancers [89]. Etoposide is classified as a topoisomerase II inhibitor. Topoisomerase II is an essential nuclear enzyme required in a number of cellular processes, including DNA replication and transcription, as well as chromosomal segregation and DNA recombination. Under normal physiological conditions, topoisomerase II relaxes supercoiled DNA by cleaving and religating both strands of the double helix through the formation of a transient covalent cleavage intermediate. Etoposide binds to topoisomerase II and acts by disrupting the cleavage-religation equilibrium and thereby increases the concentration of covalently bound DNA-topoisomerase II cleavage complexes. By blocking the religation of cleaved DNA and stabilizing the DNA-drug-enzyme intermediate, etoposide induces the accumulation of single- or double-strand DNA breaks which eventually leads to apoptosis. Thus, etoposide converts topoisomerase II into a DNA-damaging enzyme

that acts primarily in the G2 and S phases of the cell cycle when topoisomerase II is significantly expressed [90, 91].

1.9.4 Effects of etoposide on the male reproductive system

In the rat, chronic i.v. administration of etoposide for 3 months induced dose-dependent testicular alterations characterized by depletion of germ cells [92]. In addition, it has been reported that spermatogonia and spermatocytes were the major cell type undergoing apoptosis following acute exposure to etoposide [93, 94]. Previous studies have pointed out that male meiotic cells were the major target of etoposide-induced cytogenotoxicity as topoisomerase II activity is at its peak during mammalian meiosis [95, 96]. In the mouse, etoposide was shown to decrease the frequency of meiotic crossing-over and to adversely affect chromosomal segregation. Etoposide was also shown to be a potent inducer of micronuclei in male rat spermatogenic cells during meiosis [97]. In 2001, Marchetti and colleagues showed that etoposide administered at clinical relevant doses affected meiotic germ cells in which chromosomal abnormalities and aneuploidy were produced and transmitted to the progeny [98]. More recently, the same group reported that etoposide exposure of spermatogonial stem cells did not affect the frequencies of sperm with numerical abnormalities but induced a significant increase in the frequencies of sperm with chromosomal structural aberrations suggesting that etoposide may induce the persistent production of sperm

with chromosomal aberrations through its effects on spermatogonial stem cells [99].

1.9.5 Bleomycin

Bleomycin is a mixture of basic glycopeptide antibiotics isolated from *Streptomyces verticillus* that have potent antitumor activity against several types of tumors, notably malignant lymphomas, head and neck cancers, and germ cell tumors. In addition, bleomycin has proven to be an essential component in cisplatin-based combination chemotherapy for testicular cancer [100-102]. Although its precise mechanism of action is not fully known, the cytotoxicity of bleomycin against cancer cells is thought to be related to its ability to induce single- and double-stranded breaks in DNA. Bleomycin-mediated DNA degradation requires a redox-active metal ion (Fe(II) or Cu(I)), oxygen and one-electron reductant [100].

1.9.6 Effects of bleomycin on male reproductive system

Relatively few studies have investigated the effects of bleomycin alone on the male reproductive functions. In the mouse, it was shown that a single i.p. injection of bleomycin induced the killing of spermatogonia (Aal through B spermatogonia) while spermatogonial stem cells were resistant. Bleomycin was shown to induce cytogenetic defects particularly structural chromosomal aberrations. Interestingly, bleomycin was reported as specifically mutagenic in mouse spermatogonia [103-105].

1.10 Formulation of the project

To date, no study has yet addressed the possibility that the combination of bleomycin, etoposide and cisplatin could affect developing germ cells including SSCs and progeny outcome in the rat. In this thesis, BEP-induced adverse effects will be investigated on developing germ cells, fertility and progeny outcome using rat as a model. Thus, our study is rather unique as we examined the combination of these three different anticancer agents known to have multiple targets and mechanisms of action on both testicular germ cells and progeny outcome. Despite this complexity, our protocol has the advantage that the dose and time course of drug administration could be established based on the clinical BEP regimen and without the confounding factors of testicular malignancy and orchiectomy.

The general aims of this thesis were to determine the adverse effects of a combined cisplatin, etoposide and bleomycin regimen used in clinic for testicular cancer treatment on testicular function, including SSC function, fertility and progeny outcome using rat as a model and to develop a new short-term in vitro SSC assay to test the cytotoxicty of these three anticancer drugs, alone or combined on stem/progenitor spermatogonial cultures.

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Chapter 2

Chapter 2: Effects of Chemotherapeutic Agents for Testicular Cancer on the Male Rat Reproductive System, Spermatozoa, and Fertility[1]

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Running title: Testicular cancer drugs affect male germ cells

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2.1 Abstract

Testicular cancer is the most common cancer affecting men of reproductive age. Advances in treatment of the disease, which include the coadministration of bleomycin, etoposide, and cis-platinum (BEP), have brought the cure rate to over 90%. This high cure rate, coupled with the young age of patients, makes elucidation of the impact of the treatment on reproductive function, fertility, and progeny outcome increasingly important. The goal of this study was to determine the effects of BEP, in doses analogous to those given to humans, on the male reproductive system, spermatozoa, fertility, and progeny outcome in an animal model. Male Sprague-Dawley rats were treated daily with BEP for 3 cycles of 3 weeks each, for a total of 9 weeks. After 6 and 9 weeks, males were mated to 2 groups of untreated females. BEP treatment resulted in decreases in testicular and epididymal weights of 52% and 28%, respectively, when compared to control.Decreased testis and epididymis weights were accompanied by impairment of spermatogenesis and by a decrease in spermatozoal count of nearly 90% (11.9×10^7 spermatozoa per caput epididymidis in control vs 1.65×10^7 in BEP-treated rats). The percent of motile spermatozoa in the treated rats was more than 30% lower than in controls. Defects in the flagella of spermatozoa increased by more than twofold in the midpiece, and by more than sixfold in the principal piece. Paternal BEP treatment, for either 6 or 9 weeks, did not affect fertility, pre- or postimplantation loss, litter size,

or sex ratio among progeny on gestation day 21. In contrast, among the pregnancies allowed to proceed to delivery, a significant number of pups sired by males treated with BEP for 9 weeks died between birth and postnatal day 2; this was not observed in pups sired by males treated for 6 weeks. Markers of postnatal development were not affected in the surviving offspring from either group. Thus, despite the dramatic effects of the testicular cancer drug regimen on spermatogenesis, the numbers of spermatozoa, and their motility and morphology, male rats were fertile. While fetal development was apparently normal, early postnatal mortality, which may be associated with a delay in parturition, was elevated among the progeny sired by males exposed to BEP for 9 weeks.

2.2 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 (Adami et al, 1994), mortality due to the disease has been declining (Forman and Moller, 1994). Advances in the treatment of the disease, which include the coadministration of bleomycin, etoposide, and cis-platinum (BEP), have brought the 5-year survival rate to over 90% for those patients who are considered to be at good risk (Mead and Stenning, 1997). This high cure rate makes the posttreatment quality of life of testicular cancer patients a concern, and because of 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 the number of spermatozoa produced and in their motility, as well as an increase in morphologically abnormal spermatozoa (Stephenson et al, 1995). Spermatogenesis recovers in most men after 5 years (Lampe et al, 1997). However, reports on the chromatin quality of the surviving spermatozoa are conflicting: one study found an increase in aneuploidy (De Mas et al, 2001), another found no change (Thomas et al, 2004), and yet another found a decrease in aneuploidy (Martin et al, 1997). The sperm chromatin structure assay revealed no increase in DNA fragmentation (Stahl et al, 2004); however, one case report described a man whose sole semen abnormality after treatment with BEP was an increase in DNA denaturation (Deane et al, 2004).

While studies of the effects of BEP on spermatogenesis in human testicular cancer patients provide valuable information, these 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 (Petersen et al, 1999b; Jedrzejczak et al, 2004). The semen quality further decreases following orchidectomy, even before the initiation of chemotherapy (Petersen et al, 1999a). 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.

The effects of BEP treatment on progeny outcome are difficult to examine in humans. It has been suggested that fertility in patients with testicular cancer was decreased by 30% after treatment (Huyghe et al, 2004). Several reports have suggested that there is no increase in congenital malformations among the progeny of testicular cancer patients who were treated with BEP (Senturia et al, 1985; Byrne et al, 1988). Because of small sample sizes, however, these reports do not have the power to detect an increase in relative risk of less than threefold to fivefold (Lahdetie et al, 1994). Animal studies indicate that paternal treatment with cis-platinum alone can affect progeny outcome: chronic cis-platinum treatment in the rat resulted in increased pre- and postimplantation loss, a change in sex-ratio of the offspring, as well as an increase in malformed and growth-retarded fetuses (Seethalakshmi et al, 1992). The effects of etoposide or bleomycin alone, or of the BEP combination, on progeny outcome have not been examined in an animal model. Furthermore, postnatal development of pups sired by male rats treated with any of the 3 drugs has yet to be examined.

We hypothesized that even in the absence of testicular cancer and orchidectomy, the chemotherapeutic regimen used to treat testicular cancer is deleterious to the production of spermatozoa, to their

morphology and motility, and can affect fertility and progeny outcome. We tested this hypothesis in the rat model.

2.3 Materials and Methods

Chemicals

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

Animals, Treatment, and Mating Protocol

Adult male (300–350 g) and virgin female (200–225 g) Sprague-Dawley rats were purchased from Charles River Canada (St Constant, Canada) and housed under controlled light conditions (14:10 hours light:dark) in the Animal Resources Centre of McGill University. Animals were provided with food and water ad libitum. 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 Animal Research Centre protocol 4699).

Males were randomly divided into 2 groups of 10 rats each. The rats from the control group were gavaged on days 1 through 5 of each week with 1 mL of 7:3 saline (Roche, Laval, Canada): DMSO (Fischer Scientific, Fair Lawn, NJ). On day 2 of each week, control rats were given 1 mL of saline by intraperitoneal injection. The animals from the drug-treated group were gavaged on days 1 through 5 of each week with 3.0 mg/kg cis-

platinum (LKT Laboratories, St Paul, Minn) and 15.0 mg/kg etoposide (LKT Laboratories) dissolved in 7:3 saline:DMSO. On day 2 of each week, they were given an intraperitoneal injection of 1.5 mg/kg bleomycin (LKT Laboratories) dissolved in saline. This dose regimen was chosen based on the standard dose given to humans (Benedetto, 1999), adjusted for surface area according to the following formula: f x mg/kg = mg/m^2, where f equals 6.0 for the rat (Bachmann et al, 1996). This dosing regimen differs from that used in humans in that humans are treated for 1 week per cycle of 3 weeks; each course of treatment generally consists of 2 or 4 cycles.

Males were mated after 6 weeks of treatment and at the completion of the 9-week treatment. On day 6 of the week (a non-treatment day), males were placed in a cage overnight with 2 naturally cycling females in proestrous, as determined by vaginal smears. The progeny of these females were used for the analysis of fetal development on gestation day 21. Four days later, on day 2 of the subsequent week, the males were mated again. The progeny of these females were used for analysis of postnatal growth and development. Females were checked for sperm by vaginal smear on the morning after mating. This was considered to be gestation day 0.

Tissue Collection and Histology

At the end of the 9-week treatment, males were anesthetized and the ventral prostate, seminal vesicles, left testis, and left epididymis were

removed and weighed. The contralateral testis and epididymis were cleared with saline and perfused with Bouin fluid as a fixative through the abdominal aorta. The tissues were then excised, postfixed for an additional 24 hours in the same fixative, dehydrated, and embedded inparaffin. To evaluate spermatogenesis, 5-µm testis sections were cut and stained with periodic acid–Schiff, according to the manufacturer's instructions (Sigma). Sections were viewed with a Leica AS LMD microscope (Leica, Wetzlar, Germany) equipped with a RS Photometrics CoolSNAP fx digital camera (Roper Scientific, Tucson, Ariz).

The left epididymides were sectioned into caput-corpus and cauda regions. The caput-corpus epididymides 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.

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, Canada), for 2 intervals of 15 seconds separated by a 30-second interval. Heads of spermatozoa were counted using a hemocytometer to assess the absolute number of sperm per caput-corpus epididymidis (Robb et al, 1978).

Spermatozoal Motility

Spermatozoa from the cauda epididymides were used immediately for computer-assisted sperm analysis (CASA), as previously described (Slott et al, 1993), 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, at 37°C (Klinefelter et al, 1991). 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 tissues were transferred to a Petri dish containing 10 mL of medium, allowing spermatozoa to disperse into the medium. The tissues were 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 prewarmed 80-µm–deep glass cannula for CASA analysis using the HTM-IVOS system (Hamilton Thorne Research, Beverly, Mass) and version 12 of the Toxicology software (analysis speed = 60 Hz; minimum track duration = 30 frames at 60 Hz; minimum average-path velocity [VAP] = 50; minimum straightness [STR] = 80). For each animal, 4 slides, each with two 80-µm–deep chambers, were analyzed. At least 100 spermatozoa per slide were analyzed. The following parameters were determined: percent of motile

spermatozoa, percent of progressively motile spermatozoa, curvilinear velocity(VCL), straight line velocity (VSL), 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 epididymides were minced into 7 mL of phosphate-buffered saline (PBS) (Roche). The suspension was filtered, washed several times with PBS, fixed for 1 hour in 1% glutaraldehyde (MecalabLTD, Montreal, Canada) in PBS, and washed again with PBS. The resulting pellet was suspended in 1% agarose (Gibco Invitrogen) and embedded for electron microscope analysis as follows. The samples were washed 2 times in 0.1 M sodium cacodylate buffer containing 3% sucrose, pH 7.4; postfixed in 1% osmium tetroxide and 1.5% potassium ferrocyanide; and embedded in epoxy resin. Spermatozoal ultrastructure was analyzed on the electron microscope (Philips 410 Electron Microscope, Eindhoven, The 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.

Analysis of Pregnancy Outcome

On gestation day 21, sperm-positive females were sacrificed by

CO_2 asphyxiation and laparotomized. The ovaries were removed, and the numbers of corpora lutea were recorded. The uteri were removed and examined for any abnormalities, including resorption moles. The numbers of implantation sites in the uteri were recorded. The extent of preimplantation loss per litter was calculated as follows: (number of corpora lutea - number of implantation sites)/(number of corpora lutea). The number of postimplantation losses was calculated as follows: (number of implantation sites - number of live fetuses)/(number ofimplantation sites). The fetuses were removed successively, blotted, weighed, examined for external malformations, sacrificed by hypothermia, and immersion-fixed in Bouin fixative. After fixation, the fetuses were dissected and the major organs examined for internal malformations. The sex-ratio was calculated as the ratio of the number of male fetuses to the total litter size.

Postnatal Growth and Development

Pregnant females were placed alone in a cage on gestation day 20. From day 21 onward they were monitored regularly for signs of labor. Labor was considered complete when all pups were cleaned and nursing and when the vaginal area was free of blood. Upon completion of labor, pups were removed from the cage, weighed, sexed, examined for malformations, labeled by toeclipping, and returned to the mother. The pups were weighed biweekly until weaning, after which time they were weighed once per week until postnatal day 62. Pups were weaned on

postnatal day 21, at which time 4 males and 4 females from each litter were placed in separate cages, and the remaining pups were sacrificed. The pups were monitored daily for any changes, and the days of eye opening, vaginal opening, and preputial separation were recorded. Female pups were sacrificed by CO_2 asphyxiation at 10 weeks of age, and the spleen, kidneys, and ovaries were weighed. Male pups were sacrificed at 13 weeks, and the spleen, kidneys, left testis, left epididymis, seminal vesicles, and ventral prostate were weighed.

Statistical Analysis

The number of postnatal deaths was analyzed using the Fischer's exact test. All other data were analyzed using the Student's *t* test, with Bonferonni correction as appropriate. Data are presented as the mean plus or minus standard error of the mean (SEM). The level of significance was considered $P < .05$.

2.4 Results

Weights of the Male Rats and Reproductive System Tissues

During the treatment, one rat from the BEP group became sick and was sacrificed. The remaining BEP-treated rats gained less weight than the control rats, although their body weights did increase over the course of the treatment (Figure 1). 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 compared to controls. The reduction in organ weights remained significant when expressed as relative organ weight (data not shown). There was no significant effect on seminal vesicle or ventral prostate weights (Figure 2).

Testis Histology

We examined the histopathology of the testis of rats exposed to BEP for 9 weeks. Testis cross-sections of BEP-treated rats were characterized by severe atrophic and germ cell–depleted seminiferous tubules compared to controls, reflecting the decrease in testis weight (Figure 3A and B). Loss (sloughing) of immature germ cells into the lumen, giant multinucleated cell formation, along with extensive vacuolization of seminiferous tubules and Sertoli cell–only tubules were observed among the sections of treated rats (Figure 3C through F).

Spermatozoal Numbers, Motility, and Morphology

We observed a striking effect on the number of spermatozoa in the BEP-treated rats. The total number of spermatozoa per caput-corpus epididymides in the BEP-treated rats was reduced by more than 90% when compared to control rats (Figure 4). The motility characteristics of the BEP-treated spermatozoa were significantly altered (Figure 5). The overall percent of motile spermatozoa was approximately 30% lower in the BEP-treated rats compared to controls. Although the range of values for percent motile spermatozoa was relatively small among the control group (68%–77%), the values for the drug-treated rats varied

greatly; one rat had 7.5% motile spermatozoa, while another had 71% motile spermatozoa. The mean percent of progressively motile spermatozoa was unchanged in the BEP-treated group. All 3 of the velocity parameters (VAP, VCL, and VSL) were significantly decreased in the BEP-treated animals; both the VAP and the VCL decreased by approximately 10%, and the VCL decreased 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 similar values in controls. 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 6). The midpiece of a control spermatozoon consists of 9 microtubule doublet pairs surrounding 1 central doublet. Each of the 9 peripheral doublet pairs is associated with 1 outer dense fiber. Surrounding the outer dense fibers is the mitochondrial sheath (Figure 7A). In the control group, the only abnormalities seen were an abnormal spatial arrangement of outer dense fibers and the presence of 2 or more flagellar sections within 1 membrane. Some of the abnormalities observed in the midpiece cross-sections of spermatozoa from BEP-treated rats (Figure 7) were the same as the 2 abnormalities seen in the control group. Interestingly, additional abnormalities, not observed in the

control group, were found in spermatozoa from the BEP-treated animals. These included an absence of the mitochondrial sheath, an abnormal number of outer dense fibers (either too many or too few), hemilateral absence of the axoneme and the outer dense fibers, a malformed mitochondrial sheath, the presence of some normal outer dense fibers 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 fibers and debris (Figure 7). There was no increase in the percent of sperm with a cytoplasmic droplet in the BEP-treated group (data not shown).

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 6). The principal piece of a spermatozoon from a control rat (Figure 8A) consists of the same 9 plus 2 arrangement of microtubules as the midpiece; however, outer dense fibers 3 and 8 are replaced by the 2 longitudinal columns of the fibrous sheath. Surrounding the outer dense fibers 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 incidencescompared to the control group (Figure 6); these included a hemilateral absence of the outer dense fibers, with or without an intact axoneme, and a missing outer dense fiber (Figure 8).

Fertility and Progeny Outcome

In pregnancies sired by males exposed to BEP for 6 or 9 weeks, all sperm-positive females became pregnant; there were no changes in pre- or postimplantation loss, litter size, or sex-ratio (Table). In addition, there were no effects on fetal weights. There were no external malformations in either of the control groups or in the 6- or 9-week BEP-exposed groups. No internal malformations were observed in any of the major organs, such as the liver, kidneys, lungs, or heart.

The second group of females that were mated to males after 6 and 9 weeks of BEP treatment were allowed to proceed through gestation to delivery. All the females mated to males that were treated for 6 weeks with BEP were pregnant and delivered live pups between gestation days 21 and 22. The average number of pups per litter that survived past day 1 was 14.9 ± 0.65 in the 6-week BEP group and 13.1 ± 1.1 in the control group. Additionally, the growth curves for the pups sired by 6-week BEP-treated males did not differ from the pups sired by control animals (data not shown). Developmental markers (dates of eye opening, vaginal opening, and preputial separation) did not differ between control and BEP-treated groups.

All of the females mated to males exposed to BEP for 9 weeks were also sperm positive. However, unlike the females mated to males after 6 weeks of BEP treatment, only 1 of 10 sperm-positive matings resulted in a litter of a normal size (Figure 9). Of the others, we observed one mother

eat all of her pups while they were still alive, one had 1 pup that died within 1 day, 3 had litters of 5 or less, and 4 others that were sperm positive were never observed with any pups. It is noteworthy that the 1 normal-sized litter in the 9-week group was not sired by the 1 male that had normal sperm motility. Interestingly, only 2 of the 10 sperm-positive females mated to BEP-exposed males went into labor on gestation day 22 ($P \leq .002$, χ^2 analysis), compared with 7 out of 7 females mated to control males. Of the other 8 females mated to BEP-treated males, parturition occurred on gestation day 23 in 4, and for the remaining 4 females, there was no observable evidence of surviving offspring; in contrast, all females mated to control animals went into parturition on day 22 or earlier. Of the surviving pups sired by BEP-treated males, no abnormalities were observed in postnatal growth (Figure 10), developmental markers (data not shown), or organ weights (data not shown).

2.5 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. Furthermore, the risk to the progeny of testicular cancer patients treated with BEP has yet to be elucidated. The use of an animal model allows us to administer BEP in a dose 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 and to determine whether BEP treatment has the potential to adversely affect progeny outcome.

In the current study, male rats were treated continuously for 9 weeks with BEP, and the reproductive organs and sperm quality were analyzed upon the completion of treatment. The BEP-treated animals experienced a substantial decrease in the weights of both the testes and epididymides. Reduction of the testis size (weight) along with disrupted spermatogenesis are well-known side effects of cisplatin-based chemotherapy (Lampe et al, 1997; Howell and Shalet, 2001). We observed that exposure to chronic BEP chemotherapy resulted in marked alterations of seminiferous tubule histology, with severely impaired spermatogenesis. At the testicular level, 9-week chronic BEP chemotherapy induced germ cell depletion and tubule atrophy. Abnormalities in the spermatogenesis process may account for the resulting low spermatozoal count observed in that group. We found that 71% of the BEP-treated rats had a spermatozoal count that was decreased by more than 90% when compared to controls. Relatively few studies have examined the impact of BEP treatment on the number of spermatozoa in men immediately after the completion of chemotherapy. The spermatozoal concentration in men who have undergone BEP gradually improves after completion of treatment (Tomomasa et al,

2002), and thus it is difficult to compare the response in humans with the results of the current study.

In a study by Stephenson et al (1995), 57% of human patients treated with BEP had less than 50% motile spermatozoa. In the rat, motility of less than 70% is considered abnormal (Seed et al, 1996). In the current study, 86% of drug-treated rats had spermatozoal motility below 70%. In addition to the percent motility, the VAP, VSL, VCL, BCF, and ALH values were significantly altered in the BEP-treated rats. Of these parameters, VSL, VCL, and BCF have been shown to be correlated with fertility (Toth et al, 1991). This may explain why fertility remains a problem even in patients who are not oligospermic (Hansen et al, 1991).

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 morphologicallyabnormal 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 (Masuda et al, 2003) or triptolide, a diterpene triepoxide isolated from a Chinese plant (Huynh et al, 2000). These abnormalities, however, are extremely rare in control rats (Syntin and Robaire, 2001). The flagellum is formed during spermiogenesis in the testis, indicating that the increase in

abnormalities in BEP-treated rats probably reflects a defect in spermiogenesis, rather than epididymal maturation. This defect may be at the gene expression level and may occur as early as during the pachytene spermatocyte phase of spermatogenesis, as this is when the first genes required for flagellar formation are expressed (Horowitz et al, 2005). Nevertheless, a number of the flagellar defects found in selenium-deficient rats were detected only after the spermatozoa left the caputepididymidis (Olson et al, 2004), indicating that the defects we observed may also originate during spermatozoal maturation in the epididymis. In murine models, defects in the flagella of spermatozoa are frequently associated with infertility (Huttner et al, 1993; Olson et al, 2005).

To the best of our knowledge, this is the first study to assess the impact in the rat model of concurrent administration of bleomycin, etoposide, and cis-platinum. Each of these drugs, however, has previously been given individually to rats (Seethalakshmi et al, 1992; Russell et al, 2000, 2004). Of the 3 drugs, cis-platinum has been reported to affect the numbers of spermatozoa and their motility (Seethalakshmi et al, 1992). After 9 weeks of daily intraperitoneal treatment with 0.5 mg/kg cis-platinum, a decrease in sperm count of approximately 60% and a decrease in sperm motility of approximately 50% were reported in rats. The effect on motility is much greater than we observed in our study; however, the effect on sperm count is much smaller. The greater effect on motility with cis-platinum alone may be a result of the route of

administration, although it is interesting to note that the percent of sperm that were motile in the control rats in that study (56%) is well below what is considered normal (Seed et al, 1996). However, the greater effect on numbers of spermatozoa in our study is likely to be due to an additive effect of the 3 drugs and underscores the value of elucidating the impact of the combination regimen in the rat model.

As a result of the effects of BEP treatment on sperm count, motility, and morphology, we anticipated a decrease in fertility; a reduction in sperm production of more than 90% has been demonstrated to impair fertility in the rat (Robaire et al, 1984). Furthermore, changes in motility and morphology are likely to impair fertility. Interestingly, paternal exposure to cis-platinum alone has been reported to increase the incidence of fetal malformations and deaths (Seethalakshmi et al, 1992). Surprisingly, after either 6 or 9 weeks of paternal BEP treatment, there was no adverse impact on fetal development, as assessed on gestation day 21; fetal morphology was normal, as were the numbers of live fetuses per litter. First, the sperm number required to maintain fertility in BEP-treated rats was less than 10% compared to controls.Second, even though a significant proportion of the remaining 10% have decreased motility and flagellar defects, their function was not affected sufficiently to alter progeny outcome. These results are in stark contrast to experiments with cyclophosphamide, another anticancer drug. After paternal treatment with this drug, at doses that did not alter male reproductive organ

weights or sperm counts, there were increases in pre- and postimplantation loss and congenital malformations in the progeny (Trasler et al, 1985).

When the females mated to males treated with BEP for 9 weeks were allowed to deliver, a significant proportion of their pups did not survive past postnatal day 1. We observed that of 10 litters, only 1 was of a normal size. There was no decrease in litter size nor was there an increase in fetal deaths when pregnancy was terminated on gestation day 21, indicating that it is likely that many of the sperm-positive mothers that were allowed to proceed through gestation may have delivered full litters of live pups, which subsequently died and were eaten or were eaten alive. No obvious lethal defects were observedamong the fetuses examined on gestation day 21. We speculate that delayed parturition had an adverse impact on postnatal survival in the 4 litters in which this occurred. Prenatal maternal exposures to dexamethasone, diethylstilbestrol, ethylene glycol dimethyl ether, 2-methoxyethanol, or Aroclor 1254 have been reported to delay parturition in rats (White et al, 1983; Chatterjee et al, 1993; Marty and Loch-Caruso, 1998; Lee et al, 2003). Delayed parturition is frequently associated with an increase in perinatal mortality (Rands et al, 1982; Leonhardt et al, 1991; Zimmerman et al, 1991). Alternatively, the pups may have had a fatal functional deficit that we did not observe. To the best of our knowledge, this is the first study

to show an effect of paternal chemical exposure on the timing of parturition and postnatal survival of progeny.

These results clearly show that BEP treatment has a deleterious impact on the quality of spermatozoa in the male rat, resulting in a decrease in spermatozoal numbers and motility, an increase in morphologically abnormal spermatozoa, and harmful effects to the postnatal development of the progeny. These data indicate that the chemotherapeutic agents, rather than cancer or orchidectomy, may affect the quality of spermatozoa in patients who have been treated for testicular cancer.

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Figures and Legends

Figure 1. Body weight changes over the course of the 9-week treatment with bleomycin, etoposide, and cis-platinum (BEP)**,** 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. * $P < .01$.

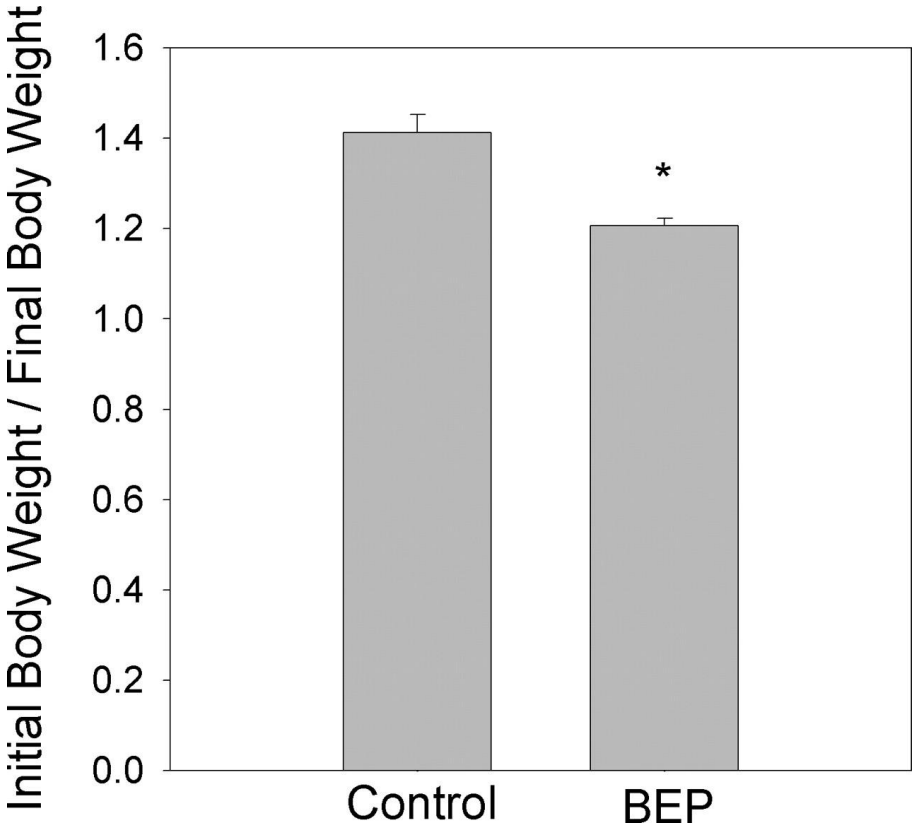
Initial Body Weight / Final Body Weight
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Control
BEP

Figure 2. Weights of reproductive organs after 9 weeks of treatment with vehicle or bleomycin, etoposide, and cis-platinum (BEP) (control, n = 8; BEP, n = 7). There was a significant decrease in the weights of the testes and epididymides of the BEP-treated animals, whereas the weights of the seminal vesicles and ventral prostate remained unchanged. ** $P < .001$.

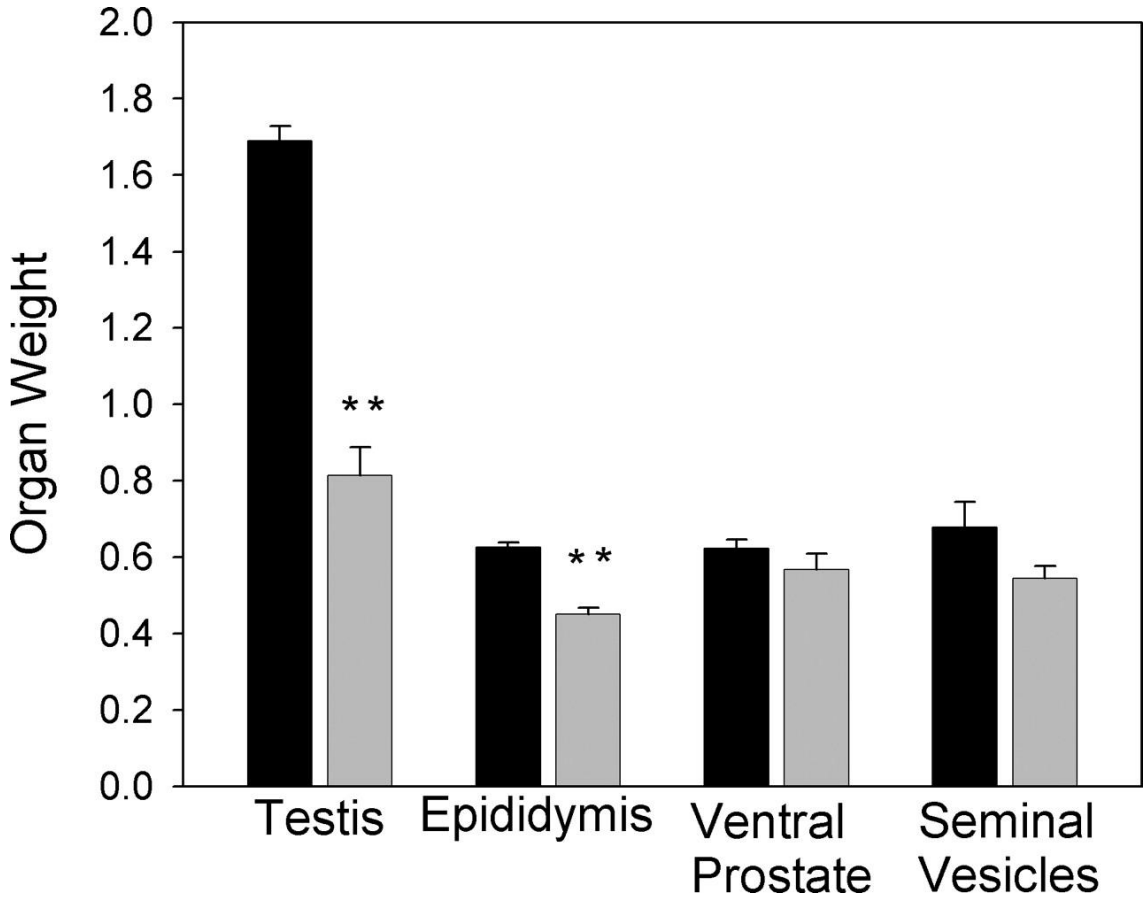
Organ Weight
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
* *
* *
Testis
Epididymis
Ventral Prostate
Seminal Vesicles

Figure 3. Histopathological examination of testis seminiferous epithelium. Representative testis sections of control **(A)** and bleomycin, etoposide, and cis-platinum (BEP)–treated rats **(B)**. Sections were stained with periodic acid–Schiff to show histology of seminiferous tubules.**(A, B)** A high proportion of tubules in the testis of BEP-treated rats **(B)** presented degenerated seminiferous epithelium and germ cells compared to controls **(A)**. Higher magnification of testis sections from control and BEP-treated rats. **(C)** Control rats showed normal seminiferous tubule organization and spermatogenesis; **(D–F)** testis sections from treated rats showed abnormal seminiferous tubules. Nine-week BEP exposure resulted in severe abnormalities, such as tubules with drastic reduction in germ cell content, sloughing of immature germ cells into the lumen (indicated by solid arrowheads), and extensive vacuolization (arrows). Scale bar = 200 μm in A and B; Scale bar = 50 μm in C–F.

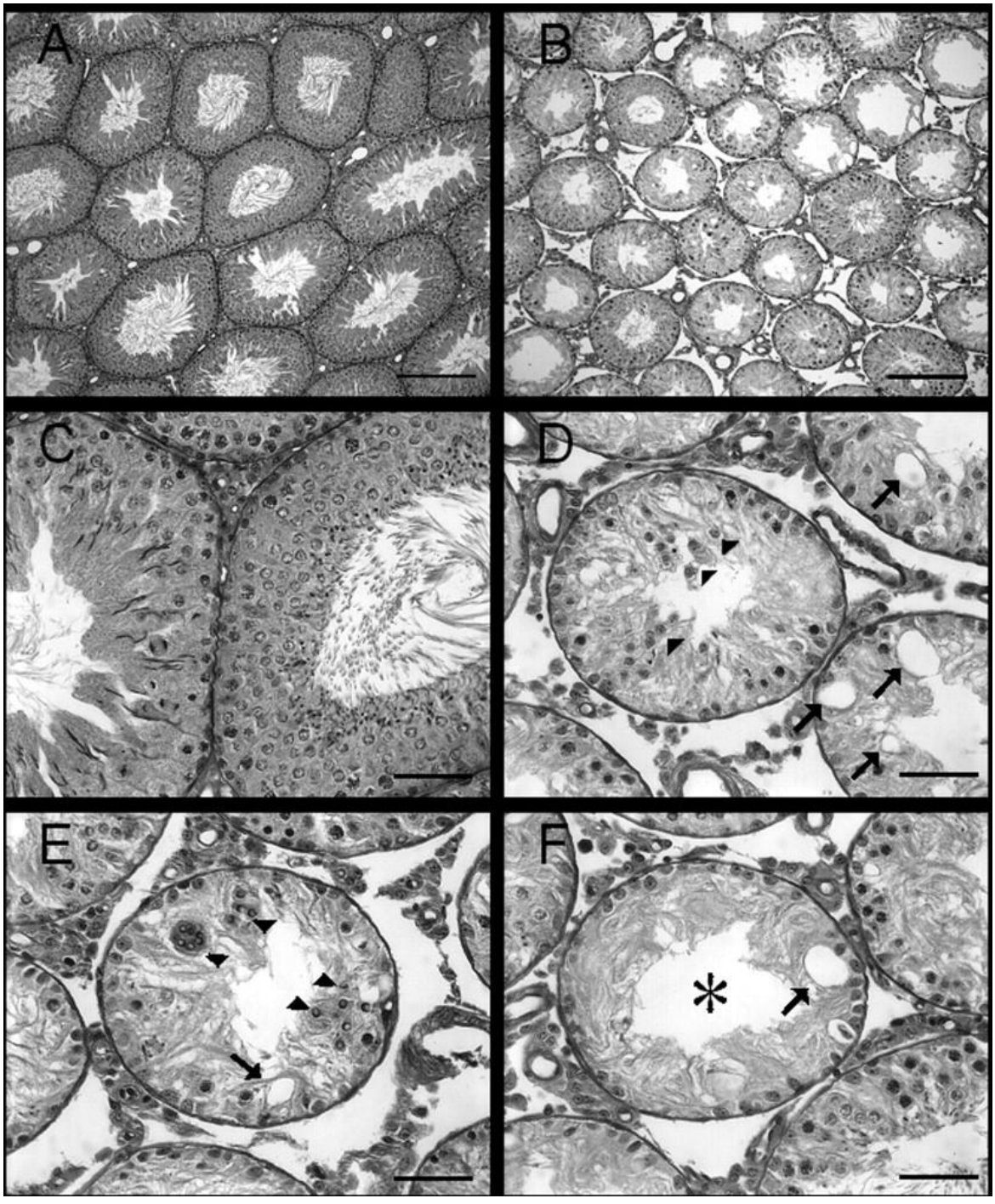
A
B
C
D
E
F

Figure 4. Spermatozoal counts in the caput-corpus epididymidis after 9 weeks of treatment with vehicle or bleomycin, etoposide, and cis-platinum (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 90%. ** $P < .001$.

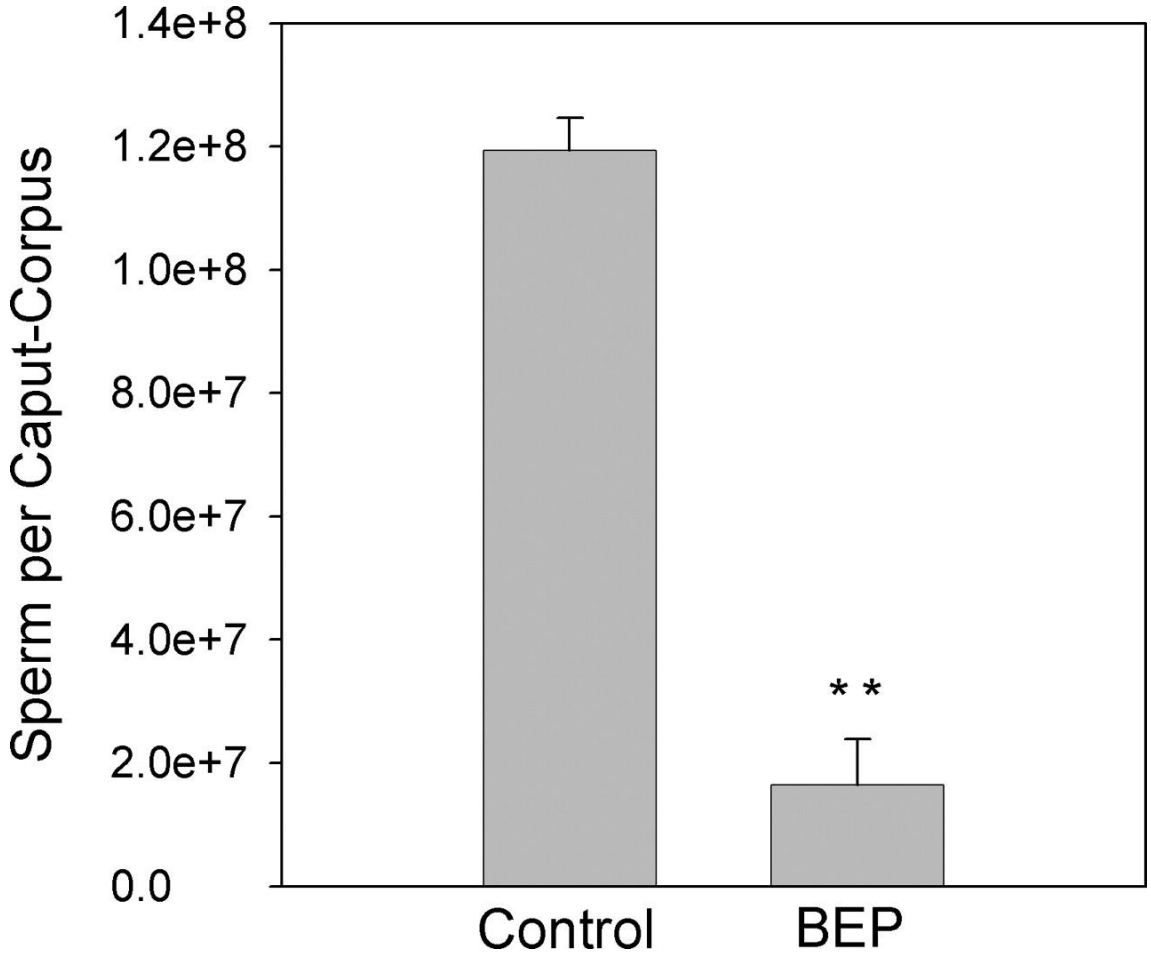
Sperm per Caput-Corpus
1.4e+8
1.2e+8
1.0e+8
8.0e+7
6.0e+7
4.0e+7
2.0e+7
0.0
* *
Control
BEP

Figure 5. Effects of bleomycin, etoposide, and cis-platinum (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). VCL indicates 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. * $P < .01$; ** $P < .05$.

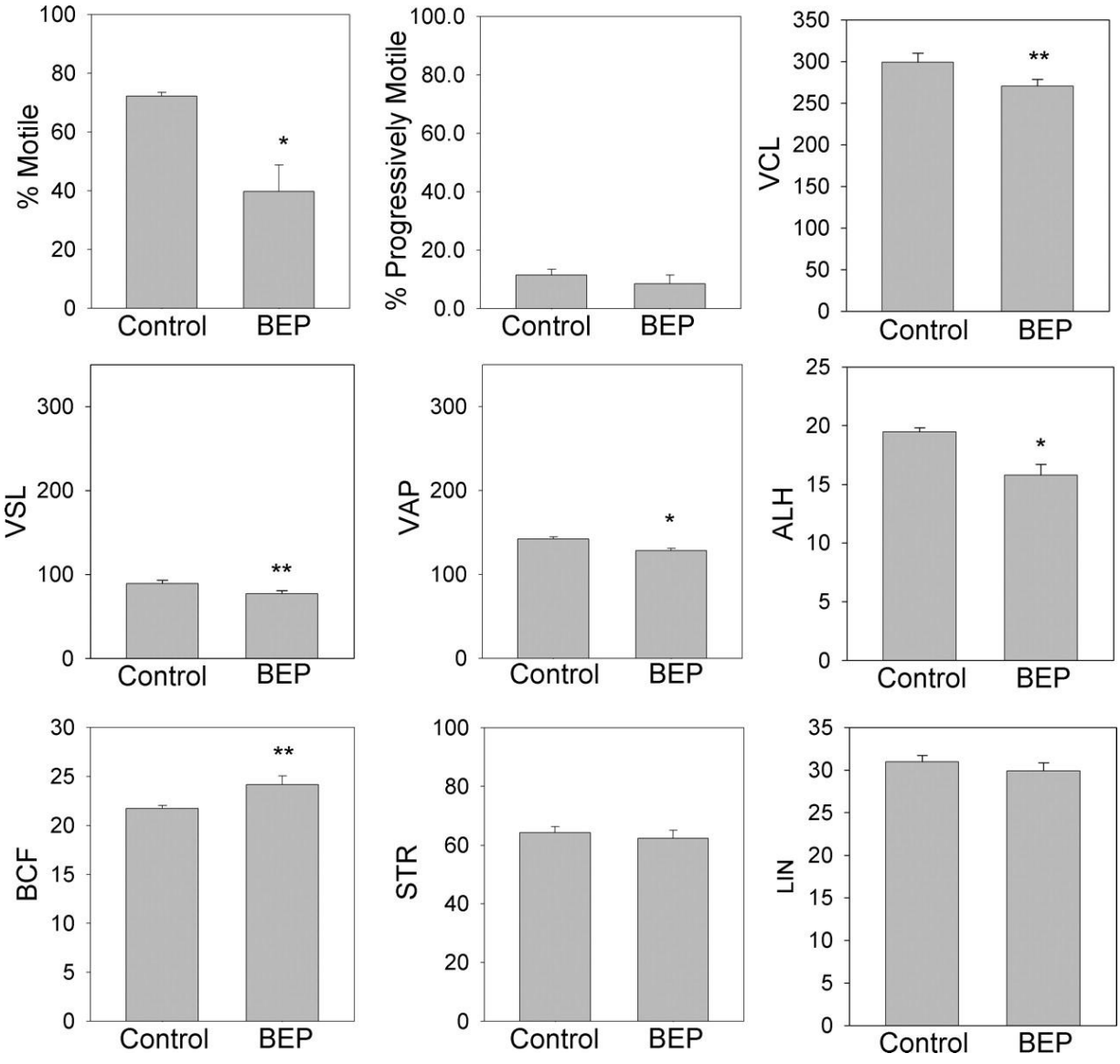
% Motile
100
80
60
40
20
0
*
Control
BEP
% Progressively Motile
100.0
80.0
60.0
40.0
20.0
0.0
Control
BEP
VCL
350
300
250
200
150
100
50
0
**
Control
BEP
VSL
300
200
100
0
**
Control
BEP
VAP
300
200
100
0
*
Control
BEP
ALH
25
20
15
10
5
0
*
Control
BEP
BCF
30
25
20
15
10
5
0
**
Control
BEP
STR
100
80
60
40
20
0
Control
BEP
LIN
35
30
25
20
15
10
5
0
Control
BEP

Figure 6. Percent abnormalities in both the midpiece and principal piece of spermatozoa from control or bleomycin, etoposide, and cis-platinum (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. * $P < .05$.

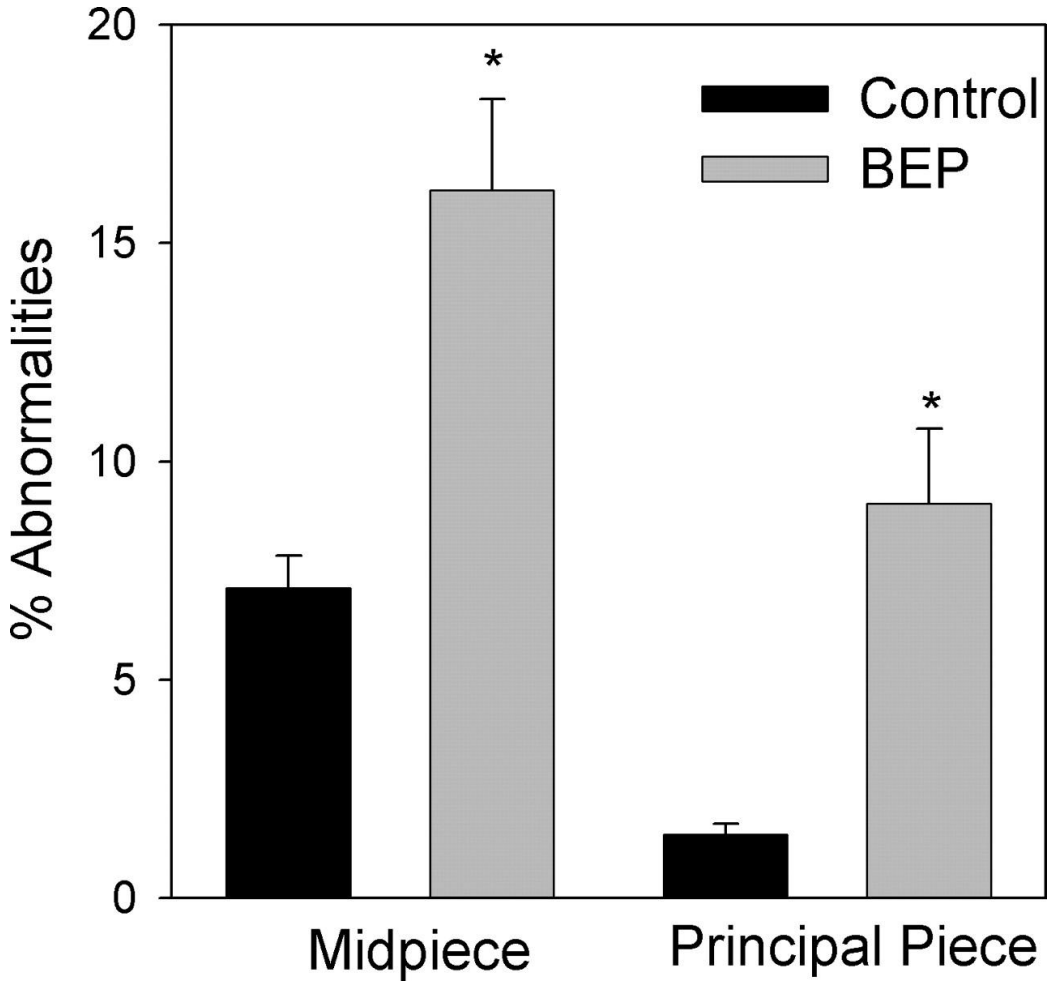
20
15
10
5
0
% Abnormalities
*
*
Control
BEP
Midpiece
Principal Piece

Figure 7. Morphology of the midpiece of a control spermatozoon, displaying the 9 plus 2 arrangement of microtubules (M), the 9 outer dense fibers (ODF), and the mitochondrial sheath (Mt) **(A)**, as well as spermatozoa from rats treated with bleomycin, etoposide, and cis-platinum (BEP) **(B–I)**. Abnormalities include an abnormal spatial arrangement of outer dense fibers **(B)**, the presence of 2 flagellar sections within 1 membrane **(C)**, absence of a mitochondrial sheath **(D)**, an abnormal number of outer dense fibers **(E)**, hemilateral absence of the outer dense fibers and axoneme **(F)**, a malformed mitochondrial sheath **(G)**, malformed outer dense fibers **(H)**, as well as a malformed midpiece **(I)**. Scale bars = 100 µm.

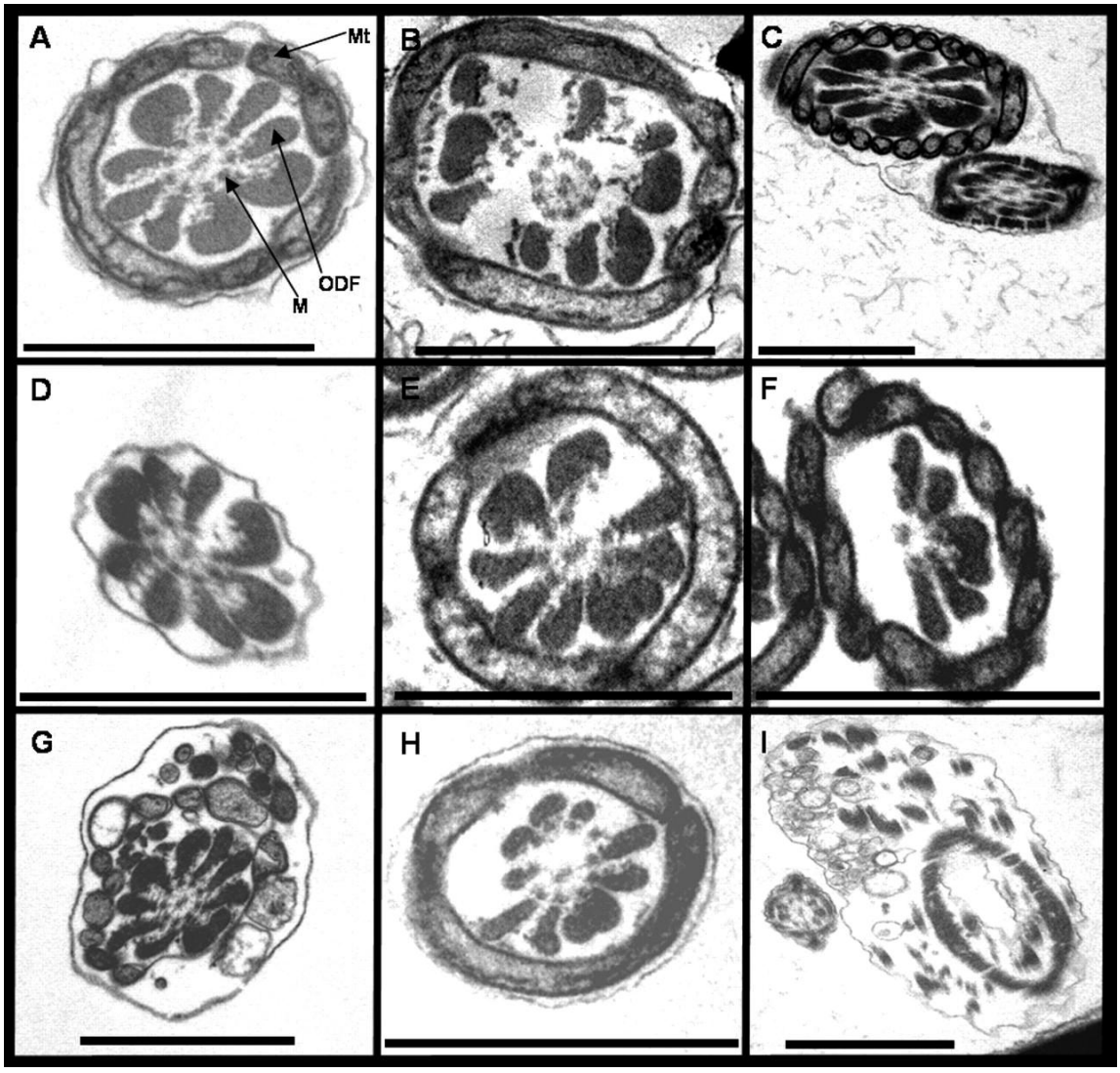
A
Mt
ODF
M
B
C
D
E
F
G
H
I

Figure 8. Morphology of the principal piece of a control spermatozoon **(A)** as well as spermatozoa from rats treated with bleomycin, etoposide, and cis-platinum (BEP) **(B–D)**. Abnormalities include the hemilateral absence of the outer dense fibers, with **(B)** or without **(C)** hemilateral absence of the axoneme, as well as an abnormal number of outer dense fibers **(D)**. Scale bars = 50 µm.

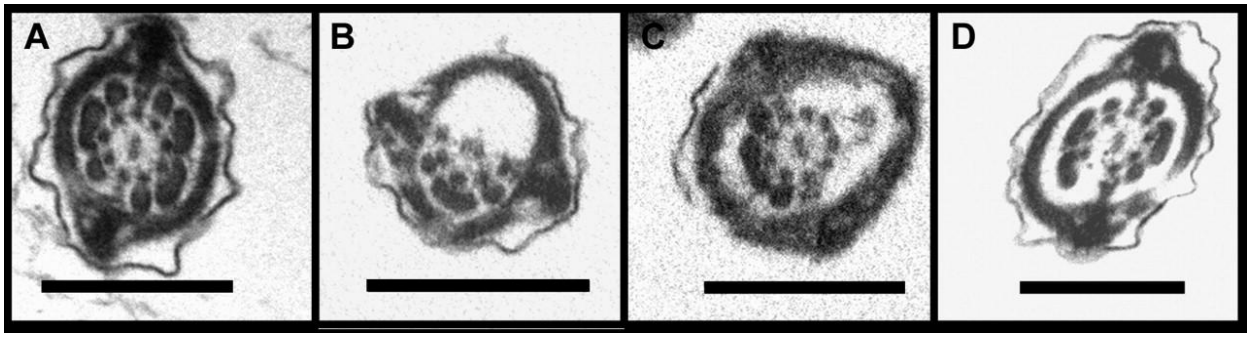
A
B
C
D

Figure 9. Numbers of pups per litter sired by males treated for 9 weeks with bleomycin, etoposide, and cis-platinum (BEP) or saline that survived past postnatal day 1. All the litters sired by control males had at least 12 pups, all of which survived. Of the litters sired by BEP-treated males, 6 did not have any pups that survived past day 1, 3 had litters of 5 or fewer pups, and 1 had a litter of 15 pups. Chi-square analysis was used to compare the number of litters with pups surviving past day 1.

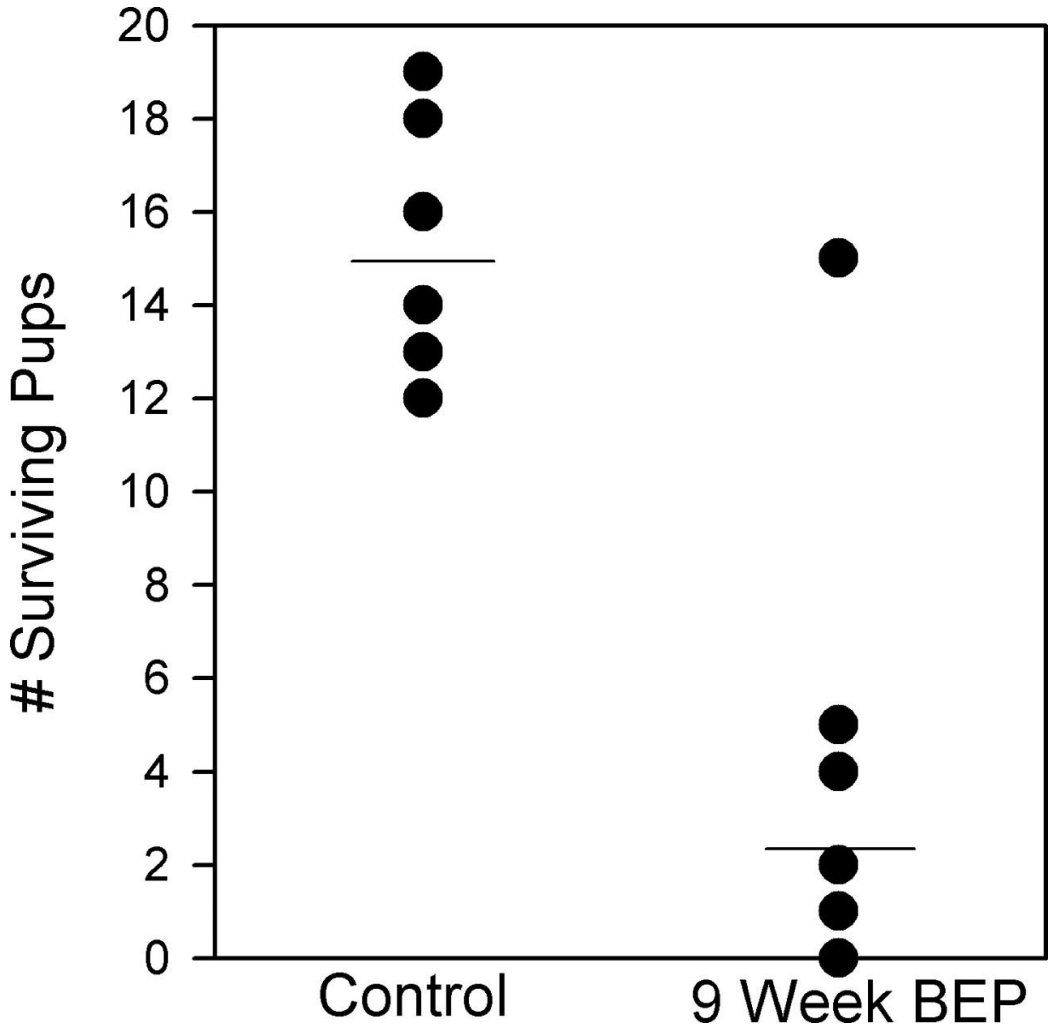
Surviving Pups
20
18
16
14
12
10
8
6
4
2
0
Control
9 Week BEP

Figure 10. Postnatal growth of surviving pups sired by males treated with bleomycin, etoposide, and cis-platinum (BEP) for 9 weeks. Pups were weighed biweekly until weaning, after which they were weighed weekly. Male and female pups are grouped separately. No difference in growth rate was observed between pups sired by control or BEP-treated males. Pups sired by males treated with BEP for 6 weeks showed a similar pattern. Values represent means ± standard errors of the mean (control, n = 7; BEP, n = 4). Inset is postnatal days 1–25.

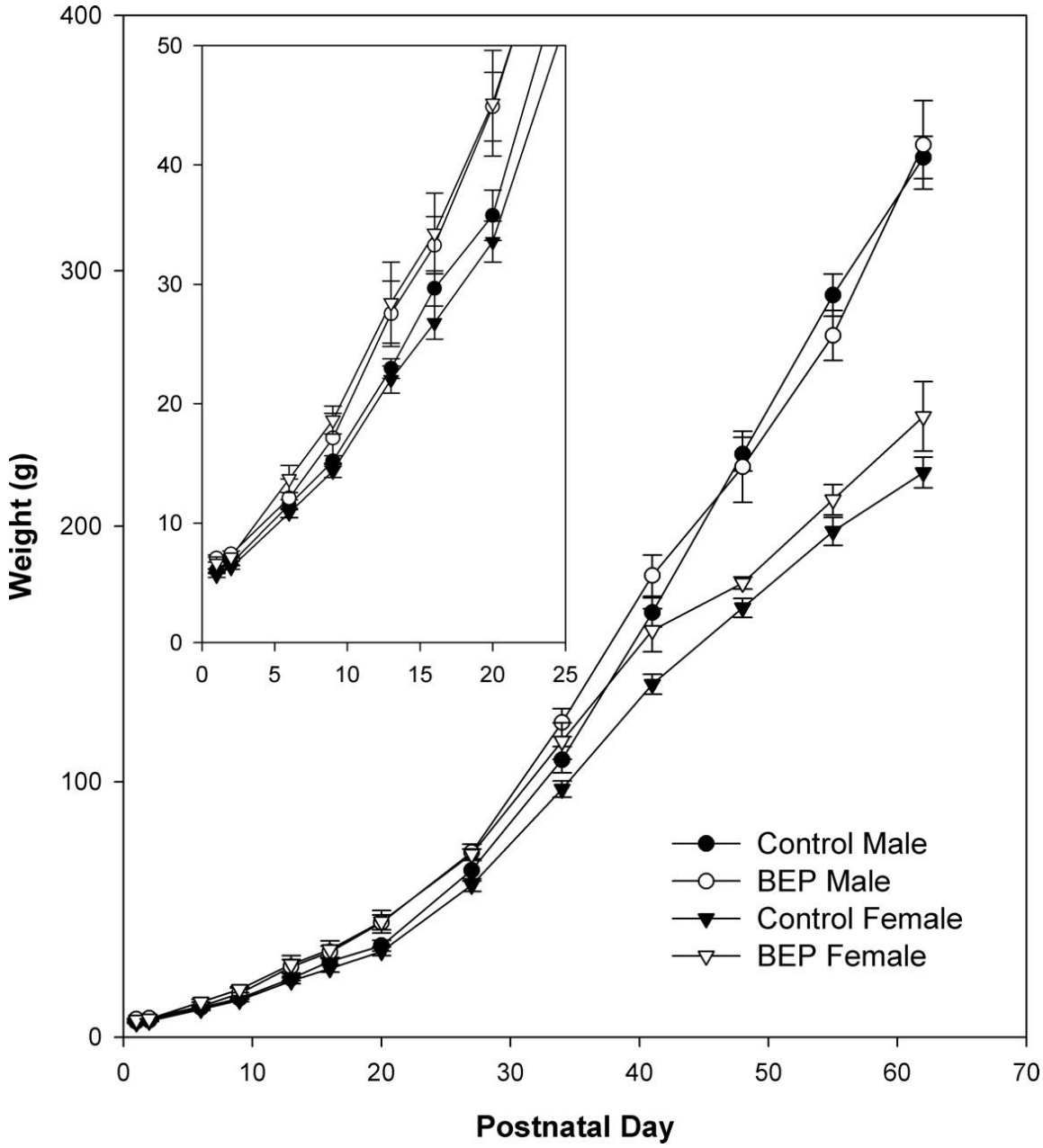
Weight (g)
Postnatal Day
Control Male
BEP Male
Control Female
BEP Female

Table. Pregnancy outcome in litters sired by males treated for 6 or 9 weeks with BEP*

	Duration of Treatment			
	6 wk		9 wk	
	Control	BEP	Control	BEP
Litter size	14.61 ± 0.54†	14.50 ± 0.73	13.53 ± 1.19	12.54 ± 0.82
Preimplantation loss	0.05 ± 0.02	0.09 ± 0.03	0.15 ± 0.06	0.17 ± 0.04
Postimplantation loss	0.01 ± 0.02	0.02 ± 0.06	0.07 ± 0.02	0.11 ± 0.04
Sex-ratio	0.48 ± 0.03	0.49 ± 0.04	0.50 ± 0.03	0.39 ± 0.05

*BEP indicates bleomycin, etoposide, and cis-platinum treatment, as defined in the "Materials and Methods" section. 6 Weeks: control, n = 18; BEP, n = 12; 9 Weeks: control, n = 15; BEP, n = 11. Preimplantation loss is defined as the number of corpora lutea minus the number of implantation sites per litter. Postimplantation loss is defined as the number of implantation sites minus the number of fetus per litter on day 20 of gestation

†All values are expressed as the mean ± SEM

CONNECTING TEXT

In Chapter 2, we developed and studied a novel animal model for the impact of the testicular cancer treatment on male germ cell development and progeny outcome by co-administrating cisplatin, etoposide and bleomycin (BEP) in a schedule and doses related to the ones used in clinic to adult male SD rats. The results presented in Chapter 2 clearly established that the co-administration of cisplatin, etoposide and bleomycin disrupts spermatogenesis, reduces sperm counts and alters spermatozoa structure. Although we demonstrated that the combination of cisplatin, etoposide and bleomycin interfered with spermatogenesis but had no detectable effects on progeny outcome at the end of the 9 week treatment, we did not explore the possibility that such treatment may indeed have prolonged adverse effects in particular on progeny outcome. We hypothesized that a reversibility study would allow us to examine progeny outcome parameters during the spermatogenic recovery phase at regular intervals once the treatment is stopped. Therefore, in chapter 3, we examined the long-term effects of a BEP subchronic treatment after its completion, during a recovery period for up to 9 weeks. The BEP subchronic treatment was designed to more closely mimic the BEP regimen used in clinic, and differs from the chronic BEP treatment we previously used as follow: cisplatin and bleomycin were administered on days 1 -5 of each 21-day cycle and given by i.p injection and oral gavage,

respectively; whereas bleomycin was still given on day 2, 9 and 16 by i.p for each cycle.

Chapter 3

Chapter 3: Reversibility of the Effects of Subchronic Exposure to Cancer Chemotherapeutics, Bleomycin, Etoposide, and Cisplatin on Spermatogenesis, Fertility, and Progeny Outcome in the Male Rat[1]

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Running title: Reversibility of BEP Effects in Rats

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3.1 Abstract

Testicular cancer is the most common cancer among young men of reproductive age. A regimen of bleomycin, etoposide, and cisplatin (BEP regimen) is the standard chemotherapy for testicular cancer. BEP has adverse effects on spermatogenic function that pose a long-term reproductive health risk to cancer survivors and their progeny. Using a rat model, we investigated the persistence of the effects of BEP on male reproductive function, fertility, and progeny outcome. Adult male Sprague-Dawley rats received a BEP regimen mimicking human clinical exposure (three 21-day cycles of etoposide and cisplatin on days 1–5 and bleomycin on days 2, 9, and 16, or vehicle). Reproductive and progeny outcome parameters were assessed at the end of BEP treatment and up to 9 weeks post-treatment, at 3-week intervals. BEP treatment reduced testicular weights and impaired spermatogenesis, characterized by abnormal testis histology and germ cell depletion. Germ cell apoptosis increased at least 3-fold in BEP-treated rats compared with controls at the end of treatment; 9 weeks posttreatment, germ cell apoptosis in BEP-treated rats did not differ from controls. BEP-exposed males were fertile; a decrease in litter size and an increase in preimplantation and postimplantation losses were observed. Preimplantation loss remained elevated in litters sired by BEP-treated males up to 9 weeks posttreatment; however, neither postimplantation loss nor litter sizes differed from controls. Thus, both germ cell apoptosis and

the postimplantation loss induced by BEP treatment were reversible. The persistence of the elevation in preimplantation loss 9 weeks after BEP treatment suggests that spermatogonia are affected.

3.2 Introduction

Testicular cancer represents the most common cancer among young men of reproductive age, and its incidence is steadily increasing (Bergström et al, 1996; Moller and Evans, 2003). The standard chemotherapeutic regimen for testicular cancer patients with metastatic disease, the BEP regimen, combines the administration of bleomycin, etoposide, and cisplatin. Over the last 2 decades, this regimen has been remarkably successful in curing testicular cancer, with reported 5-year survival rates greater than 90% (Williams et al, 1987; Einhorn, 1990; Einhorn and Foster, 2006). However, BEP treatment is associated with a transient or permanent loss of fertility. Considering that most of these patients are of reproductive age, and that their long-term survival is improved due to the effectiveness of the BEP treatment, there is an increasing interest in the reproductive health of testicular cancer survivors because of the impact on their postchemotherapy quality of life (Naysmith et al, 1998; Lambert and Fisch, 2007).

Spermatogenesis is often impaired in testicular cancer patients prior to chemotherapy as a result of the cancer itself (Agarwal, 2005). In

addition, BEP chemotherapy has substantial detrimental effects on spermatogenic function; most patients are rendered temporarily azoospermic or oligozoospermic, depending on the dose and duration of treatment (Petersen et al, 1994). Normal spermatogenesis recovers in about 50% of the patients after 2 years and in the large majority (80%) 5 years after the completion of chemotherapy (Lampe et al, 1997; Howell and Shalet, 2005; Magelssen et al, 2006). However, in some patients, sperm production does not reinitiate and permanent infertility ensues. Thus, fertility is a major concern for testicular cancer patients undergoing BEP chemotherapy.

In animal models, previous studies have shown that either acute or chronic administration of the chemotherapeutic drugs cisplatin or etoposide induces adverse effects on various male reproductive parameters shortly after exposure. For instance, subchronic administration of cisplatin over a 9-week period resulted in a decrease in reproductive organ weights, including testes and epididymides, decreased sperm motility, and increased preimplantation and postimplantation loss; malformed and growth-retarded fetuses were observed among the progeny sired by cisplatin-treated males (Seethalakshmi et al, 1992). Acute exposure to cisplatin resulted in decreased reproductive organ weights, as well as increased preimplantation loss (Huang et al, 1990; Kinkead et al, 1992). However, no studies have investigated the potential persistence of these effects,

particularly with respect to progeny outcome, after completion of treatment. Likewise, chronic etoposide administration induced drastic alterations in spermatogenesis at high doses, but the impact of such treatment on progeny outcome remains unknown (Kadota et al, 1989; Kawaguchi et al, 2000). In addition, these studies all consisted of exposure to a single drug. However, as mentioned above, the current BEP regimen relies on the synergistic effects of 3 different anticancer drugs over at least a 9-week period. Therefore, to accurately mimic the impact of this regimen, bleomycin, etoposide, and cisplatin have to be given chronically and in combination.

To date, chemotherapy for testicular cancer has not been associated with increased birth defects or abnormal progeny, but the number of subjects in these studies has been relatively small (Hartmann et al, 1999). The relative risk for abnormal progeny among the offspring of testicular cancer survivors remains poorly defined and requires further study. Furthermore, it is very difficult to predict the long-term effects of the BEP regimen on fertility and progeny outcome in patients prior to the initiation of chemotherapy as the condition of each patient may vary at the time of diagnosis and with the treatment modalities of the disease (Gandini et al, 2006). Owing to this lack of information, animal studies may provide a better understanding of how BEP affects fertility and progeny outcome in the short- and long-term. We have reported previously that the combination of bleomycin, etoposide, and

cisplatin, when chronically administered for 9 consecutive weeks to adult male rats, resulted in a drastic reduction in spermatozoal count and decreased progeny survival but, interestingly, did not compromise fertility (Bieber et al, 2006). The purpose of this study was to investigate, using the rat as a model, the long-term effects of the subchronic combined-administration of bleomycin, etoposide, and cisplatin, mimicking the clinical chemotherapeutic exposure in humans, on male reproductive functions, with emphasis on fertility and progeny outcome.

3.3 Materials and Methods

Animals and Chemicals

Adult male (350–400 g) and virgin female (200–225 g) Sprague-Dawley rats were purchased from Charles River Laboratory Inc (St-Constant, Canada). Animals were housed in the animal facility at the Animal Resources Centre, McIntyre Medical Sciences Building, McGill University (Montréal, Canada), maintained on a reversed 14 h light/10 h dark photoperiod, and given free access to rat pellet chow and water ad libitum. All animal experimentation was conducted in accordance with the principles and procedures outlined by the Canadian Council on Animal Care and with the animal guidelines of McGill University (Montréal, Canada). Cisplatin, etoposide, and bleomycin were obtained from LKT Laboratories (St Paul, Minnesota). All other chemicals were obtained from Sigma Chemical Co (St Louis, Missouri), unless otherwise specified.

Animal Groups and Treatment Regimen

Animals were divided randomly into 3 different treatment groups (n = 15 per group): a control group (0x), and 2 BEP-treated groups (0.33x and 0.5x). Dose selection was based on the human clinical regimen. In the clinic, patients undergoing adjuvant BEP chemotherapy commonly receive a daily dose of 20 mg/m^2 cisplatin and 100 mg/m^2 etoposide on days 1–5 and a weekly dose of 30 mg bleomycin. These standard doses for each drug were converted to rat doses by adjusting for body weight/surface area ratio (Bachmann et al, 1996) and represent the 1x dosage. In a 1x dosage, animals would receive 15 mg/kg body weight (bw) of etoposide, 3 mg/kg bw of cisplatin, and 1.5 mg/kg bw of bleomycin. In the 0.33x BEP group, animals received one-third of the 1x dose, or 5 mg/kg etoposide, 1 mg/kg cisplatin, and 0.5 mg/kg bleomycin. Accordingly, the 0.5x BEP animals were given 7.5 mg/kg etoposide, 1.5 mg/kg cisplatin, and 0.75 mg/kg bleomycin. Rats in the BEP groups were treated with 3 cycles of bleomycin, etoposide, and cisplatin for a total of 9 weeks. In order to closely mimic the time-schedule of the human clinical regimen, etoposide and cisplatin were administered on days 1–5 and bleomycin on days 2, 9, and 16 of each 21-day cycle. Cisplatin and bleomycin, in 0.9% saline, were given by intraperitoneal (IP) injection. Etoposide was dissolved in a 3:7 (vol/vol) mixture of dimethylsulfoxide (DMSO)-saline and administered by gavage. Age-matched rats in the control group were treated in the same manner and

received equivalent volumes of saline and DMSO. All drug solutions were prepared fresh daily before administration. For the recovery experiment, the treatment period was followed by a recovery period of 3 cycles (9 weeks) before the rats were euthanized. The 0.33x BEP dose was the maximum tolerated dose consistent with good survival during the recovery phase. Only 1 animal died in this treatment group before the end of the recovery period. The 0.5x BEP dose resulted in high mortality, with 7 out of 17 rats dying during the treatment; data from this group at the end of treatment are provided only to emphasize potential systemic and germ cell toxicity of this subchronic BEP treatment.

Tissue Collection and Preparation

At the time of euthanasia, animals were anesthetized with an i.p injection of a mixture of ketamine hydrochloride (50 mg/kg), acepromazine (1 mg/kg), xylazine hydrochloride (5 mg/kg) and 0.9% saline. Prior to perfusion, blood was collected under anesthesia; the serum was then separated and stored at –80°C. The left testis and epididymis were ligated, dissected out, weighed, and rapidly frozen in liquid nitrogen and stored at –80°C until further use. The contralateral testis and epididymis were first cleared with 0.9% saline and then perfused through the abdominal aorta with Bouin fixative for 10 minutes. Following perfusion, the testis and epididymis were excised and postfixed for an additional 24 hours in the same fixative. Tissues were dehydrated and then embedded in paraffin (Serre and Robaire, 1999).

Histology

For histological analysis, 5 µm-thick sections were cut on a microtome, mounted on microscope glass slides (Superfrost Plus microscope glass slides; Fisher Scientific Inc, Montreal, Canada) and dried overnight at 37°C. Tissue sections were deparaffinized with xylene and rehydrated through a graded series of ethanol changes; staining of sections with periodic acid-Schiff (PAS) was carried out using the Sigma PAS-kit 395B-1KT, according to the manufacturer's instructions. Sections were examined and photographed by light microscopy.

Terminal Deoxynucleotidyl Transferase–Mediated Nick-End Labeling Assay

Testicular germ cell apoptosis was assessed by terminal deoxynucleotidyl transferase (TdT)-mediated deoxy-UTP nick end labeling (TUNEL) assay using ApopTag Peroxidase In Situ Apoptosis Detection kit (S7100; Chemicon International Inc, Temecula, California), according to the manufacturer's instructions. Following TUNEL staining, sections were counterstained with Harris Hematoxylin and mounted under glass coverslips with Permount (Fisher). The sections were examined and scored under a light microscope (Leica DM LB2 microscope; Leica, Wetzlar, Germany) equipped with an RS Photometrics CoolSNAP fx digital camera (Roper Scientific, Tucson, Arizona). TUNEL-positive germ cells were quantified by counting the number of TUNEL-positive cells in each round cross-section of the seminiferous tubules. Sections from 5

animals per group were analyzed, and apoptotic germ cells were counted in at least 200 seminiferous tubules per testis section. As negative controls, a section from each animal was processed as described above, but terminal transferase was omitted from the TdT labeling buffer.

Spermatid/Spermatozoal Head Counts

A weighed portion of the decapsulated left testis was homogenized in 5 ml of 0.9% NaCl, 0.1% thimerosal, and 0.5% Triton X-100 with a Polytron (Brinkman Instruments, Westbury, New York) twice for 15 seconds, separated by a 30-second period, as previously described (Robb et al, 1978). Spermatid heads in an aliquot from each testis homogenate were counted with a hemocytometer.

Measurement of Serum Testosterone Levels

Serum testosterone concentrations were measured using a testosterone enzyme-linked immunosorbent assay (ELISA) kit (catalog no 55R-RE52151; IBL Immunobiological Laboratories, Hamburg, Germany) following the manufacturer's instructions.

Analysis of Progeny Outcome Parameters

After the completion of the 9-week BEP treatment, each male from the 0.33x BEP-treated and control groups (n = 10–15) was caged overnight with 2 selected female rats in proestrus. To assess fertility and progeny outcome parameters during the recovery phase, the BEP-treated rats were mated 3, 6, and 9 weeks after the cessation of treatment. The

following morning, vaginal smears were examined for the presence of spermatozoa to determine whether copulation had occurred; this day was defined as gestation day 0. All confirmed-pregnant females were euthanized on gestation day 20 by CO_2 asphyxiation andthen cesarean-sectioned. The ovaries were removed, and the numbers of corpora lutea (representing the numbers of ovulated oocytes) were counted. The uteri were dissected and inspected for implantation and resorption sites, and the numbers of implantation and resorption sites were recorded.

$$\text{Preimplantation loss} = \frac{\text{Number of corpora lutea} - \text{Number of implantation sites}}{\text{Number of corpora lutea}}$$

$$\text{and postimplantation loss} = \frac{\text{Number of implantation sites} - \text{Number of live fetuses}}{\text{Number of implantation sites}}$$

were calculated for each female. Finally, the fetuses were categorized as live or dead and were sexed, individually weighed, and examined for gross

external malformations. Sex ratio was defined as the number of male pups divided by the total number of pups per litter.

Statistical Analysis

All data were analyzed using SigmaStat (SPSS Inc, Chicago, Illinois). Values are presented as means ± SEM. Differences between groups were examined for statistical significance using a Student's *t* test when only 2 groups were compared or 1-way analysis of variance (ANOVA). If ANOVA indicated *P* values of .05 or below, a Bonferroni *t* test was performed to determine the significance of difference between all groups. Nonparametric Mann-Whitney tests were performed when data were not distributed normally. *P* values of .05 or below were regarded as significant.

3.4 Results

Effects of BEP Treatment on Body Weight Gain

Body weights of control and BEP-treated rats were measured daily during the 9 weeks of treatment and the recovery period. The effects of BEP treatment on body weight gain are shown in Figure 1. At the initiation of treatment, there were no significant differences in the mean body weights among the control and the 2 BEP-treated groups. After 9 weeks of treatment, male rats exposed to either 0.33x or 0.5x BEP showed significant reductions in body weight gain compared with animals from the control group (Figure 1). Rats in the 0.33x BEP exposure group gained

weight during the 9-week recovery period, but this weight gain was also significantly less than that in the control group.

Effects of BEP Treatment on Reproductive Organ Weights

Despite the reduced body weight gain, the mean testis, epididymis, and ventral prostate weights did not differ significantly between control and 0.33x BEP-treated groups at the end of the 9-week treatment period (Figure 2). In contrast, treatment with 0.5x BEP resulted in significant decreases in testis, epididymis, seminal vesicle, and prostate weights (Figure 2). However, 9 weeks after the completion of treatment, mean testis, epididymis, and seminal vesicle weights of 0.33x BEP-treated rats were not significantly different from controls (Figure 2). Interestingly, ventral prostate weights remained significantly decreased in the 0.33x BEP group compared with controls.

Testis Histology

Testis sections from control and BEP-treated rats were used for light microscopic studies. As illustrated in Figure 3 (A, D), normal histology of the seminiferous epithelium at various stages was observed in the control group, both immediately after the 9 weeks of treatment and following the recovery period. In contrast, the histological evaluation of BEP-treated rat testes demonstrated that BEP exposure affected spermatogenesis, with marked interindividual variability in response among animals in both the 0.33x and 0.5x BEP treatment groups. The

extent of response ranged from apparently normal seminiferous tubules to completely atrophic tubules, characterized by the presence of a single layer of cells consisting of vacuolated Sertoli cells and a few spermatogonia at the basal membrane of the seminiferous epithelium (Figure 3B and C). Abnormal tubules exhibited disorganized germ cell association, epithelial vacuolization, multinucleated giant cells, severe germ cell loss, sloughing of immature germ cells and tubule atrophy. In the 0.5x BEP treatment group, almost 80% of the tubules were degenerated at the end of treatment, while only 20% of the tubules were degenerated in the 0.33x BEP treatment group (Figure 3H). Nine weeks after the completion of treatment, testis histology of most BEP-treated rats revealed apparently normal spermatogenesis (Figure 3E and H). Interestingly, only a few tubules in 1 BEP-treated rat were still depopulated of germ cells and atrophied (Figure 3F), surrounded by normal tubules, suggesting that incomplete recovery of spermatogenesis may occur following BEP treatment; focal loss of functionalspermatogenesis in some seminiferous tubules is likely to reflect an effect on spermatogonial stem cells.

Spermatid Head Counts

In the 9 week BEP-treated groups, there was a dose-dependent decrease in the spermatid/spermatozoa head counts that was significant only in the 0.5x group compared to control (Figure 3G). Treatment with 0.33x BEP followed by the 9-week recovery

period resulted in a 20.6% decrease in spermatid/spermatozoa head count that was not statistically significant (*P* = .07; Figure 3G).

Effects of BEP Treatment on Serum Testosterone Concentrations

Treatment with BEP for 9 weeks did not alter serum testosterone concentrations in the 0.33x dosage group (Figure 4). Although serum testosterone concentrations were reduced in the 0.5x BEP group by nearly 50%, the difference was not statistically significant (*P* = .10), presumably due to the wide fluctuations in serum testosterone known to occur in the rat (Robaire and Bayly, 1989). In addition, serum testosterone concentrations were not different in the 0.33x BEP group compared to the control group following the recovery phase.

Effects of BEP Exposure on Germ Cell Apoptosis

As demonstrated above, BEP treatment is clearly associated with abnormal spermatogenesis and a reduction in germ cell content in the seminiferous epithelium in a dose-dependent manner. Therefore, we investigated whether testes from BEP-treated rats had an increased number of apoptotic germ cells when compared with testes from control animals, using the TUNEL assay. Under normal physiologic conditions, apoptosis occurs spontaneously in testicular germ cells, affecting principally spermatogonia and spermatocytes. As expected, testis cross-sections from control rats had few TUNEL-positive cells (Figure 4A) and were characterized by a low number of overall TUNEL-positive

tubules (Figure 4E). Whereas exposure to 0.33x BEP had no significant effect on the testis weight, an increase in both the number of TUNEL-positive tubules and the number of TUNEL-positive cells per tubule was observed compared to the control after 9 weeks of treatment (Figure 4B, E, and F). In addition, the number of TUNEL-positive tubules in the 0.5x BEP group was increased significantly compared with the control group. However, there was no significant increase in the number of TUNEL-positive cells per TUNEL-positive tubules; this may indicate that the dramatic depletion of germ cells observed in those testis sections resulted from massive apoptosis (Figure 4C, E, and F). No significant differences in either TUNEL parameter were detected between 0.33x BEP-exposed and control rats after the 9-week recovery period (Figure 4E and F). Therefore, the increase in apoptotic germ cells in the seminiferous epithelium of 0.33x BEP-treated rats was not persistent.

Reversibility of the Effects of Subchronic BEP Treatment on Progeny Outcome

The effects of paternal subchronic BEP exposure on progeny outcome at the end of treatment, and after 3, 6, or 9 weeks of recovery, were assessed by examining preimplantation and postimplantation loss, litter size, sex ratio, and fetal weights. BEP treatment (0.33x BEP group) resulted in a 5-fold increase in preimplantation loss compared to control (Figure 5A, $P < .001$);

preimplantation loss remained elevated in litters sired by BEP-treated males up to 9 weeks posttreatment. A 3-fold increase in postimplantation loss was observed in the BEP group relative to control after 9 weeks of treatment (Figure 5B; $P < .05$). Interestingly, unlike preimplantation loss, which remained persistently elevated, the incidence of postimplantation loss did not differ from the control in the BEP-treated group during the recovery period. As a consequence of the increases in preimplantation and postimplantation loss, a significant reduction in litter size was observed in the 0.33x BEP group compared with the controls (Figure 5C). The number of pups per litter remained low in the BEP-exposed group after the 3-week recovery period but did not differ from controls in the 6-week and 9-week recovery groups. Sex ratios were not altered by paternal exposure to BEP (Figure 5D). The mean weights of male and female fetuses were not affected in any treatment group, nor did exposure to BEP induce an increase in the incidence of external malformations in the fetuses (data not shown).

3.5 Discussion

In this study, we evaluated the impact of a subchronic combination treatment with bleomycin, etoposide, and cisplatin that mimics the human clinical regimen on male reproduction, fertility, and progeny outcome in a rat model in the absence of testicular cancer and orchidectomy. Our main objective was to assess whether the effects of BEP treatment on these parameters were reversible. Testicular cancer

patients often present with significant reproductive problems prior to chemotherapy due to the disease condition. Therefore, the use of an animal model allows us to administer BEP in a dose regimen that is clinically relevant, without the presence of testicular cancer or orchidectomy. To the best of our knowledge, no previous studies have evaluated the reversibility of the effects of BEP chemotherapy on spermatogenesis, fertility, and progeny outcome.

Impairment of spermatogenesis is one of the earliest signs of the adverse effects of BEP chemotherapy on testicular function as sperm counts of patients decrease following the initiation of chemotherapy (Gandini et al, 2006). In the present study, we showed that exposure to 0.33x low-dose BEP treatment caused the degeneration of germ cells, the formation of multinucleated giant cells, the shedding of immature germ cells within the lumen, and Sertoli cell vacuolization. The 0.5x BEP treatment resulted in more drastic effects on the seminiferous epithelium, with extensive germ cell depletion and tubule atrophy after 9 weeks of treatment.

In the mammalian testis, spontaneous germ cell death occurs to eliminate damaged germ cells from the seminiferous epithelium, thereby maintaining cellular homeostasis of the epithelium. In the adult rat testis, various studies have shown that drug-induced germ cell death is mediated by apoptosis (Cai et al, 1997; Blanco-Rodriguez et al, 1998; Sjöblom et al,

1998). In this study, by in situ analysis of TUNEL-positive cells, we demonstrate that the germ cell degeneration observed in adult rats exposed to BEP treatment is mediated by apoptosis. At low-dose 0.33x BEP exposure, a marked increase in apoptotic germ cells was observed at the end of the 9-week treatment; the rate of apoptosis may increase gradually with BEP exposure. In contrast, with exposure to an increased dose, 0.5x BEP, testes with the most extensive degeneration and atrophic tubules showed a relatively low number of apoptotic germ cells, presumably as a result of extensive germ cell depletion. Apoptosis is a relatively rapid mechanism of cell death; therefore, it is possible that the high dose 0.5x BEP treatment induced a more drastic increase in germ cell death in the early days of treatment, resulting in the severe reduction of germ cell content and tubule atrophy observed at the end of dosing. In addition, BEP-induced apoptosis was more pronounced in spermatogonia and spermatocytes, suggesting that BEP treatment may target actively dividing germ cells.

It is well documented that testicular cancer patients experience impaired spermatogenesis shortly after the initiation of BEP chemotherapy (Oliver, 1996). However, it is not clear whether drug-induced apoptosis is solely responsible for the loss of germ cells and subsequent decreased production of spermatozoa. Interestingly, the number of apoptotic germ cells did not appear to be markedly increased in the testis cross-sections examined following the 9-week recovery period,

demonstrating that the apoptotic germ cell death is either transitory or reversible. Taken together, the apparently normal testis histology and the absence of enhanced apoptosis in the majority of BEP-treated male rats after the recovery period suggest that, at least histologically and numerically, apparently spermatogenesis recovers effectively after BEP treatment. These results are in agreement with previous animal experiments where single drug exposures were used (Zhang et al, 2001; Seaman et al, 2003; Stumpp et al, 2004).

The action of BEP chemotherapeutics on seminiferous epithelial germ cells is the consequence of the combination of 3 different anticancer agents with 3 separate modes of action. Cisplatin alone is known to have adverse effects on testis histopathology and progeny outcome when chronically administered to rats. Cisplatin functions by damaging DNA through the formation of chemically stable DNA adducts. Cisplatin-induced DNA adducts accumulate in the testis following repeated dosing (Poirier et al, 1992); this persistence of DNA adducts in rat testes may suggest that germ cells are less efficient in eliminating adducts, thus leading to prolonged adverse effects of the drug in these cells. A dose-dependent accumulation of DNA adducts in rat spermatozoa was demonstrated following acute cisplatin exposure but, interestingly, without inducing adverse developmental effects (Hooser et al, 2000). Therefore, it is possible that cisplatin-induced DNA adducts may have accumulated in

germ cells during spermatogenesis withthe cisplatin-based BEP treatment; these cisplatin-induced DNA adducts may result in DNA strand break formation in spermatozoal DNA during the treatment period. Unrepaired DNA damage in spermatozoa after chemotherapy is a known cause of mutations or chromosomal aberrations, contributing to progeny outcome defects. Thus, we cannot exclude that the genetic integrity of spermatozoa exposed to BEP may have been compromised.

In the current study, we addressed whether the BEP treatment for testicular cancer had a prolonged negative impact on fertility and the outcome of progeny sired after paternal exposure. We showed that BEP treatment for 9 weeks caused a significant increase in the incidence of both preimplantation and postimplantation loss when rats were mated with untreated females at the end of the treatment. Interestingly, in the reversal study, BEP treatment resulted in an increase in preimplantation loss that persisted up to 9 weeks after completion of treatment.
This preimplantation loss may represent unfertilized, ovulated oocytesor the death of early embryos prior to implantation. According to the timing of spermatogenesis in rats, the abnormal progeny parameters observed at the end of the 9-week BEP treatment may represent the effects of BEP treatment on spermatozoa that were first exposed as spermatogonia and throughout their complete spermatogenic development. The persistence of preimplantation loss at 3 and 6 weeks after completion of BEP treatment may in turn reflect the consequences of BEP treatment on germ cells that

were first exposed as spermatogonia and up to the premeiotic differentiation but not during the postmeiotic steps or spermiogenesis, as the treatment had stopped by that time. Following 9 weeks of recovery, preimplantation loss remained elevated; the spermatozoa responsible for fertilizing these embryos were exposed to BEP treatment during their spermatogonial differentiation stages.

Understanding the long-term effects of BEP exposure is essential for testicular cancer patients, particularly for those seeking to have children after chemotherapy; the potential risk for abnormal progeny after paternal BEP exposure requires further studies. Based on our results, it is clear that BEP treatment has prolonged adverse effects on progeny outcome in rats. Our observations, if translated to the human clinical situation, emphasize that spermatozoa produced during BEP chemotherapy exposure may increase risk for abnormal progeny outcome.

In conclusion, we demonstrate that paternal exposure to subchronic BEP induces transient defects on spermatogenesis and exerts prolonged effects on preimplantation loss in progeny sired more than 1 spermatogenic cycle after the termination of paternal exposure to BEP.

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Figures and Legends

Figure 1. Effect of BEP (bleomycin, etoposide, and cisplatin) treatment on body weight gain. The body weight gains were recorded at the end of BEP treatment (n = 5–8/group) and after the 9-wk recovery phase (n = 10/group). Bars represent mean ± SEM; * indicates $P < .05$.

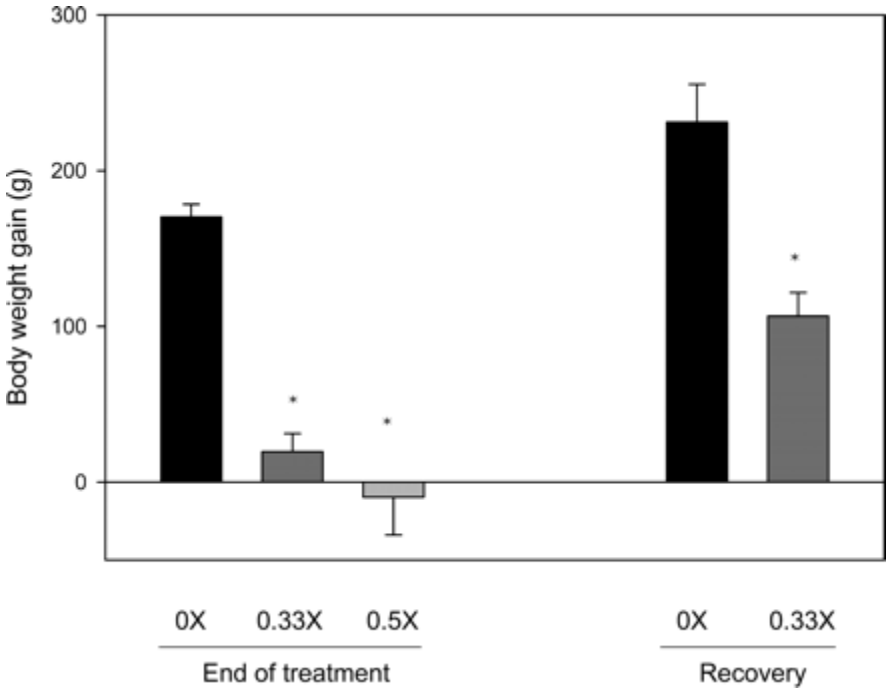

300
200
100
0
Body weight gain (g)
*
*
*
0X
0.33X
0.5X
End of treatment
0X
0.33X
Recovery

Figure 2. Reproductive organ weights. (A) Testis, (B) epididymis, (C) seminal vesicle, and (D) ventral prostate weights in control and BEP (bleomycin, etoposide, and cisplatin)-treated rats at the end of BEP treatment (n = 5–8/group) and after the 9-wk recovery phase (n = 10/group). There was a significant decrease in weights of the testes, epididymides, prostate, and seminal vesicles of the 0.5x BEP-treated animals at the end of treatment and a significant decrease in prostate weight in the 0.33x BEP-treated animals was observed after the 9-wk recovery phase. Bars represent mean ± SEM; * and ** are noted when significant differences of $P < .05$ and $P < .01$), respectively, were observed between the control and the 0.33x BEP group; § indicates a significant difference ($P < .05$) between the values for the 0.33x and 0.5x BEP groups.

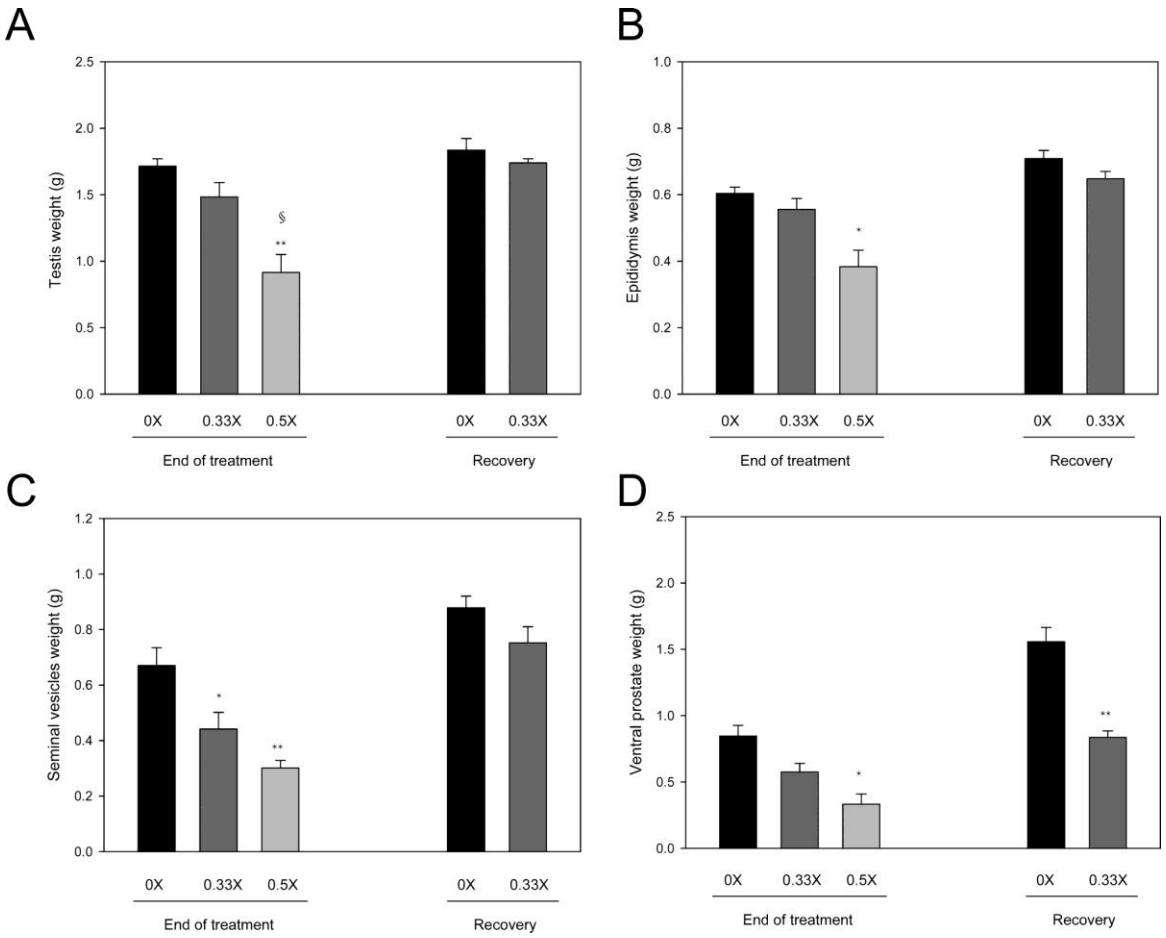

A
Testis weight (g)
2.5
2.0
1.5
1.0
0.5
0.0
§
**
0X
0.33X
0.5X
End of treatment
0X
0.33X
Recovery
B
Epididymis weight (g)
1.0
0.8
0.6
0.4
0.2
0.0
*
0X
0.33X
0.5X
End of treatment
0X
0.33X
Recovery
C
Seminal vesicles weight (g)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
**
0X
0.33X
0.5X
End of treatment
0X
0.33X
Recovery
D
Ventral prostate weight (g)
2.5
2.0
1.5
1.0
0.5
0.0
*
**
0X
0.33X
0.5X
End of treatment
0X
0.33X
Recovery

Figure 3. Histopathological examination of the testis seminiferous epithelium. Photomicrographs of testis sections from control (A, D) and BEP (bleomycin, etoposide, and cisplatin)-treated rats (B–C, E–F) stained with periodic acid Schiff (PAS). (B–C) A substantial proportion of tubules in the testis of 0.33x and 0.5x BEP-treated rats have degenerated seminiferous epithelium and germ cells after 9 wk of treatment. (E–F) Histology of seminiferous tubules from control and 0.33x BEP-treated rats after the recovery phase. (D) The seminiferous epithelium from control testis showed normal spermatogenesis. (E–F) In contrast, the seminiferous epithelium from 0.33xBEP-treated testis presented either normal or abnormal spermatogenesis. (G) Spermatid head counts, (H) Number of degenerated tubules found in testes sections, (I) serum testosterone levels from control and BEP-treated rats at the end of treatment (n = 5–8/group) and after the 9 weeks recovery period (n = 10/group). Scale bar = 50 μm. Bars represent mean ± SEM; * represents a significant difference of $P < .05$ between the control and the 0.33x BEP group; $ indicates a significant difference ($P < .05$) between the values for the 0.33x and 0.5x BEP groups.

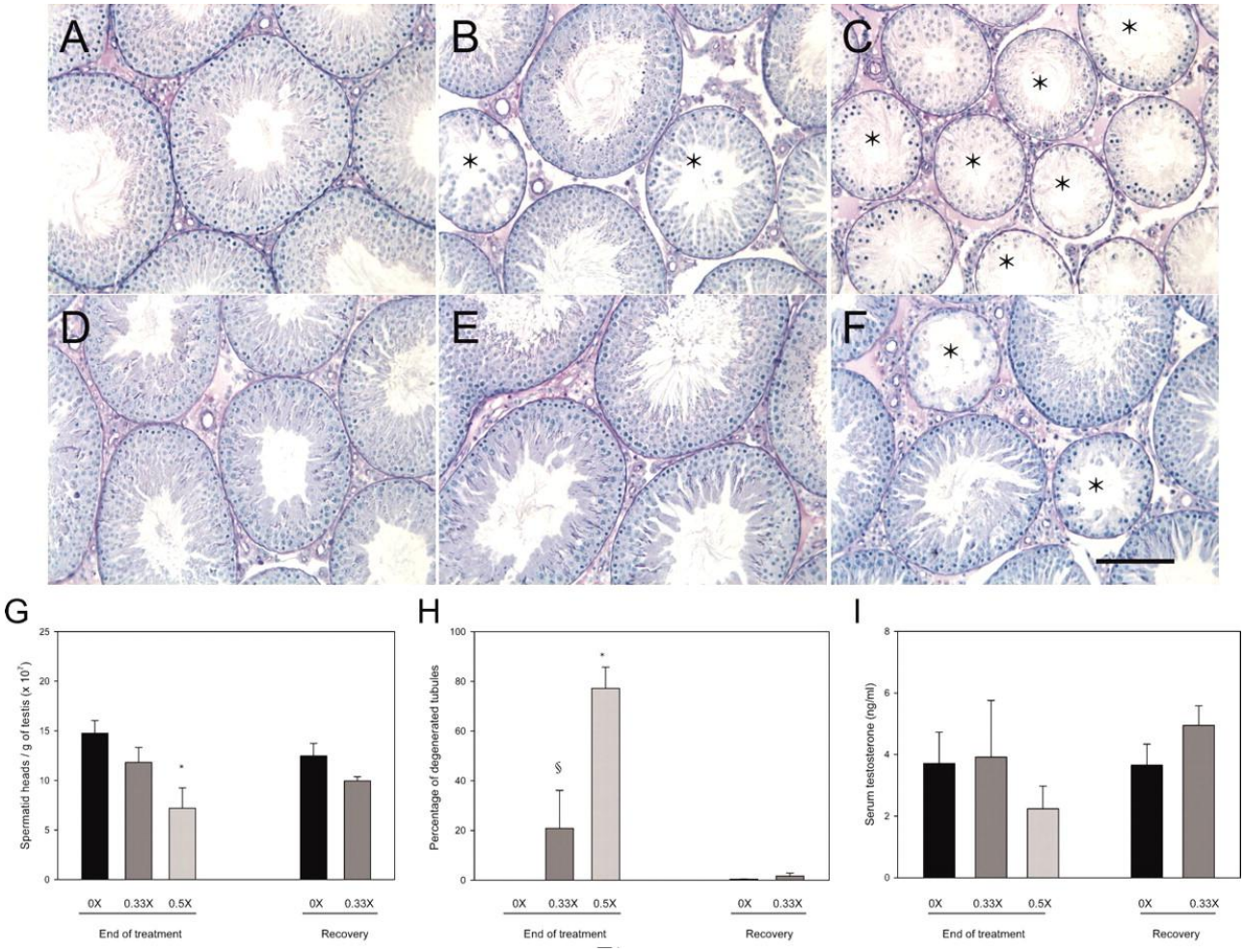

A
B
C
D
E
F
G
Spermatid heads / g of testis (x 10⁶)
0X
0.33X
0.5X
End of treatment
0X
0.33X
Recovery
H
Percentage of degenerated tubules
§
*
0X
0.33X
0.5X
End of treatment
0X
0.33X
Recovery
I
Serum testosterone (ng/ml)
0X
0.33X
0.5X
End of treatment
0X
0.33X
Recovery

Figure 4. In situ detection of apoptotic germ cells in sections of testis from control and BEP (bleomycin, etoposide, and cisplatin)-treated rats. The testis sections were subjected to TUNEL (terminal deoxynucleotidyl transferase-mediated deoxy-UTP nick end labeling) analysis to visualize and count apoptotic cells. (A–D) Photomicrographs of TUNEL-stained testes sections from control (A), 0.33x BEP (B), 0.5x BEP (C)-treated rats, negative control (D). Arrows indicate the positive TUNEL staining for apoptotic cells. (E) Percentage of TUNEL-positive tubules and (F) number of TUNEL-positive germ cells per seminiferous tubules in testis of control and BEP-treated rats at the end of BEP treatment (n = 5–6/group) and after the 9-wk recovery period (n = 8–10/group). More than 200 tubules were scored for each group. Bars represent mean ± SEM; * indicates $P < .05$.

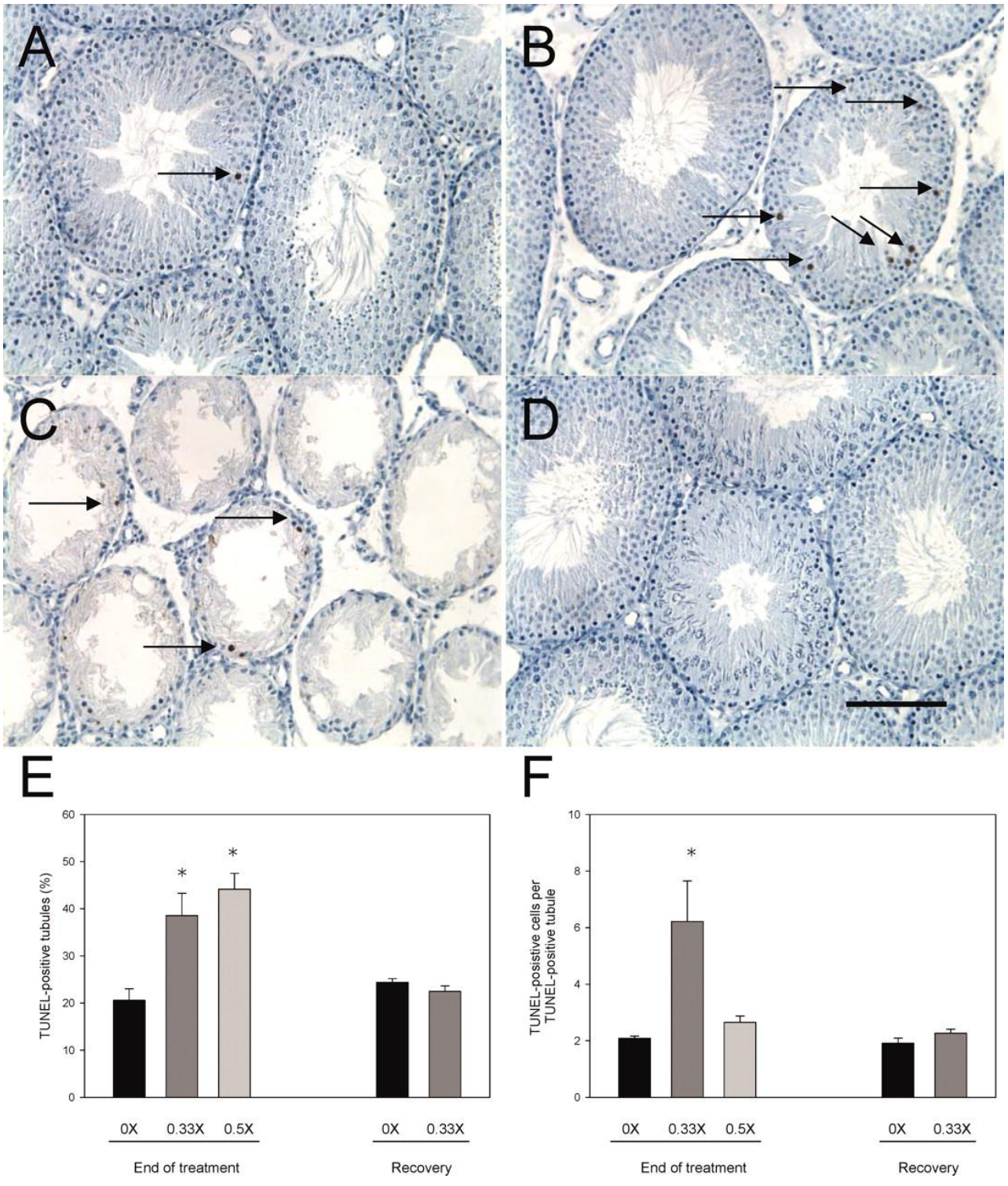
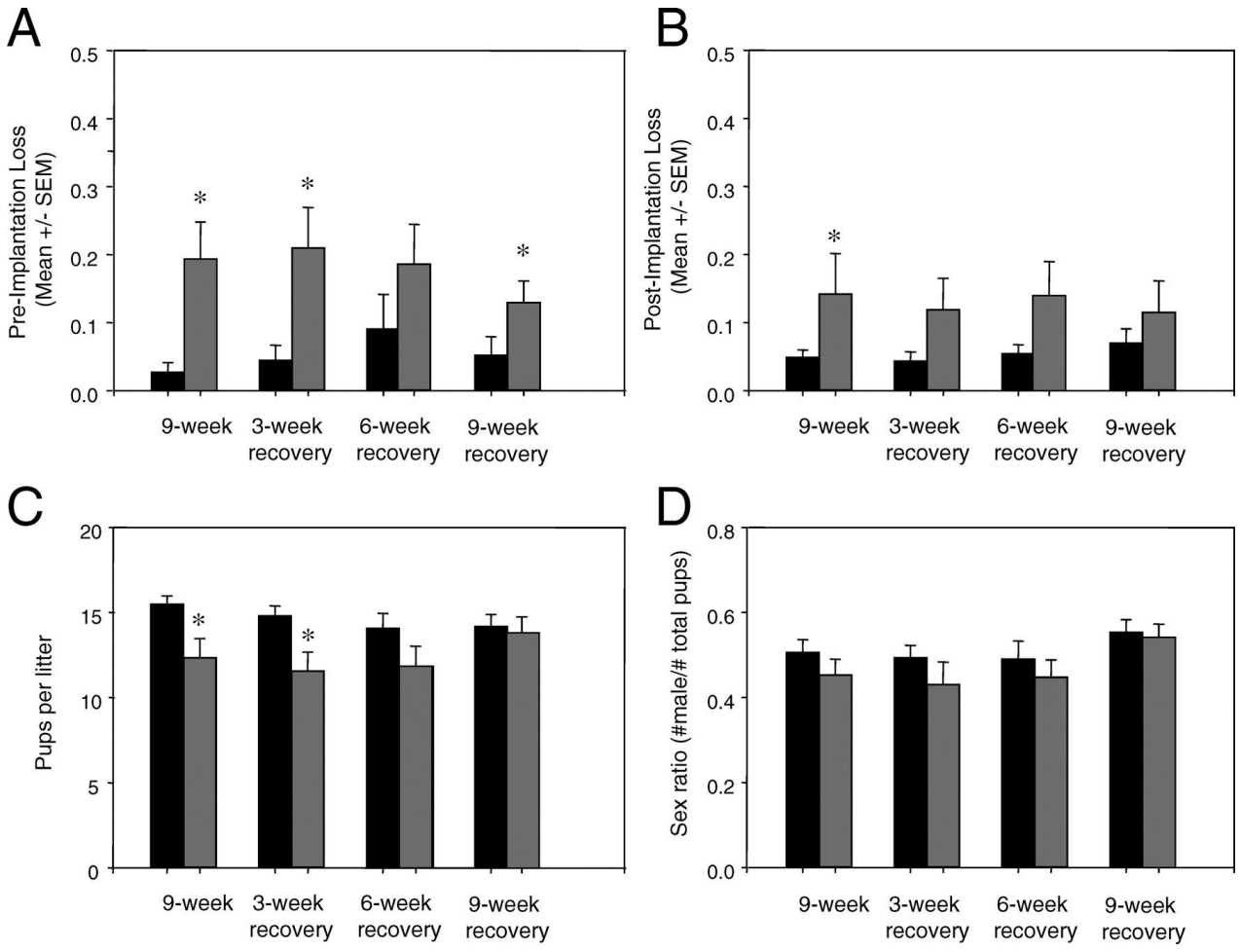

A
B
C
D
E
TUNEL-positive tubules (%)
60
50
40
30
20
10
0
*
*
0X
0.33X
0.5X
0X
0.33X
End of treatment
Recovery
F
TUNEL-posistive cells per TUNEL-positive tubule
10
8
6
4
2
0
*
0X
0.33X
0.5X
0X
0.33X
End of treatment
Recovery

Figure 5. Effects on progeny outcome after paternal treatment with vehicle (black bars) and 0.33x BEP (bleomycin, etoposide, and cisplatin; dark-grey bars) and during the recovery period. Parameters measured were (A) preimplantation loss, (B) postimplantation loss, (C) average number of pups per litter, and (D) sex ratios. Preimplantation loss was determined by calculating the difference between the number of corpora lutea and implantation sites for each female. Postimplantation loss was determined by calculating the difference between the number of implantation sites and the number of live fetuses. Bars represent mean ± SEM; n = 10–15/group; * indicates $P < .05$.


A
Pre-Implantation Loss
(Mean +/- SEM)
0.5
0.4
0.3
0.2
0.1
0.0
*
*
*
9-week
3-week recovery
6-week recovery
9-week recovery
B
Post-Implantation Loss
(Mean +/- SEM)
0.5
0.4
0.3
0.2
0.1
0.0
*
9-week
3-week recovery
6-week recovery
9-week recovery
C
Pups per litter
20
15
10
5
0
*
*
9-week
3-week recovery
6-week recovery
9-week recovery
D
Sex ratio (#male/# total pups)
0.8
0.6
0.4
0.2
0.0
9-week
3-week recovery
6-week recovery
9-week recovery

CONNECTING TEXT

In both chapter 2 and 3, we found that spermatogenesis was severely affected after exposure to the combination of bleomycin, etoposide and cisplatin. Since SSC function is crucial for the recovery of spermatogenesis after such cytotoxic insult, we asked whether SSCs represent potential targets for these anticancer agents. The unequivocal assay of spermatogonial stem cell function is the regeneration of spermatogenesis upon transplantation of SSCs into infertile recipient testis. Thus, spermatogonial transplantation allows for the definitive qualitative and quantitative analysis of SSC function by measuring length and number of donor-derived colonies of spermatogenesis, respectively. Therefore, in chapter 4, we investigated the effects of BEP treatment on SSC function using the spermatogonial transplantation assay. We used a specific transgenic rat line in which the enhanced green fluorescent protein reporter gene is exclusively expressed in the male and female germ cell lineages. Male transgenic rats were administered a cocktail of bleomycin, etoposide and cisplatin or vehicle for 9 consecutive weeks as previously described in chapter 2. Then, testis cell suspensions from control and BEP-treated rats were injected into nude mice recipient testes. We examined the effect of BEP treatment by quantifying the number and length of donor-derived colonies. Thus, a difference in the number or length of spermatogenic colonies may reflect that the BEP treatment directly interferes with SSC function.

Chapter 4

Chapter 4: Effects of Chemotherapeutic Agents for Testicular Cancer on Rat Spermatogonial Stem/Progenitor Cells[1]

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Short title: Effects of BEP on rat SSCs in vivo.

Summary sentence: Chronic exposure to bleomycin, etoposide and cisplatin in combination transiently decreases rat spermatogonial stem cell (SSC) activity.

Key words: Testicular cancer, chemotherapy, bleomycin, etoposide, cisplatin, spermatogenesis, spermatogonial stem cells, transgenic rats, enhanced green fluorescent protein, undifferentiated type A spermatogonia, GFRA1, ZBTB16.

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4.1 Abstract

Spermatogonial stem cells (SSCs) are responsible for the production of spermatozoa throughout adulthood and for the recovery of spermatogenesis following exposure to cytotoxic agents. Previously, we have shown that the combined administration of bleomycin, etoposide and cisplatin (BEP) used in the treatment of testicular cancer causes impaired spermatogenesis and reduced sperm production in the rat. However, definitive evidence about the potential impact of such chemotherapy on SSCs is still lacking. The objective of this study was to determine whether chronic exposure to BEP treatment causes adverse effects on rat SSC activity. We first investigated the effects of BEP treatment on the clonal organization of undifferentiated spermatogonia by staining whole-mount preparations of rat seminiferous tubules for GFRA1 and ZBTB16 (previously known as PLZF), two established markers of undifferentiated spermatogonia. We found that BEP treatment drastically reduced the number of A-aligned spermatogonia while sparing A-single and A-paired cells from the effect. Next, we determined the SSC activity following BEP exposure. Adult transgenic rats carrying EGFP expression in the germ line were treated with BEP for 9 weeks, and SSCs were quantified using spermatogonial transplantation. We found that BEP treatment significantly decreased SSC numbers, which were restored to the control level after a 9-week recovery period. These results demonstrate that BEP treatment transiently affects the activity of rat SSCs.

4.2 Introduction

Over the past 30 years, advances in diagnostics and therapeutics for testicular cancer have resulted in considerable improvement in the outcome of the disease. With 5-year survival rates ranging from 90 to 95%, testicular cancer is now considered a highly curable form of cancer (Feldman et al, 2008; Sabanegh and Ragheb, 2009). Standard chemotherapy regimens for testicular cancer combine the administration of bleomycin, etoposide and cisplatin (BEP) and have proven highly effective in curing testicular cancer even at an advanced stage of the disease. Given the elevated cure rates and the young age at the time of diagnosis, there is concern from a growing number of newly diagnosed testicular cancer patients regarding the potential long-term side effects of BEP treatment on their future fertility (Huddart et al, 2005; Hartmann et al, 1999; Arai et al, 1997).

It is well documented that as a direct consequence of BEP chemotherapy, nearly all testicular cancer patients become oligo- or azoospermic shortly after treatment initiation (Lampe et al, 1997; Chaudhary et al, 2003). However, after the cessation of chemotherapy, recovery of spermatogenesis occurs in about 50% and 80% of patients by 2 and 5 years, respectively (Howell and Shalet, 2005). This variability in the time at which spermatogenesis recovery occurs depends primarily on the dose and duration of the chemotherapy regimen (Petersen et al, 1994). Most importantly, the recovery of spermatogenic function is

dependent on the survival of undamaged spermatogonial stem cells (SSCs) and on their ability to regenerate populations of normal differentiated germ cells within the injured testis environment (Meistrich, 1986). Since survival of SSCs is essential to the regeneration of spermatogenesis after the completion of treatment, these cells are of major importance for the future fertility of testicular cancer survivors.

Spermatogenesis is particularly sensitive to numerous anticancer agents (Meistrich et al, 1982; Meistrich, 1993; Bieber et al, 2006). In animal models, exposure to a single chemotherapeutic agent such as cisplatin or etoposide can induce dose-dependent disruption of spermatogenesis and prolonged azoospermia (Cherry et al, 2004; Sawhney et al, 2005; Kadota et al, 1989). Our recent studies have shown that chronic and sub-chronic BEP treatments resulted in reduced spermatogenic function in rats but failed to abolish spermatogenesis completely (Bieber et al, 2006; Marcon et al, 2008). The effects of BEP treatment on spermatogenesis included markedly altered testicular histology with the elimination of differentiated spermatogonia and the subsequent depletion of meiotic and haploid germ cells, thus resulting in a drastic reduction in sperm count after 9 weeks of treatment. Despite these findings, the impact of BEP treatment on SSCs and their immediate progenitor cells has remained largely unexplored.

In rodents, SSCs constitute a small subset of the most primitive spermatogonia, the undifferentiated spermatogonia (also called

undifferentiated type-A spermatogonia). Due to their self-renewal and differentiation abilities, SSCs are responsible for the maintenance of continual spermatogenesis throughout the adult life of a male individual (Oatley and Brinster, 2006; Oatley and Brinster, 2008. In adult testes, undifferentiated spermatogonia are located on the basement membrane inside the seminiferous tubules. Based on their topographical arrangement and the presence of intercellular bridges, undifferentiated spermatogonia can be subdivided into Asingle (As; isolated spermatogonia with no intercellular bridges), Apaired (Apr; two spermatogonia connected by one intercellular bridge) and Aaligned (Aal; chains of 4, 8, 16 and occasionally 32 spermatogonia connected by intercellular bridges) (De Rooij, 2001). In recent years, molecular markers for undifferentiated spermatogonia such as ZBTB16 (also known as promyelocytic leukemia zinc finger, PLZF), NANOS2, UTF1, GDNF family receptor alpha-1 (GFRA1) and CDH1 (E-cadherin) have been identified and have facilitated greatly 67 the study of this population of germ cells during spermatogenesis (Buaas et al, 2004; Costoya et al, 2004; Hofmann et al, 2005; Grisanti et al, 2009; van Bragt et al, 2008; Tokuda et al, 2007; Sada et al, 2009; Suzuki et al, 2009). In mouse testes, recent studies have demonstrated that GFRA1 expression is restricted to a small population of undifferentiated spermatogonia (Hofmann et al, 2005; Grisanti et al, 2009; Tokuda et al, 2007). In addition, previous studies have shown that ZBTB16, a nuclear transcriptional repressor essential for SSCs self-renewal, was specifically expressed by

undifferentiated spermatogonia in rodent testes (Buaas et al, 2004; Costoya et al, 2004). In rodents, the prevailing model of spermatogonial self-renewal and differentiation defines SSCs as the As spermatogonia. However, in the absence of a specific marker, it is not currently possible to unequivocally identify SSCs among other undifferentiated spermatogonia using immunophenotypic or morphological criteria (De Rooij, 2001). At present, spermatogonial transplantation is the only biological assay for SSCs that allows us to evaluate with certainty the stem cell activity in a quantitative and qualitative manner (Oatley and Brinster, 2006). In this technique, a donor cell suspension containing SSCs is injected into the seminiferous tubules of a recipient testis with depleted endogenous spermatogenesis. SSCs then engraft and resume their function to regenerate colonies of donor-derived spermatogenesis, an activity that defines SSCs (Brinster, 2002).

In this study, we first examined the clonal organization of undifferentiated spermatogonia after chronic BEP treatment in whole-mount preparations of rat seminiferous tubules using immunostaining for GFRA1 and ZBTB16. We then assessed the activity of SSCs after exposure to BEP treatment using spermatogonial transplantation, and subsequently tested whether a nine week recovery period following the completion of BEP treatment allows for the restoration of the SSC population.

4.3 Materials and methods

Animals

The rat transgenic GCS-EGFP strain was previously described in detail (Cronkhite et al, 2005). These rats were produced in the Sprague-Dawley background and express EGFP exclusively in the germ cell lineage. Founder pairs of these rats for our colony were kindly provided by Dr. RE. Hammer, University of Texas Southwestern Medical Center, Dallas, Texas, USA. GCS-EGFP transgenic rats and subsequent progeny were identified by PCR of tail genomic DNA by using the following primers: EGFP-5-2, 5'CTGACCCTGAAGTTCATCTGCACCAC-3'; EGFP-3-2, 5' TCCAGCAGGACCATGTGATC 3'. Wild-type male Sprague-Dawley rats (SD, weighing 350-375g) were purchased from Charles River Laboratories (St. Constant, QC, Canada). Both strains were maintained under controlled conditions of photoperiod (light on, 0700 hr; lights off, 1900 hr), temperature (~22°C), and humidity (~55%) with free access to food and water. Male nude (Ncr nu/nu) mice were purchased from Taconic Farms (Germantown, NY) and used as recipients in transplantation experiments (Ryu, 2005). All animal protocols were in accordance with the guidelines of the Canadian Council for Animal Care and Use and approved by the McGill University Institutional Animal Ethics Committee.

Animal treatments and tissue sample collection

GCS-EGFP rats were randomly divided into 4 groups: 2 control groups and 2 treated groups (3-6 animals per group). Rats in the first

control and treated groups were euthanized immediately at the end of the 9-week BEP treatment, while the remaining rats were euthanized 63 days after the completion of the treatment. As previously described (Bieber et al, 2006), bleomycin was administered by intraperitoneal (i.p) injection once a week while etoposide and cisplatin were given by daily gavage. Bleomycin (1.5 mg/kg; LKT Laboratories, St Paul, MN) was dissolved in sterile saline, etoposide (15 mg/kg; LKT Laboratories) and cisplatin (3 mg/kg; LKT Laboratories) were dissolved in 3:7 DMSO and sterile 0.9% saline. The doses of bleomycin, etoposide and cisplatin used were equivalent to the doses clinically used in human patients after adjusting for body weight and surface area (Bieber et al, 2006). Rats in the control groups received equal volumes of vehicle. No animal died during the entire treatment or recovery periods. Rats were euthanized by CO2 inhalation at scheduled times. Testes, epididymides, seminal vesicles, and ventral prostate were removed and their weights were recorded.

Immunohistochemistry

Rat testes were embedded in Cryo-OCT compound (Fisher Scientific) and frozen in liquid nitrogen. Testis sections (5 μm) were cut using a cryostat (Leica Microsystems Inc., ON, Canada) and mounted on glass slides. Sections were fixed in 100% methanol (-20°C) for 15 min, rinsed with phosphate-buffered saline (PBS) once and incubated in blocking buffer (3% bovine serum albumin (BSA), 0.1% Triton X-100 in PBS pH 7.4) for 20 min at room temperature (RT). Sections were then

incubated overnight at 4°C with a polyclonal goat anti-rat GFRα1 primary antibody (cat. no. AF 560; R&D Systems, Minneapolis, MN) at a 1:100 dilution in the blocking solution. The following day, sections were washed three times 134 with PBS and then incubated with a rabbit anti-goat Alexa Fluor 488 secondary antibody (Invitrogen Molecular Probes, Carlsbad, CA) at a 1:200 dilution for 1h at room temperature. From the step using the secondary antibody onwards, all procedures were done in the dark. Sections were washed three times in PBS, nuclear DNA was counterstained with 4',6-diamidino-2-phenylindole (DAPI, 0.1 µg/ml in PBS), and then were mounted with cover slips using Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA, USA). Images were acquired using a fluorescence microscope (Leica AS LMD) equipped with a RS Photometrics CoolSNAP fx CCD camera (Roper Scientific, Tucson, AZ).

Whole-mount immunofluorescence staining of rat seminiferous tubules

The testes from control (wild-type SD) and BEP-treated rats (n = 3/group) were decapsulated and rinsed in PBS after removal of any visible blood vessels. Seminiferous tubules were gently teased apart in PBS; segments of tubules (~1-3 cm) were dissected and directly fixed in St Mary's fixative (95% ethanol, 2% acetic acid, 3% water). For immunostaining, segments of seminiferous tubules were cut into smaller fragments of 2-3 mm in length, transferred to a 1.5 ml microcentrifuge tube, rehydrated in PBS and incubated for 20 min in blocking buffer.

Tubule fragments were incubated with a primary antibody in blocking buffer overnight at 4°C, washed in PBS, and incubated with a secondary antibody. For double labelling, seminiferous tubules were incubated with 1:50 mouse monoclonal anti-ZBTB16 (OP128, Calbiochem, San Diego, CA, USA) and 1:100 GFRA1 (R&D Systems) in blocking buffer overnight at 4°C. Tubules were washed in PBS three times and incubated with a secondary antibody for 1h at RT. Nuclear DNA was counterstained with DAPI. Tubule pieces were transferred onto microscope glass slides (Superfrost, Fisherbrand, Fisher Scientific Co.), mounted in Vectashield Mounting Medium (Vector Laboratories) and sealed under coverslips. Tubule fragments remained in suspension throughout the staining procedure.

Preparation of Donor Cell Suspensions

Testis cells from control and BEP-treated GCS-EGFP rats were used as donor cells in xenotransplantation experiments. Single-cell suspensions from donor rat testes were collected by a two-step enzymatic digestion, as previously described (Bellvé et al, 1977) with modifications (Aguilar-Mahecha et al, 2001). Briefly, both rat testes were dissected, rinsed in sterile phosphate-buffered saline (PBS), then decapsulated to remove the tunica albuginea and placed in Dulbecco's modified Eagle's medium (DMEM, Gibco, Invitrogen, Burlington, ON, Canada) containing 0.5 mg/ml collagenase Type IA (C9891; Sigma Chemicals Co, St. Louis, MO) and incubated at 34°C in a shaking water bath for 15 min. Tubules

were allowed to sediment and were washed three times with DMEM to remove interstitial cells. Tubules were then incubated in DMEM containing 0.5 mg/ml trypsin (type I; T8003; Sigma) and 1 μg/ml DNase I (DN-25; Sigma) under the same conditions as above. Fragments of seminiferous tubules (2-3 mm in length) were then mechanically dissociated into a single cell suspension using a flamed-tip glass Pasteur pipette. The dispersed cells were washed twice with DMEM, resuspended in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS) and subsequently filtered through 40-μm nylon mesh (BD Biosciences, San Jose, CA). Cell viability was determined by trypan blue exclusion, and cell counts were done using a hemacytometer.

Recipient Preparation and Transplantation Procedure

Male nude mice were injected intraperitoneally with a single 180 dose of busulfan (40 mg/kg body weight; Sigma) at 4–6 weeks of age to deplete endogenous germ cells, as described previously (Ryu et al, 2005). Approximately 6 μl of donor testis cell suspension was injected per testis through the rete testes at the time of transplantation at least 5 weeks after busulfan treatment. Three to four recipient mice were used per donor and time point. Recipient mice were euthanized no less than 12 weeks after SSC transplantation. Recipient testes were excised, their tunica albuginea removed and placed in PBS for analysis of EGFP+ colonies. For every testis, the number of donor-derived EGFP+ colonies of spermatogenesis was counted using an epifluorescence stereomicroscope (Leica MZ10 F);

colony numbers reflect the number of SSCs (Oatley and Brinster, 2008; Brinster, 2002). Each individual segment of recipient seminiferous tubule containing an EGFP+ colony was carefully separated from other tubules, fixed with 10% neutral buffered formalin and kept in the dark until further analysis. Images of individual EGFP+ colonies were acquired using a fluorescence microscope (Leica AS LMD) and then analyzed for colony length and fluorescence using ImageJ (NIH, National Institutes of Health).

Immunochemistry of donor-derived colonies

Segments of recipient seminiferous tubules containing an EGFP+ colony were fixed with 10% neutral buffered formalin, dehydrated and embedded in paraffin. Sections (5 μm) were incubated with anti-GFP antibody (Millipore, Billerica, MA) at 1:100 dilution overnight at 4°C. After washing three times in PBS, sections were incubated with a secondary antibody for one hour in the dark, then counterstained with DAPI and mounted with Vectashield. For negative controls, the primary antibody was omitted.

Statistical analysis

All results are expressed as mean ± s.e.m. Intergroup analyses were done with unpaired Student's *t*-test using SPSS (SPSS, Chicago, IL). $P \leq 0.05$ was interpreted as statistically significant.

4.4 Results

Body and testicular weights

The mean body weights following BEP treatment with and without a recovery period were not significantly different from control values (Supplemental Fig. 1A), suggesting no overt systemic toxicity of the BEP treatment. However, after 9 weeks of chronic treatment, the weights of BEP-treated testes were significantly decreased to about 60% of the testis weight in control rats (Supplemental Fig. 1B). Following 9 weeks of recovery, testicular weight had fully recovered to control values. These results are consistent with our previous studies (Bieber et al, 2006; Marcon et al, 2008) in which a reduction in testicular weight was concomitant with germ cell depletion and lowered sperm counts, both indicating that BEP treatment severely impaired spermatogenesis.

Immunostaining of undifferentiated spermatogonia in testes of control and BEP-treated rats

To investigate the impact of BEP treatment on the population of undifferentiated

spermatogonia, we first determined whether GFRA1, a marker of mouse stem/progenitor spermatogonia (i.e., As, Apr and Aal spermatogonia), was expressed in the testes of control adult rats. A previous study (Fouchécourt et al, 2006) reported that GFRA1 transcripts and protein expression were detected in pachytene and round spermatocytes and in Sertoli cells within the rat testis, suggesting that GFRA1 expression might differ between mouse and rat. However, our analysis using immunofluorescence staining conducted on adult rat testes revealed that

GFRA1 labelled exclusively germ cells located at the basement membrane of seminiferous tubules (Fig. 1 a-b). These cells were sparsely distributed at the periphery of the tubules as individual cells or clusters of cells. In addition, GFRA1 was immunolocalized along cell membranes of positive cells (Fig. 1 c-d), a staining pattern previously described in mouse undifferentiated spermatogonia (Grisanti et al, 2009; Tokuda et al, 2007). In BEP-treated testes, spermatogenesis was impaired; nonetheless, GFRA1+ cells were still detected in tubules of BEP-treated rats (Fig.1 e-f). As in control testis sections, cells positive for GFRA1 were located on the basement membrane of treated seminiferous tubules. These results show that GFRA1-positive spermatogonia were detected in both control and BEP-treated rat testis sections, indicating that BEP treatment did not completely eliminate GFRA1-positive spermatogonia.

Whole-mount immunofluorescence of rat seminiferous tubules

To examine the impact of BEP treatment on the clonal organization of undifferentiated spermatogonia, immunofluorescence staining of whole-mount preparations of rat seminiferous tubules was conducted to detect GFRA1-expressing spermatogonia. In testis cross sections, it is not possible to establish whether a GFRA1+ cell is single or part of a chain. However, in whole mounts, the topographical arrangement of undifferentiated spermatogonia can be determined based on their clone size through the presence or absence of intercellular cytoplasmic bridges (i.e., As, Apr, and Aal) (Grisanti et al, 2009; Tokuda et al, 2007). As

illustrated in Fig. 2a and b, GFRA1+ spermatogonia from control rat tubules were observed as single, paired or chains of aligned cells, in a pattern characteristic of undifferentiated type A spermatogonia in which SSCs reside (De Rooij, 2001; Tokuda et al, 2007). This result indicates that GFRA1-expressing cells can be defined as undifferentiated spermatogonia in the rat. Interestingly, some single and paired cells extended long and fine cytoplasmic processes whereas others possessed only small cytoplasmic spikes (Fig. 2b). In contrast, GFRA1+ cells that comprise chains of 4, 8 or 16 cells were usually connected through a broader surface of cell membrane. A majority of GFRA1+ spermatogonia were single, paired and aligned 4 and 8 cells while chains of 16-32 interconnected cells were observed less frequently. Moreover, whole-mount immunostaining using an antibody to ZBTB16, a known marker for undifferentiated spermatogonia, revealed that ZBTB16 was exclusively expressed in a subset of cells having characteristics of As, Apr and Aal spermatogonia, indicating that ZBTB16 was also expressed in rat undifferentiated spermatogonia (Fig. 2 c, d).

To further confirm that GFRA1 protein expression was restricted to undifferentiated spermatogonia in the rat, we undertook the double labelling of whole-mounted seminiferous tubules using both GFRA1 and ZBTB16 antibodies. As shown in Fig.2 e, f and Suppl. Fig. 2 g-i, GFRA1/ ZBTB16 double labelling demonstrated that almost all undifferentiated spermatogonia including As, Apr and Aal expressed both proteins in the

adult rat testis. We found that >99% of GFRα1-positive As to Aal spermatogonia co-expressed ZBTB16 and that only few ZBTB16+ cells, mostly As, did not express GFRA1 (Fig.2 e, f, arrow). In addition, GFRA1 and ZBTB16 were also detected in the same population of germ cells in whole-mount seminiferous tubules from postnatal day 7 (PND7) pup testes (Suppl. Fig.2 a-c). Since at this stage of postnatal testis development, the only germ cells present in the seminiferous epithelium are undifferentiated spermatogonia (Malkov et al, 1998), this result further validates the conclusion that both GFRA1 and ZBTB16 are markers of undifferentiated type A spermatogonia in the rat testis. At PND28, we also observed that As, Apr and Aal clones of undifferentiated spermatogonia were positive for both GFRA1 and ZBTB16 (Suppl. Fig.2 d-f). Using whole-mount preparations, we then counted the number of GFRA1+ / ZBTB16+ cells according to their clonal arrangement.

Through the quantification of ZBTB16+ spermatogonial clones we found that the

proportion of Aal-4, 8 and 16 clones was dramatically reduced in tubules from rats exposed to chronic BEP treatment compared to control animals (Fig. 3 a, b), indicating that these types of spermatogonial clones were highly sensitive to treatment. In contrast, the percentage of As and Apr clones was increased in tubules of BEP-treated testes (Fig. 3c). Thus, GFRA1+ undifferentiated spermatogonia survived mainly as As and Apr clones.

BEP-treated testes were smaller and their weight was significantly decreased when compared to controls. To account for the shrinkage of seminiferous tubules, ZBTB16+ cells were counted in BEP-treated tubules according to the tubule surface area. We found that the number of ZBTB16-expressing spermatogonia per surface area of tubule was drastically reduced following BEP treatment, indicating the loss of undifferentiated spermatogonia (Suppl. Fig.3).

Reversibility of the BEP effects on SSC activity

To evaluate the effects of BEP treatment on the number and activity of SSCs, we transplanted testicular germ cells from control and BEP-treated GCS-EGFP rats into recipient nude mouse testes. Three months after transplantation, colonies of donor-derived EGFP+ spermatogenesis were clearly recognizable in recipient testes under a fluorescence stereomicroscope, demonstrating that SSCs from rat donor-cell suspensions had repopulated recipient seminiferous tubules (Fig. 4 a, b). The quantification of donor-derived EGFP+ colonies demonstrated that a 9-week BEP treatment significantly decreased the number of SSCs capable of generating colonies to less than half of the control level (n = 38-39 testes, $P \leq 0.05$) (Fig. 4c, left panel). To investigate whether SSC numbers recovered after the treatment was terminated, we transplanted donor testis cells from control and BEP-treated rats after a 9-week recovery period. The numbers of rat EGFP+ colonies were similar between control and BEP-treated rats (Fig. 4 c, right panel; n = 20-21

testes, P = 0.356). These data demonstrate that the 9-week BEP treatment significantly depleted SSCs capable of initiating spermatogenesis and that the SSC population size had recovered by 9 weeks after the completion of the treatment.

The length of colonies has been used as an indication of the SSC potential to reconstitute spermatogenesis (proliferation and production of differentiated cells) (Nagano et al, 1999). We measured this parameter to examine whether the 9-week BEP treatment affected the regeneration capacity of SSCs (Fig. 5a). As seen in Fig. 5a, the analysis of colonies obtained 3 months after transplantation revealed that BEP-treated SSCs generated colonies that were significantly shorter than those of the control group. The average colony length was 5.47 ± 0.63 mm (range: 1.05 to 15.92 mm) in the control group (n = 154 colonies, 12.83 colonies/testis on average) whereas the average length of colonies derived from BEP treated rats after 9 weeks of treatment was 3.61 ± 0.34 mm, ranging from 0.75 to 13.8 mm (n = 166, 11.06 colonies/testis on average). Therefore, the regeneration ability of SSCs was significantly affected by the BEP treatment. Importantly, the average colony length was similar in both groups when analyzed after 9 weeks of recovery, indicating normal activity was restored in BEP-treated SSCs. Furthermore, histological analyses showed that rat spermatogenesis progressed normally to the elongated spermatids/spermatozoa stage in the recipient testes after transplantation at the completion of treatment (Fig. 5 b, c). These results indicate that the

SSCs that survived the treatment retained the ability to regenerate complete spermatogenesis.

4.5 Discussion

Chemotherapeutic treatments can cause severe adverse effects on the fertility of male cancer patients, leading to temporary or permanent azoospermia. Several past studies have documented such adverse effects on male germ cells and spermatogenesis in rodent models (Meistrich et al, 1982; Trasler et al, 1987; Bieber et al, 2006); however, only a few studies have investigated the potential impact of an anticancer drug (busulfan) on the regenerative capacity of SSCs (Kanatsu-Shinohara et al, 2003; Hermann et al, 2007). Here, we evaluated the effects of chronic co-administration of bleomycin, etoposide and cisplatin (BEP) on rat SSC activity. Consistent with our previous studies, administration of BEP to adult GCS-EGFP transgenic rats resulted in the anticipated decrease in testis weight and abnormal testis histology due to depletion of germ cell populations (Bieber et al, 2006). However, spermatogenesis was not completely abolished following BEP treatment, suggesting that SSCs survived. We then showed that BEP treatment disrupted the clonal organization of GFRA1+ and ZBTB16+ undifferentiated spermatogonia in whole-mounts of seminiferous tubules and that the overall number of undifferentiated spermatogonia was drastically reduced. Finally, using spermatogonial transplantation, we found that BEP treatment significantly

reduced SSC numbers and the length of spermatogenic colonies. However, SSCs activity was restored after a 9 week recovery period.

In adult rodent testes, SSCs are a subpopulation of undifferentiated type-A spermatogonia, that consists of As, Apr and Aal. Several markers of these cells have been described in the mouse (Buaas et al, 2004; Costoya et al, 2004; Hofmann et al, 2005; Grisanti et al, 2009; van Bragt et al, 2008; Tokuda et al, 2007; Sada et al, 2009; Suzuki et al, 2009). However, there is limited information about the specificity of these markers for rat spermatogonia and whether spermatogonia expressing these markers are affected following cytotoxic perturbations. Using whole-mount immunofluorescence staining of seminiferous tubules, we showed that both ZBTB16 and GFRA1 are expressed in undifferentiated type-A spermatogonia, including As, Apr and chains of Aal 4-16 in the adult rat testis, confirming the conservation of these markers among rats, mice and non-human primates (Hermann et al, 2009). In particular, ZBTB16 was expressed by most undifferentiated spermatogonia in both mice and rats. However, a difference was noted in the expression pattern of GFRA1 between mice and rats. In mice, expression of GFRA1 is known to be restricted predominantly to As and Apr spermatogonia (Grisanti et al, 2009; Tokuda et al, 2007; Nakagawa et al, 2010). In contrast, in the rat, GFRA1 is expressed broadly in all subtypes of undifferentiated spermatogonial clones. Interestingly, we observed that

a small fraction of ZBTB16+ spermatogonia, predominantly As, did not express GFRA1, which indicates that rat undifferentiated spermatogonia are a heterogeneous cell population. These results suggest that different subpopulations of As exist in the rat, as in the mouse model (Nakagawa et al, 2010).

BEP treatment drastically reduced the number of ZBTB16-expressing spermatogonia per surface area of tubule, demonstrating the overall loss of undifferentiated spermatogonia.Interestingly, the treatment severely depleted the more advanced clones of undifferentiated spermatogonia (e.g. Aal spermatogonia) and more than 90% of undifferentiated spermatogonia that survived the treatment were As and Apr. These results suggest that As and Apr spermatogonia were more resistant to BEP treatment. However, we cannot exclude the possibility that some of the As or Apr spermatogonia observed after treatment result from the fractionation of Aal clones.

Such fragmentation of spermatogonial clones was previously observed using live imaging during steady-state spermatogenesis and following busulfan exposure in mice (Yoshida et al, 2007; Nakagawa et al, 2010).

Previous studies on the recovery of spermatogenesis after exposure to cytotoxic agents were generally based on sperm count and histological evaluation of the seminiferous epithelium (Meistrich et al, 1982; Meistrich, 1993, Marcon et al, 2008). In this study, we focused on SSCs and determined the effect of BEP on this cell population using

spermatogonial transplantation. Our results demonstrate that BEP treatment significantly diminished SSC numbers and the length of donor-derived colonies, suggesting that the BEP-induced reduction of SSC activity was both quantitative and qualitative. Nonetheless, it is notable that approximately 46% of SSCs survived BEP treatment; this is far higher than the survival rate of SSCs in busulfan-treated mice as Kanatsu-Shinohara et al. (2003) have reported that a single injection of busulfan at 15 mg/kg depleted over 95% of SSCs within 3 days. Since we used a BEP dose comparable to that commonly used in clinical settings, our results suggest that this therapeutic regimen allows a significant proportion of SSCs to survive, leading to a high percentage of patients who spontaneously regain fertility. This is consistent with our observation that SSC numbers were similar between BEP-treated and control donors after a 9 week recovery period.

It is unclear why SSCs produced only a limited length of colonies when transplanted at the end of the BEP treatment. Since complete spermatogenesis was established upon transplantation of these cells, surviving SSCs must have retained the regeneration capacity. This assumption is supported by our observation that SSCs of BEP-treated donors produced a similar length of colonies after 9 weeks of recovery as those of control donors. Therefore, it is conceivable that the onset of regeneration by BEP-exposed SSCs may have been delayed. These SSCs experienced BEP exposure and the mechanical stress that is

inherent to spermatogonial transplantation; thus, a period of time may have been necessary for SSCs to recover from such stresses and to initiate regeneration of spermatogenesis. Further studies are necessary to address this issue.

In conclusion, we have demonstrated that exposure to BEP chemotherapy reduced both the size and functional capacity of the rat SSC population; however, these effects are transient as complete recovery appears to occur after termination of treatment. Recent advances in the techniques of cryopreservation and transplantation of spermatogonial stem cells developed in rodent models suggest that, in the future, the therapeutic use of human SSCs will help restore fertility in male cancer patients. Therefore, investigating the effects of chemotherapy regimen on SSC activity may eventually help to characterize the underlying mechanisms controlling SSC survival and provide new insight in the processes leading to recovery of spermatogenesis in cancer patients following chemotherapy.

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Figures and legends

Figure 1. Immunofluorescence localization of GFRA1 expression in adult control and BEP treated rat testis cryosections. Testis cross-sections were stained with anti-GFRA1 antibody (green), and DAPI (blue nuclei). (a, b) Representative images showing localization of GFRA1- positive spermatogonia at the base of seminiferous epithelium in contact with the basal membrane; GFRA1-positive cells represent only a small subset of germ cells (arrows indicate GFRA1+ cells). Individual as well as clusters of GFRA1-positive cells were observed. Note that no staining was observed in the testicular interstitium or in meiotic or post-meiotic cells. (c, d) Higher magnification of GFRA1 staining showing cell surface localization of GFRA1 on undifferentiated spermatogonia in control testis. (e, f) Localization of GFRA1 staining in a BEP treated rat testis section. Although the more advanced differentiated germ cells are depleted, GFRA1-positive spermatogonia are still detected in the seminiferous tubules after BEP treatment (n=3 testes examined). Bars: 50μm in (a, b, e and f); 25 μm in (c and d).

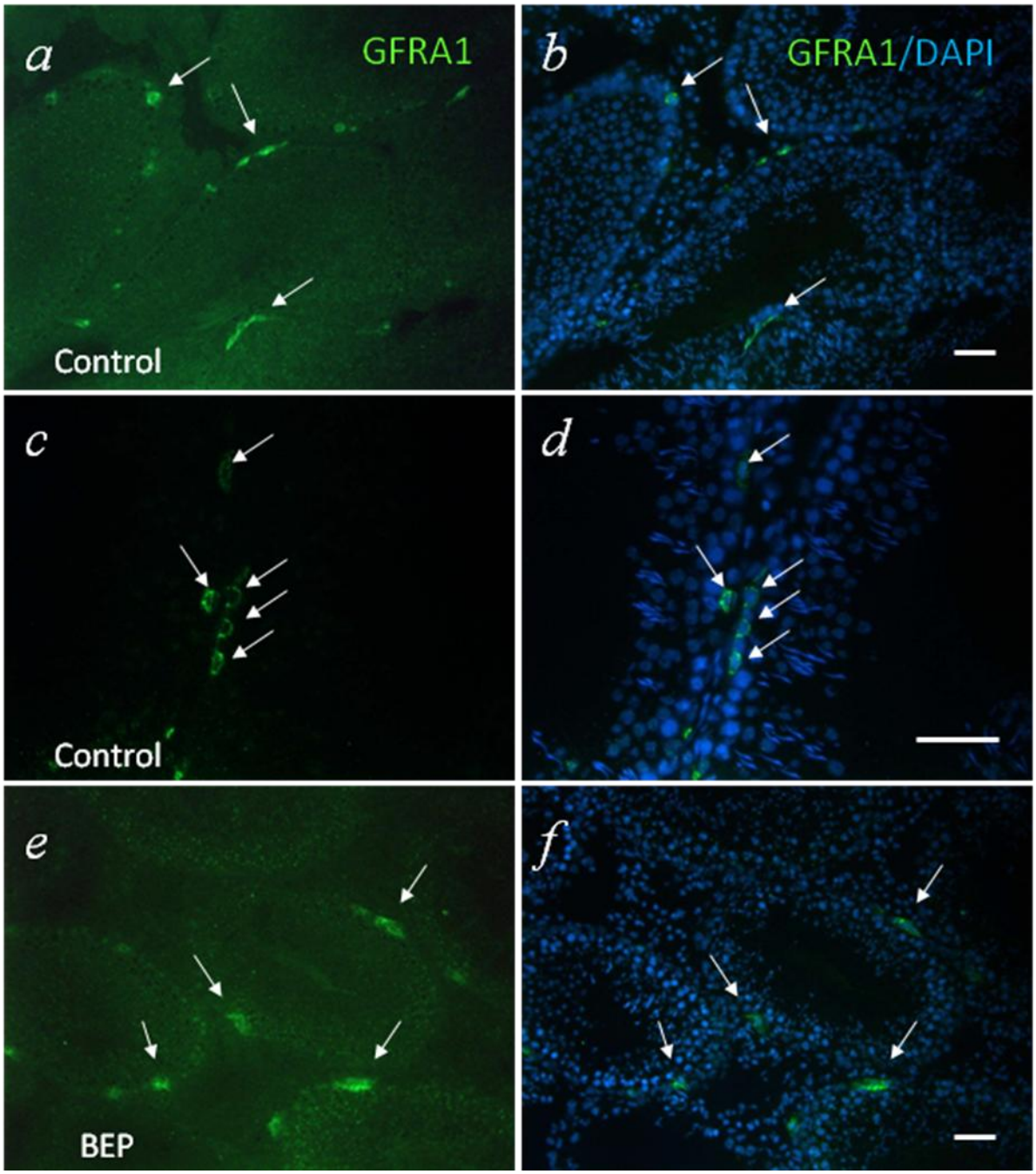

a
GFRA1
b
GFRA1/DAPI
Control
c
d
Control
e
f
BEP

Figure 2. Topographical arrangement of GRFA1- and ZBTB16-positive undifferentiated spermatogonia in whole mounts of rat seminiferous tubules. GFRA1 staining delineates the cellular boundaries of undifferentiated spermatogonia (intercellular bridges), distinguishing clones of As, Apr and Aal. ZBTB16 labels nuclei. (a, b) Representative images of whole-mount GFRA1 staining of rat seminiferous tubules at low and high magnifications. (c, d) Representative images of rat seminiferous tubules stained with anti-ZBTB16 antibody at low and high magnifications. ZBTB16 staining reveals cell arrangements that are similar to those seen with GFRA1 staining. (e, f) ZBTB16 (red) and GFRA1 (green) are co-expressed in the same population of undifferentiated spermatogonia in adult rat testes. ZBTB16 is expressed in the nuclei of undifferentiated spermatogonia while GFRA1 is expressed 527 at the cytoplasmic membrane. The arrow indicates a ZBTB16-positive/GFRA1-negative cell. Bars: 50μm in (a, c); 25 μm in (b, d, e and f).

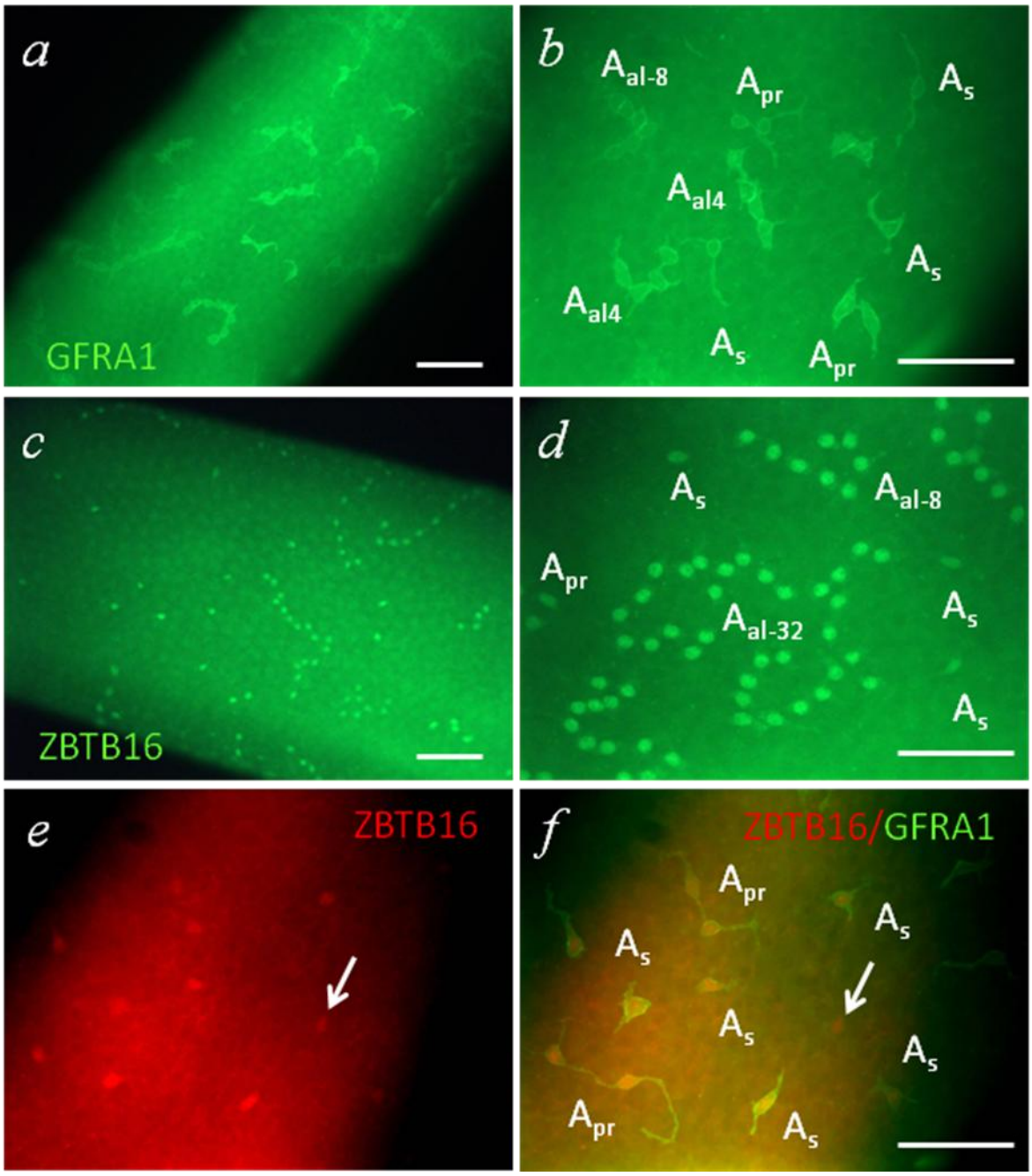

a
GFRA1
b
A al-8
A pr
A s
A al4
A s
A al4
A s
A pr
c
ZBTB16
d
A s
A al-8
A pr
A al-32
A s
A s
e
ZBTB16
f
ZBTB16/GFRA1
A pr
A s
A s
A s
A s
A pr
A s

Figure 3. BEP treatment affects the clonal organization of undifferentiated spermatogonia. (a, b) Representative images of whole-mount tubules stained with anti-ZBTB16 antibody from control (a) and BEP-treated testis (b). (c) Quantitative analysis of ZBTB16-positive As, Apr and Aal spermatogonial populations. Bar: 50μm in (a, b).

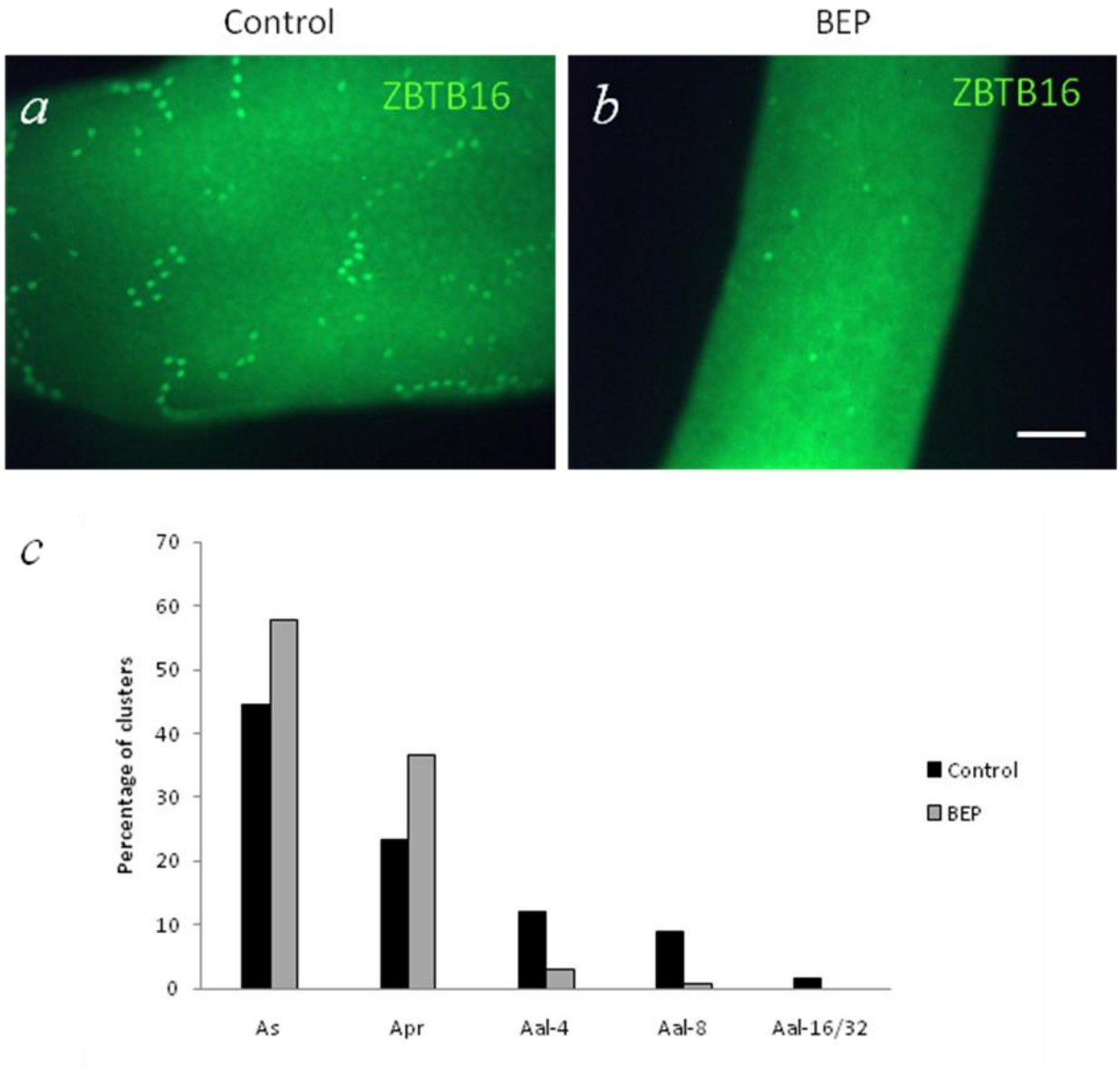
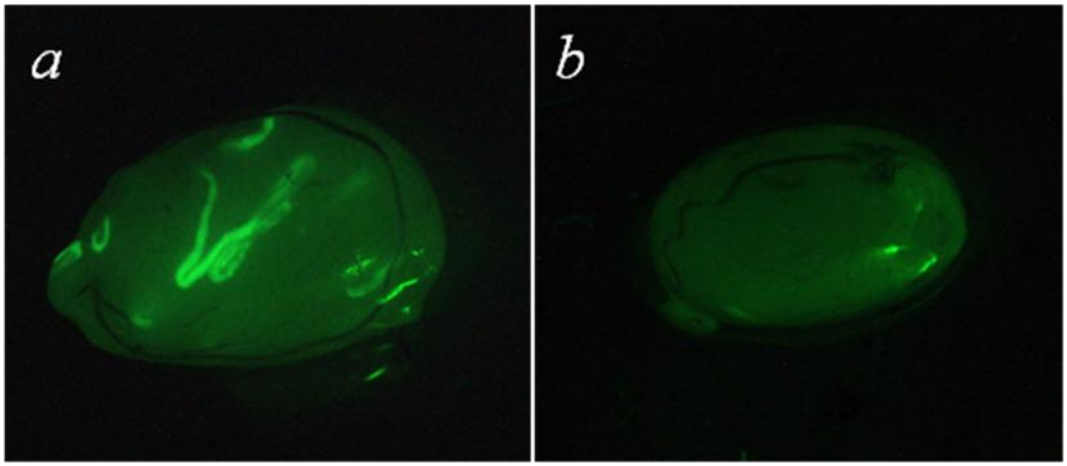

Control
BEP
a
ZBTB16
b
ZBTB16
c
Percentage of clusters
70
60
50
40
30
20
10
0
As
Apr
Aal-4
Aal-8
Aal-16/32
Control
BEP

Figure 4. BEP treatment decreases rat SSC activity. (a, b) Representative images of recipient nude mouse testes transplanted with rat EGFP+ testicular germ cells: (a) control, (b) BEP-treated. (c) SSC quantification using spermatogonial transplantation after BEP treatment. The number of donor-derived EGFP-positive colonies is indicated per of 106 donor testis cells BEP treatment significantly decreased SSC numbers. No significant difference was observed between the two groups after a 9-week recovery period. An asterisk indicates a significant difference ($P \leq 0.05$).


a
b

c

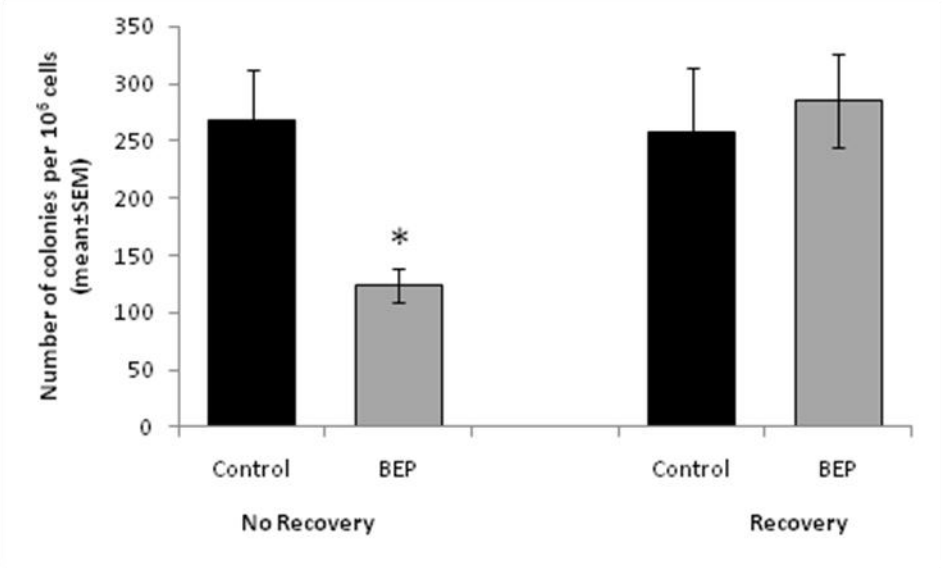
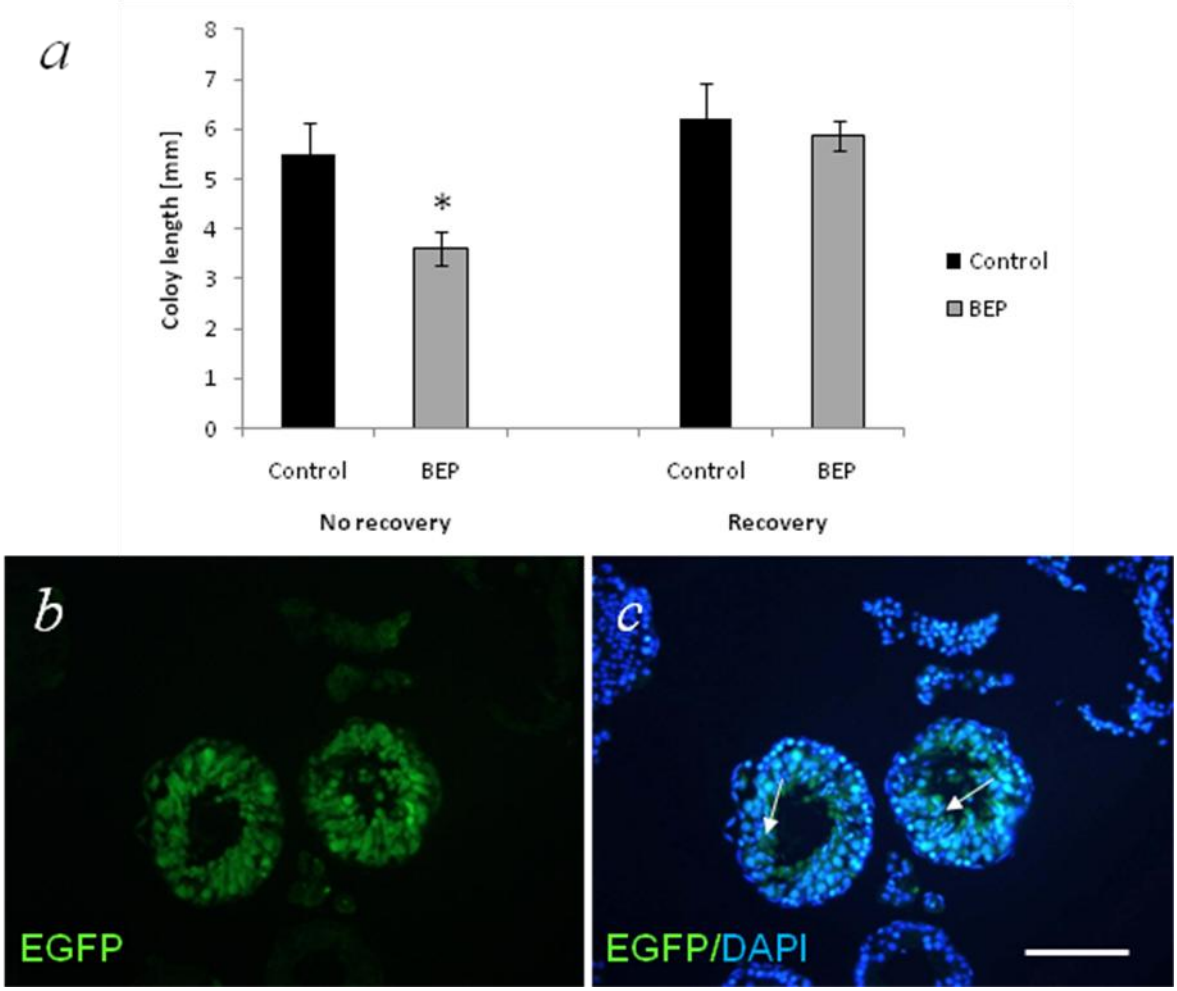
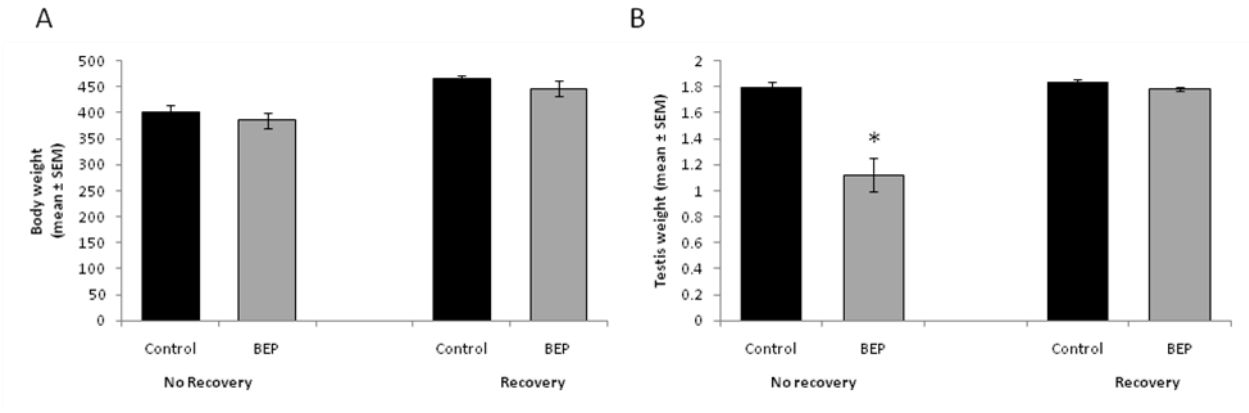

Number of colonies per 10^5 cells (mean±SEM)
350
300
250
200
150
100
50
0
*
Control
BEP
Control
BEP
No Recovery
Recovery

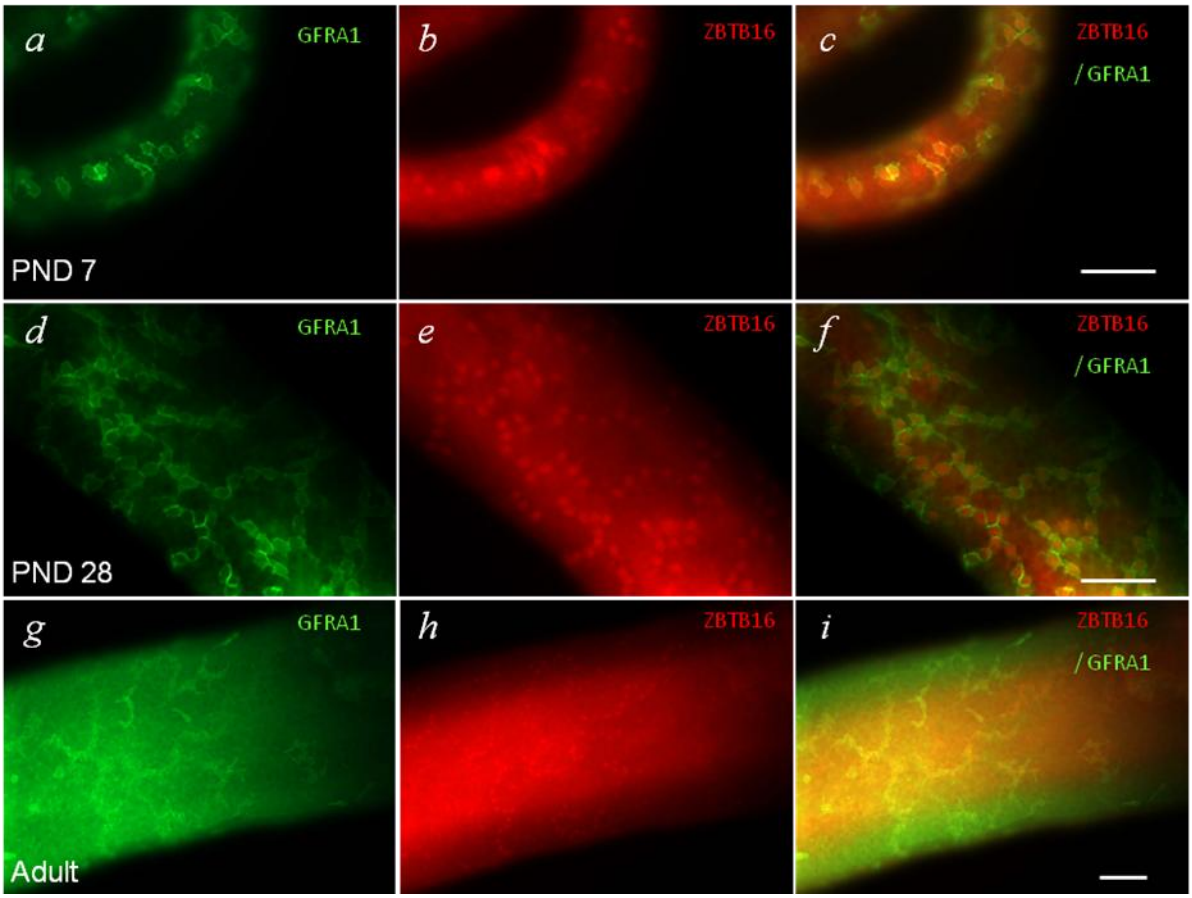
Figure 5. The length of colonies arising from control and BEP-treated rat EGFP+ SSCs after transplantation. (a) Average colony length of control and BEP-treated groups: (left) at the time of treatment completion, (right) after recovery. (b) Histology of GFP-positive colonies arising from BEP-treated SSCs at the end of the treatment. (c) The same section as in b but counterstained with DAPI (blue). The heads of elongated spermatids are seen (arrows). Bar: 50μm.


a
Coloy length [mm]
8
7
6
5
4
3
2
1
0
*
Control
BEP
Control
BEP
No recovery
Recovery
Control
BEP
b
EGFP
c
EGFP/DAPI

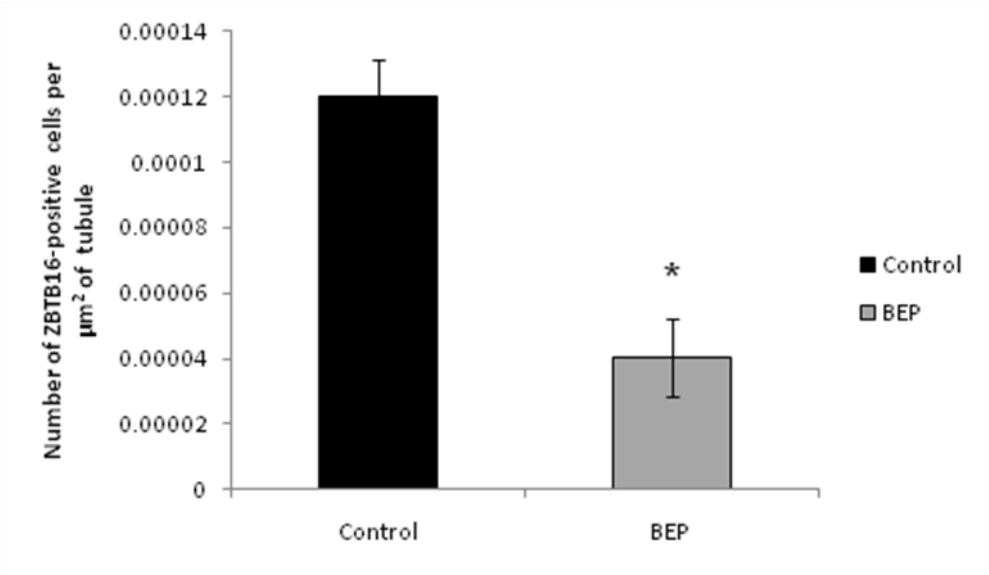
Supplemental Figure 1. Effects of BEP treatment on body and testis weights. (A) Body weights of control and BEP-treated rats after 9 weeks of exposure (no recovery, n = 6) and following a 9 week recovery period (n=3). (B) Testis weights of corresponding animals. Testis weights in BEP-treated rats were significantly decreased compared to the 549 control group. An asterisk indicates a significant difference.


A
Body weight (mean ± SEM)
500
450
400
350
300
250
200
150
100
50
0
Control
BEP
Control
BEP
No Recovery
Recovery
B
Testis weight (mean ± SEM)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
Control
BEP
Control
BEP
No recovery
Recovery

Supplemental Figure 2. Expression of GFRA1 and ZBTB16 in the developing and adult rat testis. Co-immunostaining of whole mounts of seminiferous tubules with anti-GFRA1 (green) and anti-ZBTB16 (red) antibodies at PND7 (a-c), PND28 (d-f) and in the adult (g-i). Bars: 50μm in (g-i); 25 μm in (a- f).


a
GFRA1
b
ZBTB16
c
ZBTB16
/ GFRA1
PND 7
d
GFRA1
e
ZBTB16
f
ZBTB16
/ GFRA1
PND 28
g
GFRA1
h
ZBTB16
i
ZBTB16
/ GFRA1
Adult

Supplemental Figure 3. Quantification of ZBTB16-positive spermatogonia per surface area (μm2) of seminiferous tubules (n > 10). An asterisk indicates a significant difference (P ≤ 0.05).


0.00014
0.00012
0.0001
0.00008
0.00006
0.00004
0.00002
0
Number of ZBTB16-positive cells per µm² of tubule
*
Control
BEP
Control
BEP

CONNECTING TEXT

In the preceding chapter, we have investigated the effects of the chronic administration of cisplatin, etoposide and bleomycin in combination on the population of rat stem/progenitor spermatogonia in vivo using the spermatogonial transplantation assay. The results suggested that SSCs are transiently affected by the treatment. Although spermatogonial transplantation is the gold standard assay to test SSC activity, the nature of the assay itself greatly limits further investigation of the effects on SSCs of these anticancer drugs alone or in combination at different doses. Instead, we used cultures of rat stem/progenitor spermatogonia as a new model to assess in vitro cytotoxicities. In serum-free medium and in the presence of glial cell line-derived neurotrophic factor (GDNF) and fibroblast growth factor-2 (FGF-2), rodent stem/progenitor spermatogonia can be cultured and expanded on a feeder cell layer, as cell aggregates termed clusters. Over the years, this in vitro culture assay has become a valuable tool to study SSC biology. In addition, the count of individual clusters formed in culture has been used as a semi-quantitative measure of SSC activity in vitro [19]. In chapter 5, we first developed a fluorescence-based assay using cultures of rat $EGFP^+$ stem/progenitor spermatogonial clusters. $EGFP^+$ cluster numbers were counted and their area measured via automated fluorescence microscopy and image analysis. Then, the experimental application of this assay was explored by investigating the cytotoxic effects of cisplatin,

etopside and bleomycin, alone or combined on stem/progenitor spermatogonia cultured in vitro. Thus, SSC cluster cultures could provide a new way of testing toxicity of anticancer drugs or other suspected testicular toxicants in vitro.

Chapter 5

Chapter 5: Development of a Short-term Fluorescence-Based Assay to Assess the Toxicity of Anticancer Drugs on Rat Stem/Progenitor Spermatogonia In Vitro[1]

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Running title: Anticancer drugs and rat spermatogonia

Summary sentence: Rat stem/progenitor spermatogonia culture can be used as a new in vitro model for studying the toxicity of chemotherapeutic drugs.

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anticancer drugs, enhanced green fluorescent protein, germ cells, spermatogenesis, spermatogonial stem cells, toxicity, toxicology, transgenic rats.

5.1 Abstract

In vitro culture of rodent spermatogonial stem cells (SSCs) has become an important asset in the study of mammalian SSC biology. Supported by added growth factors, SSCs divide in culture and form aggregates of stem/progenitor spermatogonia, termed clusters. Recent studies have shown that serial passaging of clusters results in long-term maintenance and amplification of the SSC pool and that this culture system can also be used for short-term semiquantification of SSC activity. Here, we report the development of an automated assay to assess the activity of rat stem/progenitor spermatogonia in vitro and its application for investigating the cytotoxicity of chemotherapeutic drugs on these cells. Cultures of EGFP-expressing rat spermatogenic cells allowed us to determine the number and two-dimensional surface area of clusters using an automated fluorescence imaging system, thereby providing quantitative data of SSC activity. Using this assay, we examined the germ cell toxicity of three drugs that are routinely used in testicular cancer therapy, namely, bleomycin, cisplatin, and etoposide, alone and in combination. All three drugs showed a significant and dose-dependent reduction of cluster number and surface area, indicating their adverse effects specific to spermatogonia. The inhibitory concentration at which cluster number and surface area are inhibited by 50% (IC_{50}) was the lowest with etoposide and the highest with cisplatin, implying that etoposide was most toxic to spermatogonia in vitro. These results suggest that the SSC culture should

provide an effective and efficient system to assess the germ cell toxicity of various drugs and chemical compounds.

5.2 Introduction

Spermatogonial stem cells (SSCs) are responsible for the lifelong production of spermatozoa throughout adulthood and for the transmission of genetic information to the next generation. Importantly, SSCs play a crucial role in the regeneration of spermatogenesis following cytotoxic insults such as cancer chemotherapies [1, 2]. However, evaluating the potential adverse effects of chemotherapeutic agents on spermatogonial stem/progenitor cells remains a challenging task, in part because of the absence of specific SSC markers and the complexity of spermatogenesis.

Most studies investigating the toxicity of anticancer agents on spermatogenesis and stem/progenitor spermatogonia have focused on whole-animal models [3, 4]. Although such in vivo approaches provide important toxicological information in a physiological context, they have several limitations. Cytotoxic effects on spermatogonia have been examined frequently using histology [3, 5–7], a laborious step for toxicological studies of this cell population. While cytotoxic effects on SSCs can be evaluated using spermatogonial transplantation [8, 9], this technique requires the appropriate preparation of recipient animals and the skillful microinjection of a germ cell suspension into the seminiferous tubules of individual recipient testes. In addition, measurement of SSC activity is done retrospectively, at least 2 mo after transplantation, by

manually counting donor-derived colonies of spermatogenesis formed in recipient testes [10–12]. Hence, the transplantation assay is both labor intensive and time consuming and not amenable to assess the toxicity of multiple drugs at a range of doses.

In vitro culture systems have been recently developed that allow the long-term maintenance and expansion of rodent SSCs [13–16]. In such systems, SSC-enriched germ cells are seeded on a feeder layer in a serum-free medium supplemented with glial cell line-derived neurotrophic factor (GDNF) and fibroblast growth factor 2 (FGF2). Under these conditions, SSC-enriched germ cells divide and grow into tightly connected aggregates of cells termed clusters. These clusters consist of both SSCs and proliferating daughter spermatogonia that are phenotypically indistinguishable from each other [13,14]. Importantly, clusters of stem/progenitor spermatogonia can be expanded and maintained in culture through serial passaging for several months. Furthermore, transplantation of cluster cells into recipient testes at any time during the culture period results in the formation of spermatogenic colonies, confirming the presence of functional SSCs among cluster cells [17, 18]. More recently, Yeh et al. showed that the number of clusters formed in vitro strongly correlated with the number of spermatogenic colonies produced following transplantation of these clusters into infertile recipient testes [19], demonstrating that SSC activity can be detected in a semiquantitative manner and in a short period of time by counting cluster

numbers. Thus, the mouse cluster culture was proposed as a SSC detection method that is an alternative to spermatogonial transplantation.

In this report, we examined whether cluster cultures could provide an alternative approach for evaluating the potential adverse effects of pharmacological agents, such as anticancer agents, on the spermatogonial stem/progenitor cell population in vitro. The goal of the current study was to develop a reproducible, short-term, and automated in vitro toxicity assay for rat stem/progenitor spermatogonia using cisplatin, etoposide, and bleomycin as model chemotherapeutic drugs. A transgenic rat strain in which the expression of enhanced green fluorescent protein (EGFP) is restricted to the germ cell lineage was used to establish cultures of rat $EGFP^{+}$ clusters [20]. We developed a new method to enumerate clusters using an automated fluorescence imaging system and applied this in vitro assay to evaluate the adverse effect of the three anticancer drugs, alone or in combination, on clusters of stem/progenitor spermatogonia.

5.3 Materials and Methods

Chemicals

All chemicals were obtained from Sigma Aldrich unless otherwise indicated. Cell culture reagents, including media, fetal bovine serum (FBS), trypsin, and human FGF2 were purchased from Invitrogen. Human recombinant GDNF and rat recombinant GFRα1-Fc fusion protein (GFRA1) were from R&D Systems. Cisplatin, etoposide, and bleomycin were purchased from LKT Laboratories.

Animal Care and Use

Homozygous SD-*Tg(ROSA-EGFP)2–4Reh* transgenic rats that exclusively express enhanced green fluorescent protein (EGFP) in the germ cell lineage (also termed GCS-germ cell specific) were kindly provided by R.E. Hammer (University of Texas Southwestern Medical Center, Dallas, TX) [20]. GCS-EGFP male pups on Postnatal Days 7–8 were used as donors of testis cells to induce cluster culture. Nude male mice (NCr-nu/nu) between 4 and 6 wk of age were purchased from Taconic Farms. The animals were maintained on a 12L:12D cycle with free access to rat chow and sterile water. All animal experiments were conducted in accordance with the regulations and policies of the Canadian Council on Animal Care and approved the McGill University Committee on Animals (protocol 4699).

Single-Cell Suspension Preparation

Homozygous GCS-EGFP rat pups were euthanized, and their testes were immediately dissected, freed of their tunica and placed into ice-cold phosphate-buffered saline (1× PBS, Ca^{2+}/Mg^{2+} free). Single-cell suspensions were prepared using a two-step enzymatic digestion as previously described [21]. Briefly, decapsulated testes were first incubated for 15 min at 37°C into Dulbecco modified Eagle medium (DMEM; Invitrogen) containing 1 mg/ml collagenase (type IA, C9891; Sigma). The supernatant containing mostly testicular interstitial cells was decanted, and the sedimented seminiferous tubules were resuspended with 0.5 mg/ml

trypsin (type 1, T8003; Sigma) and 1 μg/ml DNAse I (type 1, DN-25; Sigma) in DMEM and incubated for an additional 15 min at 37°C prior to mechanical dissociation to a single-cell suspension using a flamed-tip glass Pasteur pipette. Remaining tissue debris was removed by filtration through a 40-μm nylon mesh cell strainer (BD Biosciences). Dissociated cells were centrifuged at 500 × *g* for 5 min, resuspended in DMEM/10% FBS, and then immediately subjected to fluorescence-activated cell sorting (FACS). For each experiment, the testes of five to eight pups were used.

FACS

EGFP-expressing undifferentiated spermatogonia were isolated by FACS. Cell sorting was done using a FACSAria flow cytometer (Becton Dickinson Biosystems), and results were analyzed using Cell Quest software (Becton Dickinson Biosystems). During the acquisition process, forward and side-scatter plots were used to exclude cellular debris from the histogram analysis plots. On sorting, we obtained a clear bimodal distribution with an $EGFP^+$ cell peak about five times smaller than the peak for $EGFP^-$ cells (Supplemental Fig. S1, all Supplemental Data are available online at www.biolreprod.org). Cells were sorted under sterile conditions at about 50 cells/sec, collected in 15-ml Falcon tubes containing DMEM/10% FBS, and maintained on ice until seeding.

Establishment of Rat Cluster Cell Lines

Sorted $EGFP^+$ spermatogonia were cultured for cluster induction following protocols as previously described [14, 18]. The $EGFP^+$ cells were

counted using a hemacytometer and an inverted fluorescence microscope (Leica). $EGFP^+$ spermatogonia were plated at a density of 0.5–1.25 × 10^5 cells/cm^2on feeder cells in 24-well tissue plates with 1 ml/well of rat serum-free medium (RSFM). Feeder cells were prepared by treating STO (SIM mouse embryo-derived Thioguanine- and Oubain-resistant SNL76/7) fibroblasts with mitomycin C, as described previously, and seeding at 5 × 10^4 cells/cm^2 [13,14]. GDNF, GFRA1, and FGF2 (referred to hereafter as growth factors) were added to RSFM at concentrations of 40, 300, and 1 ng/ml, respectively. Under these conditions, $EGFP^+$ spermatogonia developed into cell aggregates termed clusters by Day 6 after seeding. Visual inspection using a fluorescence microscope confirmed that all clusters were EGFP-positive. For long-term maintenance in culture, rat clusters were cultured as described previously with a medium change every other day and passaged onto a new feeder layer every 6–7 days [14, 18]. All cultures were maintained in a 37°C atmosphere in a humidified chamber with 5% CO_2 in air.

Serial Dilution Assays

Serial dilution assays were done to measure the number of cluster-forming cells within the population of $EGFP^+$-sorted spermatogonia. Serial dilutions of FACS-isolated rat $EGFP^+$ cells were made in 200 µl of RSFM to obtain a final number of cells ranging from 100 to 20000 cells per well and plated in 96-microwell plates (CLS3904, Corning Inc.). Following 6

days of cultures, the number of clusters per well was determined and plotted against the number of cells initially seeded per well on Day 0.

Drug Treatments

For toxicity assays, 5000 dissociated cells from cultured clusters were plated in black-walled, flat/clear-bottom, half-area 96-microwell plates (CLS3882; Corning Inc.) in a 0.1-ml volume of RSFM supplemented with growth factors. Chemotherapeutic drugs were added to cluster cultures on Day 4 postplating, and the effects of drugs were assessed 48 h later. The stock solution of each drug was prepared in 100% dimethylsulfoxide (DMSO; Fisher Scientific) and then diluted into RSFM to the desired concentrations prior to use. The concentration of the drug in the stock solution was 40 mM for cisplatin, 50 mM for etoposide, and 100 mM for bleomycin. The final DMSO concentration in the media of all drug assays was 0.1 % (v/v). Control cultures received 0.1% DMSO only.

Immunofluorescence Staining of Rat Clusters

Clusters were directly fixed in culture plates (96-well flat/clear-bottom black plates) following medium removal with 10% neutral-buffered formalin without washing and stored at 4°C in the dark. For immunostaining, clusters were incubated with blocking buffer (3% [w/v] bovine serum albumin and 0.1% [v/v] Triton X-100 in PBS pH 7.4) for 20 min and then incubated with primary antibodies diluted in blocking buffer overnight at 4°C. The following primary antibodies were used: mouse monoclonal anti-GFP antibody (catalog no. MAB 3580), rabbit polyclonal

anti-phospho-histone H3 (Ser10) (catalog no. 06–570), and rabbit polyclonal anti-phospho-H2A.X (Ser139) (catalog no. 07–164), all from Millipore. ZBTB16 (also known as mouse monoclonal anti-promyelocytic leukemia zinc finger or PLZF) (catalog no. OP128), was from Calbiochem, and mouse monoclonal anti-human RET (catalog no. MAB718) was from R&D Systems. On the next day, following three washes with PBS, clusters were incubated with secondary antibodies for 45 min at room temperature in the dark. Secondary antibodies used were Alexa Fluor 594-conjugated goat anti-mouse IgG, Alexa Fluor 594 goat anti-rabbit IgG (Molecular Probes), and sheep anti-mouse FITC-conjugated secondary antibody (GE Healthcare). Finally, the nuclei of cells in clusters were counterstained with 4,6 diamidino-2-phenylindole (DAPI; 0.1 μg/ml in PBS), and then VECTASHIELD Mounting Medium (Vector Laboratories) was added to each well. Images were acquired using an inverted epifluorescence microscope (Leica).

Automated Imaging System and Cluster Parameter Analysis

The images of clusters in 96-microwell plates were acquired automatically using the ImageXpress^{MICRO} imaging system (Molecular Devices) equipped with a 10× Plan Fluor 0.30 NA objective (Nikon) and a CoolSNAP$_{HQ}$ digital CCD camera (Roper Scientific) at maximum resolution (696 × 520 pixels/image, 1.29-micron/pixel resolution, with a 2 × 2 camera bin). For each well of a 96-microwell full-area plate (CLS3904, Corning Inc.), 48 pictures were captured, whereas in experiments using 96-

microwell half-area plates (CLS3882, Corning Inc.), 30 pictures were captured. Subsequently, cluster numbers and surface area were analyzed using MetaXpress software (Molecular Devices). Clusters were defined as objects within the range of a minimum width of 35 μm and a maximum width of 85 μm, corresponding to groups or aggregates of at least six cells [19]. Cluster surface area was defined as the total area of all pixels inside the two-dimensional or projected perimeter of clusters that have fluorescence intensity above the set threshold value (intensity above local background 300 gray level). Counts were automatically exported to Excel. For dual-imaging channel experiments (two different fluorophores), channel 1 was used to measure the fluorescence of EGFP to identify discrete clusters and channel 2 to measure the fluorescence of Alexa Fluor 594-conjugated secondary antibody.

Transplantation of Cultured Rat Cluster Cells

Transplantation of rat EGFP cluster cells was done as previously described [14]. Briefly, nude mice were injected with busulfan (44 mg/kg) at 4–6 wk of age to eliminate endogenous spermatogenesis and then used as recipients at least 5 wk after the busulfan treatment. Cultured rat $EGFP^+$ clusters were dissociated to single cells by trypsin digestion and resuspended in injection medium (DMEM, 10% FBS, 1 mg/ml DNase I, and 0.04% trypan blue). Approximately 7 μl of cluster cell suspension were injected into each recipient testis. Three months after transplantation,

recipient testes were collected, and $EGFP^+$ donor-derived colonies of spermatogenesis were visualized under a fluorescence stereomicroscope.

Statistical Analyses

For serial dilution assay, we calculated the root square (r^2) of the Pearson product moment correlation coefficient using Microsoft Excel. In drug testing experiments, each anticancer agent was tested in triplicate in at least three independent experiments using different cluster cultures. Dose-response curves were plotted, and IC_{50} values (concentration of drug inducing a 50% decrease in cluster number or cluster surface area) were calculated using nonlinear regression analysis.

5.4 Results

Establishment and Maintenance of EGFP-Expressing Rat Stem/Progenitor Spermatogonia Cultures

The development of an automated cluster-based assay system primarily requires cultures of cluster cells carrying a fluorescent marker transgene. Taking advantage of the restricted expression of EGFP in the germ cells of GCS-EGFP transgenic rats, we first isolated stem/progenitor spermatogonia (>99% purity) from Postnatal Day 7–8 testis cell suspensions using FACS (Supplemental Fig. S1) [20]. At this young age, undifferentiated spermatogonia are the only germ cells present in the seminiferous epithelium [21, 22]. To generate $EGFP^+$ clusters, single dissociated $EGFP^+$ spermatogonia were seeded on a feeder layer of STO

cells in a serum-free medium supplemented with GDNF, GFRA1, and FGF2 [14]. In the presence of growth factors, $EGFP^+$ spermatogonia divided and developed into clusters of tightly connected cells by Day 6 (Fig. 1, a–d), whereas cells within the EGFP-negative fraction sorted by FACS did not form clusters (Supplemental Fig. S2). In the absence of growth factors, $EGFP^+$ spermatogonia survived but did not form clusters during the 6 days of the culture period (Fig. 1, e and f). Cultures of $EGFP^+$ spermatogonia were subsequently maintained by passaging clusters onto new STO feeder layer every 6–7 days for up to five passage generations until used for assays.

To confirm the undifferentiated state of $EGFP^+$-cluster cells in culture, we analyzed the expression of undifferentiated spermatogonial markers, ZBTB16 and RET proteins [23–26]. Using immunofluorescence, ZBTB16 staining was clearly localized in the nuclei of cluster cells, whereas the RET staining profile demonstrated a cytoplasmic membrane distribution. Thus, $EGFP^+$-cluster cells were comprised of undifferentiated spermatogonia (Supplemental Fig. S3).

We assessed the presence of functional SSCs in $EGFP^+$ cluster cultures using the spermatogonial transplantation assay. To this end, single-cell suspensions from cultures of rat $EGFP^+$ clusters were transplanted into nude mouse testes. Three months later, we observed EGFP-positive colonies in recipient testes (Supplemental Fig. S4, a and b). Further immunohistochemical analysis of $EGFP^+$ colonies revealed that

rat elongated spermatids/spermatozoa were formed within mouse tubules, indicating that complete spermatogenesis was regenerated from donor cluster cells (Supplemental Fig. S4, c–e). Together, these results demonstrate that functional SSCs were maintained in rat $EGFP^+$ cluster cultures.

Determination of the Rat Spermatogonial Cluster-Forming Efficiency Using Automated Fluorescence Microscopy

In order to determine the sensitivity of the automated cluster-based assay, we assessed the relation between the number of $EGFP^+$ cells placed in culture and that of clusters developing using a serial dilution assay. At Day 0, $EGFP^+$ spermatogonia were plated in 96-well plates in serial dilutions that ranged from 0 to 20000 cells per well. After 6 days of culture, images of clusters were acquired using the ImageXpress^{MICRO} imaging system and analyzed for cluster numbers and area using MetaXpress, as described in *Materials and Methods*. As shown in Figure 2A, the number of clusters formed increased with an increase in the number of cells initially seeded at Day 0. Data obtained for cluster numbers and area were then analyzed using linear regression analysis. As seen in Figure 2, B and C, strong linear correlations between the cluster numbers and the number of cells initially plated per well and between cluster area and number of cells seeded per well were obtained with both r^2 correlation coefficients above 0.99. Thus, the slope of the regression line obtained from the cluster numbers reflects the proportion of

cluster-forming cells present in the population of rat EGFP$^+$ cells initially seeded. Under our culture conditions, we found that on average one cluster emerged from every 45 EGFP$^+$ spermatogonia. The slope of the regression line calculated from cluster area may reflect the proliferation of seeded cells.

Notably, we did not observe a plateau phase even at the highest cell density examined (20000 cells/well) for either parameter, indicating that the cluster growth did not reach a saturation level within this range of cell densities. The linearity in both cluster number and surface area was maintained even at a very low cell density (100 cells input/well), demonstrating the high sensitivity of the automated cluster assay (Fig. 2, B and C). In addition, the strong linearity observed between cell inputs and cluster numbers/areas suggest that the majority of cluster are of clonal origin (i.e., one cluster originates from a single cluster-forming cell) [19]. However, since suspensions of dissociated cells tend to group themselves if seeded at a high density, it is also possible that the formation of some clusters begins with such cell aggregations. Since there is a possibility that the maximum number of cells seeded (i.e., 20000 cells/well) could be reaching a plateau, we selected a density of 10000 cells/well for further assays. Taken together, these results demonstrate that automated fluorescence microscopy (ImageXpress^{MICRO} imaging system) can be used to rapidly count cluster numbers and analyze cluster surface area and to determine cluster-forming activity.

Comparison of Automated vs. Manual Cluster Quantification

Using the previously mentioned serial dilution experiments, we then compared the results of manual cluster counts to the results obtained using the automated imaging system. For manual counting, clusters were visually detected under a fluorescent microscope, and their number was recorded. We then used linear regression to evaluate cluster numbers as a function of cell dilutions. The cell surface area was difficult to assess manually, particularly when the culture contains a high number of clusters; therefore, we used only cluster numbers as assay readouts. Both quantification methods generated similar results with a strong linear correlation between cluster numbers and cell dilution per well (Supplemental Fig. S5). Hence, data obtained with the automated imaging system were in good agreement with those from the manual counts of clusters, further attesting to the reliability of the automated assay system and its ease of use.

γH2AX Immunofluorescence Staining and Automated Quantification of γH2AX-Positive Cluster Cells

In eukaryotic cells, histone H2AX (official symbol H2AFX), a variant of the nucleosome core histone H2A, is rapidly phosphorylated in response to DNA double-strand breaks induced by ionizing radiation and cytotoxic agents such as anticancer drugs [27–29]. Phosphorylated histone H2AX at serine 139, designated as γH2AX, forms distinct nuclear foci along megabase chromatin domains at or nearby the sites of DNA

damage. Hence, a diffuse γH2AX staining is often indicative of cells experiencing DNA damages and potentially undergoing apoptosis [30]. Using an anti-γH2AX antibody, we determined the number of DNA-damaged cells within $EGFP^+$ clusters (i.e., double positive for γH2AX and EGFP). After 6 days in culture, we observed that a small subset of cluster cells strongly expressed γH2AX and that these cells were almost exclusively located at the outer edge of the clusters (Fig. 3, a and b). In serial dilution experiments, we found that the number of γH2AX-positive cells per cluster tended to increase at higher cell concentrations, but there was no significant difference across the cell concentrations examined, suggesting that the proportion of DNA-damaged cells in a cluster is relatively constant (Fig. 4A). On average, 0.5 γH2AX-positive cells were observed per cluster, regardless of the number of clusters that developed when at least 2000 cells were seeded per well. These results, obtained using the automated imaging system, indicate that a small number of cells retains DNA-damage after 6 days of culture, and these cells are located primarily at the periphery of a cluster.

Detection of Phospho-Histone H3Ser 10 and Evaluation of Cluster Cell Mitotic Activity

Phosphorylation of histone H3 at serine 10 is a well-characterized marker for mitosis that identifies cells in G2/M [31, 32]. Therefore, we determined the mitotic activity of cluster cells using an antibody that specifically recognizes histone H3 phosphorylated at serine 10 and

counted the number of positive cells within EGFP$^+$ clusters using the automated imaging system. Cells immunopositive for phosphorylated histone H3 (pH3) were observed in a subset of cluster cells, and, in clear contrast to γH2AX staining, the localization of pH3-positive cells was not restricted to cells at the periphery of clusters but also was found within the cluster structure (Fig. 3, c and d). We then quantified the numbers of cells stained for pH3 per cluster in serial dilution experiments. We found that the number of pH3-positive cells per cluster remained constant regardless of the number of cells seeded per well (Fig. 4B); a statistical difference was found only between 100 and 20000 cells. On average, one cell per cluster was found to be proliferating when at least 2000 cells were seeded per well. Thus, these results indicate that a small fraction of mitotically active cells are consistently present within clusters regardless of the total number of clusters per well.

Effects of Chemotherapeutic Drugs on Rat SSC Clusters

Having established an automated cluster assay system, we then applied it to test the effects of anticancer drugs on stem/progenitor spermatogonia in vitro. To this end, we established dose-response curves for cisplatin, etoposide, and bleomycin, either alone or in combination. Rat EGFP$^+$ clusters were cultured for 4 days under the standard SSC culture condition with RSFM supplemented with growth factors and then exposed to drugs for an additional 2 days (Fig. 5A). Here, we used concentration ranges from 0.01 to 100 μM, depending on the drug. Cluster numbers and

surface area were measured on Day 6 using the automated imaging system. The results obtained were compared to control cultures where only vehicle was added. Microscopic examination of the clusters revealed that rat $EGFP^+$ clusters exhibited a striking change in morphology already 1 day after treatment with cisplatin. In contrast to control clusters that showed a tightly packed alignment, cisplatin-treated clusters were clearly smaller and surrounded by detaching cells (Fig. 5B, a and b). Clusters exposed to etoposide and bleomycin showed a similar morphological change (data not shown). These observations suggest that each of these drugs elicits rapid cytotoxicity in cluster cells.

To assess the cytotoxic effects quantitatively, we determined both cluster numbers and surface areas using the automated system following exposure of clusters to the three drugs at different concentrations. As seen in Figure 6, each of the three drugs tested reduced cluster numbers and surface area in a dose-dependent manner. The results showed varied kinetics of cytotoxic effects among cisplatin, etoposide, and bleomycin (Fig. 6, A–C). Cisplatin did not significantly affect cluster numbers and surface area at 0.1 μM but reduced both parameters gradually with increasing doses. In contrast, etoposide and bleomycin showed more potent effects; both cluster numbers and surface area had declined drastically at 0.1 μM and remained at low levels across the higher doses examined. Based on these dose-response curves, we determined IC_{50} values (μM) for each drug (Table 1). The highest IC_{50} value measured

was for cisplatin (1.88 μM), and the lowest IC_{50} was obtained with etoposide (0.023 μM); the IC_{50} value for bleomycin was intermediate at 0.197 μM. A similar trend of IC_{50} values was seen when the values were calculated on the basis of cluster area. Hence, these results indicate that etoposide is the most toxic to cluster cells in vitro, while cisplatin appears the least toxic.

Since cisplatin induces DNA strand breaks that may trigger apoptosis and decrease cell proliferation if left unrepaired [30, 33], we further characterized the adverse effects of cisplatin on cluster cultures by staining them for γH2AX and phosphorylated histone H3 at serine 10. As shown in Figure 7A, after 2 days of cisplatin treatment, the number of γH2AX-positive cells per cluster significantly increased at 0.1 μM cisplatin compared to control and decreased after treatment with 10 μM cisplatin ($P < 0.05$), suggesting that DNA-damaged cells were rapidly lost from clusters. Similarly, the numbers of pH3-positive cells per cluster were also significantly decreased with increasing concentration of cisplatin, indicating that some cluster cells remained mitotically active even at higher concentrations of cisplatin. We found a significant difference between the number of pH3-positive cells at 0.01 and 0.1, 5 and 10 μM cisplatin ($P < 0.05$) (Fig. 7B). Thus, using specific antibody labeling and automated image analysis, this cluster assay allowed us to study quantitatively the effects of chemotherapeutic drugs on cluster cells in terms of DNA damage and mitotic activity.

Finally, we examined the combined effects of bleomycin, etoposide, and cisplatin (BEP). These drugs were mixed at equimolar concentrations to give a combined concentration from 0.001 to 0.1 μM; thus, for 0.1 μM BEP, each drug was added at a concentration of 0.033 μM. At 0.001 and 0.01 μM BEP, the cluster number did not show a significant difference from the control number; however, 0.1 μM BEP significantly reduced the cluster number to ~50% of control. The results of cluster area were in parallel to those of cluster numbers. It is noted that 0.1 μM BEP contained etoposide at 0.033 μM, which is similar to the IC_{50} for this drug, implying that the significant effect observed at 0.1 μM BEP may be caused largely by etoposide. These results indicate that although each drug has cytotoxic effects on spermatogonia in vitro, there appears to be no synergistic effect; combined BEP toxicity does not become evident until the concentration of one of the three drugs reaches the IC_{50}.

5.5 Discussion

In this study, we first developed a new fluorescence-based assay to assess SSC activity using cultures of rat $EGFP^+$ spermatogonia. Taking advantage of their EGFP expression, we used automated fluorescence microscopy and image analysis as a novel method to rapidly and reproducibly enumerate clusters formed by $EGFP^+$ stem/progenitor spermatogonia in vitro. We then demonstrated that such a culture system, combined with automated fluorescence microscopy, can be used to assess the effects of anticancer drugs, cisplatin, etoposide, and

bleomycin, alone or in combination, on the number and surface area of rat stem/progenitor spermatogonial clusters.

Although the need for developing in vitro methods as alternative strategies to animal testing is gaining greater attention in the field of reproductive toxicity [34], few methods are available. A variety of in vitro culture systems for testis germ cells have been described previously in the literature [35, 36]. However, few of these methods have been used to directly assess the adverse effects of anticancer drugs or other toxicants on stem/progenitor spermatogonia. A proposed alternative approach for in vitro toxicity testing using testis germ cells involves the use of immortalized testis cell lines [37,38]. Although these cell lines may have some characteristics of undifferentiated spermatogonia, their ability to restore complete spermatogenesis has not yet been demonstrated. Primary cultures of germ cells or cultures of seminiferous tubule segments provide other alternative approaches, but they have limited longevities in vitro and therefore require repetitive use of experimental animals to supply testicular tissue fragments or cells [36]. Instead, the culture system used in this study displays several distinct advantages. Rat $EGFP^+$ stem/progenitor spermatogonia cultures can be expanded and maintained for long periods [14] and therefore can be used to continuously supply cells required for assays. Since these cells can be cryopreserved, target cells for the assay can be available at any time. Further, rat $EGFP^+$ cluster cultures retain many in vivo properties of stem/progenitor

spermatogonia. The regeneration of $EGFP^+$ colonies of spermatogenesis within recipient testes after transplantation demonstrated the presence of functional SSCs among cultured germ cell clusters. The stem/progenitor phenotype of rat $EGFP^+$ cluster cells was further confirmed by analyzing the expression of known undifferentiated spermatogonial cell markers, GNDF receptor RET and transcription repressor ZBTB16 [9, 18, 19]. Taken together, we propose that our culture system provides a suitable model of early spermatogenesis in which the effects of toxic compounds on stem/progenitor spermatogonia can be assessed directly.

Despite their utility, cluster cultures also have some limitations. Quantitative cluster-forming assays for rodent SSCs require clusters to be manually counted using a bright-field microscope. The use of direct observation to enumerate clusters is a time-consuming and labor-intensive procedure in which consistent objectivity is difficult to achieve. This greatly limits the number of assay conditions, such as the number of different drugs and their concentrations that can be tested.

In this study, we took advantage of the restricted EGFP expression in the germ cell lineage of GCS-EGFP transgenic rats and established $EGFP^+$ cluster cultures using previously reported culture conditions [14]. To date, SSC culture systems have been developed for several rodent species, including mouse, hamster, and rat [13–16, 39]. However, rat is a well-established model in reproductive toxicology; the toxicity of several anticancer drugs to testis germ cells was characterized previously using in

vivo rat models, although data on the specific response of spermatogonia to anticancer drugs are often limited [4, 42]. To the best of our knowledge, no previous study has reported the use of a rat SSC culture system in toxicity studies. The ease and rapidity of accurately counting rat $EGFP^+$ clusters in culture using the automated imaging system prompted us to use this culture system to assess anticancer drug toxicity in vitro.

Cisplatin, etoposide, and bleomycin are used clinically in a combination chemotherapy regimen (BEP regimen) for the treatment of patients with metastatic germ cell tumors. It is well documented that one of the major side effects of this BEP regimen is damage to the spermatogenic system, resulting in a transient or permanent decrease in sperm production in patients shortly after treatment [3, 40, 41]. Previous studies in animal models have also well documented the effects of these three anticancer agents individually on spermatogenesis [7, 42, 43]. In addition, we previously showed in the rat that a combined regimen of cisplatin, etoposide, and bleomycin induced disruption of spermatogenesis that resulted in a drastically decreased sperm production rate and altered progeny outcome [44, 45].

Here, using the rat $EGFP^+$ cluster culture system, we showed that all three drugs have concentration-dependent toxic effects on cluster numbers and surface area. Thus, the concentration at which toxicity was observed varied significantly among the drugs used. To gain more insight into the mechanisms of cisplatin toxicity, γH2AX labeling has been

employed to demonstrate that a subpopulation of cluster cells exhibit diffuse-nuclear staining indicative of DNA-damaged cells, whereas pH3 staining provided a measure of the numbers of mitotically active cells within clusters. After drug treatment, γH2AX-positive cells that are experiencing DNA damage likely undergo cell death. It is interesting to note that both γH2AX-positive cells and detaching cells are found in periphery of a cluster (Fig. 5 and Supplemental Fig. S6). These observations suggest that drug treatments likely induce apoptosis of cluster cells (i.e., spermatogonia and SSCs), leading to a decline in cluster number and surface area. This possibility is supported by previous in vivo studies in which cisplatin was shown to induce germ cell apoptosis with targeted cells including spermatogonia [5, 46]. Similar results were also obtained when etoposide alone or a combination of bleomycin, etoposide, and cisplatin was given to rats [7, 45]. Alternatively, the drug treatments may affect the expression of cell adhesion molecules that could also lead to detachment of cluster cells, in addition to apoptosis induction, resulting in the reduction of cluster number and surface area. Further studies are necessary to clarify these possibilities. Nonetheless, our study indicates that the rat $EGFP^+$ SSC assay may be useful for cytotoxic testing as well as for investigating mechanisms of toxicity.

Our data clearly indicate that etoposide is more toxic to clusters of stem/progenitor spermatogonia cultured in vitro than bleomycin and cisplatin. Although several investigations have reported that in vivo

cisplatin or etoposide exposures are often associated with impaired spermatogenesis, no comparative data are available on their specific effects on stem/progenitor spermatogonia either in vitro or in vivo. In addition, most of these in vivo studies have used different routes of administration and durations of treatment. Since our study is the first report about drug effects imposed specifically on spermatogonia in vitro, a direct comparison of past studies with our current results is difficult. In order to assess our in vitro results with in vivo data, experiments involving transplantation of cluster cells after treatment will be required to determine the toxicity of these anticancer drugs specifically on SSCs. The results obtained in this study will allow us to primarily identify the doses of individual drugs to be examined alone and in combination by the transplantation assay.

In conclusion, our results indicate that the automated fluorescence-based SSC assay developed in this study may provide new insights into the adverse effects of anticancer drugs on stem/progenitor spermatogonial activity in vitro. It may also offer unique opportunities for screening assays using libraries of small molecules to search for molecules that may improve proliferation or survival of stem/progenitor spermatogonia in vitro. We anticipate that such a fluorescence-based cluster assay will serve as a useful tool for assessing the potential cytotoxicity of new drugs and environmental toxicants. In light of a recent report on in vitro culture conditions for the propagation of human SSCs [47], human SSC culture

should also be applicable to cytotoxic studies, as shown in this study, and could also be extended to a patient-specific toxicological analysis on undifferentiated spermatogonia in the future.

Acknowledgments

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Figures and Legends

Figure 1. Rat EGFP+ SSC clusters derived from pup testes on Day 6 of culture. **a**–**d**) Single dissociated rat spermatogonia which constitutively express EGFP form clusters when cocultured with STO cells under serum-free conditions in the presence of GDNF, GFRA1, and FGF2 after 6 days. Low-magnification fluorescence (**a**) and merged phase contrast with fluorescence (**b**) photomicrographs of rat $EFGP^{+}$ clusters. Higher magnification of fluorescence (**c**) and merged phase contrast with fluorescence (**d**) images of an individual $EGFP^{+}$ cluster. All individual cells within clusters appeared $EGFP^{+}$, clearly distinguishing them from feeder cells. **e** and **f**) FACS sorted $EGFP^{+}$ spermatogonia cultured without growth factors. Bars = 100 µm (**a**–**f**).

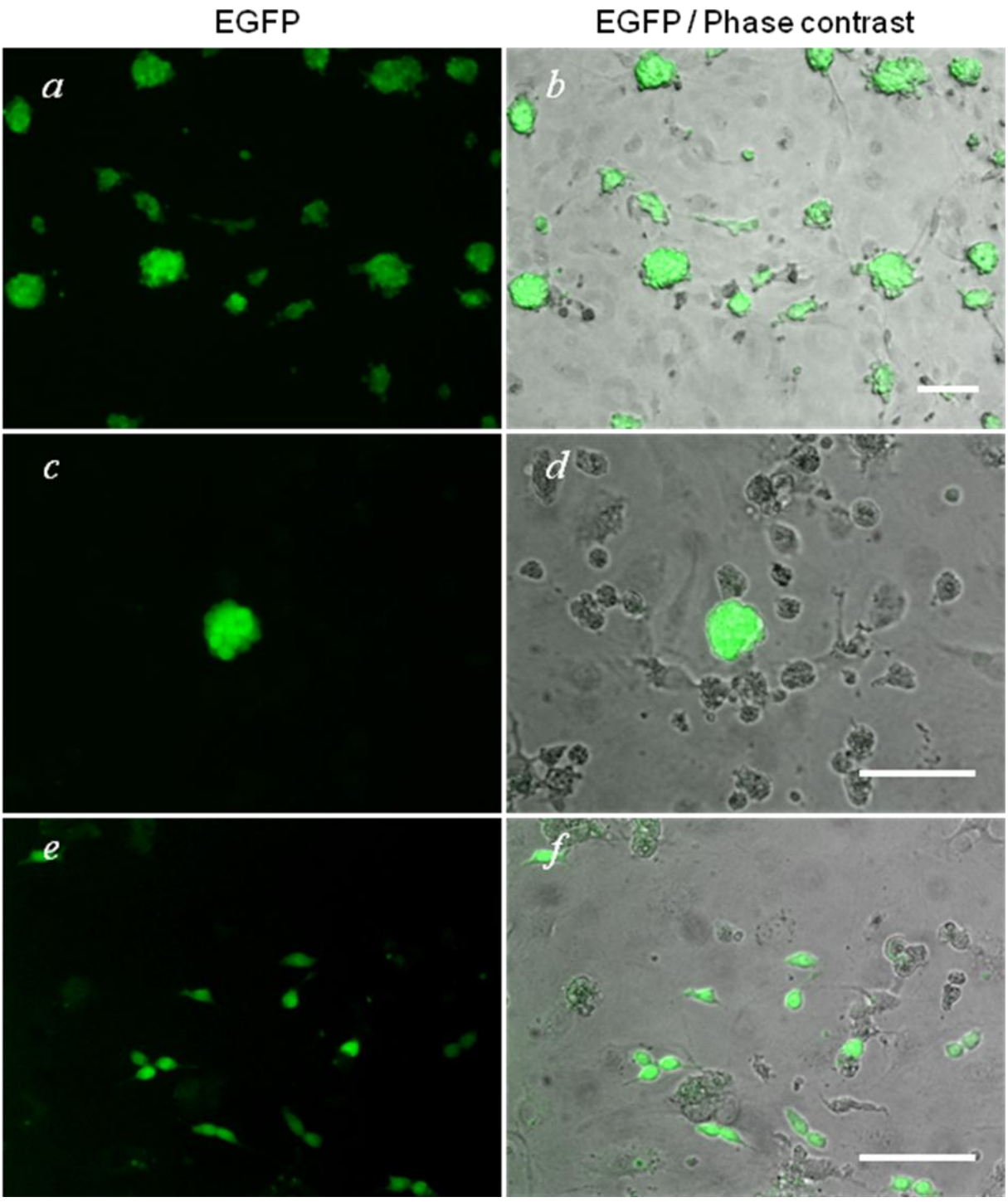

EGFP
EGFP / Phase contrast
a
b
c
d
e
f

Figure 2. Quantification of cluster numbers and surface area using automated fluorescence microscopy and image analysis. $EGFP^{+}$spermatogonia were plated at increasing densities (0–20000 cells/well) in RSFM supplemented with growth factors in 96-well plates and cultured for 6 days. **A**) Representative composite image of one replicate from a serial dilution experiment. A total of 48 images per well were acquired at 10× magnification with the $ImageXpress^{MICRO}$ imaging system. An increasing number of clusters was observed with increasing numbers of cells seeded per well. **B**, **C**) Cluster numbers and surface area were analyzed using MetaXpress software. **B**) Cluster numbers after 6 days of culture (Y axis) were plotted against the initial numbers of cells seeded per well at Day 0 (X axis). A strong linear correlation between the two parameters was observed. **C**) Cluster surface area (Y axis) was plotted against the numbers of seeded cells per well at day 0 (X axis). A clear linear correlation between the two parameters was detected. Data are means ± SEM values of three replicates (n = 5 experiments).

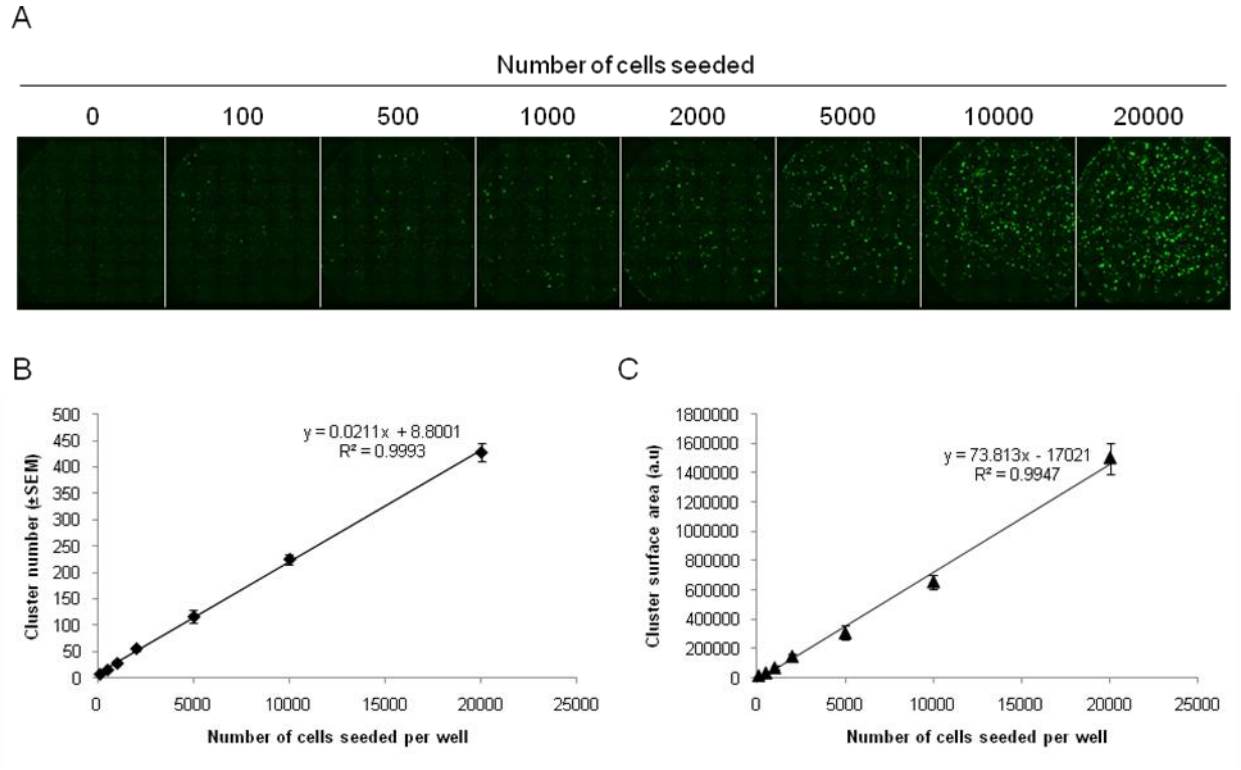

A
Number of cells seeded
0
100
500
1000
2000
5000
10000
20000
B
y = 0.0211x + 8.8001
R² = 0.9993
Cluster number (±SEM)
Number of cells seeded per well
C
y = 73.813x - 17021
R² = 0.9947
Cluster surface area (a.u)
Number of cells seeded per well

Figure 3. Immunofluorescence analysis of rat EGFP+ clusters. Clusters were fixed and costained with anti-GFP antibody and antibodies to either γH2AX (**a** and **b**), or pH3ser10 (**b** and **c**). **a**) Representative γH2AX immunofluorescence staining of clusters (red). **b**) Merged images of γH2AX staining with $EGFP^+$ clusters (green) shows that γH2AX stained cells (indicated by arrows) occurred mainly at the edge of clusters. **c**) Immunofluorescence staining of clusters using phosphorylated H3 antibody (red). **d**) Overlay of the pH3ser10 staining in $EGFP^+$clusters (green) showing pH3-positive cluster cells. pH3ser10-stained cells were noticed in both the inner and the outer edge parts of clusters. Bar = 100 μm.

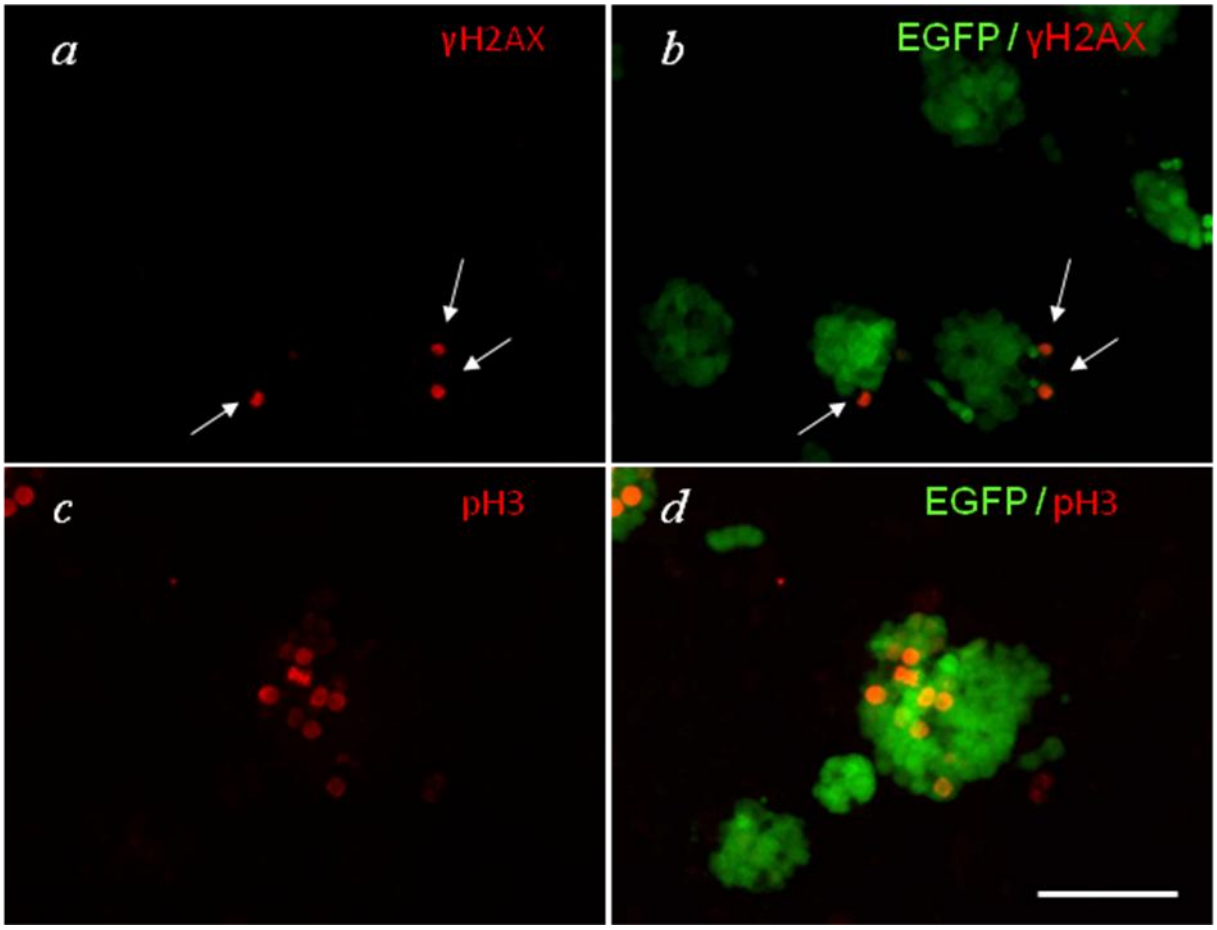
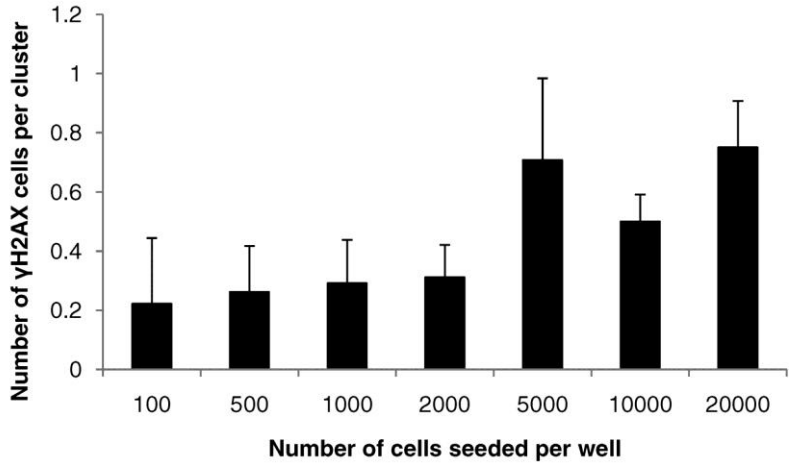

a
γH2AX
b
EGFP / γH2AX
c
pH3
d
EGFP / pH3

Figure 4. Quantifications of γH2AX- and pH3-positive cells in clusters grown in serial dilution experiments. **A**) The number of γH2AX-positive cells per clusters was analyzed using automated imaging. There was no significant difference across the cell concentrations examined. **B**) Number of pH3ser10-positive cells per clusters. A statistical difference was found between 100 and 20000 cells (*$P < 0.05$). Values represent mean ± SEM from three independent experiments.

A


Number of γH2AX cells per cluster
1.2
1
0.8
0.6
0.4
0.2
0
100
500
1000
2000
5000
10000
20000
Number of cells seeded per well

B

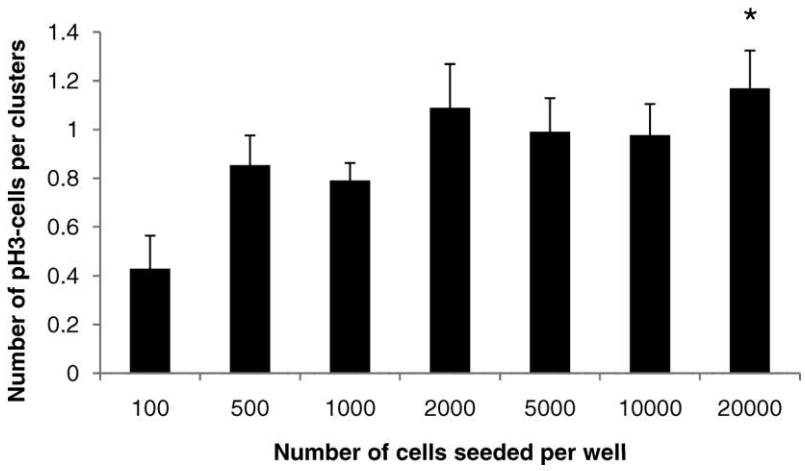
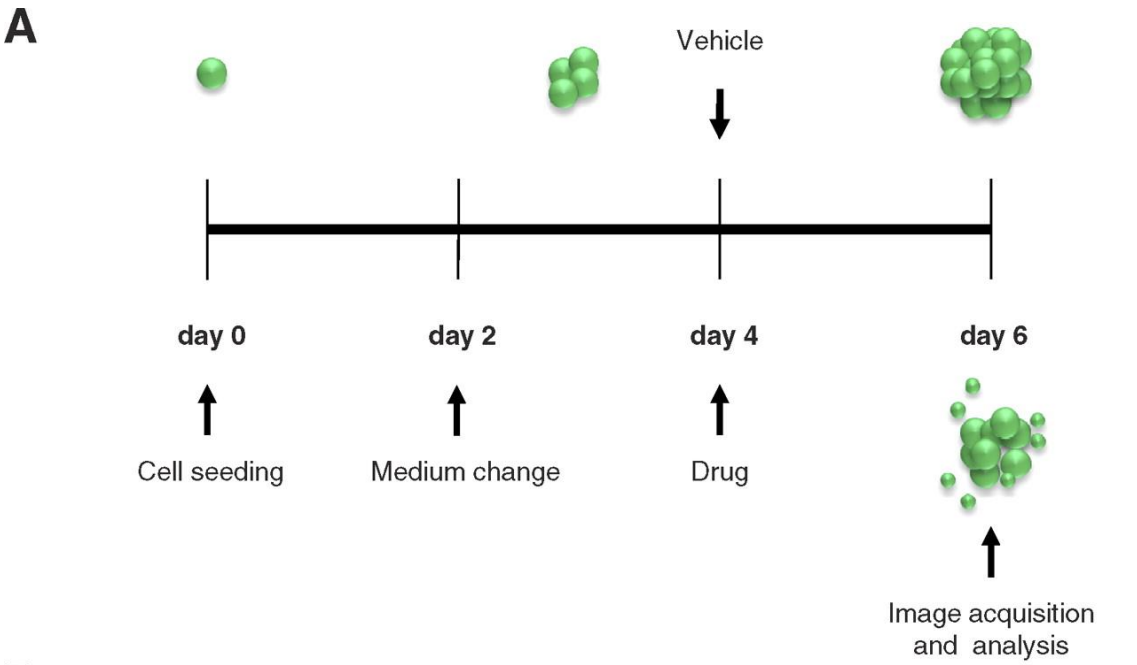

Number of pH3-cells per clusters
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
100
500
1000
2000
5000
10000
20000
Number of cells seeded per well

Figure 5. Rat EGFP+ SSC culture as an in vitro toxicity assay. **A**) Schematic diagram of the timeline for drug exposure in the rat $EGFP^+$ cluster cytotoxicity assay. **B**) A decrease in cluster surface area and abnormal cluster morphology was evident in clusters incubated with cisplatin after 24 h of treatment. Control (**a**) and cisplatin-treated for 24 h (**b**) $EGFP^+$ clusters. Insets show an enlarged cluster from control and treated well, respectively. Bar = 50 μm.


A
Vehicle
day 0
day 2
day 4
day 6
Cell seeding
Medium change
Drug
Image acquisition
and analysis

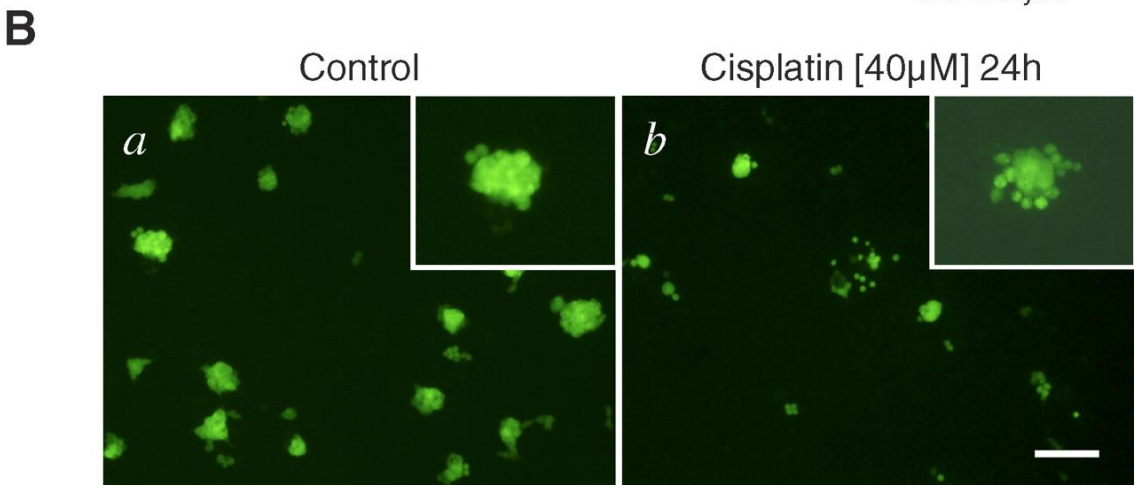

B
Control
Cisplatin [40μM] 24h
a
b

Figure 6. Effects of chemotherapeutic agents, cisplatin, etoposide, and bleomycin on in vitro cultured rat EGFP+ clusters. For cytotoxicity assays, clusters were first grown for 4 days without drug. At Day 4, culture medium was changed and replaced by a fresh one with drug or vehicle. Following culture for 2 days, rat $EGFP^+$ clusters were fixed and then counted using an automated fluorescence microscope, as described in *Materials and Methods*. The cytotoxicity of each drug for rat $EGFP^+$ clusters was assessed by plotting concentration-response curves for the cluster numbers (*left axis*) and surface area (*right axis*). Clusters in 96-well plates were treated with cisplatin (**A**), etoposide (**B**), bleomycin (**C**), or a combination of the three drugs (**D**). Cluster number and surface area were determined 48 h later. Data shown represent the average of three independent experiments, each containing three replicates. The data are presented as the means ± SEM.

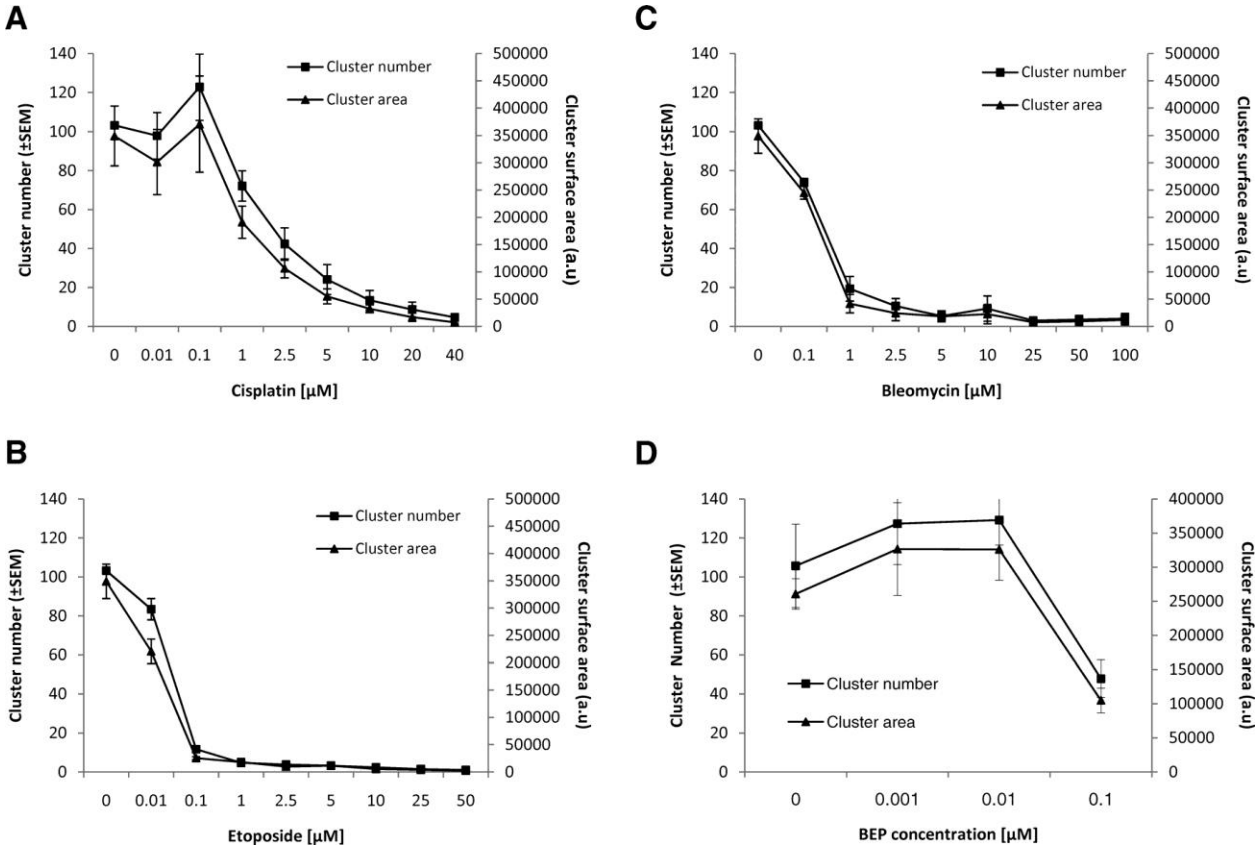
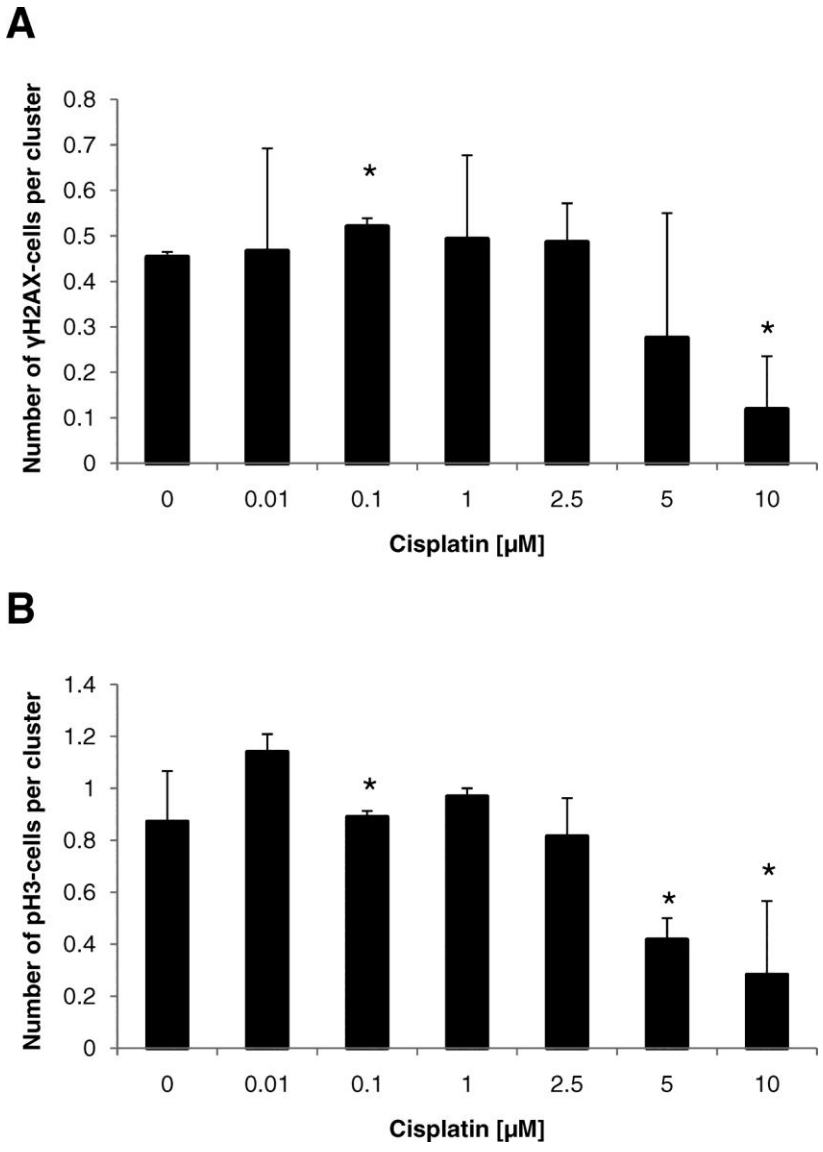
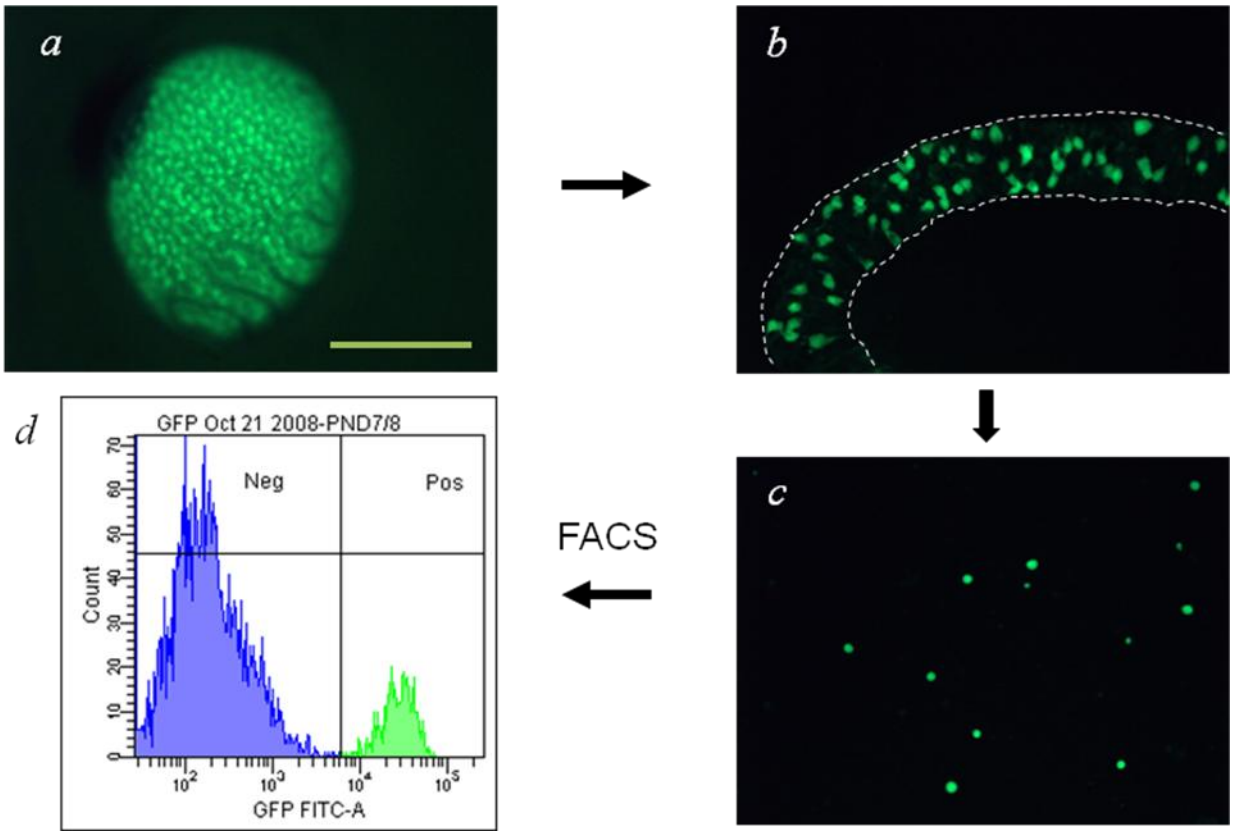

A
Cluster number
Cluster area
Cluster number (±SEM)
Cluster surface area (a.u)
0 0.01 0.1 1 2.5 5 10 20 40
Cisplatin [μM]
C
Cluster number
Cluster area
Cluster number (±SEM)
Cluster surface area (a.u)
0 0.1 1 2.5 5 10 25 50 100
Bleomycin [μM]
B
Cluster number
Cluster area
Cluster number (±SEM)
Cluster surface area (a.u)
0 0.01 0.1 1 2.5 5 10 25 50
Etoposide [μM]
D
Cluster number
Cluster area
Cluster Number (±SEM)
Cluster surface area (a.u)
0 0.001 0.01 0.1
BEP concentration [μM]

Figure 7. Assessment of cluster DNA damage and mitotic activity following cisplatin exposure. **A**) Immunostaining of phosphorylated histone H2AX-positive cells per cluster after cisplatin exposure (0–10 μM).**B**) Average number of pH3 ser10-positive cells per cluster after treatment with cisplatin at various doses [0–10 μM]. Data are shown as means ± SEM. *$P < 0.05$.

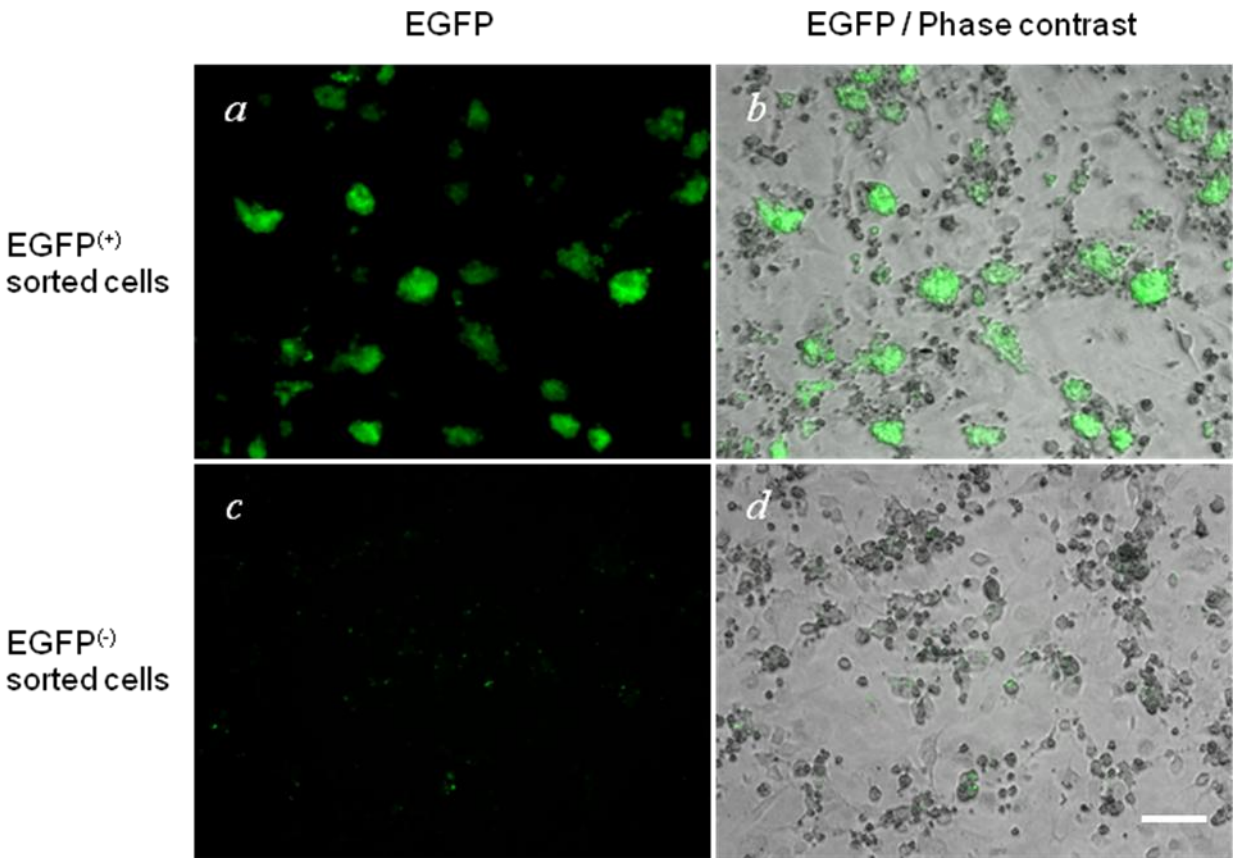

A
Number of γH2AX-cells per cluster
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
*
0
0.01
0.1
1
2.5
5
10
Cisplatin [μM]
B
Number of pH3-cells per cluster
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
*
0
0.01
0.1
1
2.5
5
10
Cisplatin [μM]

Supplemental Figures and Legends

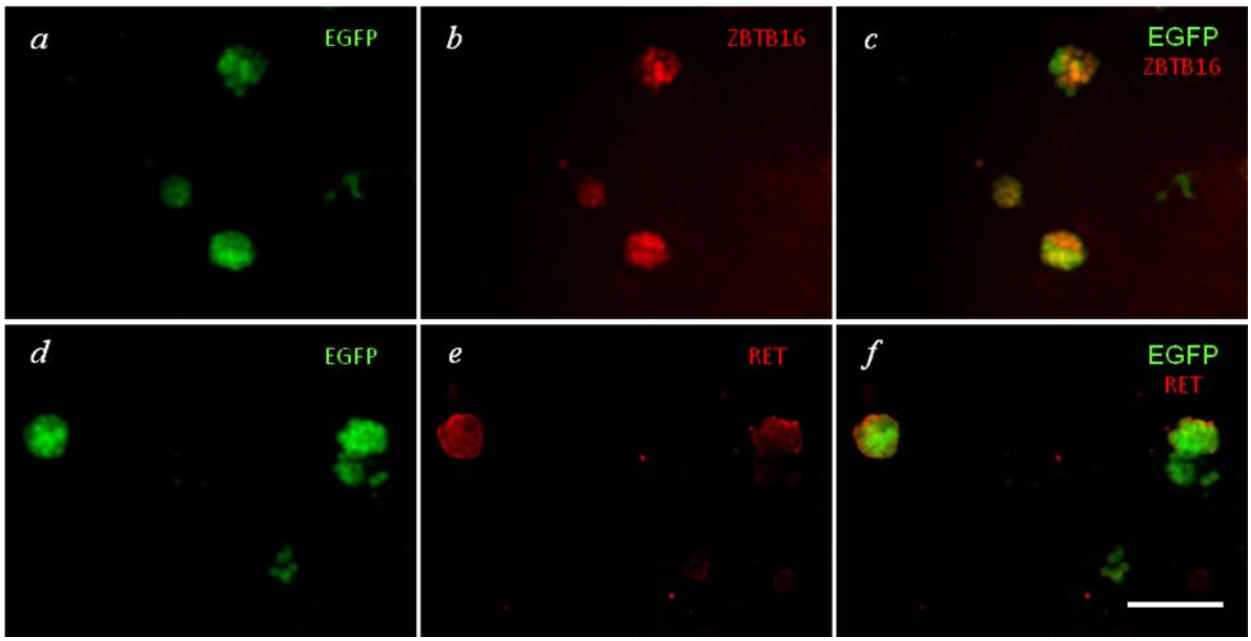
Supplemental Figure S1. Isolation of rat EGFP(+)- undifferentiated spermatogonia from PND7/8 pup testes by FACS. A brief overview of the strategy used to purify PND7/8 undifferentiated spermatogonia expressing EGFP. The lack of definitive cell surface markers for SSCs has hindered the purification of pure population of SSCs. However, we established an alternative approach based on FACS purification of undifferentiated spermatogonia from GCS-EGFP transgenic rats. **a**) Postnatal Day 7/8 GCS-EGFP expressing testis. **b**) Whole-mounted seminiferous tubule from PND7/8 GCS-EGFP testis. Only undifferentiated spermatogonia are EGFP-positive. **c**) Single cell suspension of GCS-EGFP testicular germ cells. **d**) Representative FACS profile from testicular germ cell suspension of GCS-EGFP germ cells.


a
b
c
d
GFP Oct 21 2008-PND7/8
Neg
Pos
Count
GFP FITC-A
FACS

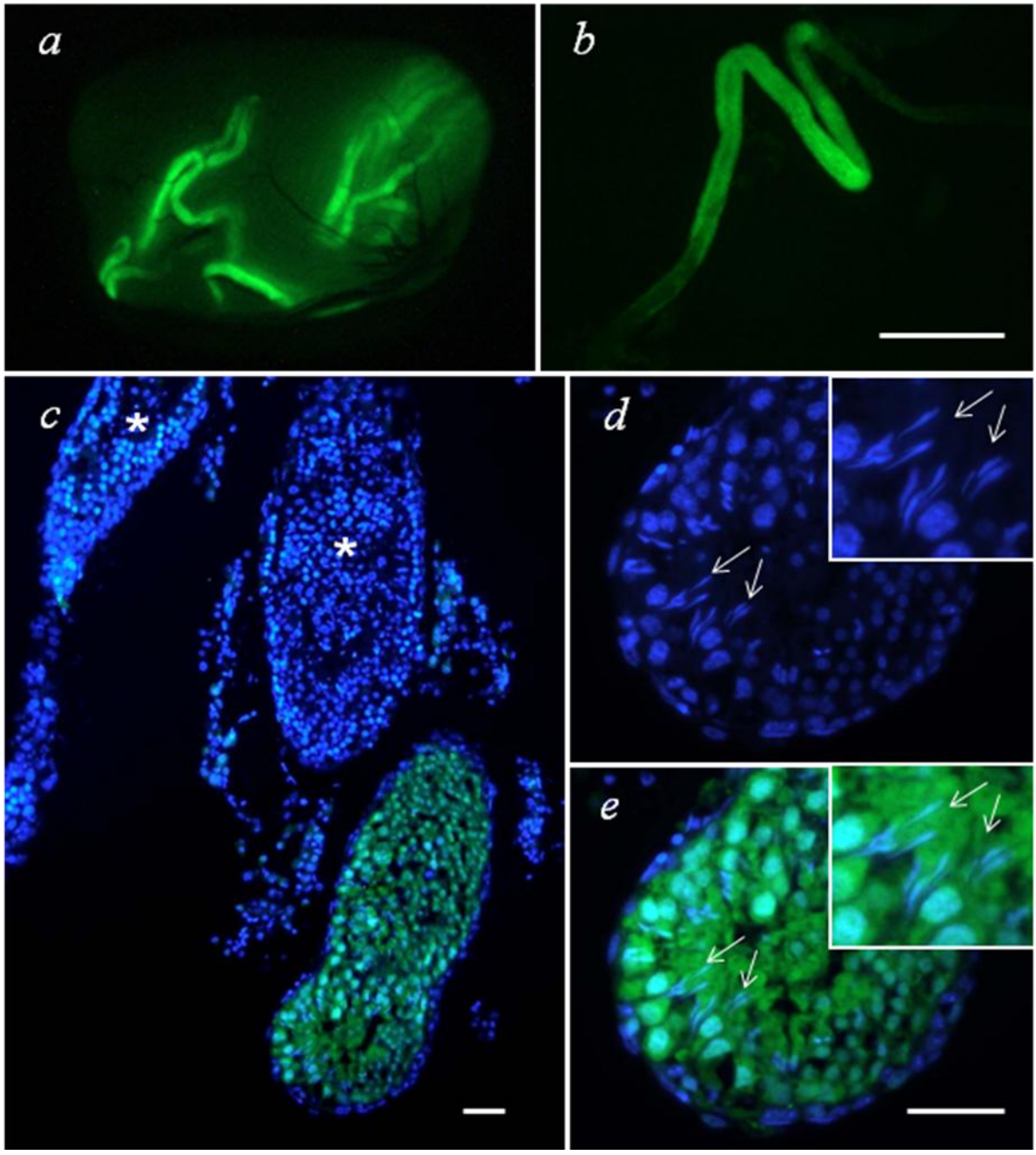
Supplemental Figure S2. Rat EGFP+ clusters developed only from EGFP-positive sorted cells. **a**, **b**) EGFP(+) sorted cells form clusters when cultured with GDNF, GFRA1, and FGF2 after 6 days. **c, d**) EGFP(-) sorted cells were cultured under the same conditions. However, after 6 days of culture, no clusters were observed. Bar = 100 μm.


EGFP
EGFP / Phase contrast
EGFP(+) sorted cells
EGFP(-) sorted cells
a
b
c
d

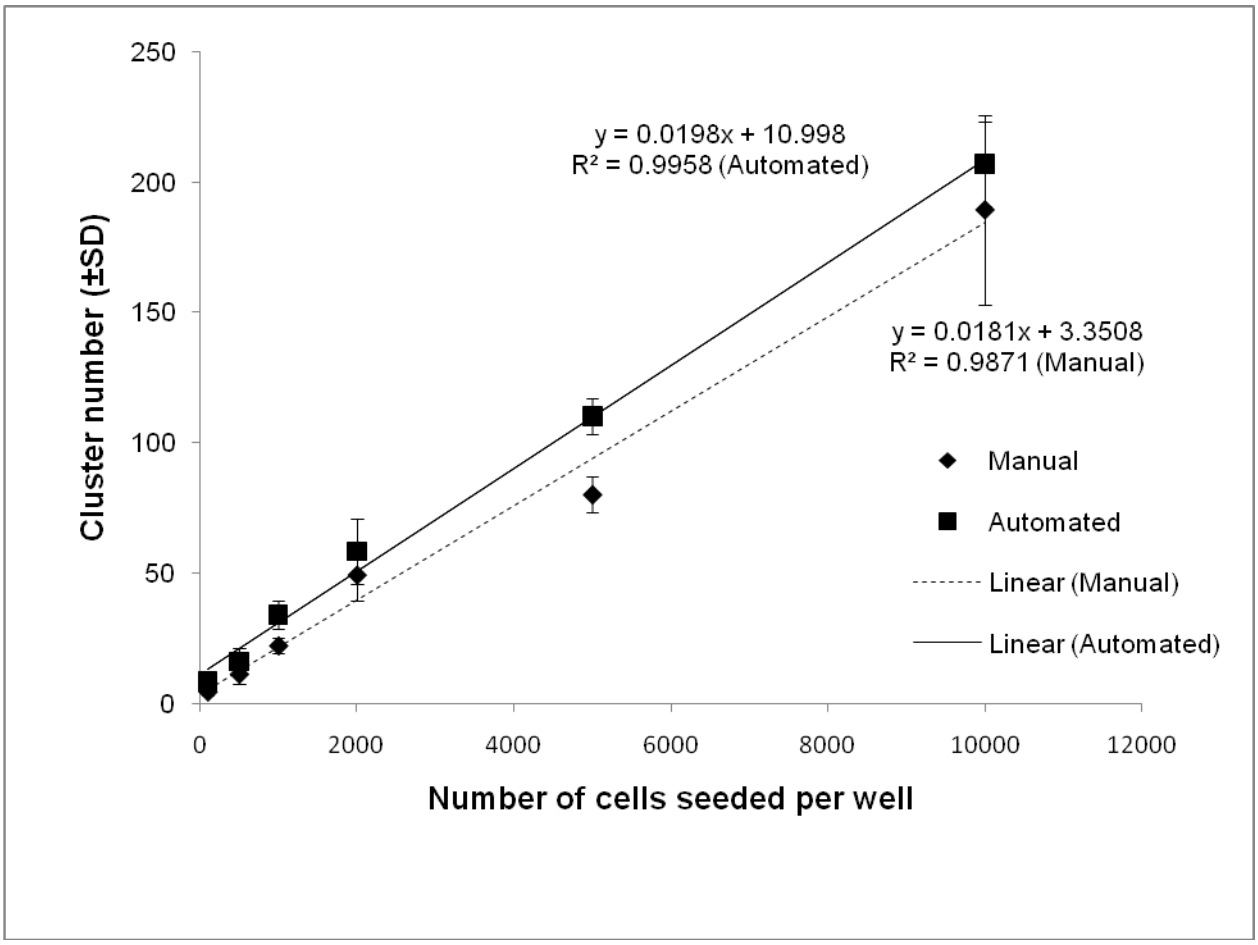
Supplemental Figure S3. Phenotypic characterization of rat EGFP+ clusters. Cultured rat EGFP+ clusters express (**a**-**c**) ZBTB16, also known as PLZF, a transcriptional repressor essential for self-renewal, and (**d**-**f**) the GNDF receptor c-RET; in vivo the expression of both markers is restricted to undifferentiated spermatogonia. Bar = 100 μm.


a
EGFP
b
ZBTB16
c
EGFP
ZBTB16
d
EGFP
e
RET
f
EGFP
RET

Supplemental Figure S4. Colonization of busulfan-treated nude mouse testes by cultured rat EGFP+ clusters. To test the ability of rat EGFP clusters to form colonies of spermatogenesis, single-cell suspensions of rat cultured EGFP+ cluster cells were transplanted into the seminiferous tubules of busulfan-treated immunodeficient mouse testes. Three months after transplantation, recipient testes were collected and analyzed for spermatogenic colony formation. **a**) Fluorescence photomicrograph of a recipient testis showing colonization of seminiferous tubules by EGFP-cluster cells. Each individual EGFP-positive colony represents the clonal expansion of a single SSC. **b**) Representative image of an individual EGFP+ colony of spermatogenesis derived from cluster cells following transplantation. **c**–**e**) Cross-sections of paraffin-embedded segments of seminiferous tubules containing a cluster-derived EGFP+ colony; (**c**) Merged DAPI and GFP images showing colonized and non-colonized tubules (*). **d**, **e**) Generation of rat spermatogenesis in mouse tubules. Note that elongated spermatids/spermatozoa were observed (arrows) within tubules, demonstrating that complete spermatogenesis was resumed. Thus, rat EGFP-positive spermatogenic colonies with functional spermatogenesis were clearly detected after transplantation of cultured rat cluster cells, indicating SSC activity among cultured cluster cells. Original magnification x2.5 (**a**); bars = 1 mm (**b**) and 100 μm (**c**–**e**).


a
b
c
*
*
d
e

Supplemental Figure S5. Manual and automated counts of clusters per well showed very similar linear relationships, suggesting that results obtained with the automated system faithfully reflect the actual number of clusters.


y = 0.0198x + 10.998
R² = 0.9958 (Automated)
y = 0.0181x + 3.3508
R² = 0.9871 (Manual)
Manual
Automated
Linear (Manual)
Linear (Automated)
Cluster number (±SD)
Number of cells seeded per well
0
50
100
150
200
250
0
2000
4000
6000
8000
10000
12000

Supplemental Figure S6. γH2AX immunofluorescent staining of cisplatin-treated rat EGFP+ clusters. Clusters were co-stained with anti-GFP and anti-γH2AX antibodies after 48h treatment with 0.01 μM (**a**, **b**), 0 .1 μM (**b**, **c**) and 1 μM (**e**,**f**) cisplatin. **a**, **c**, and **e**) Representative γH2AX staining of cisplatin-treated clusters (red). **b**, **d**, and **f**) Merged images of γH2AX staining with EGFP+ clusters (green) indicating that γH2AX stained cells (arrows) located at the edge of clusters. Bar = 100 μm.

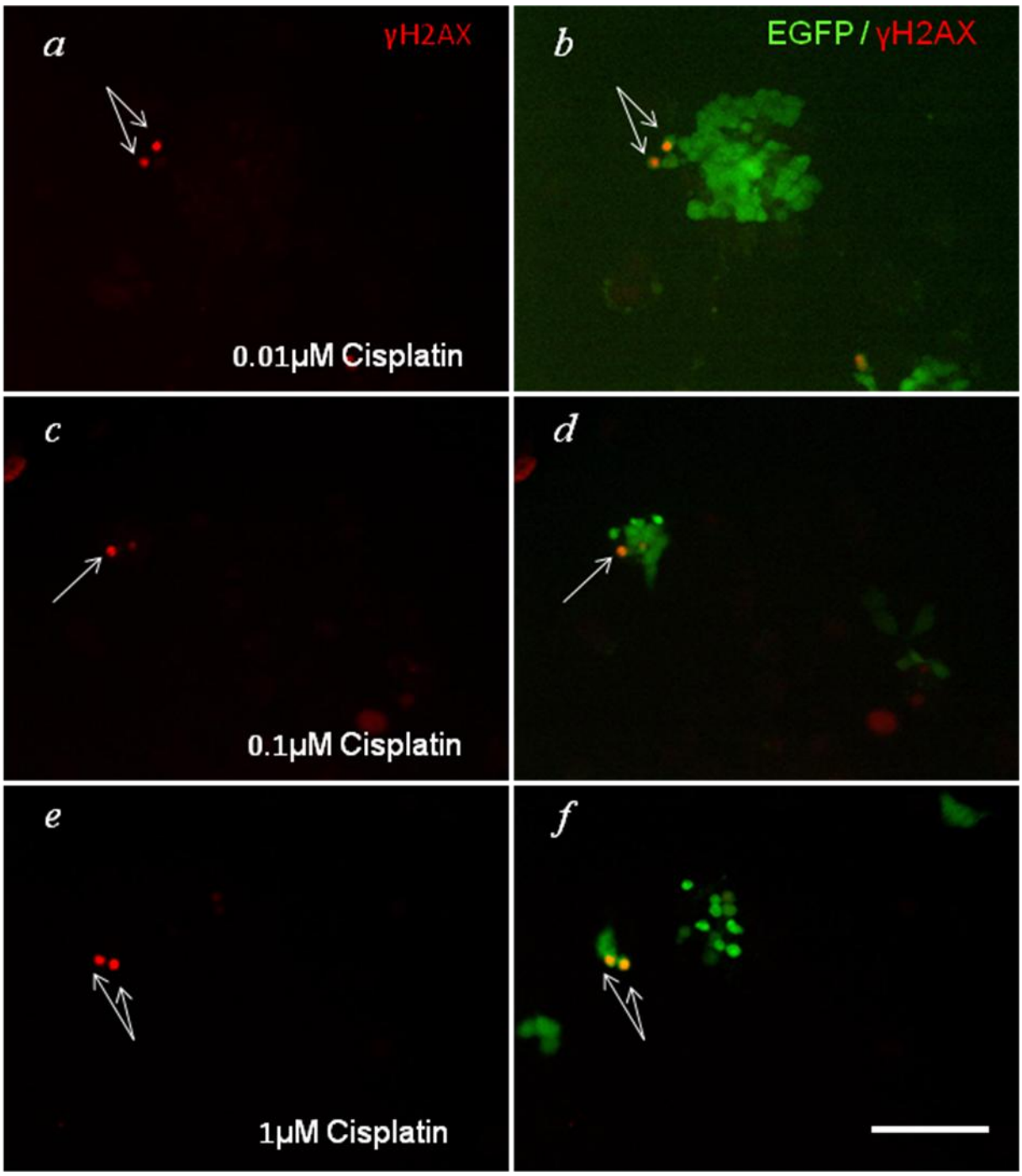

a
γH2AX
0.01μM Cisplatin
b
EGFP / γH2AX
c
0.1μM Cisplatin
d
e
1μM Cisplatin
f

Table 1.Comparison of IC_{50} values of three anticancer drugs with rat $EGFP^+$ stem/progenitor spermatogonia based on cluster numbers and cluster total area.

Anticancer agent	IC_{50} (μM)	
	Cluster numbers	Cluster area
Cisplatin	1.88 ± 0.54	1.40 ± 0.49
Etoposide	0.023 ± 0.0028	0.015 ± 0.005
Bleomycin	0.197 ± 0.015	0.241 ± 0.13

[a] Values are means ± SEM (n= 3)

[b] No significant differences were observed between cluster number and cluster surface area.

Chapter 6

Discussion

Over the years, advances in diagnostics and therapeutics have drastically improved life expectancy of men diagnosed with testicular cancer. As a result, long-term survival is now achieved in a majority of patients primarily due to effective chemotherapy. BEP regimen has become the first choice of chemotherapy due to its excellent efficacy and constant efforts have been made to optimize this treatment regimen in order to maintain its efficacy while minimizing toxicity and reducing duration of therapy. However, fertility after BEP chemotherapy is often impaired with most men experiencing azoospermia or oligozoospermia as common outcomes. Consequently, there has been an increasing interest in the issues affecting post-treatment quality of life of testicular cancer survivors. Since there is yet insufficient information on the health of testicular cancer offspring, we initiated these studies to understand how BEP chemotherapy potentially affects spermatogenesis, fertility and progeny outcome using the rat as an animal model. Furthermore, animal model studies provide a unique opportunity to account for chemotherapy-related effects alone without those of the cancer itself on male reproductive function and progeny outcome.

Establishment of an animal model for BEP regimen

The main objective of this thesis was to determine the adverse effects of the co-administration of bleomycin, etoposide and cisplatin based on the BEP regimen used in clinic for testicular cancer on the male rat reproductive function and progeny outcome. Toxicological studies in

animal models have been previously done to determine the adverse effects of bleomycin, etoposide or cisplatin on the male reproductive system when administered individually [1-5]. However, to the best of our knowledge, experimental use of these drugs in a schedule and doses mimicking the BEP regimen in such models had not been undertaken prior to our studies. In the second chapter of this thesis, we first established and described a novel animal model to evaluate the impact of the 9 weeks chronic BEP regimen on the male reproductive system, fertility and progeny outcome using adult Sprague-Dawley rats. According to the timing of spermatogenesis in the rat, a 9 weeks chronic administration allows for the exposure of developing germ cells throughout the entire spermatogenic process. Therefore, we anticipated that germ cells of the seminiferous epithelium to be particularly sensitive to the combination of these three drugs. In fact, when BEP treatment was administered to adult male rats, we observed a substantial decrease in testicular and epididymal weights concomitant with a decrease in the number of spermatozoa counted in the epididymis. The decrease in testis weight reflected the depletion of germ cells within the seminiferous epithelium. At the histological level, spermatogenesis was significantly affected when developing germ cells were exposed during 9 consecutive weeks to the BEP cocktail. The BEP treatment of adult male rats also resulted in decrease sperm motility, and increase in abnormal spermatozoa. Furthermore, although previous studies have shown that testosterone

production was affected by cisplatin [1, 6], the weights of other reproductive organs such as the seminal vesicles and ventral prostate were not significantly decreased, suggesting that the androgen production was not altered by the BEP treatment. Surprisingly, despite the abnormal spermatogenesis and drastic decrease in sperm count (nearly 90%), fertility and progeny outcome parameters including pre-and post-implantation loss, litter size, sex ratio, and fetal weights at gestational day 21 were similar to controls after BEP treatment. However, a significant number of pups sired by males treated with BEP showed early postnatal mortality but with no obvious fetal morphological abnormalities, suggesting that a delay in parturition may have occurred or that the progeny had functional abnormalities that were detected by the dam but were not easily visualized. These results clearly demonstrate, for the first time, that the BEP treatment has deleterious effects on the male reproductive system.

The studies done in chapter 2 did not establish whether the effects of BEP treatment were reversible. Therefore, in chapter 3, we investigated the long-lasting effects of a subchronic BEP treatment on the male reproductive system and progeny outcome. As expected, subchronic BEP treatment also reduced testicular weights and impaired spermatogenesis, characterized by abnormal testis histology and germ cell depletion. Furthermore, TUNEL staining of testis cross sections indicated that germ cells were undergoing apoptosis at a higher rate after BEP treatment whereas after 9 weeks of recovery germ cell apoptosis was similar in

control and BEP-treated testes. For instance, previous studies have shown that acute and chronic administration of cisplatin to mice resulted in increased germ cell apoptosis [7, 8], however, none of these studies had investigated the level of germ cells apoptosis several weeks after treatment. Consistent with the human scenario, spermatogenesis and sperm count recovered following cessation of BEP treatment. However, we observed that pre-implantation loss remained elevated in litter sired from BEP-treated males up to 9 weeks into the recovery period, suggesting that spermatogonia may be affected.

Based on the results presented in chapters 2 and 3, already two follow up studies have been done in our laboratory [9, 10]. In the first one, sperm samples collected from control and 0.3, 0.6 and 1X BEP-treated rats in chapter 2 were further analyzed for chromatin integrity using TUNEL, COMET and acridine orange-based assays [9]. Results of this study revealed that BEP treatment induced increased numbers of DNA strand breaks detected in spermatozoa from BEP-treated rats when compared with control spermatozoa. More importantly, sperm chromatin integrity was only significantly affected after 9 weeks of treatment with the highest dose of BEP, the 1x dose, indicating that spermatogonia were damaged and that this damage was left unrepaired and could later be detected in mature spermatozoa. To date, several methods have been reported to assess sperm chromatin quality in animal models and in clinical settings [11-14].

In the second study, the effects of BEP treatment on the gene expression profile of germ cells were investigated [10]. Spermatogenesis is a highly regulated process that requires precise gene expression in germ cells and Sertoli cells. Previous studies done in our laboratory showed that acute and chronic exposures to a single alkylating agent cyclophosphamide resulted in the alteration of the gene expression of stress response genes in male germ cells [15, 16]. Based on these studies, germ cell exposure to BEP treatment may also induce altered gene expression that may in turn interfere with normal development of spermatozoa. In fact, the effects of BEP treatment on gene expression in round spermatids have later been investigated using DNA microarrays [10]. Interestingly, BEP treatment was shown to significantly up- and down-regulate subset of genes involved in stress response.

However, in chapters 2 and 3, we did not establish whether spermatogonial stem cells (SSCs) were specifically affected by the BEP treatment. In chapter 4 and 5, particular emphasis was then placed on the characterization of the impact of BEP regimen specifically on SSCs in vivo and on stem/progenitor spermatogonia in vitro. In the mammalian testis, spermatogonial stem cells are the “parent” cells of all germ cells; they continuously produce millions of spermatozoa every day throughout adult life via self-renewal and differentiation into committed progenitor cells. SSCs are responsible for the transmission of genetic information to the next generation and, importantly, for the regeneration of spermatogenesis

following cancer chemotherapy. Since SSCs are potential targets of the BEP regimen, we next examined SSC activity following BEP treatment using spermatogonial transplantation.

Specific markers for SSCs do not currently exist, but a few molecular markers have recently been identified for undifferentiated spermatogonia, of which that SSCs are a sub-group, such as ZBTB16, GRFA1, NANOS2, UTF1 and CDH1 (E-CAD) [17-25]. In chapter 4, we used immunostaining of GRFA1 and ZBTB16 on whole-mounted seminiferous tubules to detect undifferentiated spermatogonia in control and BEP-treated testes. We observed that BEP treatment drastically reduced the number of A-aligned spermatogonia, while sparing A-single and A-paired from the effect. In human, BEP chemotherapy often induces azoospermia or oligozoospermia. Since little is known about the phenotypic and molecular characteristics of human undifferentiated spermatogonia, it would be of considerable interest to investigate the expression of these markers in testes from untreated and BEP-exposed patients.

In mice, the stem cell potential of undifferentiated spermatogonia expressing GFRA1 and ZBTB16 has been clearly demonstrated using techniques such as gene targeting and spermatogonial transplantation [17, 18, 21].However, the presence alone of GFRA1/ ZBTB16-positive spermatogonia after BEP treatment did not insure that SSCs had retained their ability to self-renew or their capacity to regenerate spermatogenesis.

Previous studies on the recovery of spermatogenesis after exposure to cytotoxic chemotherapeutic agents were generally based on sperm count and histological evaluation of damage induced to the germ cells of the seminiferous epithelium [3, 5]. Nonetheless, the effects on spermatogenesis or sperm counts might also reflect how the SSC environment responds to cytotoxic treatment, thereby failing to provide specific information on the capacity of SSCs per se to regenerate spermatogenesis. Damage to the immediate SSC environment, Sertoli cells, peritubular myoid cells or any other supportive constituents of the seminiferous epithelium such as the extracellular matrix components might indirectly alter the SSC ability to restore spermatogenesis. As seen in studies with radiation therapy, while isolated spermatogonia survive exposure, they fail to differentiate and to regenerate germ cell population due to damage to the somatic environment, leading to long-term or irreversible infertility [26, 27]. Therefore, the impact of BEP treatment on SSCs can only be dissociated from any potential adverse effects on supporting SSC environment through the transplantation assay in a heterologous recipient system previously unexposed to chemotherapy.

Using the spermatogonial transplantation assay, we demonstrated that BEP-induced decrease in GFRA1/ ZBTB16-positive spermatogonia was accompanied by a significant decreased in SSC colonization activity, indicating that although affected by BEP treatment, SSCs could survive and restore spermatogenesis. This observation was further confirmed by

the fact that no significant differences were seen in the number and length of spermatogenic colonies after the nine week recovery between control and BEP-treated testes, indicating that spermatogenesis can fully recover from surviving SSCs. Thus, it is likely that higher doses of the BEP cocktail might be required to produce more profound and permanent damages to SSCs.

As mentioned previously, BEP treatment may also affect constituents of the SSC niche. In the mammalian testis, stem cell fate and function are governed by an ensemble of physicochemical and biological factors from their local environment or niche [28, 29]. Among these factors, GDNF has been identified as a critical component for the maintenance of spermatogonial stem cells in vivo and in vitro [30, 31]. In a previous study [32], the level of GDNF expression in aging mouse testes was investigated using quantitative real-time RT-PCR analysis. Results showed that GDNF expression drastically declined in aging-infertile mice, suggesting an abnormal Sertoli cell function. Interestingly, in busulfan-treated testes, GDNF expression displayed a large increase, indicating that Sertoli cells responded to the germ cell loss by increasing the GDNF production. In future work, it would be interesting to assess the effects of BEP treatment on the GDNF gene expression.

With a view to developing new toxicity testing tools, we reasoned that the effects of BEP treatment could be further investigated with the help of in vitro spermatogonial stem/progenitor cultures. In chapter 5, we

described the development of a new fluorescence-based assay for rat stem/progenitor spermatogonia using SSC culture system and its application to evaluate anticancer drug toxicities. In the remainder of this chapter, I will first discuss the advantages and disadvantages of stem/progenitor spermatogonia cultures for use in a short-term in vitro cytotoxicity assay to screen toxic compounds and then describe some of the possible follow up experiments.

6.1 Advantages of SSC culture systems for toxicity testing in vitro

Reproductive toxicology studies such as those described in chapter 2, 3 and 4 are time-consuming, labor-intensive and particularly expensive because of the large number of animals to be used and their prolonged housing. Considering the cost and time-consumption associated with the transplantation procedure, an in vitro system with the ability to assess toxicity of pharmaceutical and environmental compounds on stem/progenitor spermatogonia would be of considerable interest. In addition, cell-based culture systems offer several advantages for assaying the cytotoxicity of anticancer drugs; they provide a rapid, efficient and cost effective assay system to investigate cytotoxic effects of the large number of pharmaceutical and environmental chemicals. The recent ability to culture rodent stem/progenitor spermatogonia for long periods of time may provide a valuable tool to evaluate the toxicity potential of any xenobiotic in vitro.

6.2 Culture systems for stem/progenitor spermatogonia for toxicity testing

The development of in vitro cell-culture systems for the long-term maintenance and propagation of rodent SSCs has provided a relatively simple and robust means to study the biology of SSCs [31, 33-37]. In such culture systems, SSCs divide and form three-dimensional aggregates of stem/progenitor spermatogonia, termed clusters. In a recent study, Yeh and colleagues used such culture system as a short-term in vitro assay for SSCs and demonstrated that cluster numbers in culture faithfully reflect numbers of functional SSCs determined by transplantation in recipient testes [38]. Based on this study, it was suggested that SSC culture systems could be used as an alternative approach to determine SSC activity in a semi-quantitative manner simply by counting clusters. However, these in vitro culture systems have not been previously to our knowledge applied to estimate toxicity of anticancer drugs on stem/progenitor spermtogonia. Our proposal was to develop a new in vitro assay based on stem/progenitor spermatogonial cultures in order to evaluate the toxicity of bleomycin, etoposide and cisplatin, the three anticancer drugs used in the BEP regimen.

To this end, we first established cultures of rat $EGFP^+$ stem/progenitor spermatogonia. We isolated populations of $EGFP^+$ SSC-enriched testis cells from single-cell suspensions of GCS-EGFP transgenic postnatal pup testes using FACS. Several methods including FACS, MACS sorting or differential plating have been reported to isolate

SSC-enriched testis cell populations from rodents [31, 33, and 37]. Importantly, previous studies have demonstrated that the fractionation of testis germ cells to enrich for stem cells and remove testicular somatic cells is essential to establish cultures of stem/progenitor spermatogonia [31, 33 and 39]. Albeit yielding relatively small number of cells, our current FACS-based strategy has enabled us to isolate almost pure populations of stem/progenitor spermatogonia (> 99% $EGFP^+$ cells) and thereby rapidly initiate our cultures.

In vitro toxicity assays generally necessitate relatively large numbers of cells [40]. As reported by Ryu et al., 2005, rat SSCs can be expanded for a long period in vitro when cultured on STO feeder cells, in a serum-free medium containing growth factors (GDNF, GFRa1 and FGF2) [33]. Therefore, to collect enough cells to do subsequent assays, isolated EGFP-expressing testis cells were cultured for at least 5 weeks using the conditions previously described [33]. As expected, we observed the formation of germ cell clusters composed of EGFP-expressing stem/progenitor spermatogonia after 6 days of culture.

Several culture parameters may influence cluster growth and proliferation such the type and concentration of growth factors, pH and oxygen tension [33]. In future studies, these parameters could be varied in order to define culture conditions that are optimal for rat SSC expansion and thereby rendering rat stem/progenitor spermatogonial cultures more amenable to large-scale experiments.

6.3 Automated analysis

In the assay described by Yeh et., al 2007, clusters were counted manually using a bright-field microscope [38]. However, the use of direct observation as a means of enumerating clusters is time-consuming and labor intensive. In fact, if it is to be used in a cytotoxicity assay, this way of counting clusters would greatly limit the number of assay conditions such as the number of different drugs and their concentrations that could be reasonably analyzed at once by a single observer. To address this situation, we reasoned that an automated imaging system could be used to detect and count $EGFP^{+}$ clusters cultured in 96-well plates. We employed the $ImageXpress^{MICRO}$ imaging system from Molecular Devices to capture images of clusters cultured in 96-well plates. For quantitative analysis, images were analyzed via the MetaXpress software by using masks specifically design to discriminate fluorescence signal intensity and size in order to count the number of rat $EGFP^{+}$ clusters per well. To our knowledge, this was the first description of an automated fluorescence-based method to enumerate clusters of stem/progenitor spermatogonia in culture.

Such an in vitro assay has a number of important variables, including initial seeding density of cells and the intrinsic ability of seeded cells to regenerate clusters that may influence the readouts. Based on our serial dilution experiments, we determined that 5000 cells per well as the suitable cell seeding density since it is within the linear range and

produces a sufficient number of clusters for automated microscopy and image analysis at 6 days after plating; therefore we used this plating density as starting cell density in our cytotoxicity studies. However, it is likely that higher seeding densities may facilitate cell-cell interactions thereby promoting cell aggregation and cluster formation. Thus, the seeding of constant number of cells per well with equivalent cluster forming ability is necessary between experiments to ensure reproducibility and to prevent biased results.

At the time of analysis, clusters appeared well separated from each other and distributed evenly over the area of each well. However, since clusters are likely to be moving entities within culture wells, there is still the possibility that some clusters might have been resulting from the fusion of one or more clusters. Although our serial dilution experiments showed that a strong linear correlation existed between cluster numbers and the number of cells initially seeded, suggesting that clusters were of clonal origin, further studies will be required to ascertain that cultured rat $EGFP^+$ clusters are indeed clonal.

6.4 Feeder-free cultures

Currently, clusters of rat stem/progenitor spermatogonia are often cultured and maintained adherently on a layer of STO feeder cells. Although mitotically arrested with Mitomycin C, feeder cell functions may be affected by the anticancer drugs we have tested. Therefore, data obtained from cluster count and surface area can represent both direct as well as indirect

effect on cultured spermatogonia and feeder cells. In this regard, the development of culture protocols, where clusters of stem/progenitor spermatogonia could be efficiently propagated and maintained under feeder-free conditions would be extremely useful to avoid such caveat. To date, one study has attempted to develop feeder-free culture conditions for spermatogonial stem cells [44]. In other stem cell culture systems, such as human embryonic stem cells, feeder cells were replaced by specific substrates including collagen, laminin or extracellular matrix preparations (Matrigel) [41]. Recently, Kanatsu-Shinohara and colleagues have developed a new technique for culturing mouse SSCs in the absence of feeder cells without adding serum to the culture medium [52]. Hypothesizing that rat SSCs might be cultured using similar conditions, this would imply that effects of drug treatment on rat stem/progenitor spermatogonia could be directly assessed.

6.5 Experimental design

In the present work, we assessed the cytotoxicity of three anticancer agents, cisplatin, etoposide and bleomycin on cluster cultures of rat stem/progenitor spermatogonia using only one experimental design. However, many variations in experimental design including starting cell concentration, timing and duration of exposure may be applied to this assay.

In our assay, single-cell suspensions from cluster cultures were seeded into 96-well plates at day 0 and grown under standard conditions

for 4 days prior to the addition of the test compounds for 48 h. In such design, the clusters in culture must be dissociated into single cell suspensions before plating; consequently, cluster cells are subjected to procedures including trypsinization, detachment from feeder cells, reattachment and then regrowth. Since these procedures might be expected to interfere with the outcome of the cytotoxic assay, we chose to add the test drugs after 4 days of culture under normal conditions so that the clusters were allowed enough time to resume their growth.

For instance, protocols for investigating the impact of anticancer drugs on cluster growth could be also developed. As an example, dissociated cluster cells could be cultured for 24 hours, a period of time sufficient for cluster cells to reattach to the feeder cells and resume growth; then test compounds could be added to the culture medium. After one day of exposure, the medium would be discarded; the clusters rinsed and incubated with normal medium for the rest of the culture period. This experimental design would allow for clusters to recover from the toxic exposure for an additional 4 days under normal culture conditions. Thus, the design of our cluster-based assay can be modeled in various ways to gain further insights on the impact of drugs on stem/progenitor spermatogonia in vitro.

6.6 In vitro - in vivo correlations

As a proof of principle, we have demonstrated that rat SSC cultures could be used to assess anticancer drug toxicities in vitro. However, an

important remaining question is whether this assay could be used to reliably predict the toxicity of anticancer drugs on SSCs in vivo. In chapter 5, we exposed clusters of stem/progenitor spermatogonia to bleomycin, etoposide and cisplatin and determined IC_{50} values for each of these drugs based on both the number of clusters and their surface area. However, to date, we cannot ascertain whether the 50% decreases in cluster numbers (IC_{50} value) determined in vitro for each drug will correspond to a 50% decrease in stem cell activity in vivo. Since clusters consist not only of SSCs, but also of a large number of progenitor spermatogonia, there is a possibility that the drug-associated decreases in cluster numbers and area may reflect a predominant loss of progenitor cells rather than stem cells. To determine whether the SSC activity of cells kept in culture is affected by drug exposure, in vivo transplantation assays are required. By combining in vitro propagation of SSCs followed by in vivo transplantation into host testes, previous studies have identified several genes contributing to SSC self-renewal in mouse [35, 36]. Using spermatogonial transplantation, we could determine unequivocally the effects of anticancer drugs on SSCs after in vitro exposure. In future experiments, cluster cultures could be exposed to concentrations of drugs corresponding to their respective IC_{50} values, and then transplanted into recipient testes. If the decrease in number of colonies obtained after transplantation correlates well with the reduction in cluster numbers, this would demonstrated that our assay can be used to assess SSC activity.

6.7 Other types of markers

It is likely that cluster cells initially respond to drug treatment (bleomycin, etoposide and cisplatin) by sensing DNA damage, then undergoing cell cycle arrest, and finally inducing apoptosis-leading to a reduction in cluster numbers and area. Microscopy images of clusters taken after 24 hours of drug exposure showed the presence of many detaching cells with abnormal morphology lining around clusters thereby indicating the detachment of cells from clusters shortly after drug exposure. Hence, it is possible that cell aggregation of clusters offers a certain degree of protection to cells hidden within the aggregate.

In the present thesis, cell proliferation of stem/progenitor spermatogonia within clusters was measured by labelling proliferating cluster cells with an anti-phosphorylated histone H3 at serine 10 antibody and quantifying the number of pH3-positive cells per cluster using automated image analysis. Similarly, DNA-damaged cells in clusters were detected using an anti-phosphorylated histone H2AX antibody and then automatically counted. Interestingly, we observed that the distribution for pH3- and pH2AX-positive cells differs within clusters. While the pH3-positive cells were located throughout the clusters, pH2AX-positive cells appeared to be preferentially confined to the outer edges. Using those markers, we observed low overall numbers of both pH3 and pH2AX-positive cells per clusters.

A variety of methods are available for measuring cellular proliferation or apoptosis endpoints in in vitro cytotoxicity assays. Although it would have been interesting to measure the proliferation and apoptotic activity of rat cluster cells by means of other markers or assays, we were limited in our experimental setup by the presence of feeder cells. Therefore, cell proliferation assays that require cell disruption such as ATP or, MTT assays, could not be applied to our assay.

For instance, other markers such as cleaved-caspase 3, TUNEL staining or Annexin V binding assay could be used to assess apoptosis index in clusters. Thus, we have shown that it is possible to measure the number of proliferating and DNA-damaged cells in cluster cells cultured in 96-well plates with or without exposure to anticancer drugs. Yet, detailed analysis of the underlying mechanisms of drug effects was beyond the scope of this study and clearly requires further work.

6.8 Other species –same approach

Conveniently, throughout this thesis, we were able to use the rat as an animal model and source of cells for our in vivo and in vitro experiments, respectively. SSC culture systems have also been developed for other rodent species such as mouse and hamster [31, 42]. Therefore, evaluating the cytotoxicity potential of pharmaceutical compounds such as anticancer drugs on SSCs should be adaptable for use with any of these other rodent SSC culture systems. For instance, since transgenic mice that express the marker gene enhanced GFP have

been previously generated, cultures of mouse $EGFP^+$ clusters could be used in our in vitro toxicity assay [43, 44]. In fact, mouse SSC cultures may also be a useful model to test the toxicity of anticancer drugs. One major advantage to use mouse SSC cultures for in vitro cytotoxic assays is that mouse SSCs have a much shorter doubling time of 5 to 6 days compared to the 10-21 days for rats; that, combined with the ease of culture would allow for the production of large numbers of cells more rapidly. In addition, since the culture conditions used to maintain mouse and rat SSCs are very similar, treatment of mouse clusters would enable the comparison of anticancer drug effects between these two species and potentially provide further confirmation of the results we obtained in our rat in vitro studies.

6.9 Culture of human spermatogonial stem cells and potential applications in clinic for reproductive interventions and in toxicity screening

Over the past decades, survival rates achieved in childhood cancer have continuously improved owing to advances in cancer diagnostics and therapeutics [45, 46]. Consequently, an increasing number of survivors are currently among the young adult population. Male survivors will potentially face adverse effects including infertility as a result of their cancer treatments. Infertility is a devastating side/late effect of chemotherapeutic treatments particularly in the case of disease-free patients that wish to start a family [47]. Sperm banking or semen cryopreservation before initiating cancer therapy is often considered as the obvious choice for

preserving fertility of adult male patients, but unfortunately, this cannot be an option for younger patients such as prepubertal boys as they do not yet produce semen or mature spermatozoa. Recently, autologous (self-donated) transplantation of human SSCs has been proposed as a potential clinical application for restoring spermatogenesis in infertile cancer survivors following their treatments [48, 49]. Although being a well established method in various species such as mice, rats, pigs, goats and dogs, the benefit of human SSC transplantation for restoring fertility in cancer survivors still remains hypothetical. Yet, cryopreservation of testicular tissues or germ cell suspensions that contain SSCs may represent the only alternative for future fertility restoration therapy childhood cancer patients. In a potential future scenario, testicular tissues from prepubertal boys could be collected and stored prior the initiation of cancer treatments. Once their treatment completed and cancer-free, patients could then have their own SSCs transplanted into their testes to regenerate spermatogenesis. Interestingly, Ginsberg and colleagues have recently reported that testicular tissues from prepubertal boys diagnosed with cancer can be retrieved with minimal surgical stress and subsequently cryopreserved prior to cancer treatment and that such procedures were well accepted by a majority of parents [49]. However, the volume of testicular tissue harvested by diagnostic biopsy is usually low (50- 100 mg), meaning that only a limited number of SSCs, possibly not sufficient for transplantation, would possibly be retrieved from it.

Therefore, in vitro expansion of human SSCs will be a prerequisite for the production of large number of SSCs necessary for transplantation. Since the success of human SSC transplantation will likely depend on obtaining suitable number of SSCs through in vitro expansion, research efforts are currently made toward developing human SSC culture from testicular biopsies. Recently, Sadri-Ardekani et al., 2009, have succeeded in the attempt to propagate and expand human adult SSCs in vitro from testicular biopsies [50]. Using testicular cell cultures, they obtained germ cell clusters similar to those observed in mouse or rat cultures. More importantly, these human germ cell clusters were subcultured on human placental laminin-coated dishes for up to 28 weeks, thereby demonstrating that human clusters can be maintained and propagated even in the absence of feeder cells. Like in rodents, cultures of human SSCs may also provide a valuable tool for drug toxicity testing. For example, human clusters could be exposed to anticancer drugs, stained with a fluorescent dye (DAPI or propidium iodide) and then analyzed using automated fluorescence microscopy in the same manner we used for rat clusters.

6.10 Final Conclusions

The results presented in this thesis have contributed to understand how the BEP treatment adversely affects male rat reproductive function, progeny outcome and in vivo SSC activity. Furthermore, the development of a new in vitro method-based on cultured stem/progenitor spermatogonia using an automated microscopy and image analysis has provided a fast,

robust and reproducible method to assess toxicity of the three anticancer drugs used in the BEP regimen, suggesting that this assay may provide the basis for a model for stem/progenitor spermatogonial toxicity in vitro. In future studies, it would clearly be of significance to expand the testing of additional anticancer drugs in order to identify drugs with the highest levels of toxicity to spermatogonial stem/progenitor cells. In addition, one potential application of such assay could be the identification of compounds regulating self-renewal and differentiation of SSCs using high-throughput screening of chemical libraries. Finally, with the recent discovery of culture conditions for human SSCs, it is likely that in the near future, human SSC culture will be used in such in vitro screening assays.

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications. It comprises of six chapters, four of which are manuscripts written in the form they were or will be submitted. Published articles were reproduced with the permission from their copyrights holders.

I. Bieber AM, **Marcon L**, Hales BF, Robaire B. Effects of chemotherapeutic agents for testicular cancer on the male rat reproductive system, spermatozoa, and fertility. J Androl. 2006 Mar-Apr;27(2):189-200.

II. **Marcon L**, Hales BF, Robaire B. Reversibility of the effects of subchronic exposure to the cancer chemotherapeutics bleomycin, etoposide, and cisplatin on spermatogenesis, fertility, and progeny outcome in the male rat. J Androl. 2008 Jul-Aug;29(4):408-17.

III. **Marcon L**, Zhang X, Hales BF, Robaire B and Nagano MC. Effects of chemotherapeutic agents for testicular cancer on rat spermatogonial stem/progenitor cells. *Submitted to Journal of Andrology*.

IV. **Marcon L**, Zhang X, Hales BF, Nagano MC and Robaire B. Development of a short-term fluorescence-based assay to assess the toxicity of anticancer drugs on rat spermatogonial germ cells *in vitro*. Biol Reprod. 2010 Aug 1;83(2):228-37.

APPENDIX