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Journal of the National Cancer Institute

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Abstract

Some chemotherapy regimens include agents that are mutagenic or clastogenic in model systems. This raises concerns that cancer survivors, who were treated before or during their reproductive years, may be at increased risks for abnormal reproductive outcomes. However, the available data from offspring of cancer survivors are limited, representing diverse cancers, therapies, time-to-pregnancies, and reproductive outcomes. Rodent breeding data after paternal exposures to individual chemotherapeutic agents illustrate the complexity of factors that influence the risk for transmitted genetic damage including agent, dose, endpoint, and the germ-cell susceptibility profiles that vary across agents. Direct measurements of chromosomal abnormalities in sperm of mice and humans by sperm FISH have corroborated the differences in germ-cell susceptibilities. The available evidence suggests that the risk of producing chromosomally defective sperm is highest during the first few weeks after the end of chemotherapy, and decays with time. Thus, sperm samples provided immediately after the initiation of cancer therapies may contain treatment-induced genetic defects that will jeopardize the genetic health of offspring.

Introduction

The continuing search for cancer cures has produced chemotherapies that have significantly increased survival among certain cancer groups. Over 70% of individuals now survive childhood cancer and this proportion continues to increase [1]. As more survivors of childhood cancers and cancers in the reproductive years regain their fertility after treatment, there are concerns that the therapy may have induced germ-line mutations that increase the risks of birth defects, genetic diseases or cancer among the children of cancer survivors. These concerns are motivated by two major lines of evidence: (a) doses used for human chemotherapies are in the ranges known to be mutagenic in animal models [2] and (b) several chemotherapies have been shown to induce chromosomal abnormalities in sperm of treated patients [3, 4].

Studies evaluating genetic diseases among the offspring of cancer survivors who received chemo- or radio-therapies have found little evidence for elevated risks of chromosomal abnormalities [5] or genetic diseases in the offspring [6-10]. Although these findings are reassuring, the offspring data have major limitations [11]: (a) they include patients who received both mutagenic and nonmutagenic regimens with broad differences in drug regimens, doses, exposure duration, etc. with small numbers of children born to survivors of any specific treatment regimen, (b) the endpoints evaluated in pregnancies and offspring have been diverse, with few data for any one endpoint, and (c) most patients were treated as children so that pregnancies under investigation occurred long after treatment and do not reflect pregnancies that may result from exposure to germ cells at more sensitive time windows of gametogenesis. These variables

have precluded reliable estimates of relative reproductive risks for survivors of treatments with specific chemotherapeutic agents.

The generally negative human offspring data need to be better reconciled with the generally positive data for mouse breeding studies, as well as rodent and human sperm FISH assays for induced chromosomal abnormalities. The purpose of this paper is to provide a brief overview of (a) the rodent breeding data for heritable effects after paternal exposure to chemotherapeutic agents, (b) the human and rodent data for chemotherapy-induced chromosomal abnormalities in sperm, and (c) to identify areas for additional research and clinical recommendations regarding germ cell susceptibilities after cancer chemotherapy.

Spermatogenesis and types of genetic damage transmitted via sperm

Spermatogenesis is a highly regulated differentiating system, both temporally and spatially. The spermatogenic stem cells differentiate through division of spermatogonia (mitotic divisions) to form spermatocytes (meiotic cells) that undergo two meiotic divisions to give rise to spermatids (haploid postmeiotic cells) that mature into functional sperm. The kinetics of spermatogenesis are well established for men and several mammalian species [12] and are remarkably constant within species, so that the time between treatment and sampling of sperm can be used as a surrogate for sampling effects of chemotherapy on specific spermatogenic cell types. The longer the time interval between treatment and sampling, the earlier in spermatogenesis the effects are being sampled.

Physiological damage of chemotherapy to male germ cells, which has been strongly associated with fertility, is primarily monitored by parameters of semen quality. Exposure to more than 100 chemicals, individually or as mixtures, including chemotherapeutics are known to induce detrimental effects on sperm morphology, number, and motility [13]. However, we understand very little about the effects of chemotherapy on the mechanisms and frequency of sperm defects that might increase the risks of genetic or chromosomal abnormalities among offspring (Table 1) [14]. Sperm defects induced by chemotherapy in model systems include whole- and segmental chromosomal aneuploidies that can results in complete or partial trisomy in offspring, respectively. The molecular targets for whole chromosomal aneuploidy (e.g., centromere, microtubules, chromosome pairing) are not thought to involve mutational mechanisms. *De novo* segmental aneuploidy in germ cells, on the other hand, involves double-strand DNA breaks that can arise spontaneously, especially in post-meiotic cells [15], or can be induced by exposure to mutagens [16, 17]. Sperm carrying defects in the imprinting profiles are theoretically important because altered expression of a paternal gene during critical stages of development might result in abnormal development or defects. Trinucleotide repeat length variation appear to be inducible in male germ cells after exposures to ionizing radiation [18] or environmental pollution [19], but there is no information on the effects of chemotherapeutic agents. More research will be needed to understand the underlying mechanisms of induction and their heritable consequences.

Animals breeding tests for heritable effects of chemotherapeutic agents

As most dramatically demonstrated in rodents, when males are treated with a mutagen and mated with unexposed females, the deleterious effects on reproduction can be profound, including infertility, lethality during development, as well as heritable chromosomal translocations, malformations or cancer among offspring. Over the past 40 years, more than 30 chemicals have been tested in mice for germ cell mutagenicity using three major tests [2, 20]: dominant lethal (DL), heritable translocation (HT) and specific locus mutation (SLM). DL measures the induction of unstable chromosomal aberrations that lead to the embryonic death of the progeny of treated males, however, other genetic and epigenetic mechanisms cannot be excluded. The HT and SLM tests measure the induction of chromosomal reciprocal translocations and gene mutations in the offspring of treated males, respectively. Table 2 lists the chemotherapeutic agents that have been tested in at least two of these breeding tests (see [2] for an in-depth discussion of all the germ cell mutagenicity results with chemotherapeutic agents).

Several points can be drawn from these studies regarding differential susceptibilities of male germ cells to chemotherapeutic agents. First, mutagenic chemotherapies generally induce a wide spectrum of lesions resulting in various chromosomal abnormalities and/or gene mutations. Second, most chemotherapeutic drugs induce positive results in all three tests. One notable exception is 6-mercaptopurine which affected only preleptotene spermatocytes, inducing DL but not HT or SLM [21]. Third, there are differences among the various phases of spermatogenesis in the sensitivity of induction of transmissible genetic damage. With the exception of etoposide, all treatments produced the highest response, if not exclusively, in postmeiotic cells. The high sensitivity of postmeiotic cells is probably related to the reduced DNA repair

capacity of late spermatids and sperm when compared with early spermatids and the other spermatogenic cell types [22]. Therefore, unrepaired DNA damage induced in these late stages of spermatogenesis may be transmitted. Also, protamines, basic proteins that replace histones during postmeiosis [23], can be preferential targets for alkylating agents. For example, acrylamide is a weak inducer of genotoxic effects in somatic [24] and female germ cells [25], but it is one of the most potent clastogens in male germ cells [26, 27].

Although much has been learned from rodent breeding tests, they are very expensive requiring thousands of animals, and they provide little information on the underlying mechanism(s) of action. Below, we describe two additional techniques (zygote PAINT/DAPI and sperm FISH), as direct methods for investigating germ-cell sensitivity profiles and mechanisms of action that may lead to abnormal reproductive outcomes after chemotherapy.

Mouse PAINT/DAPI assay for transmitted chromosomal abnormalities

The metaphase plate of mouse first-cleavage zygotes provides the first opportunity [28-30] for detecting cytogenetic defects in parental chromosomes after fertilization. We improved the classic cytogenetic analysis of mouse zygotes by combining DAPI staining with chromosome-specific painting probes (PAINT) for the simultaneous detection of numerical as well as stable and unstable chromosomal aberrations [31]. The PAINT/DAPI procedure was recently used to demonstrate that therapeutic doses of etoposide affected primarily male meiotic germ cells producing unstable structural aberrations and aneuploidy, effects that were transmitted to the

progeny [32]. This was the first report of an agent for which paternal exposure led to an increased incidence of aneuploidy in the offspring.

The PAINT/DAPI method has been used for investigating germ-cell stage susceptibility, the pattern of chromosomal aberrations in the zygote, and the type of abnormal reproductive outcomes induced by various mutagens, including the chemotherapeutic agents, cyclophosphamide, etoposide and melphalan [27]. These studies have confirmed the high sensitivity of male postmeiotioc germ cells to mutagens and have shown that induced chromosomal damage and premutational lesions are carried to the zygote where they are converted into chromosomal abnormalities that are associated with specific abnormal reproductive outcomes, in terms of the germ-cell stage sensitivity, proportion of affected zygotes, and types of outcomes.

Information obtained from human sperm FISH assays

During the 1990's, FISH technology was adapted for the detection of chromosomally defective sperm, and its relevance has improved with the availability of chromosome-specific DNA probes for clinically relevant aneuploid syndromes (i.e., 21, 18, 13, X and Y) [4]. Using sperm FISH, small exposure effects can be detected by studying large numbers of sperm in small number of patients. New sperm FISH methods have been recently developed for the detection of aneuploidy as well as structural aberrations [33], but essentially all of the information for chemotherapeutic effects is limited to aneuploidy outcomes (Table 3).

In one of the larger studies, Robbins et al., [3] used an X-Y-8 sperm-FISH assay to study eight cancer patients treated with NOVP chemotherapy and found an

approximate 5-fold increase in sperm with disomies and diploidies. The aneuploidy effects were transient, however, declining to pretreatment levels within ~100 days after the end of the therapy. Another analysis of NOVP patients using an X-Y-21-18 sperm FISH assay found significant, yet also transient, 2- to 14-fold inductions for most clinically relevant of sperm aneuploidies, suggesting that NOVP therapy increased the risk of fathering a child with any one of the major clinical aneuploidy syndromes [4]. A significant increase in the frequency of diploidy and disomy for chromosomes 16, 18 and XY was induced in testicular cancer patients treated with the PEB regimen [34]. In a study of the effects of BEP chemotherapy, sperm from 8 testicular cancer patients were assessed before, and 2–13 years after treatment [35], showing no significant treatment-related increase in the frequency of chromosomal abnormalities. In a different study, sperm chromosomal abnormalities were assessed in cancer patients before, during, and after BEP therapy using probes for chromosomes 1, 12, X, and Y [36], showing a significant increase in the frequency of XY disomic sperm with treatment. Taken together, these limited data are consistent with the statement that treatment-induced aneuploidy effects in sperm, when they occur, are transient with no long-term effects. However, the generality of this statement is unknown, because so few treatment regimens have been evaluated for sperm aneuploidy, and essentially none have been evaluated for treatment-induced chromosomal structural aberrations in sperm.

Information from mouse sperm FISH studies

Most chemotherapeutic regimens consist of combination of drugs. Therefore, animal models were developed to evaluate the relative risk of individual drugs for the

induction of genetic and chromosomal damage in sperm. Several multicolor sperm FISH assays have been developed to detect numerical abnormalities and chromosome structural aberrations in mouse sperm [37, 38]. However, to date, only four chemotherapeutics have been studied with the mouse sperm-FISH assay for aneuploidy and diploidy induction. As shown in Table 4, taxol was tested at the maximum tolerated dose and the increase of disomic sperm was marginally significant [39]; vinblastine gave inconclusive results in repeated experiments in an inter-laboratory comparison [40]; while etoposide and merbarone, both topoisomerase II inhibitors, showed significant increases in the frequencies of diploid and hyperhaploid sperm [41].

Etoposide is currently the only agent for which it is possible to compare the response to chemotherapeutic agents between rodents and humans. It induced significant increases in the frequencies of diploid and aneuploid sperm of both mice [41] and young human patients [34].

Evidence for susceptibilities of differentiating male germ cells to chemotherapies and clinical implications

The evidence from sperm, zygote, and breeding studies demonstrates that chemotherapy can induce significant increases in the frequencies of sperm with chromosomal abnormalities (aneuploidies, structural abnormalities, as well as prelesions) that can lead to abnormal reproductive outcomes, and that male germ cells differ in their susceptibility to damage. For aneuploidies, induced increases in ejaculated sperm diminish with increasing time after exposure, suggesting that cancer patients have only a transient risk for producing abnormal offspring after chemotherapy. However, very few

chemotherapeutic agents have been evaluated for chromosomal aberrations and gene mutations in sperm, and further studies are needed for these sperm endpoints and others listed in Table 1.

Our knowledge of the mechanisms underlying the susceptibility differences among types of male germ cells is very limited. There is evidence for gene mutations that the nature of the mutations is more dependent upon the germ-cell stage than upon the chemical itself [42]. The time-dependent risks for specific types of abnormal reproductive outcomes may differ depending on the type of chemotherapeutic agents employed and time from the end of therapy.

We do not know how many cancer drugs produce transient chromosomal abnormalities in male meiotic and post meiotic germ-cell stages and persistent gene mutations in spermatogonial stem cells. Using the cumbersome animal breeding tests, only a few chemicals have been found to cause mutations in spermatogonial stem cells [2]. The animal breeding tests show that most mutagens are effective in differentiating germ cells (Table 2), namely spermatocytes and spermatids [43]. Genetic damage to spermatids is a special case, because genetically damaged spermatids are known to develop into mature sperm that are fully capable of fertilizing eggs despite the presence of DNA damage [2, 32]. Taken together, the available data from animal breeding and human sperm studies suggest that it may be ill advised to cryopreserve sperm within the first few weeks after the start of chemotherapy, even though sperm counts and motility are still high. Postponing fertilization for at least three months and up to a year following chemotherapy is likely to reduce the risk of fathering an abnormal reproductive outcome [44] for those agents that do not produce stem cell mutations. Better methods are needed

to assess the risk of chemotherapy exposures on mutations in stem cells, because these, if they occur, may persist throughout the reproductive life of the cancer survivor. Modern molecular assays for specific gene mutations in sperm, such as those recently applied to age effects in men [45, 46], may provide a new approach to assess susceptibility and persistence in sperm from treated cancer patients.

Although the primary responsibility of the physician is to try to achieve remission and cure, the increasing effectiveness of modern anti-cancer treatments increases the importance of understanding whether drug regimens can induce elevated frequencies of sperm with gene mutations and/or chromosomal abnormalities. Furthermore, young cancer survivors and their parents deserve counseling regarding the possibility that chemotherapy may have detrimental effects on the future ability to father healthy progeny, especially for samples obtained within the first few weeks after receiving treatment when highly susceptible meiotic and postmeiotic cells have progressed to the ejaculate.

Acknowledgements

This work was performed under the auspices of the US DOE by the University of California, LLNL under Contract No. W-7405-Eng-48 with funding support from NIEHS Superfund P4ZES04705, NIH ES 09117-03 and NIEHS IAG Y1 ES 8016-5.

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Table 1: DNA and chromosomal alterations that can be transmitted by sperm

• **Aneuploidy**	• **Premutational lesions**
- sex chromosomes	- DNA adducts
- autosomes	- protamine adducts
	- single and double strand breaks
• **Structural aberrations**	
- duplications/deletions	
- rearrangements	• **Nucleotide repeats**
- chromosome breaks	
	• **Gene mutations**
• **Epigenetic modifications**	
- imprinting	

Table 2: Dominant Lethality (DL), Specific Locus Mutation (SLM) and Heritable Translocation (HT) analyses of anticancer drugs in mice

Chemical	**DL**	**SLM**	**HT**	**Cell Types with Peak sensitivity**
Adriamycin	-	ND	-	
Chlorambucil	+	+	+	Postmeiosis, Round spermatids
Chlormethine	+	+	ND	Postmeiosis, Sperm
Cisplatin	-	ND	-	
Cyclophosphamide	+	+	+	Postmeiosis
Etoposide	+	+	+	Pachytene spermatocytes
Melphalan*	+	+	+	Postmeiosis, Round spermatids
6-Mercaptopurine	+	ND	-	Preleptotene spermatocytes
Mitomycin C*	+	+	+	Preleptotene spermatocytes
Myleran	+	+	ND	Postmeiosis, Sperm
Procarbazine*	+	+	+	Postmeiosis, Round spermatids
Trophosphamide	+	+	+	Postmeiosis, Sperm
Vinblastine sulphate	-	ND	-	

* Treatment also induced mutations in spermatogonia
ND = not done

Table 3 Summary of human sperm FISH analyses of cancer patients who received various chemotherapy regimens

Chemotherapy Regime	**Chromosomes in FISH Assay**	No. of Patients	**Samples and collection times**	**Results**	**Reference**
BEP bleomycin, etoposide, cisplatin	1,12, X, Y	**1**	3 samples, pre-, during post-CT	**Increase of disomy XY**	Martin et al., 1999
BEP bleomycin, etoposide, cisplatin	1,12, X, Y	**8**	8 samples pre- and 8 samples post-CT	No significant effect	Martin et al., 1997
BEP bleomycin, etoposide, cisplatin	X, Y, 16, 18	**5**	1 sample each after CT	Increase of disomy XY, 16, 18 and diploidy	De Mas et al., 2001
MACOP-B methotrexate, doxorubicin, cyclophosphamide, vincristine, prednisone	1,12, X, Y	**1**	1 sample three years after CT	No significant effect	Martin et al., 1995
NOVP Novanthrone, Oncovin, Vinblastine, Prednisone	X,Y,8	**8**	1 sample each after CT	5-fold increase of disomies and diploidy	Robbins et al., 1997
NOVP Novanthrone, Oncovin, Vinblastine, Prednisone	X, Y, 18, 21	**8**	1 sample each after CT	4-to-10-fold increase of disomies and diploidy	Frias et al., 2003

Table 4: Summary of sperm FISH analyses of mice treated with specific chemotherapeutic agents

Chemical tested	**Chromosomes used in Assay**	**Results**	**Reference**
Etoposide	XY8	Disomy, Diploidy induced	Attia et al., 2002
Merbarone	XY8	Disomy, Diploidy induced	Attia et al., 2002
Taxol	XY8	No statistically significant effect	Adler et al., 2002
Vinblastine	XY8	No statistically significant effect	Schmid et al., 2001