

# Sperm mRNA as a Biomarker of Testicular Toxicity

This thesis has been submitted in partial fulfillment of the requirements for the Bachelor of Science degree in Environmental Science at Brown University.

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# ABSTRACT

**Background:** For the past 50 years there has been a rise in incidence of male reproductive disorders including low sperm counts, infertility, cryptorchidism, hypospadias, and testicular cancer. The rapid industrialization of the 20<sup>th</sup> century and the introduction of thousands of synthetic chemicals into the environment are seen as major causes for non-genetic male reproductive disease. Numerous epidemiologic studies have linked specific toxicants with testicular disorders, leading to the hypothesis known as the testicular dysgenesis syndrome. The testis is composed of seminiferous tubules in which spermatogenesis takes place. Sertoli cells line the epithelium of the tubules and have the integral function of supporting and regulating germ cell development. Spermatogenesis is a process organized into three developmental phases: proliferative, meiotic, and spermiogenic, where diploid spermatogonia eventually give rise to haploid spermatozoa. Untranslated mRNA in sperm can serve as a “footprint” of spermatogenesis because many of the transcripts are involved in transcription, cell proliferation, and signal transduction. Sperm protamine-1 (PRM1) and bcl2 mRNA have been found in elevated amounts in infertile men. Protamine-1 replaces histones in chromatin condensation, and bcl2 is an apoptosis antagonist. 2,5-Hexanedione is a Sertoli cell toxicant and metabolite of n-hexane, an industrial solvent. It causes germ cell loss and testicular atrophy via the mechanism of altered microtubule assembly. The aim of my study was to examine whether sperm mRNA could be a reliable biomarker of testicular toxicity by comparing alterations in sperm mRNA with functional and histological endpoints of toxicity to 2,5-hexanedione exposure.

**Materials and Methods:** Adult male rats were exposed to 0.33% 2,5-HD in drinking water for 9 weeks (n=10), and compared to controls (n=10). Testis and caput epididymal sperm counts were performed, and tissue sections were cut for histological analysis. Cauda epididymal sperm were isolated, RNA was extracted, and processed for quantitative RT-PCR to determine the expression of PRM1 and bcl2.

**Results:** Rats in the treatment group had significantly lower body weight, lower caput epididymis weight, and lower testis spermatid head counts. Histological analysis showed mild focal atrophy and spermatid head retention in the treatment group. Quantitative RT-PCR showed an increase in sperm mRNA for PRM1 and bcl2 in rats exposed to 2,5-HD.

**Discussion:** The increased sperm mRNA for both genes is consistent with previous human studies, and shows that sperm mRNA is a biomarker of testicular toxicity. This elevated mRNA content could be the result of abnormal mRNA retention and disturbed protein translation, or an adaptive response of the germ cells to Sertoli cell 2,5-HD-induced toxicity. As a biomarker of testicular toxicity, sperm mRNA has significant implications for public health programs as a biomonitoring tool.

**Future Directions:** This experiment should be repeated with a lower dose but longer exposure duration to induce minimal testicular injury. Other toxicants with different cellular targets should be explored to examine target-dependent differences in sperm mRNA content. Because this study was performed on adult rats and would be most translatable to adult men, a different experiment could be conducted with in utero exposures to explore the TDS hypothesis. Adult sperm mRNA content could be evaluated after a prenatal exposure to a toxicant to test for long lasting effects.

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## LIST OF ABBREVIATIONS

BCL2.....	B-Cell Leukemia/Lymphoma-2
BSA.....	Bovine Serum Albumin
DEPC.....	Diethylpyrocarbonate
2,5-HD.....	2,5-Hexanedione
HBSS.....	Hank's Basic Salt Solution
HEPES.....	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPRT.....	Hypoxanthine phosphoribosyltransferase
PCR.....	Polymerase Chain Reaction
PRM1.....	Protamine-1
RT.....	Reverse Transcription
TUNEL.....	Terminal deoxynucleotidyl transferase-mediated digoxigenin- UTP Nick End Labeling

# INTRODUCTION

During the past century, reports gathering data from multiple sources have revealed a long-term trend of declining sperm counts and a rise in testicular cancer incidence (1, 2). Other related disorders of the male reproductive tract have been rising in incidence as well, such as cryptorchidism and hypospadias. Cryptorchidism is when one or both testes fail to descend into the scrotum, and hypospadias is a birth defect in which the urinary opening is abnormally placed.

Declining sperm counts also raises fertility concerns. Infertility currently affects up to 17% of American couples, up from 12% in 2002 (4). Male-factor infertility accounts for 50% of these cases. While genetics explains a very small percentage of male-factor infertility, over 90% of these cases are idiopathic, having no identifiable cause (5). In this vast majority, the phenotype of infertility is restricted to the testis, which implies that infertility is related to the imbalance of gene expression, not to deletions or mutations of genes, which would be present in somatic cells as well (6).

## **Role of the Environment**

Due to the rapid expansion of industry and introduction of thousands of new chemicals this past century, the environment likely plays a large role in the concurrent incidence of male reproductive disorders. Of the 15,000 high-production-volume synthetic chemicals in current use, over half have never undergone any toxicological testing (7). Consequently, their effects on human health remain unknown until a case arises that provides a direct association between a toxicant and specific disease.

Occupational exposures often provide the best link between an exposure and disease because they involve a specific group of people that have a specific disease, or higher incidence of that disease than in the general population. For example, in the 1970's, banana plantation

workers in Costa Rica were exposed to extremely high levels of the pesticide 1,2-dibromo-3-chloropropane (DBCP) due to unsafe handling practices, which resulted in the sterilization of 1,500 men (8). A 1984 study found that men involved in farmwork were 6 times as likely to develop testicular cancer as men who do not work around farms (9).

Children are especially vulnerable to toxicants for multiple physiological reasons. Pound for pound, children breathe more, eat more, and drink more than adults do (7). Their metabolic pathways are immature, and because their bodies develop rapidly, their organ systems are more susceptible to injury. Numerous studies have linked toxicant exposure in children to reproductive disease. For example, prenatal phthalate exposure can cause a decreased anogenital distance in young boys (10). For males this distance should be longer than for females, so decreased anogenital distance is an endpoint of adverse male reproductive development manifested as feminization.

Because of the early onset of many of these diseases, and the gathering data that links them to toxicants, prenatal exposure has been proposed as the relevant etiologic period of exposure. This hypothesis is called testicular dysgenesis syndrome (TDS). Evidence for this hypothesis comes from the fact that boys with cryptorchidism and/or hypospadias are at greater risk of developing testicular cancer later in life (11). The underlying cause is disturbed gonadal development in the embryo (12). Spermatogenesis is the sequence of events by which spermatogonial stem cells develop into meiotic spermatocytes, followed by haploid spermatids, which finally develop into spermatozoa (13).

## Anatomy of the Testis

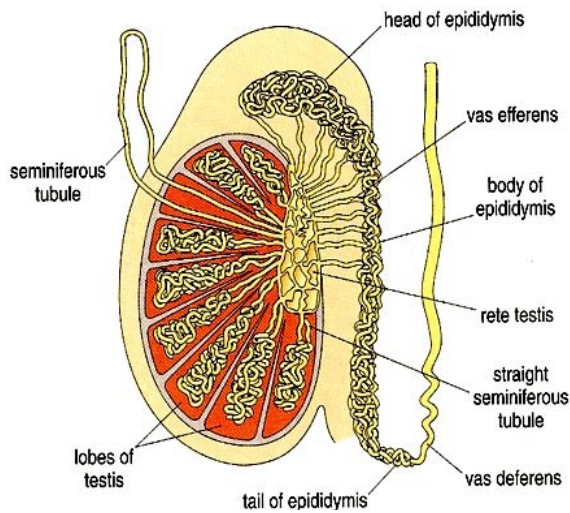


Figure 1. The testis and epididymis.

epididymis and mature along the length of the epididymis until they reach the cauda (tail).

### The Sertoli Cell

The Sertoli cell is critical for the process of sperm production, and because of its supportive role, is also known as the “Nurse” cell. As such it has several major functions (15). First, it maintains the place of the germ cells, which are attached to it throughout their development. Second, it can phagocytose residual bodies and other degraded

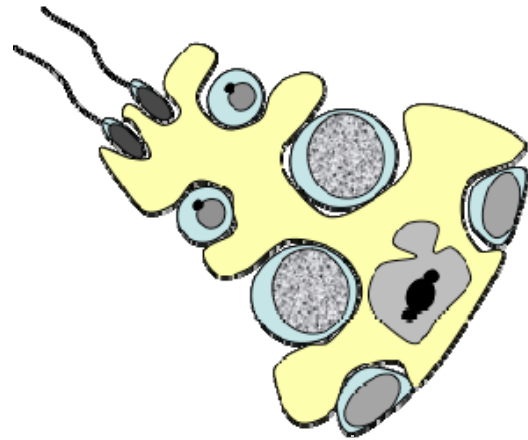


Figure 2. The Sertoli cell with attached germ cells

cells. Third, it forms tight junctions with adjacent Sertoli cells, which is called the blood-testis barrier because it protects the developing sperm. This barrier separates the epithelium into an adluminal compartment and a basal compartment. The basal compartment contains spermatogonia and early spermatocytes, and the adluminal compartment contains later spermatocytes and spermatids (16). The Sertoli cell also delivers nutrients to each of the

germinal cells, such as sugars, amino acids, and lipids. It also secretes a seminiferous fluid that includes many different proteins and hormones that regulate spermatogenesis. Signals from the Sertoli cell regulate germ cell proliferation, differentiation, and death (14).

Beyond the basic functions of maintaining cell shape and forming cytoplasmic extensions, the cytoskeleton of the Sertoli cell has functions specific to the seminiferous epithelium. One of the primary components of the cytoskeleton, microtubules are especially important as their function is perturbed by exposure to certain toxicants, and this impacts how the entire process of sperm production is carried out. These microtubules run parallel to the long axis of the Sertoli cell and help anchor germ cells to the Sertoli cells and translocate developing germ cells from the basal compartment to the adluminal compartment (17).

## Spermatogenesis

Spermatogenesis is the process by which germ cells divide and differentiate, eventually leading to spermatozoa. The entire process takes approximately 64 days in humans (18).

There are three major developmental phases in the cycle: proliferative, meiotic, and spermiogenic (Fig. 3) (14). In the proliferative phase, there are three types of spermatogonia: stem cell, proliferative, and differentiating. While spermatogonia increase their cell population by one thousandfold, it is important that there is a reserve of spermatogonia that do not commit to development in order that spermatogenesis stays on a regular

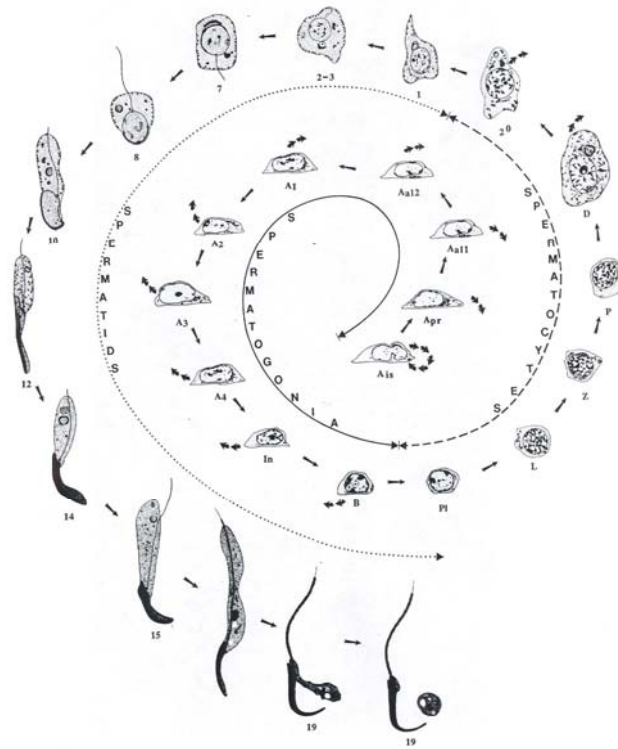


Figure 3. The Spermatogenic cycle, including the 3 major cell types: spermatogonia, spermatocytes, and spermatids. (14)

schedule. These stem cells do not undergo mitosis as much as the other two types, and as a result are less vulnerable to injury from toxicants. However, the complete loss of stem cells leads to irreversible atrophy. These stem cells divide to give rise to proliferative spermatogonia, which further divide to form differentiating spermatogonia. These differentiating spermatogonia then proceed to the meiotic phase of the cycle.

In the meiotic phase, diploid spermatocytes undergo the very long processes of meiosis I and meiosis II to eventually produce haploid spermatids (14). During meiosis, chromosomes are

recombined, which mixes the genetic material. Because the chromosomes are halved in each of the two meiotic divisions, the germ cell number is quadrupled.

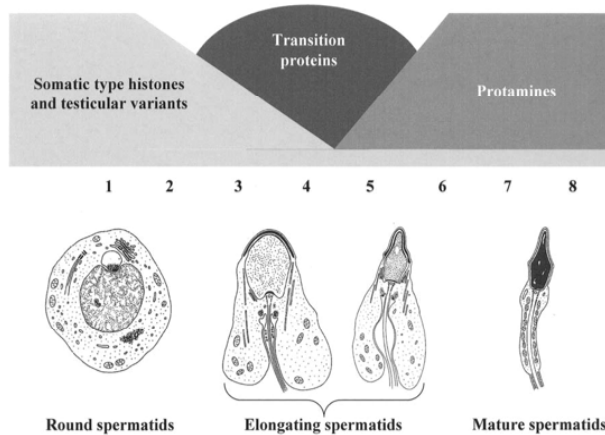


Figure 4. Histone-to-protamine replacement in eight steps, in humans. (13)

reduces the spermatid's size to approximately 25% of its original size. As the spermatids elongate and mature, the nucleus condenses by the mechanism of histone-to-protamine replacement (Fig. 4). DNA wraps around histones and protamines, which aid in packing the vast amounts of genetic material into a microscopic volume.

Because the cellular machinery for transcription and translation shut down as spermatids mature, any mRNA that remains in sperm was transcribed earlier in spermatogenesis. It was previously assumed that mRNA transcripts would be unable to survive the condensation of spermatids into spermatozoa.

### Sperm mRNA

Because many testis-specific mRNAs are synthesized and translated under strict control, the mRNAs found in mature spermatozoa could be untranslated remnants that we can use as a fingerprint of spermatogenesis (6). Therefore, it is necessary to know just what particular mRNAs are found in sperm before they can be investigated to determine how their altered

The final spermiogenic phase is different from the previous two phases because there is no cell division involved (14). Instead, young spermatids evolve through morphologic transformations into spermatozoa. Some of these changes include formation of the flagellum and acrosome, and elimination of cytoplasm, which

amounts elucidate mechanisms of injury. Microarray data has been very useful in describing the myriad of transcripts in sperm. Microarrays are ordered sets of cDNAs that are arranged on a plate; probing allows for the discovery of gene expression in the sample to be studied (6). Of the 5,000 different mRNA transcripts found in sperm, 25% of them encode for proteins involved in transcription and regulation of transcription (5). Most of the mRNAs in sperm are involved in cell proliferation, signal transduction, and oncogenesis, all of which are related to spermatogenesis (6). These surviving mRNA transcripts are therefore a useful indicator of gene activity during the critical steps of spermatogenesis, and could give information about mechanisms of testicular injury.

Several genes have altered amounts in the sperm of infertile men compared to fertile men. In a study of motility-impaired sperm and normal sperm, testis-specific protein 1 (TPX-1) and lactate dehydrogenase C (LDHC) levels were significantly higher in normal sperm (19). Another example of altered gene expression in sperm involves protamine-1 (PRM1) and B-cell leukemia/lymphoma-2 (bcl2), which are both in elevated amounts in infertile men (20). PRM1 and bcl2 have roles that are more relevant to the mechanism of toxicity explored in the present experiment, and their properties will be described in the following section.

### **PRM1 and Bcl2**

Because of the stage of the cycle during which these genes are expressed, PRM1 and bcl2 will indicate different mechanistic events. PRM1 is specific to spermiation, and bcl2 is associated with apoptosis of earlier germ cell types, spermatocytes in particular.

#### *PRM1.*

During spermiogenesis, nuclear condensation is achieved by replacing histones with protamines (13). The phosphorylation of protamines 1 and 2 results in chromatin condensation

and termination of gene expression. In humans, male fertility is contingent on a PRM1 to PRM2 ratio of 0.8-1.2 in mRNA levels (21). A study using a mouse model found that a decrease in the protein amount of either protamine leads to disrupted nuclear formation, resulting in abnormal sperm function (22). However, it is specifically PRM1 that has lower mRNA levels in infertile men (23). Abnormal protein synthesis has been found to be associated with abnormal mRNA retention, which suggests that protamine translational regulation may contribute to protamine deficiency in infertile men (21). A correlate of the protamine ratio, *bcl2* blocks apoptosis and has been shown to be in quantities 10-fold the normal amount in infertile men (20).

### *Bcl2.*

Apoptosis is programmed cell death, an important mechanism that prevents damaged cells from proliferating, and also controls cell populations (24). The *bcl2* family includes both pro- (such as *Bax*) and anti-apoptotic effector proteins, but the original *bcl2* is anti-apoptotic. It is the ratio of these agonists and antagonists that determines the cell's resistance to apoptosis (24). In mice, an early and massive wave of apoptosis is required in order for spermatogenesis to proceed normally; high levels of *bcl2* protein in germinal cells has been shown to produce sterility in mice, due to the prevention of apoptosis early in the the germ cell differentiation sequence (25).

*Bcl2* is regulated by paracrine signaling from the Sertoli cell. Under stimulation by the pituitary-derived follicle-stimulating hormone (FSH), the Sertoli cell upregulates stem cell factor (SCF), which binds to the c-kit receptor on spermatogonia and results in germ cell proliferation (26). SCF directly inhibits transcription of the pro-apoptotic *Bax*, but does not affect *bcl2* expression (27). SCF does, however, upregulate the pro-survival *Bcl-w* and *Bcl-xl*.

While many different genes are involved in apoptosis, including the Fas system and p53, bcl2 is the only mRNA that has been seen in sperm so far. PRM1 and bcl2 have been studied in comparisons of fertile and infertile men, but thus far no animal studies have explored their expression following toxicant-induced testicular injury. PRM1 is also an ideal gene to follow because it is highly abundant in mature spermatozoa (19). The toxicant of interest in the present study is 2,5-hexanedione (2,5-HD), which has been studied in our lab for over two decades.

### **2,5-Hexanedione**

2,5-hexanedione is the ultimate metabolite of n-hexane, which is ubiquitous in the environment because it is a common industrial solvent and a major component of gasoline (28). Therefore, people are exposed to it most commonly through inhalation, though it can also be ingested. This is especially a concern for people who live near toxic waste sites, because n-hexane can leach into the soil and contaminate groundwater. The Occupational Safety and Health Administration set a permissible exposure limit (PEL) of 500 ppm, even though n-hexane causes nerve damage at this limit (29). In the rat, testicular injury occurs at levels of 2,5-HD exposure that are below neurotoxic levels (28). Thus in occupational settings, it is likely that 2,5-HD may be at levels high enough to cause testicular injury in humans. However, there has never been a reported case of n-hexane-related testicular injury in humans (30).

Though it is a Sertoli cell toxicant, germ cells are affected because of the integral role that the Sertoli cell plays in the health of germ cells. The Sertoli cell is the first cell that manifests altered function during *in vivo* exposure to 2,5-HD, thus 2,5-HD is termed a Sertoli cell toxicant (30).

*Pathology.*

Manifestations of injury are secondary alterations in the testis that are used to distinguish Sertoli cell toxicants from toxicants with other cellular targets (30). 2,5-HD causes several pathological effects: pyrrolation of testis tubulin, vacuolation, spermatid head retention, decreased seminiferous tubule fluid secretion, decreased seminiferous tubule diameter, germ cell apoptosis, and apical shedding of germ cells. First, pyrrolation of testis tubulin begins 2 weeks after initiation of 2,5-HD exposure (31). Second, unpublished data from our lab show that retained spermatid heads are the earliest morphological indicator of injury in the testis, appearing within two weeks of initiation of a low dose of 2,5-HD (32). Rather than being released from the seminiferous epithelium, step 19 spermatids (refer to Fig. 2) were instead pulled down to the basal epithelium and then phagocytosed by the Sertoli cell (32).

Third, vacuoles form from damaged Sertoli cell organelles that combine. At a high dose of 2,5-HD, vacuoles form 4 weeks after the initiation of treatment (33). Seminiferous fluid secretion stops 2 days after the appearance of basal vacuoles (34). This endpoint is measured by ligating the efferent ducts of one testis, and comparing its weight to that of the opposite, unligated testis (30). The cessation of this fluid precedes major germ cell morphological changes, suggesting that this fluid is key to the health of the germ cells, an idea supported by the “nurse” role of the Sertoli cell.

Germ cell apoptosis is made visible by TUNEL (Terminal deoxynucleotidyl transferase-mediated digoxigenin-UTP Nick End Labeling), which stains fragmented DNA in paraffin cross-sections of the testis (30). Analyzing germ cell apoptosis is a more specific endpoint because it gives more information about the mechanism of toxicity by revealing which germ cell types were affected. Finally, loss of germ cells, especially of elongated spermatids and spermatocytes, occurs 5 weeks after the initiation of exposure to 2,5-HD (33). This loss is progressive, and after

7 weeks tubules are marked with severe atrophy, which is defined as loss of at least 50% of testis weight (31).

Before these histopathologic signs of toxicity are visible, changes occur at the molecular level, which elucidate the mechanisms of toxicity.

### *Mechanisms.*

The subcellular target of 2,5-HD is tubulin, which self-assembles into microtubules. *In vitro* incubation of 2,5-HD-treated tubulin showed abnormally rapid assembly after 2 weeks of exposure, which resulted in more abundant, but shorter microtubules (31). Abnormal microtubule formation causes the cytoskeleton to ineffectively support the Sertoli cell's microenvironment for germ cells. As a result, germ cell loss commences, and with sustained exposure to 2,5-HD, testicular atrophy occurs.

The mechanism of germ cell loss is apoptosis, which is induced through paracrine signaling from the Sertoli cell. Germ cells contain Fas, the receptor of the tumor necrosis factor family, and it initiates apoptosis when bound to its ligand FasL, which is produced by the Sertoli cell (35). 2,5-HD induces the upregulation of both FasL and of Fas, which precedes apoptosis by a week. Other toxicants with other cellular targets also cause an increase in Fas expression, so Fas has been proposed as a marker of injury (36). 2,5-HD and other toxicants compromise the Sertoli cell's supportive capacity, so the Sertoli cell responds by initiating apoptosis of germ cells in order to bring their numbers to a manageable size.

### **Experimental Design**

Among the various endpoints of 2,5-HD toxicity, the latest discovery of retained spermatid heads encouraged the investigation of spermatozoa for other endpoints of toxicity. Having been released from the seminiferous epithelium, they were exposed to the same

conditions as the retained heads, and thus may have molecular markers that would describe the toxic effects. Therefore, the released spermatids likely have altered gene expression as a response to Sertoli cell dysfunction because they were exposed to 2,5-HD throughout their development.

The rat is the most commonly used animal for reproduction and toxicology studies, so we use it here (29). The aim of this experiment is to determine the extent to which the sperm mRNA content of two specific genes is altered in association with known functional and histopathologic biomarkers of testicular injury.

# MATERIALS AND METHODS

## **Animals**

Adult male Fischer 344 rats (Charles River Laboratories, Wilmington, MA) weighing 150-250 g were housed at 21°C on a 12-hour light/dark schedule with access to water and Purina Rodent Chow 5001 *ad libitum*. All procedures involving animals were performed in accordance with the guidelines of Brown University's Institutional Animal Care and Use Committee in compliance with National Institute of Health guidelines.

## ***In Vivo* Exposure**

2,5-HD was administered as a 0.33% solution in the drinking water *ad libitum* for 9 weeks (n=10). The control for 2,5-HD treatment was drinking water *ad libitum* (n=10). To assess mRNA content, rats were killed 1 day after the end of exposure.

## **Surgical Sperm and Testis Retrieval**

All chemicals used were ordered from Sigma-Aldrich (St. Louis, MO), unless otherwise noted. Animals were killed by carbon dioxide inhalation. Immediately after death, sperm were obtained from the cauda epididymides of control and 2,5-HD-treated rats by gently dicing the cauda into mm<sup>3</sup>-sized pieces and collecting the sperm that moved into the media (470 mL HBSS, 2.1 g HEPES, 1 g BSA, 0.45 g D-glucose, 0.175 g sodium carbonate, 0.5 mL sodium pyruvate). Both testes and epididymides were weighed. The right testis was placed in Formalin for histopathology, and the left testis was frozen for spermatid head counts. The caput was also frozen for spermatid head counts.

## **Testis Spermatid Counts**

The left testis was detunicated and placed in 10 mL of Saline-merthiolate-triton (SMT). SMT is composed of 0.9% NaCl (9g/L), 0.01% Merthiolate or Thimerosal (100 mg/L), and

0.05% Triton-X-100 (0.5 mL/L). The testis was then homogenized to remove degraded spermatids. The homogenate was diluted in 9 mL of SMT and loaded into two hemocytometers. Spermatid heads were counted in the squares of the hemocytometer and then the counts from the four chambers were averaged. The number of spermatid heads per gram of tissue was calculated as:

$$[(\text{mean \# of sperm heads counted}) \times (\text{squares factor}) \times (\text{hemocytometer factor}) \times (\text{dilution factor})] / (\text{tissue weight in grams})$$

The squares factor was 5, the hemocytometer factor was  $10^4$ , and the dilution factor was 100, which equaled the total volume of SMT added in milliliters.

### **Caput Spermatid Counts**

The caput was removed from the epididymis and placed in 10 mL of SMT. The caput was then homogenized, and the homogenate was diluted in 4 mL of SMT and loaded into two hemocytometers. Spermatid heads were counted using the same method as for the testis, and the concentration was calculated using the same above equation. The squares factor and hemocytometer factor were the same as above, except the dilution factor was 50.

### **Histology**

Testes in 10% neutral buffered Formalin were kept at 4°C before processing. Cross sections (1 mm thick) from the middle of the testis were placed in the embedding resin 2-hydroxyethyl methacrylate (Technovit 7100, Kulzer, Germany). Sections (3 µm thick) were cut for light microscopic histological analysis. Sections were stained with period acid-Schiff's reagent and hematoxylin (PASH). To determine the extent of morphological alterations in the testis, sections were examined digitally using Aperio ScanScope (Aperio Technologies, Vista, CA).

## **Sperm Isolation**

Sperm was collected, gently pipetted over a 50% Percoll solution, and centrifuged at 1000 rpm. The pellet of viable sperm was removed and washed with media via centrifugation at 1000 rpm for 20 seconds. All sperm from the control group or 2,5-hexanedione-treated group were pooled in a lysis buffer (Invitrogen, Carlsbad, CA).

## **RNA Extraction and cDNA Synthesis**

Total RNA was immediately isolated with Invitrogen's Micro-to-Midi Total RNA kit (Carlsbad, CA). The sample was lysed, homogenized, DNase-treated, washed, and then eluted in RNase-free water. The sperm RNA was then immediately reverse-transcribed using Invitrogen's RNA Amplification System kit (Carlsbad, CA). The complimentary DNA (cDNA) reactions were carried out on a BioRad MJMini Personal Thermal Cycler.

In preparation for first-strand synthesis, 9  $\mu\text{L}$  of total RNA and 1  $\mu\text{L}$  of T7-Oligo(dT) Primer was incubated at 70°C for 10 minutes. The reaction mix for first strand synthesis consisted of 4  $\mu\text{L}$  of 5X First Strand buffer, 2  $\mu\text{L}$  of 0.1 M DTT, 1  $\mu\text{L}$  of 10 mM dNTP Mix, 1  $\mu\text{L}$  of RNaseOUT, and 2  $\mu\text{L}$  of SuperScript III Reverse Transcriptase. The first strand synthesis reaction incubated at 46°C for 2 hours, and then at 70°C for 10 minutes to inactivate the reverse transcriptase. The reaction mix for second strand synthesis consisted of 91  $\mu\text{L}$  of DEPC water, 30  $\mu\text{L}$  of 5X Second Strand buffer, 3  $\mu\text{L}$  of 10 mM dNTP mix, 4  $\mu\text{L}$  of DNA Polymerase I, 1  $\mu\text{L}$  of DNA Ligase, and 1  $\mu\text{L}$  of RNase H. The second strand synthesis reaction incubated at 16°C for 2 hours.

The quality of the RNA and DNA was checked using the NanoDrop® ND-1000, a full-spectrum (220-750nm) spectrophotometer that measures 1  $\mu\text{L}$  samples with high accuracy and

reproducibility. The control rat concentration of cDNA was 753 ng/ $\mu$ L, and for the 2,5-HD treated rats 553 ng/ $\mu$ L.

## RT-PCR

PRM1	Forward	5'-TGTGGCCTTCTTTGAGTTCG-3'
	Reverse	3'-ATCCACAGAGCGATGTTGTC-5'
Bcl2	Forward	5'-CAAGTCCACCAAACTCCTG-3'
	Reverse	3'-TTAGCAGGCTCCTGTTTTCC-5'
HPRT	Forward	5'-GCAGACTTTGCTTTCCTTGG-3'
	Reverse	3'-TTCGAGAGGTCCTTTTCACC-5'

Table 1 shows the primers used to detect the cDNA for PRM1, bcl2, and the reference gene HPRT. Messenger RNA sequences were found using the Entrez-Gene database and

appropriate primers for the mRNA sequence were chosen using the Primer3 program. To verify that there would be no primer-dimers, the DNA Fold program was used to predict the structure of the mRNA sequence with primers, and the Amplify program also checked for primer-dimers. Primers were ordered from Invitrogen and finally tested for primer-dimers using PCR, and run on a 2% agarose gel to ensure that they detected the correct base-pair location in the cDNA.

Each PCR reaction for the control and 2,5-HD treated cDNA consisted of 5  $\mu$ L of cDNA, 12.5  $\mu$ L of 2X Syber Green, 1  $\mu$ L of the forward primer, 1  $\mu$ L of the reverse primer, and 5.5  $\mu$ L of DEPC water, making a total volume of 25  $\mu$ L.

Reactions were performed in triplicate for

Cycle	Repetitions	Step	Temp.	Time (mins)
1	1	1	95°C	0:30
2	1	1	95°C	3:00
3	50	1	95°C	0:10
		2	59.5°C	0:45
4	1	1	95°C	1:00
5	1	1	55°C	1:00
6	70	1	60 to 94.5°C	0:10
		Increase temperature after cycle 2 by 0.5°C		

accuracy. The PCR reactions were carried out on a BioRad iCycler iQ Multicolor Real-Time PCR Detection System thermalcycler. Table 2 shows the program of heating steps for the reactions. The relative levels of PRM1 and bcl2 were quantitated and normalized to HPRT using the BioRad iQ5 Optical System Software, Version 2.

# RESULTS

## In Vivo Exposure

As a result of 2,5-HD exposure, rats in the treatment group had significantly lower body weight and caput epididymis weight (Table 3). These rats also had a much lower count of homogenization-resistant testis spermatid heads (Table 4).

Treatment group	Body wt. (g)	Testis weight (g)		Epididymis weight (g)		Detunicated Left Testis	Caput Epididymis
		Left	Right	Left	Right		
Control (n=10)	312 ± 3.79	1.58 ± 0.02	1.53 ± 0.01	0.46 ± 0.01	0.46 ± 0.01	1.46 ± 0.03	0.56 ± 0.03
2,5-HD (n=10)	293 ± 2.99*	1.53 ± 0.02	1.49 ± 0.02	0.44 ± 0.01	0.43 ± 0.01*	1.34 ± 0.03*	0.35*

Note. Mean ± SEM; \*, p < 0.05 by t-test

	Control	2,5-HD
Testis spermatid heads/g ( $\times 10^{-8}$ )	1.75 ± 0.05	0.96 ± 0.02*
Caput Epididymis sperm/g ( $\times 10^{-8}$ )	2.37 ± 0.14	2.26 ± 0.05

Note. Mean ± SEM; \*, p < 0.05 by t-test

## Testicular Histopathology

2,5-HD exposure produced mild focal atrophy (Fig. 6), manifested as germ cell loss and retained spermatid heads (Fig. 7) in the basal epithelium of the seminiferous tubules. This agrees with unpublished data from our lab that showed that rats exposed to 0.33% 2,5-HD for 18 days had significantly increased occurrence of retained spermatid heads (32).

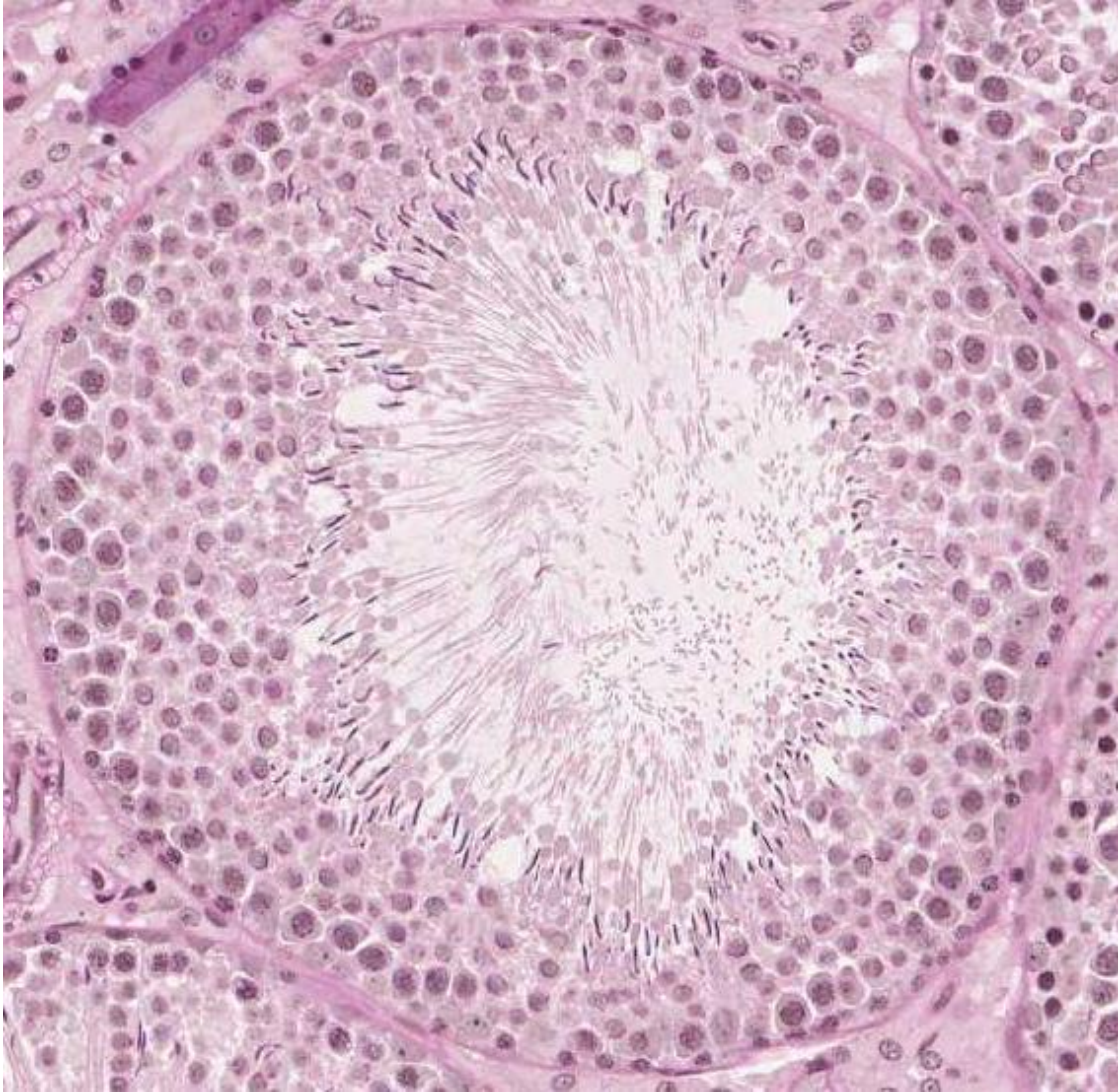


Figure 5. Cross-section of a seminiferous tubule of a control rat, showing normal germ cell development.

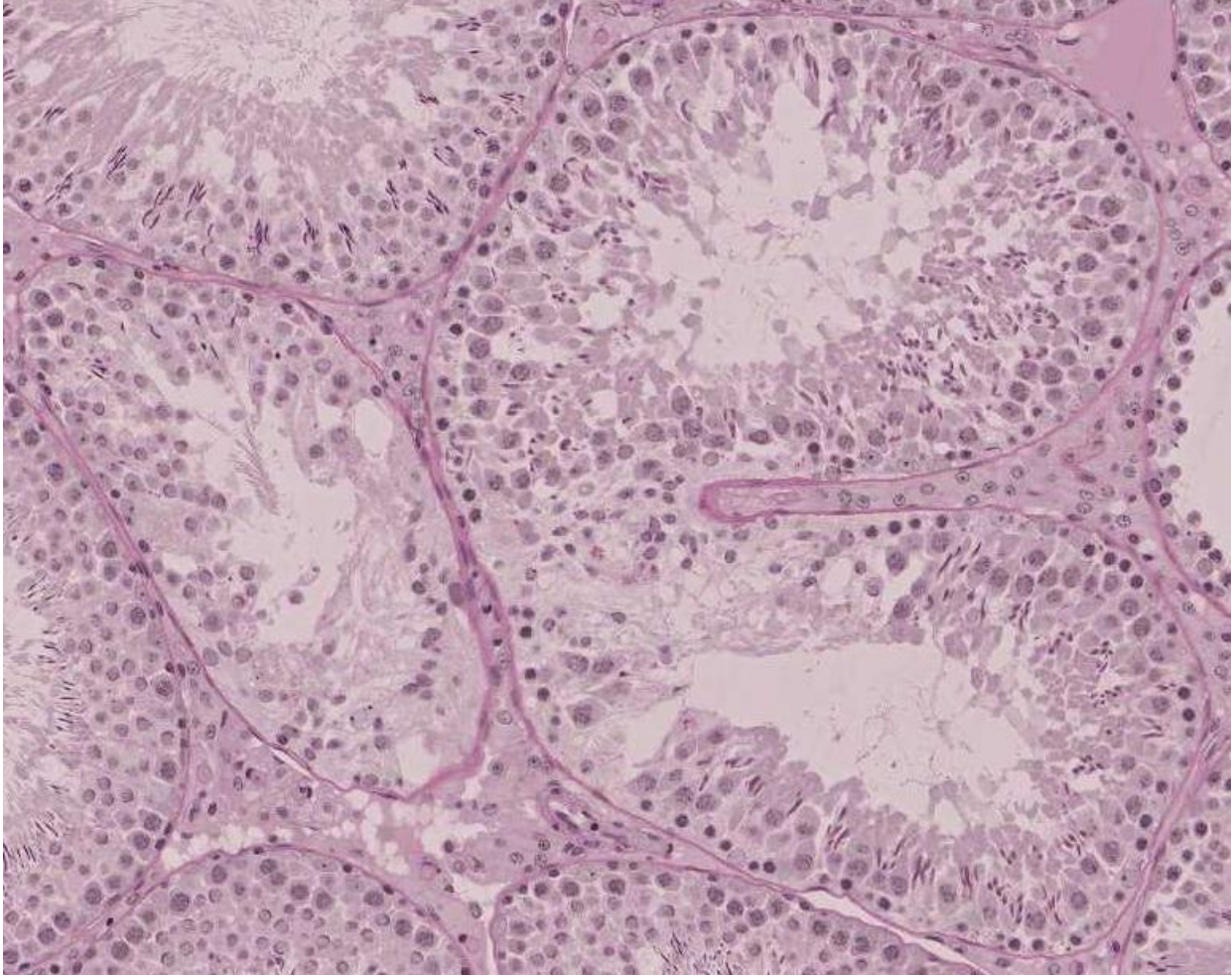


Figure 6. Focal atrophy apparent as germ cell loss following 2,5-HD exposure.

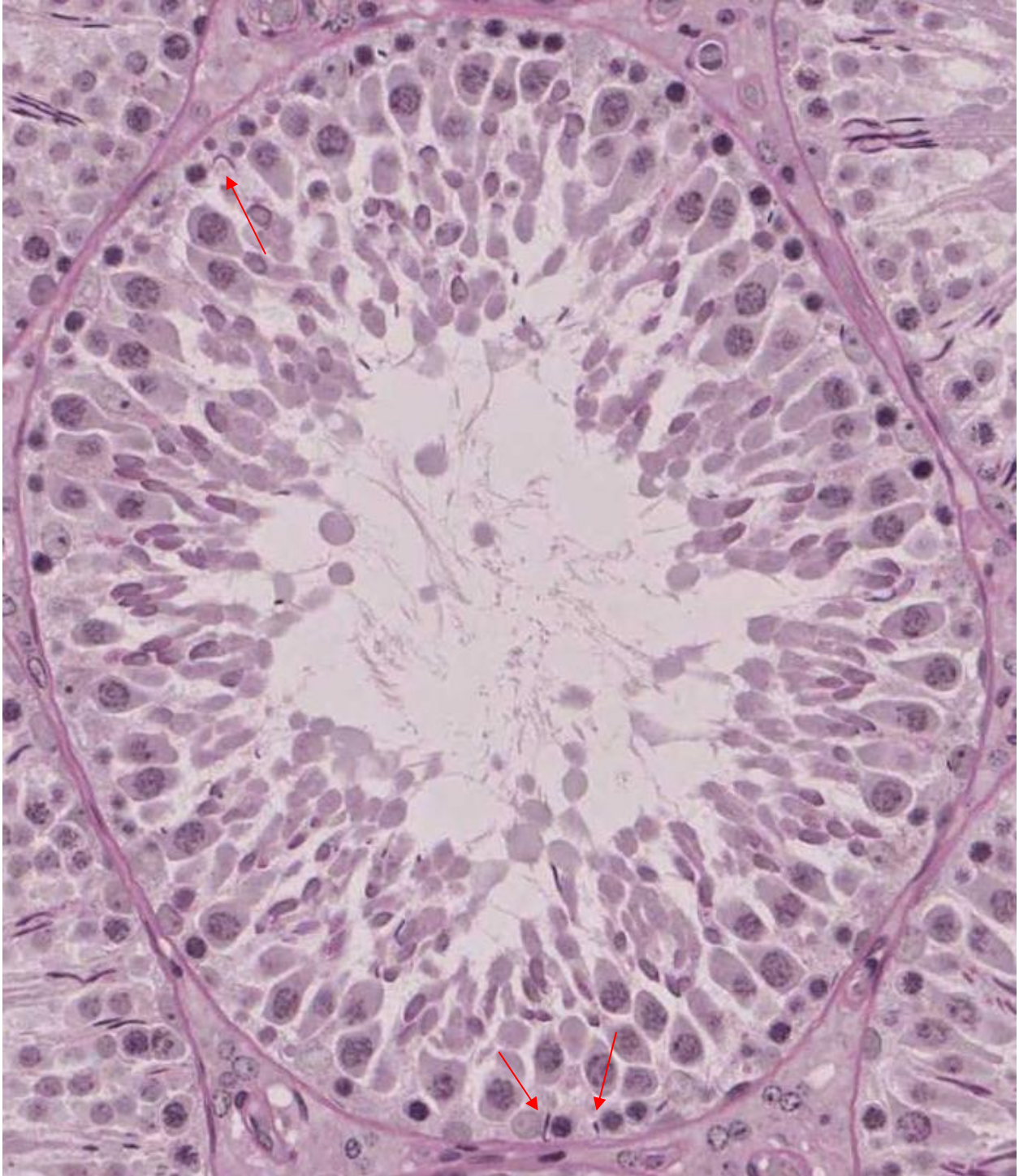


Figure 7. Retained spermatid heads in the basal epithelium in 2,5-HD rats.

## RT-PCR

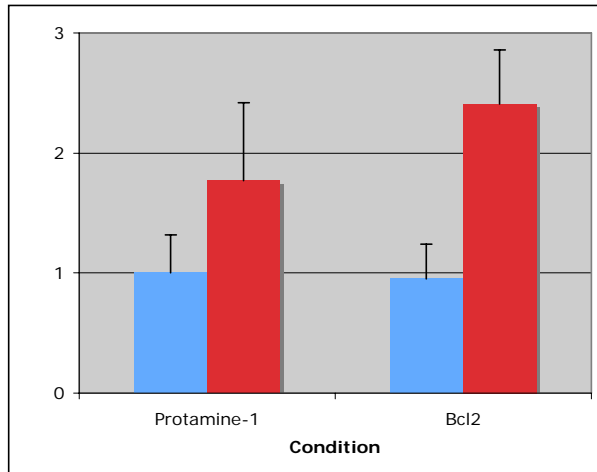


Figure 8. Quantitative RT-PCR for PRM1 and bcl2. Control, blue bar; 2,5-HD, red bar; normalized to HPRT; technical triplicates, mean  $\pm$  SD.

Because we were concerned about the extremely small amount of mRNA content in sperm, the sperm from all 10 rats was pooled into one sample for each group. Therefore, though we had 10 rats, the experiment consisted of only one the RT-PCR reaction conducted in triplicate. As a consequence, we were not able to perform statistical analysis on our results. The standard deviations shown

as error bars in Figure 7 come from the triplicate reactions for each condition and gene. Both genes are over-represented in the 2,5-HD treatment group sperm compared to controls (Fig. 7). PRM1 was expressed 1.77-fold in the 2,5-HD group, and bcl2 was expressed 2.41-fold.

## DISCUSSION

Because this is a pilot study to evaluate sperm mRNA content, the aim was to find a low dose of 2,5-HD that produced minimal phenotypic alterations in the testis. Eight weeks is the approximate length of time for a cycle of spermatogenesis in the rat, so a nine-week exposure was chosen to ensure that sperm were exposed to the toxicant throughout their development.

RT-PCR produced exciting results, as not only was there a marked difference in mRNA content, but the changes observed correlated with Steger et al.'s work with PRM1 and bcl2 (20). The mechanism of bcl2 increase is probably related to the induction of a protective response in the germ cell to the stress produced by an abnormal Sertoli cell. The Fas system is well-studied in the testis, but a closer look into the bcl2 system in the testis will reveal more about the complex pathways that induce and inhibit apoptosis. The Fas system is a marker of apoptosis in the testis, but so far mRNA transcripts for that family have not been found in sperm. Bcl2, but not Bax, is in sperm in abundance, so it can be analogous to Fas as an indicator of the state of apoptosis in the testis.

At step 19, the transition protein-to-protamine conversion should be nearly completed or finished. At this step spermatids are retained, so there could be a relationship between protamine development and the fate of the mature spermatids. Abnormal protamine assembly could signal to the Sertoli cell an unhealthy spermatid, which the Sertoli cell would then phagocytose. Aberrant mRNA retention has been proposed as the mechanism by which PRM1 mRNA levels increased, so these untranslated transcripts are an indicator of abnormal protamine-1 protein levels and altered histone-protamine conversion (21).

Biomonitoring programs have been very useful tools in assessing exposure in populations in order for public health interventions to target certain populations. For example, the heavy

metals mercury, lead, and cadmium are all measured in blood serum, and benzene, n-hexane, and polycyclic hydrocarbons are all measured in urine (37). As rapid, simple, and noninvasive analytical procedures, measuring these toxicants has made it much easier and less expensive to make exposure assessments. The results from our study show that it is possible that sperm mRNA could be biomarkers of exposure, if marked differences in gene expression were seen among different toxicants. Currently, sperm provides markers of effect, as sperm counts and motility are used to diagnose azoospermia and infertility. Sperm mRNA could potentially elucidate the mechanism of testicular injury, and from there, the exposure.

The benefits of biomonitoring data include establishing longterm trends of exposure/effect, identification of geographical regions where people have much different effect or exposure characteristics than the general population, and providing more robust exposure data in epidemiologic studies (37). Longterm trends of PBDE exposure were key to its eventual banning in some European countries. Several studies have revealed large geographical differences in semen quality across the United States. For example, sperm concentration and motility were lower in fertile men from mid-Missouri relative to those from New York, Minnesota, and California (38). Epidemiologic studies often have difficulty obtaining strong exposure data, especially if they are using hospital records or other materials subject to bias. Coupled with other information, sperm mRNA could be a powerful tool for recording incidence of testicular injury.

## FUTURE DIRECTIONS

As a pilot study, this experiment should be replicated for statistical strength and with experimental modifications. A lower dose of 2,5-HD would induce milder phenotypic alterations, such as spermatid head retention, because it is the earliest morphological sign of toxicity. 2,5-HD has dose-dependent effects, so that with a higher dose-rate, more severe injury occurs. The presence of testicular atrophy was too severe of an endpoint for the aims of this study. The duration of exposure should also be lengthened to allow more time for germ cell stress to take effect. The apoptotic gene *bcl2* was upregulated, but not as much as in Steger et al.'s study (20). Interspecies differences of course could also account for this discrepancy, though the reproductive system among mammals is highly conserved. Key to determining the reliability of sperm mRNA as a biomarker, is to experiment with other toxicants, in particular ones with different cellular targets. X-irradiation and DBCP are both germ cell toxicants, though DBCP is more clinically relevant because it is a synthetic chemical.

Because we exposed adult rats to the toxicant, this study is most translatable to adult-onset of testicular toxicity. However, because of the complex exposures that people receive in the environment, it is difficult to say whether a man's infertility or cancer was brought on by prenatal exposure or exposure later in life. Past animal studies with 2,5-HD and epidemiologic studies of occupational exposures show toxicity in adults, while studies describing testicular dysgenesis syndrome mostly focus on children. It would be interesting to examine TDS in the animal model; adult pregnant rats would be exposed to a toxicant, and then the testis development of her pups could be observed through adulthood. A time course of molecular and histological examination of these rats would give more information about prenatal exposures and how this manifests after puberty.

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