The Stage-Specific Apoptotic Response of the Rat Testis to Low Dose Co-Exposures

by

Natasha Rene Catlin

B.S., Salve Regina University; Newport, RI, 2008

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Department of Biology and Medicine at Brown University

PROVIDENCE, RHODE ISLAND

May 2014
This dissertation by Natasha Rene Catlin is accepted in its present form by Department of Biology and Medicine as satisfying the dissertation requirement for the degree of Doctor of Philosophy.

Date__________________ ______________________________

Kim Boekelheide, M.D., Ph.D., Advisor

Recommended to the Graduate Council

Date__________________ ______________________________

Richard Freiman, Ph.D., Reader

Date__________________ ______________________________

Surendra Sharma, Ph.D., Reader

Date__________________ ______________________________

Eric Suuberg, Ph.D., Reader

Date__________________ ______________________________

Robert Chapin, Ph.D., Outside Reader

Approved by the Graduate Council

Date__________________ ______________________________

Peter Weber, Ph.D., Dean of the Graduate School

iii
EDUCATION

Graduate Program in Pathobiology, Brown University, Providence, RI
Ph.D. Pathobiology     October 2013
Advisor: Kim Boekelheide, M.D./Ph.D.
Examined the mechanisms behind the attenuation in apoptosis that is seen with x-radiation and 2,5-hexanedione co-exposure in the testis. Developed a streamlined approach of laser capture microdissection to examine gene alterations in the spermatogonia, which are targeted by these exposures. This toxicant sensitive cell population has been examined with apoptosis specific PCR arrays. An adaptive survival response has been identified in the low dose x-ray exposed spermatogonia, which is accentuated with the addition of HD to the exposure.

Department of Biology and Biomedical Sciences, Salve Regina University, Newport, RI
B.S., Biology, Chemistry minor May 2008
Advisor: Steven Symington, Ph.D.
Studied the effects of deltamethrin (a pyrethroid insecticide) on Ca\textsubscript{v}3.2, a human T-type voltage sensitive calcium channel that we expressed in \textit{Xenopus laevis} oocytes. Two-electrode voltage clamp electrophysiology was used to characterize the function of the deltamethrin exposed calcium channel.

LABORATORY SKILLS
- Microarrays
- Real-time PCR arrays
- Laser capture microdissection
- RNA amplification
- Rodent surgeries
- Immunohistochemistry/Immunofluorescence
- Western blotting
- DNA, RNA, and protein isolation
- Transillumination-assisted seminiferous tubule dissection
- Molecular cloning
- Two-electrode voltage clamp electrophysiology
- \textit{Xenopus laevis} oocyte defolliculation and cRNA microinjection

HONORS AND AWARDS
2013  1st place, Northeast Society of Toxicology Research Poster Competition
2012  Finalist, Student Oral Presentation Competition at Northeast Society of Toxicology annual meeting
2011  2nd place, Northeast Society of Toxicology student travel award
2008  Student award for campus leadership through Student Association of Interdisciplinary Life Sciences
2008 Society of Toxicology, Undergraduate Toxicology Education Travel Award
2008 2nd place, Northeast Society of Toxicology Research Poster Competition
2007-08 Rhode Island Summer Undergraduate Research Fellowship
2007 1st place, 2nd Annual BioNES Student Presentation Competition

PROFESSIONAL AND HONOR SOCIETIES
Sigma Xi Honor Society (May 2011 to Present)
Society of Toxicology (February 2009 to Present)
Northeast Society of Toxicology (February 2009 to Present)
Women in Toxicology Special Interest Group, Society of Toxicology (February 2009 to Present)
Mixtures Specialty Section, Society of Toxicology (February 2009 to Present)
Reproductive and Developmental Toxicology Specialty Section, Society of Toxicology (February 2009 to Present)

COMMITTEE WORK
Superfund Research Program Student Representative (September 2010 to Present)
New England Society of Toxicology (NESOT) Senior Student Representative (February 2012 to May 2013)
ILSI Health and Environmental Sciences Institute, Subcommitteee on distinguishing adverse from non-adverse/adaptive effects. (March 2010 to Present)
President, Salve Regina University Student Association for interdisciplinary Life Sciences (SAILS) Club (August 2007 to May 2008)

PUBLICATIONS

PLATFORM PRESENTATIONS
Catlin N, Sandrof M, Campion SN, Boekelheide K. “Co-exposure to Low-dose Model Testicular Toxi


**POSTER PRESENTATIONS**


Catlin N, Mutanguha E, Symington SB. “Pyrethroid inhibition of the mammalian T-type voltage-sensitive calcium channel (Cav3.2).” Northeast Regional Society of Toxicology, Shrewsbury, MA. October 24, 2008.

Catlin N, Mutanguha E, Symington SB. “Pyrethroid inhibition of the mammalian T-type voltage-sensitive calcium channel (Cav3.2).” Rhode Island Network for Molecular Toxicology (RI-INBRE) Summer Undergraduate Research Program. University of Rhode Island, Kingston, RI. August 4, 2008.


TEACHING EXPERIENCE
2013 Brown University’s The Harriet W. Sheridan Center for Teaching and Learning Certificate III: Professional Development.

2012 **Guest Lecture**, Integrated Medical Sciences I- General Pathology (BIOL3645), Brown University; Lecture title: “Laser capture microdissection.”

2012 **Guest Lecture**, Nursing Research (NUR 253), University of Rhode Island: College of Nursing Lecture title: “Toxicology: *in vivo* vs. *in vitro*.”

2010 **Teaching Assistant**, Environmental Health and Disease (BIO182), Professor Mary Hixon. Prepared and graded quizzes and exams, led student exam reviews, lectured on Neurotoxicology.


2008 **Mentor**, Student: Edwin Mutanguha, Biology and Chemistry Major I taught Edwin oocyte defolliciation and microinjection, and electrophysiology to aid him in taking over my undergraduate research project.

REFERENCES
Kim Boekelheide, MD, Ph.D.
Professor, Brown University Department of Pathology and Laboratory Medicine 401-863-1783 • kim_boekelheide@brown.edu

Sarah Campion, Ph.D.
Principal Scientist, Developmental and Reproductive Toxicology Pfizer Worldwide Research and Development 860-715-4177 • sarah.campion@pfizer.com

Steven Symington, Ph.D.
Professor Salve Regina University Department of Biology and Biomedical Sciences 401-341-3249 • steven.symington@salve.edu
Acknowledgements

I would like to thank the chair of my committee Dr. Surendra Sharma, along with the rest of my committee, Dr. Richard Freiman and Dr. Eric Suuberg, for all your guidance and support over the past years. I would also like to thank Dr. Robert Chapin, for agreeing to be an outside reader for my thesis and for all of our poster chats at various conferences, where you tried to help me unravel what all my data meant.

I would also like to thank Brown University and my funding sources. None of this would be possible without an awesome and supportive graduate program and the wonderful people behind it all. In particular I would like to thank my funding sources (the Superfund Research Program Training Grant and the Pathology Training Grant), which without, all this incredibly expensive research would not have been possible.

Thank you Kim for being an excellent mentor who has encouraged me to persist through difficult experiments until they work and to be a more independent thinker. I really appreciate every opportunity you have given me to succeed during this process.

Sue Huse deserves a GIANT thank you. You made the final push to analyze data smooth, through your awesome knowledge of everything stats and R related. You are an amazing person and I still do not understand how you can get everything
that you have to do, done. Thank you so much for your attempt to impart some statistical analysis knowledge on me.

Thank you to my lab, “Team Testis” both present and past. Having all of you around has certainly made my Ph.D. experience one of the best times of my life. There were some days when I have laughed so hard that I cannot imagine ever being happier than that. When I go to a new boring lab, I will constantly remember our crazy inappropriate conversations, fun-filled music-in-the-lab days and our many coffee breaks. Foxtrot 3 and Caramello, thank you for inducting me as an honorary member into “The Fun Side”, I will yodel loudly again soon! Thank you to everyone for being my work family and for helping me when it was needed. I could probably write a book alone on how everyone has helped me, but to be succinct I would like to give special thanks to the following:

- Sue, my lab mom, you helped me whenever help was needed and answered all my stupid lab questions. I will never forget my first day when I ran to you after my first confusing meeting with Kim and asked what the heck plastic, paraffin or frozen sections meant. Thank you for everything over the past years.

- Ed and Dan, you have been a fountain of post-doc knowledge and I knew that you would always have an answer for any question I threw at you.

- Maggie, the queen of Westerns, you were an unbelievable help with all of the westerns I had to do. I am pretty sure I would not have been able to do them as well without such an incredible teacher to show me.

Many thanks to my LADIES: Camelia and Sarah. Camelia, you have been with me from the very beginning of this process and I would not have had it any other way. I know that whenever anything happened, good or bad, you would be there for
me to listen to me cry, complain, or get excited. Thank you so much for everything! Sarah, you will forever be my baymate! Thank you for answering my many questions related to our project, listening to me complain about how much I hated it sometimes and most of all being one of my ladiess. I know that with both of you, this is just the beginning of life long friendships and I look forward to the future.

Dr. Symington, thank you for starting this all. I would have never ended up here if it had not been for you asking our class if any students were interested in doing some research. The idea of getting my PhD started with you, so I credit a lot of what I have accomplished with you. Thank you for giving me the opportunity to fall in love with toxicology.

My deepest gratitude goes to my family. Thank you for being there for me along every step of the way. Mom and Dad, you have brought me up to never take no for an answer and have taught me to always reach for my dreams. To my younger siblings, Kimmie, Nic and Willie, I love all of you and have always wanted to make you proud of your older sister. Thank you to my baby kitties, Zoe and Linus who kept my lap warm and made me smile while spending many a Saturday writing. Derek, thank you for putting up with me while I wrote this beast. You stuck with me through my crazy mood swings and bouts of anxiety and I cannot express how much gratitude I have for you taking care of me through it all. Thank you for taking the time to teach me how to use LaTex and then fixing my coding “operator error” problems when I had them. Without your help, I would have suffered many more headaches from this whole dissertation writing process. Most of all, you helped me get through many many many hours of LCM by giving me the Harry Potter audiobooks. I think those were the only things that made me able to sit at that microscope for six hours every day for what felt like an eternity.
Preface

The work presented in this dissertation was performed in the laboratory of Kim Boekelheide, M.D., Ph.D. I performed all of the experiments presented herein, with the following exceptions:

Chapter 2 was a collaborative effort of the authors, who collectively formed a committee through International Life Sciences Institute (ILSI) Health and Environmental Sciences Institute (HESI). This committee was formed to join together a group of diverse scientists for discussion on how an adaptive effect can be differentiated from an adverse effect in the light of emerging data from new technologies, and how these data can be applied to risk assessment. This chapter was included because it reflects the immense difficulty of differentiating an adaptive from an adverse response within large datasets, particularly from gene expression studies. Toxicity pathways were determined to be of great importance within the differentiation of adaptive and adverse effects, since relevant pathways of toxicological concern are typically key biological events in the ultimate development of adverse effects following toxicant exposure. As much of my dissertation focused on differentiating low-dose pathway effects of multiple model testicular toxicants, the discussions carried out with this committee provided much insight into the difference between adaptive and adverse effects.

The microarray experiments presented in Chapter 3 were performed by Janan Hensley and Kevin Gaido in their laboratory at the Hamner Institutes for Health Sciences in Research Triangle Park, NC. Sarah Campion performed the microarray analysis in Kim Boekelheide’s lab, with direction from Andy Houseman, Yunxia Sui and Zhijin Wu.
# Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Vitae</strong></td>
<td>iv</td>
</tr>
<tr>
<td><strong>Acknowledgments</strong></td>
<td>viii</td>
</tr>
<tr>
<td><strong>Preface</strong></td>
<td>xi</td>
</tr>
<tr>
<td><strong>1 Introduction</strong></td>
<td>1</td>
</tr>
<tr>
<td>1.1 Thesis Significance and Aims</td>
<td>2</td>
</tr>
<tr>
<td>1.2 The Testis</td>
<td>4</td>
</tr>
<tr>
<td>1.2.1 Sertoli Cell Function</td>
<td>8</td>
</tr>
<tr>
<td>1.2.2 Spermatogenesis</td>
<td>9</td>
</tr>
<tr>
<td>1.3 Testicular Toxicity</td>
<td>14</td>
</tr>
<tr>
<td>1.3.1 2,5-Hexanedione</td>
<td>16</td>
</tr>
<tr>
<td>1.3.2 Carbendazim</td>
<td>18</td>
</tr>
<tr>
<td>1.3.3 X-irradiation</td>
<td>20</td>
</tr>
<tr>
<td>1.3.4 Complex Exposures</td>
<td>21</td>
</tr>
<tr>
<td>1.3.5 Low Doses of Toxicants</td>
<td>24</td>
</tr>
<tr>
<td>1.4 Apoptosis</td>
<td>27</td>
</tr>
<tr>
<td>1.4.1 Normal Apoptosis During Spermatogenesis</td>
<td>31</td>
</tr>
<tr>
<td>1.4.2 Apoptosis During Testicular Injury</td>
<td>31</td>
</tr>
<tr>
<td><strong>2 Identification and Characterization of Adverse Effects in 21st Century Toxicology</strong></td>
<td>54</td>
</tr>
<tr>
<td>2.1 Declaration of Author’s Roles</td>
<td>55</td>
</tr>
<tr>
<td>2.2 Abstract</td>
<td>56</td>
</tr>
<tr>
<td>2.3 Introduction</td>
<td>57</td>
</tr>
<tr>
<td>2.4 Characterization of Biological Response: Defining an Adverse Effect</td>
<td>59</td>
</tr>
<tr>
<td>2.5 Placement of the Effect Within a Biological System: Relevant Pathways of Toxicological Concern and the DMA Case Study</td>
<td>62</td>
</tr>
<tr>
<td>2.6 Characterization of Adversity: Moving From the Science to Risk Assessment Application</td>
<td>67</td>
</tr>
</tbody>
</table>
List of Tables

1.1 Summary of Apoptotic Regulators ........................................ 36
3.1 Genes with significant HD effects ........................................ 91
3.2 Genes with significant CBZ effects ....................................... 93
A.1 Supplemental Table 1 ......................................................... 185
A.2 Supplemental Table 2 ......................................................... 186
# List of Figures

1.1 Testis Structure ............................................. 6
1.2 Testicular Histology ........................................ 7
1.3 Spermatogenesis ............................................. 10
1.4 Spermatogenesis Stages ..................................... 13
1.5 Toxicant Summary Table .................................... 15
1.6 Low-dose Exposure Hierarchical Clustering ............... 25
1.7 Cellular Apoptosis Pathway Model ......................... 29

2.1 Present and future testing paradigms ...................... 61
2.2 Dose transitions for adverse toxicant response ........... 66
2.3 Future state of toxicity testing ............................ 74

3.1 Co-exposure effects on gene expression ................... 92
3.2 Gene expression 3 h after HD and CBZ co-exposure ....... 96
3.3 Gene expression 24 h after HD and CBZ co-exposure ..... 97
3.4 Localization of Loxl1 protein 24 h following toxicant exposure 100
3.5 Quantification of Loxl1 staining ........................... 101

4.1 Laser capture microdissection methods schematic ......... 118
4.2 Effect of amplification on measured gene expression .... 121
4.3 Consistency of RNA input method across samples ....... 123
4.4 Consistency of RNA input method within samples ....... 124
4.5 Effect of RNA input concentration on threshold cycle values. 126

5.1 Exposure paradigm ........................................... 141
5.2 Heatmap displaying hierarchical clustering of qRT-PCR data 147
5.3 Dose response comparison of 0.5 Gy x-ray to both 1 and 2 Gy x-ray 149
5.4 Western blot analysis of DR5 in staged seminiferous tubules and whole testis ........................................ 150
5.5 Western blot analysis of Casp7 in staged seminiferous tubules and whole testis ........................................ 151
5.6 Proposed mechanism of testicular apoptotic response to x-ray exposure 156

6.1 Model of x-ray induced germ cell apoptosis over time ...... 168
6.2 Model of the adverse and adaptive response to x-ray dose over time 170
6.3 Stage specific effects of toxicant exposure ................ 178
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>HD</td>
<td>2,5-hexanedione</td>
</tr>
<tr>
<td>CBZ</td>
<td>Carbendazim</td>
</tr>
<tr>
<td>X-ray</td>
<td>X-irradiation</td>
</tr>
<tr>
<td>STF</td>
<td>Seminiferous Tubule Fluid</td>
</tr>
<tr>
<td>BTB</td>
<td>Blood-Testis Barrier</td>
</tr>
<tr>
<td>LH</td>
<td>Luteinizing Hormone</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicular Stimulating Hormone</td>
</tr>
<tr>
<td>LOEL</td>
<td>Lowest Observable Effect Level</td>
</tr>
<tr>
<td>NOEL</td>
<td>No Observable Effect Level</td>
</tr>
<tr>
<td>LD50</td>
<td>Lethal Dose, 50%</td>
</tr>
<tr>
<td>CCNG1</td>
<td>Cyclin G1</td>
</tr>
<tr>
<td>PUMA</td>
<td>p53 Up-regulated Modulator of Apoptosis</td>
</tr>
<tr>
<td>AEN</td>
<td>Apoptosis Enhancing Nuclease</td>
</tr>
<tr>
<td>LCM</td>
<td>Laser Capture Microdissection</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative Real-time PCR</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling</td>
</tr>
<tr>
<td>DR5</td>
<td>Death Receptor 5</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas-associated Death Domain</td>
</tr>
<tr>
<td>DED</td>
<td>Death Effector Domain</td>
</tr>
<tr>
<td>DISC</td>
<td>Death Inducing Signaling Complex</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF-related Apoptosis Induced Ligand</td>
</tr>
<tr>
<td>TRID</td>
<td>Truncated Intracellular Domain</td>
</tr>
<tr>
<td>TRUNDD</td>
<td>Truncated Death Domain</td>
</tr>
<tr>
<td>MEHP</td>
<td>Mono-(2-ethylhexyl) Phthalate</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear Factor κB</td>
</tr>
<tr>
<td>NRC</td>
<td>National Research Council</td>
</tr>
</tbody>
</table>
RPTC Relevant Pathways of Toxicological Concern
RRR Relevant Responses for Regulation
TSCA Toxic Substance Control Act
ILSI International Life Sciences Institute
HESI Health and Environmental Sciences Institute
USEPA U.S. Environmental Protection Agency
DMA Dimethylarsinic Acid
MOA Mode of Action
WoE Weight - of - Evidence
Macrod1 MACRO domain containing 1
Dpp7 Dipeptidylpeptidase 7
Shank3 SH3 and multiple ankyrin repeat domains 3
Clca2 Chloride channel calcium activated 2
Clca4l Chloride channel calcium activated 4-like
Loxl1 Lysyl oxidase-like 1
Tubb3 Tubulin beta 3
HPRT Hypoxanthine-guanine phosphoribosyltransferase
ANOVA A one-way analysis of variance
LO Lysyl oxidase
SD Standard Deviation
Abstract of “The Stage-Specific Apoptotic Response of the Rat Testis to Low Dose Co-Exposures” by Natasha Rene Catlin, Ph.D., Brown University, May 2014

Human exposure to mixtures of environmental chemicals occurs daily, leading to challenges regarding risk assessment due to limited information on these interactions. This dissertation developed methods for studying low dose pathway effects. Through these improved methods, data was generated on pathway transition points to be applied to risk assessment with the ultimate goal of informing regulatory decision making. It was hypothesized that, based upon distinct cellular pathway activation, the mechanisms underlying model toxicant (HD, CBZ, and x-ray) co-exposures would allow for distinction of a transition point between adaptation and adversity in the continuum of effects.

At high doses, 2,5-hexanedione (HD) and carbendazim interact synergistically, while HD attenuates x-irradiation (x-ray) induced germ cell apoptosis. These studies provide an informative starting platform and underline the necessity of mixtures studies since combined toxicant effects cannot be accurately predicted. However, since only a small percentage of the testis cell population is affected, the co-exposure effects of targeted testicular cell types needed to be examined. This was achieved through the development of an optimized laser capture microdissection method to increase RNA yield for large scale use with PCR pathway arrays. Optimization of this method revealed an amplification bias in low abundance transcripts when un-amplified and amplified LCM-derived RNA were compared. This resulted in the recommendation that a screening procedure be used to exclude low abundance transcripts from data analysis and comparisons.

The optimized LCM method was used in conjunction with an apoptosis PCR array, to understand the influence HD has on x-ray exposure. X-ray exposed spermatogonia employ an adaptive survival response, through decreased pro-apoptotic
transcripts, in response to downregulated anti-apoptotic transcripts. Addition of HD to low doses of x-ray accentuates pro-apoptotic signaling, indicating that high dose induced attenuation of germ cell apoptosis does not persist in the low dose range. These studies provide a reliable and effective tool for understanding the biology underlying the distinction between an adaptive versus an adverse cellular response to toxicant insults and can be applicable to both risk assessment for low dose exposures and future studies.
Chapter One

Introduction
1.1 Thesis Significance and Aims

Humans are exposed to complex mixtures of chemicals in the environment every day, leading to a variety of challenges regarding risk assessment due to the limited information that exists on the interactions between environmental contaminants. The toxicity of chemical mixtures depends on the interactions between the chemicals that make up the mixture and their molecular targets, resulting in additive, antagonistic or synergistic effects. The complexity of mixture studies is compounded when the varyingly sensitive cell types within the target organ are differential targets of the individual components of a mixture. Little is known about the interactions between environmental contaminants and the consequences of co-exposure to multiple toxicants. The studies presented here provide much needed information on the effects of a co-exposure to prevalent model toxicants at environmentally relevant doses. Determining the effects of mixtures on the testis (a model organ) and establishing the methodology for studying them is critical for further understanding of their effects on the human population.

The homeostasis and function of the testis relies greatly on Sertoli cell-germ cell interactions. This cell-to-cell co-dependency can be disrupted by exposure to one or more toxicants that specifically target these two different cell types. By studying the environmentally relevant low dose interactions of model toxicants that target the same model organ (testis), but different cellular and molecular targets, the mechanisms underlying the interactions of mixtures can be better understood. The studies presented in this dissertation will also provide a tool for understanding the biology underlying the distinction between an adaptive versus an adverse cellular response to a toxicant insult. The goal of this dissertation was to further investigate the interactions of 2,5-hexanedione (HD), carbendazim (CBZ), and x-irradiation
(x-ray) on the germ cell response to injury. A further experimental goal was to differentiate between adaptive and adverse cellular responses to co-exposures. It was hypothesized that, based upon distinct patterns of cellular pathway activation, the mechanisms underlying model toxicant (HD, CBZ, and x-ray) co-exposures would allow for distinction of a transition point in the continuum of adaptive versus adverse effects.

Specific Aim 1: Elucidate the underlying gene effects and the mechanism of the enhanced pathology observed with HD and CBZ co-exposure.

Specific Aim 2: Characterize the stage-specific alterations in gene expression within the apoptotic pathway caused by low dose co-exposure to HD and x-ray, using laser-capture microdissection to enrich for cell-type specific responses.

Specific Aim 3: Determine the transition from an adaptive to an adverse response through the apoptotic pathway in the comparison of low doses to high doses of HD and x-ray.
1.2 The Testis

The testis is the organ responsible for the production of both androgens and spermatozoa. It is structurally composed of seminiferous tubules and an interstitial compartment, all of which are encased in the tunica albuginea. The seminiferous tubule is surrounded by a basal lamina and contractile peritubular myoid cells, which play a role in the movement of fluid and sperm through the seminiferous tubule lumen [1]. The seminiferous tubules are arranged into tight coils with convoluted and straight sections, which loop and converge at the rete testis. Many interacting cell types make up the seminiferous tubule compartment, including Sertoli cells and germ cells. The interstitial compartment contains blood and lymphatic vessels, macrophages, and Leydig cells. The Leydig cells are responsible for testosterone production within the testis, which regulates the proper development of germ cells throughout the process of spermatogenesis [1]. The organization of the testis and the cellular composition of the seminiferous tubule epithelium are shown in Figure 1.1.

The Sertoli cell is a somatic, terminally differentiated cell with a stable adult population that stops dividing during puberty [1]. It is a polarized, elongated cell type extending from the basal lamina to the lumen of the seminiferous tubule [2]. The cytoskeletal network of the Sertoli cell is made up of actin filaments, intermediate filaments and microtubules that allow it to serve as a major structural component of the seminiferous tubule [2]. The attachment of Sertoli cells to germ cells is largely supported by Sertoli cell cytoskeletal function, and maintained by cell-cell junctions [3]. In addition, the microtubules serve to move the maturing germ cells towards the lumen and to secrete nutrients and other factors into the adluminal compartment that are necessary for healthy germ cell development. Germ cells progress through the process of spermatogenesis with the support of Sertoli cells. Sertoli cells can
only support a constant number of germ cells, illustrated by studies demonstrating that the number of germ cells is typically proportional to the number of functioning Sertoli cells [4]. Also, because the number of Sertoli cells is fixed and unchanging after puberty, they remain extremely resistant to cell death [3]. Cross-sections of seminiferous tubules show several testicular cell types and features, such as a Sertoli cells, Leydig cells, and germ cells during different stages of their development (Figure 1.2 [5]).
Figure 1.1: Testis Structure. (A) Drawing of the internal structure of the testis (grey) surrounded by the tunica albuginea, which contains the seminiferous tubules (brown) attached in loops to the rete testis (tan). The testis is connected to the epididymus (reddish brown) where sperm mature and are stored. (B) A cartoon depiction of a seminiferous tubule in cross section, depicting the Sertoli cells (light yellow) and the layers of developing germ cells (green). (C) Close up view of the interaction of the fingerlike cytoplasmic projections of Sertoli cells with the germ cells.
Figure 1.2: Testicular Histology. A light micrograph of a rat seminiferous tubule portion, showing the cell associations. The star indicates the lumen of the tubule, while the cross indicates the interstitial space. The scale bar represents 25µm. Below the micrograph are individual cell types that comprise the cell associations, magnified to illustrate the cellular morphology (scale bar represents 3 µm) [5].
1.2.1 Sertoli Cell Function

One of the major functions of the Sertoli cell is to provide structure and support for the germ cells throughout their development [2]. They have fingerlike cytoplasmic processes that surround the developing germ cells with adherens junctions, allowing for close interaction between the two cell types [1]. Sertoli cells also specifically regulate the biochemical surroundings of different developmental stages of germ cells through targeted secretion of seminiferous tubule fluid (STF). STF secretion is androgen dependent, is regulated by the presence of specific germ cells in the seminiferous epithelium, and requires normal functioning of the Sertoli cell microtubule dependent transport pathway [3, 6]. Secretion of STF into the adluminal compartment bathes the germ cells in transport and bioprotective proteins, proteases, protease inhibitors, hormones, and growth factors [2, 4, 7, 8]. Secretion of STF causes a lumen to form within the center of the seminiferous tubule, which acts as a pathway for the movement of mature elongate spermatids to the cauda epididymis [1, 7]. Lack of support from the Sertoli cells, in the form of inhibited STF secretion, can result in detrimental effects on the developing germ cell populations [9].

Another major function of the Sertoli cell is to compartmentalize the seminiferous tubule epithelium, through the formation of the blood-testis barrier (BTB) [1]. The BTB is formed by tight junctions between adjacent Sertoli cells, just above the spermatogonial layer in the seminiferous epithelium [10]. This exterior basal compartment, containing only the spermatogonia and preleptotene spermatocytes, is separated from the meiotic and differentiating germ cells within the adluminal compartment by the BTB [10]. This provides an effective mechanism for the Sertoli cells to modulate the movement of xenobiotics and immunological cell types into the adluminal compartment, where most of the differentiating germ cells reside [8, 11].
In addition to the major functions noted above, Sertoli cells are also important in the phagocytosis of degenerating germ cells and in the regulation of the spermatogenic cycle of germ cell development [1].

1.2.2 Spermatogenesis

Spermatogenesis is the androgen-dependent, synchronized development of the germ cells from spermatogonial stem cells into spermatozoa. It is a complicated process that is divided into three phases: the proliferative phase, the meiotic phase, and the differentiation phase (Figure 1.3) [1]. Germ cells in the proliferative phase include undifferentiated and differentiated spermatogonia. The undifferentiated spermatogonia are stem spermatogonia (A_{isolated}, A_{is}) and their proliferative daughter cells, A_{paired} (A_{pr}) and A_{aligned} (A_{al}) [1]. There are typically two spermatogonial stem cells (A_{is}) per 100 Sertoli cells, which divide to either renew their populations or become A_{pr} spermatogonia [12, 13]. The differentiated spermatogonal types are A_1, A_2, A_3, A_4, Intermediate (In) and B cells [1]. Each class of spermatogonia arises from the previous class by mitotic cell division, with the exception of the transition of A_{al} to A_1, which occurs through a differentiation step [1]. The differentiated spermatogonia have synchronized proliferation during specific epithelial stages [14]. After cell division of the spermatogonia, telophase is incomplete, causing the germ cells to develop as a clonal unit connected by intercellular bridges [13, 15].
Figure 1.3: Spermatogenesis. The development of mature male gametes begins with spermatogonia, the most immature germ cells, which undergo several mitotic divisions to become spermatocytes. The spermatocytes then undergo meiosis to progress into haploid round spermatids. Through a series of differentiation steps, termed spermiogenesis, these latter cells become elongated spermatids.
Type A spermatogonia further differentiate into Type B spermatogonia, which divide to become preleptotene spermatocytes. The preleptotene spermatocytes move across the BTB, complete S-phase, and enter meiosis [1][15]. During prophase I, the spermatocytes transition through the leptotene, zygotene, pachytene, and diplotene phases of meiosis, a process that lasts about three weeks and allows for crossing over to occur [1]. Following the extended prophase I, metaphase, anaphase, and telophase of meiosis I are completed giving rise to haploid secondary spermatocytes. Meiosis II quickly follows Meiosis I to separate sister chromatids and produce haploid round spermatids, which undergo terminal differentiation [15]. This differentiation phase, referred to as spermiogenesis, occurs over a period of approximately three weeks in the rat. During this time, the spermatids develop a flagellum and an acrosome, undergo nuclear shaping and condensation, all while eliminating the majority of their cytoplasm to ultimately become spermatozoa [1]. Spermiation, or the release of spermatozoa into the seminiferous tubule lumen, is an active process that involves the degradation of specialized cellular junctions [3].

The entire process of spermatogenesis is regulated by the hypothalamic – pituitary – Leydig cell endocrine axis [3]. The pituitary gland secretes the gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH), to promote spermatogenesis, upon stimulation by gonadotropin-releasing hormone (GnRH) from the hypothalamus. Binding of LH to Leydig cell surface receptors triggers the production and release of testosterone, a hormone necessary for healthy spermatogenesis, while FSH directly stimulates the seminiferous tubule to initiate spermatogenesis during puberty.

Germ cell development demonstrates a histologically-apparent synchrony referred to as stages, through which the developing germ cells move away from the basement membrane towards the lumen of the seminiferous tubule [1]. In rats there are 14
designated stages (Figure 1.4A [16]), based on the morphological development of spermatids [17]. Cell associations describe the progression of changes seen in the seminiferous epithelium, and define the stages. A cell association includes spermatogonia, spermatocytes, and spermatids, but is most readily identified by the appearance of the developing acrosome in the spermatids [1]. The spermatogenic cycle refers to the temporal progression of the stages through development. Although the number of spermatogenesis stages varies between species, the spermatogenesis cycle remains constant within species and strains [17][19]. Stages occur in a cyclical consecutive order, in a wave-like pattern within the seminiferous tubule (Figure 1.4B). Because of the convolution of the tubule, a cross section of the testis will reveal many seminiferous tubules at differing stages. These stages are sequentially ordered (in roman numerals) along the length of the seminiferous tubule, which begins and ends at the rete testis, forming a loop. The stages progress from the rete testis in descending order along both ends of the seminiferous tubule, to the site of reversal where the descending stages meet, and reverse.
Figure 1.4: Spermatogenesis Stages. (A) A composite histological image of each of the fourteen stages of rat spermatogenesis, arranged in a circle to reflect the continuous development and movement of the cells (Adapted from [16]). (B) Illustration of the wave of spermatogenesis stages in the rat seminiferous tubule that progresses in an orderly fashion.
1.3 Testicular Toxicity

Germ cell damage can occur as a result of direct or indirect toxicant insult, through alterations to the germ cells or in the cells responsible for germ cell developmental support, such as the Sertoli cell and Leydig cell. Disruption of Sertoli cell function can have detrimental effects on germ cells by impairing the BTB, which in turn allows immune cells and toxicants to reach the developing germ cells. Toxicants can also cause alterations in microtubule-dependent functions within the Sertoli cells, leading to changes in the transport of STF and hindering movement within the adluminal compartment. Two toxicants that indirectly affect germ cell development by impairing Sertoli cell function are 2,5-hexanedione (HD) and carbendazim (CBZ). X-ray, on the other hand, is an example of a direct germ cell toxicant. The extensive existing literature on these three toxicants has revealed both their mechanisms of action and exposure-induced testicular injuries, making them ideal candidates for model toxicants and co-exposure studies (Figure 1.5).
<table>
<thead>
<tr>
<th>Toxicant</th>
<th>Target</th>
<th>Mode of Action</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,5-hexanedione</td>
<td>Sertoli cell</td>
<td>Accelerated microtubule formation</td>
<td>Decreased STF Secretion ↓ Germ cell apoptosis</td>
</tr>
<tr>
<td>Carbendazim</td>
<td>Sertoli cell</td>
<td>Inhibited microtubule polymerization</td>
<td>Sertoli cell cytoplasm and germ cell sloughing</td>
</tr>
<tr>
<td>X-irradiation</td>
<td>Germ Cell</td>
<td>DNA damage in rapidly dividing spermatogonia</td>
<td>Germ cell apoptosis</td>
</tr>
</tbody>
</table>

Figure 1.5: Toxicant Summary Table.
1.3.1 2,5-Hexanedione

2,5-Hexanedione (HD) has been identified as the main metabolite of n-hexane in human urine \[20, 21\], while 2-hexanol is the major metabolite in animals exposed to n-hexane \[22\]. n-Hexane is a minor component of gasoline and is a common industrial solvent found in glues, varnishes, paints, and inks \[20\]. Exposure to n-hexane most frequently occurs in industrial workers through inhalation \[20, 23–25\], and in individuals who deliberately inhale glues or lacquers containing n-hexane vapors \[20, 26–28\]. Absorption of n-hexane occurs mainly through the respiratory system and is subsequently metabolized to the γ-diketone, HD, through cytochrome P450 dependent ω-1 hydroxylation and oxidation in the liver \[2, 20, 24\].

At subneurotoxic doses, HD is a testicular toxicant that interacts with the protein lysyl ε-amines to form pyrroles (reactive 5-membered heterocyclic aromatic ring compounds). Subsequent pyrrole oxidation leads to the formation of covalently cross-linked tubulin, which has altered microtubule nucleation properties \[29–34\]. Microtubule damage can be detrimental to the testis, since they are an important cytoskeletal component of the Sertoli cell and act as “tracks” for secretory vesicle transport for apically destined proteins produced by the Sertoli cell \[35–38\]. In addition, increases in the nucleation time in microtubule formation can lead to changes in cell function. For example, after HD exposure the nucleation time is enhanced, changing the number and length of Sertoli cell microtubules, leading to alterations in the cytoskeleton \[32\]. This ultimately disrupts the Sertoli cell’s ability to support developing germ cells through the secretion of proteins and seminiferous tubule fluid \[6, 9, 39\], resulting in germ cell loss and testicular atrophy \[32\].

In the timeline of exposure and effect, Blanchard et al. illustrated with DNA fragmentation studies that it is not until the fifth week of 1% HD exposure that...
the germ cells begin to undergo apoptosis, with apoptosis first occurring within spermatids and quickly followed by some spermatocytes and spermatogonia at six weeks [40]. Histopathological examination of HD induced testicular injury reveals an increase in the number and size of basally located membrane bound vesicles in Sertoli cells [33, 41] and an increase in germ cell apoptosis, both of which lead to subsequent germ cell sloughing into the seminiferous tubule lumen [40].

Retained spermatid heads are the spermatids that do not undergo spermiation, but are instead engulfed by Sertoli cells and moved to the basal compartment for degradation [42]. Quantification of these retained spermatid heads is the most sensitive indicator for HD exposure and has been used to establish the phenotypic lowest observable effect level (LOEL) (0.21% HD) and no observable effect level (NOEL) (0.14% HD) of HD exposure [42]. The rate of HD exposure, rather than total dose alone, has also shown importance in the manifestation of testicular injury [43]. Examination of HD recovery at 75 weeks has demonstrated that testicular injury remains mainly irreversible, with severely atrophic testes containing very few post-spermatogonial germ cells [12]. Most of the remaining germ cells are undifferentiated type A spermatogonia, although a few In and B spermatogonia remain in the seminiferous epithelium following HD treatment, indicating the occurrence of a block in spermatogenesis with spermatogonial progenitor cells failing to differentiate beyond spermatogonial stages (Type A$_2$-A$_4$) [44].

Stage specificity is another important factor in the occurrence of testicular injuries following HD exposure. Peak germ cell loss occurs seven weeks after the initiation of HD exposure [45], mostly in spermatogenesis stages X-XIV where type A$_2$-A$_4$ spermatogonia are found [44]. In the testis, elongated spermatids, spermatocytes, and round spermatids, in order of susceptibility, are the most sensitive cell types to HD-induced germ cell apoptosis [45]. With HD exposure, Sertoli cell vacuoles are
induced most often in the meiotic metaphase stages I and XII-XIV \[17, 41\]. Also, changes are seen in elongate spermatids in stages II-VIII, and there are basally located abnormal elongate spermatids in stages IX and X \[9, 42\]. High doses of HD (1%) also influence the frequency and duration of spermatogenesis stages, with significant decreases in the percent of tubules in stage VII and small increases in stages III, V-VI and IX-X \[41, 46\]. Changes in the frequency and duration of spermatogenesis stages leads to an increase in the spermatogenesis cycle (12.4 to 13.4 days) and because the entire spermatogenic process requires four cycles, the total duration of spermatogenesis can increase from approximately 50 to 54 days with HD exposure \[41\].

1.3.2 Carbendazim

Carbendazim (CBZ) is a metabolite of the systemic benzimidazole fungicide, benomyl \[47, 48\]. Benomyl is used to control plant disease in fruits, vegetables, field crops and ornamental plants by disrupting fungal microtubule polymerization assembly in a manner similar to colchicine \[49–52\]. Exposure to CBZ decreases both the rate and the stability of microtubule assembly, through its binding to the $\beta$-tubulin subunit of the $\alpha\beta$-tubulin heterodimer \[53\]. In vitro, CBZ acts as an effective inhibitor of microtubule assembly of brain and testis tubulin, by interfering with tubulin polymerization in microtubule formation \[50\]. The lethal dose, 50% (LD50) of CBZ is relatively high in rats at 10 g/kg body weight, although a dose of 100 mg/kg is enough to induce a testicular lesion \[54, 55\]. CBZ and its metabolites do not accumulate in the tissues of rats and the compound is primarily excreted through the urine in exposed mammals \[56, 57\].

The effects of CBZ on the male reproductive system have been well characterized.
Exposure effects within the testis develop rapidly and are directly correlated to the administered dose \[58\]. This rapid onset of testicular injury begins with sloughing of the seminiferous epithelium occurring as soon as one hour post exposure, leading to infertility one to five weeks later \[51, 58\]. CBZ induced testicular damage is hypothesized to occur through massive sloughing of the germ cells and the apical cytoplasm of Sertoli cells, thereby occluding the efferent ducts and preventing the movement of the STF from the testis to the epididymis \[58–60\]. The outcome of impaired STF passage is an increase in testis and epididymis weights, as well as an increase in seminiferous tubule diameter \[53\]. Nakai and Hess have proposed a mechanism for CBZ-induced sloughing, whereby the Sertoli cells with disrupted microtubules are not able to maintain their shape and as a result, retract their cytoplasm basally, causing the Sertoli cells to break at the apical cytoplasm \[61\]. This proposed mechanism is supported with recovery studies in rats exposed to CBZ, where the rats developed persistent infertility, with severe seminiferous tubule atrophy frequently accompanied by “Sertoli cell only” syndrome \[51, 62, 64\]. Other effects have also been observed, including significant decreases in sperm counts and motility \[62\], abnormal spermatogenesis \[60\], chromosomal aberrations \[65, 66\], and dose-dependent increases in germ cell apoptosis \[53\]. Studies with CBZ and colchicine have identified extensive microtubule depolymerization in isolated rat seminiferous tubules \[49\].

Similar to HD exposure, stage specificity is also a factor following CBZ exposure. Germ cell sloughing as a result of CBZ exposure in rats occurs in a stage specific manner in late stage VI to early stage VII and in stages XIII to XIV \[59\]. No changes have been found in Sertoli cells or with sloughing of the seminiferous epithelium in stages II to V and in stage VIII \[59\]. Similar to these findings are those from a seminiferous tubule transillumination study, where the premature release of spermatids by the Sertoli cells occurred one to four hours following CBZ exposure in stages VII,
1.3.3 X-irradiation

There are several sources of human exposure to ionizing radiation, including both naturally occurring environmental sources, such as cosmic radiation. There are man-made radiation sources as well, including diagnostic medicine, occupational exposure and even accidental exposures [68, 69]. Occupational radiation usually results in exposure to dose levels less than 0.5 Gy, whereas diagnostic medicine and radiation therapy can expose individuals to doses greater than 1 Gy [68, 69]. Humans are approximately three times more sensitive to radiation than rodent models [70], and even a low dose of 0.35 Gy can induce aspermia in men, which can become permanent after doses of 2 Gy or higher [71, 72]. Higher doses in the human testis, ranging from 4 - 6 Gy can cause a loss in total sperm production for about two years, if not permanently [73]. In studies with mice, a single dose of 0.5 Gy can induce subtle changes, while a single dose of 1 Gy induces more drastic changes, with an 80-90% reduction of the ability of spermatogonia to produce spermatocytes and spermatids [74, 75].

Testicular sensitivity to x-irradiation (x-ray) was first observed in 1903 [72]. The testis contains multiple stages of differentiating male germ cell types, resulting in differences in susceptibilities of the germ cells to radiation-induced death depending on their stage of development, chromatin condensation, nucleoprotein composition and their capacity to repair damage [76, 78]. X-ray induces free-radical alterations in DNA of proliferating differentiating cells, such as the A₁-A₄, intermediate, and B spermatogonial subtypes [79, 82]. While most spermatogonia become damaged after x-ray exposure, the undifferentiated type A spermatogonia have been found
to be more resistant to moderate doses of radiation \[83\]. The Sertoli cells, spermatoocytes, spermatids, and spermatozoa tend to be the most radio-resistant cells due to their lack of active mitosis, although infrequent apoptosis can occur in these cells after exposure to x-ray \[75\] \[84\]. Because the spermatocytes, spermatids, and spermatozoa are more radioresistant, the testis is able to maintain fertility for about three weeks before sterility occurs due to the death of a large portion of the differentiating spermatogonia \[84\] \[86\]. Allan et al. were the first to describe that x-ray induced spermatogonial cell death in the testis was a result of apoptosis \[79\]. Most spermatogonia undergo apoptosis between 9 and 18 hours following x-ray exposure and peak apoptosis occurs at 12 hours, to doses ranging from 0.5 Gy to 5 Gy \[74\] \[76\]. Abuelihija et al. found a block in differentiation in the transition from Type A spermatogonia to Type B spermatogonia, when several rat strains exposed to x-ray contained atrophic tubules with only type A spermatogonia \[87\].

X-ray induced apoptosis of spermatogonia is stage-specific, with a dose-dependent increase seen in stages I-IV and VII-VIII \[74\]. After doses of 0.5 Gy and 5 Gy x-ray, there are higher numbers of abnormal spermatogonia in stages I-VI in mice. Ten days following 5 Gy exposure in mice, the spermatogonial population is sparse with severe depletion of spermatocytes up to the pachytene phase in stage VIII \[75\] \[88\]. Similar stage-specific effects are seen in rats \[72\], demonstrating the conserved effects of the stage specificity of x-ray exposure.

### 1.3.4 Complex Exposures

The production and marketing of approximately 70,000 commercial and industrial chemicals leads to the potential of an exponential variety of mixtures that humans can be exposed to on a daily basis. Because exposure to any single toxicant rarely
occurs independently of other chemical exposures, it is prudent to examine toxicant effects in the context of complex mixtures or co-exposures that more adequately represent the true experience of exposure. In 1939, Bliss emphasized the importance of studying mixtures when he said “the effect of the mixture cannot be assessed from that of individual ingredients, but depends upon knowledge of their combined toxicity when used in different proportions. One component synergizes or antagonizes the other” [89]. The Food Quality Protection Act was passed in 1996, which requires the cumulative risk testing of chemicals with similar mechanisms of toxicity due to the growing numbers of complex chemical interactions. Chemicals can act through independent mechanisms of toxicity, resulting in co-exposure effects that are simply additive. However, when chemicals target the same organ system, the interactions among the targeted cell types within that organ may lead to complex interactive effects. The interaction of chemicals with similar targets or mechanisms of toxicity can result in addition, synergism (the effect is greater than the additive effect of two or more chemicals), or antagonism (the effect is lower than the additive effect of the chemicals) [90, 91].

In an effort to begin tackling the complex studies of mixed exposures, many laboratories have started the assessment of mixtures through the combination of a few toxicants with well known modes of action. By beginning with well studied toxicants, the knowledge gained can ultimately be applied to many more compounds that share similar modes of action and cellular targets. Examples of mixtures studies in the testis that have been performed to date include a study in rats of steroidogenesis inhibition by Aroclor1248, a commercial mixture of polychlorinated biphenyls [92], and co-administration of endocrine disrupting chemicals and the interactions that may occur on a common pathway of effect [93]. A co-exposure study with two germ cell toxicants, oxaliplatin and x-ray, found that the two exposures do not interact in
an additive or synergistic way and that they use differing pathways to achieve germ cell apoptosis [94].

Other studies that have focused on the exploration of testicular toxicity induced by combinations of well-characterized toxicants have uncovered unpredictable mixture interactions and effects. Given the opposing effects of the two Sertoli cell toxicants (HD and CBZ) on microtubule function, it would be expected that their combined exposure would negate each other. This is surprisingly not the case, where the toxicity of HD and CBZ combined exposure is exacerbated [95]. When compared to either CBZ or HD exposure alone, it was found that the combination of HD and CBZ caused additive and greater than additive effects in vacuolization and sloughing, respectively [95].

In co-exposure studies with x-ray and HD, the germ cell loss induced by x-ray exposure is attenuated with simultaneous exposure to HD [96]–[98]. These studies identified a stage specificity of germ cell apoptosis suppression by HD pre-treatment that occurs throughout stages I-VI of spermatogenesis, with the greatest increases in apoptosis occurring within stages II and III [96]. Microarray analysis in whole testis revealed the cell cycle and apoptosis genes underlying the HD induced attenuation of x-ray effect, including cyclin G1 (Ccng1), p53 up-regulated modulator of apoptosis (Puma) and apoptosis enhancing nuclease (Aen) [97]. Having observed attenuation of genes involved with positive regulation of apoptosis in whole testis tissue and the identification of stage-specific effects on histological analysis of germ cell apoptosis, HD attenuation of x-ray induced germ cell apoptosis was further examined through laser capture microdissection (LCM) [98]. The coupling of LCM of spermatogenesis stages I-VI with quantitative real-time PCR (qRT-PCR), illustrated a significant attenuation in x-ray induced Fas expression when combined with a priming exposure to HD [98]. These studies show the immense complexity that is inherent with co-
exposure studies within an already complex multicellular tissue, like the testis.

1.3.5 Low Doses of Toxicants

Lower dose exposures remain largely unstudied, with most of the assumptions about low dose effects based on high dose extrapolations \([99]\). Studying the effects of toxicants within the low dose range is difficult because many of the common tools for assessing toxicological effects are no longer applicable. Most often, the doses studied produce effects that cannot be measured analytically as they fall below the limits of detection. Increasingly, such is the case with gene array platforms, which are inherently noisy and lack sensitivity. This lack of sensitivity was apparent in our preliminary efforts to explore the low dose effects of co-exposures, using the Affymetrix rat genome platform for low dose extrapolations. Heat maps were generated from the array data (Figure 1.6), which demonstrated clear differences between the control groups and treated groups, especially with x-ray exposure. However, after the data were analyzed and corrected for multiple comparisons, there were no significantly altered transcripts following either exposure. With the relative insensitivity of this platform, it is difficult to differentiate between noise and actual transcript effects, especially those with low fold changes (below 2-fold). Such small changes induced by low doses of toxicants can still have biological significance, particularly when they involve an entire critical cellular pathway, such as apoptosis. In addition, traditional measurements of toxicity such as histology become less useful, as low doses may not produce overt toxicity. As low dose exposures often produce widespread low level changes in genes within an entire pathway, new tools and methods need to be developed, particularly analytic methods, to elucidate the effects of pathway responses to low dose exposures.
Figure 1.6: Low-dose exposure hierarchical clustering. Hierarchical cluster analysis of differentially expressed genes after x-ray and HD exposure. RNA was isolated from whole testis removed from animals that were exposed to either HD (A) or x-ray (B) and processed on Affymetrix Rat 1.0 ST gene chips. Unsupervised hierarchical clustering and heat maps of subsets of significant transcripts were performed using Partek software analysis. Yellow represents upregulation and blue represents downregulation. Colors below the heat maps indicate biological replicates with green, red and blue representing controls, 0.1 Gy and 0.5 Gy in (A), respectively. In (B) the purple, red, blue and green colors represent controls, 0.125%, 0.2% and 0.33%, respectively.
Radiation is an excellent candidate for studying low dose-effects, since its exposure does not involve drug metabolism and the outcome of exposure in the testis has been well documented. The importance of studying radiation exposure is highlighted by the fact that humans are exposed to low doses of radiation on a daily basis. X-ray exposure effects, such as apoptosis and prevention of cell cycle progression, are not detected in doses less than 0.25 Gy [99]. Exposure to a “priming” or “adapting” low dose of x-ray (1-25 cGy) is sufficient to reduce the effects caused by a later higher x-ray dose, or “challenge” exposure [99]. An “adaptive” response has also been described, where cells that are pre-exposed to low-dose radiation are protected from subsequent high-dose radiation genomic and cytological effects [68, 100]. In fractionated x-ray experiments, Cai et al. have shown that pre-treating cells with low doses of x-ray (0.2 Gy) protected them in subsequent exposures to non-radiation agents, such as hydrogen peroxide or chemotherapeutics [101].

To determine what an adaptive response is, there first needs to be a definition of what an adaptive effect is, as well as and understanding of how an adaptive effect becomes adverse. Several definitions of an adaptive effect in the literature were compiled and put into the context of toxicology by Keller et al., who defined it as “the process whereby a cell or organism responds to a xenobiotic so that the cell or organism will survive in the new environment that contains the xenobiotic without impairment of function” [102]. The point where this adaptive effect transitions into “a change in morphology, physiology, growth, development, reproduction, or life span of a cell or organism, system, or (sub)population that results in an impairment of functional capacity, an impairment of the capacity to compensate for additional stress, or an increase in susceptibility to other influences”, is when the effect can be deemed an adverse effect [102]. Given these definitions of adaptive and adverse responses, the response detected in the low-dose x-ray studies detailed above are in
line with what has been defined as an adaptive response, where the cells pre-exposed to low doses of x-ray are able to resist the damage normally inflicted by a higher dose of x-ray.

1.4 Apoptosis

Programmed cell death characterized by a reduction in cell volume, cell membrane blebbing, chromatin condensation, cytoplasmic vacuolization, and disassembly of the cell into membrane bound vesicles, was first described as “apoptosis” in 1972 by Kerr et al. [103]. Cells undergoing apoptosis are able to die without causing damage to surrounding cells and tissues. Histological markers such as terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), and morphological characteristics like DNA laddering were used to identify apoptotic cells before gene expression studies examined the genes underlying apoptosis. Apoptosis is activated via two main pathways; an intracellular driven pathway with “stress sensors” like p53 and an extracellular pathway that works through ligand binding to cell surface receptors like Fas and death receptor 5 (DR5) (Figure 1.7). The intrinsic pathway (mitochondrial) and the extrinsic pathway (death receptors) are activated through different mechanisms, although they are not mutually exclusive. The apoptosis activation pathway employed depends on factors like tissue type, target cell developmental stage and the source of the insult. Once apoptosis has been activated, regardless of activation pathway, there are three phases: an initiation phase, a signaling phase, and an execution phase [104]. In the testis, the spermatogonia undergo apoptosis following exposures to toxicants such as radiation, but they do not exhibit typical morphological characteristics of apoptosis [74] [105].
Activation of the main cellular response to stress involves the localization of stabilized p53 to the nucleus, where it can activate or repress the transcription of the genes controlling cell cycle arrest, DNA repair, senescence and apoptosis \[106-108\]. The focus here will be on the role of p53 in apoptosis. Approximately 200-400 genes are known to be transcriptionally regulated by p53 expression, and it is hypothesized that it regulates the expression of many more target genes. Included in the transcriptional targets of p53 are the mediators of apoptosis, which include Fas, Bcl2-associated X protein (Bax), and DR5, among others \[109\].
Figure 1.7: Cellular apoptosis pathway model. Initiation of apoptosis begins with the binding of ligands (TRAIL, TNF, or FasL) to their respective receptors (DR4/DR5, TNFR1 or Fas), which are up-regulated through p53. This leads to the activation of the caspase cascade, ultimately resulting in cellular apoptosis. Adapted from El-Deiry et al. [109].
The apoptosis caspase cascade is activated by p53 through up-regulation of Bax, Fas and DR5 expression, with simultaneous repression of B-cell CLL/lymphoma 2 (Bcl2). The ratio of Bax to Bcl2 is critical in maintaining the balance in apoptosis, and is controlled by p53. p53-mediated up-regulation of Bax and down-regulation of Bcl2 results in the release of mitochondrial cytochrome c, which then interacts with apoptotic peptidase activating factor 1 (Apaf1) to activate caspase-9 and the triggering of the caspase cascade including the downstream caspases-3, -6 and -7[110,111]. The mechanism by which p53 acts upon Fas involves the translocation of Fas from the golgi to the cell surface[109]. The Fas transmembrane receptor, also known as APO protein 1 (Apo1), trimerizes following exposure to FasL and through its cytoplasmic death domain, recruits the adaptor Fas-associated Death Domain (FADD)[112,114]. The Death Effector Domain (DED) of FADD is responsible for the recruitment of the initiator caspase-8 to the Death Inducing Signaling Complex (DISC), triggering the amplification of the apoptotic signal and activation of downstream executioner caspases[110,115,118]. Other death receptors, like the TNF receptor, trigger apoptosis through the adaptor TNFRSF1A-associated via death domain (TRADD), but can also promote survival through c-Jun N-terminal kinase JNK (JNK) and TNF receptor-associated factor 2 (Traf2). DR5 is also a pro-apoptotic death-domain containing member and is in the TNF-related apoptosis induced ligand (TRAIL) receptor family, which also includes DR4 and the two “decoy” receptors Truncated Intracellular domain (TRID) and Truncated Death Domain (TRUNDD)[109]. TRAIL receptor signaling is similar to Fas signaling, in that it involves downstream caspase activation, but it does not appear to require FADD[109]. However, the mechanism by which the TRAIL receptor DR5 induces apoptosis, as well as how p53 regulates DR5 expression, remains unclear[109].
1.4.1 Normal Apoptosis During Spermatogenesis

Spermatogenesis involves a precise balance between cell survival, proliferation, and differentiation. During spermatogenesis, the germ cells clonally expand before they differentiate and mature into spermatozoa [119]. The germ cell expansion is excessive and without a “thinning out” process, the supportive capacity of the Sertoli cells would be overwhelmed. In the rat, approximately 75% of potential germ cells must undergo apoptosis to maintain the balance of germ cell density and the supportive capacity of the Sertoli cells [120]. In addition to culling excessive numbers of germ cells, apoptosis is required to eliminate aberrant germ cells containing genetic defects [121, 122]. This routine apoptosis occurs in the differentiating type A spermatogonia [123–125] and in spermatocytes during their meiotic divisions [1, 126]. Once the germ cells undergo apoptosis in the testis, the Sertoli cells quickly phagocytize them [100]. Cell density related apoptosis and DNA damage induced apoptosis might follow separate pathways in their activation [127, 128]. Sertoli cells express FasL, which can initiate apoptosis in the germ cells expressing Fas. This cell-to-cell interaction is used to limit the germ cell population and can be altered by toxicant exposure or testicular stress [119].

1.4.2 Apoptosis During Testicular Injury

The testis is a target organ for many environmental toxicants, and the different cell types comprising the testis respond to injury differently. Toxicant exposures typically culminate with germ cell apoptosis, due to the loss of nutritional and structural support from the Sertoli cells and the hormonal support from the Leydig cells that germ cells are dependent on. The apoptotic pathway is complex and how germ
cells undergo apoptosis as a result of toxicant exposure can manifest in different ways depending on the resulting testicular injury. The roles of the main apoptotic mediators in testis germ cell apoptosis, as a result of exposure to different toxicants, are detailed below and summarized in Table 3.1.

p53

Although p53 is not expressed in spermatogonia during normal spermatogenesis, it does play a role in germ cell meiosis and is present in primary spermatocytes [88, 99, 129–131]. During testicular stress, p53 is up-regulated to initiate either the extrinsic or intrinsic pathways of apoptosis, repair pathways, or G1S/G2M cell cycle arrest [88, 132]. This is illustrated with enhanced p53 expression following radiation exposure, which contributes to its role as a key molecular mediator of apoptosis [129, 131]. Following x-ray doses greater than 0.5 Gy, p53 activates downstream Fas death receptor [105] and mitochondrial pathways of apoptosis [133], leading to a 30 - 50% loss of differentiating spermatogonia. Fas localization to the plasma membrane from Golgi complex stores is mediated by p53 in a transcription independent manner [134, 135]. Caspase-7 is downstream of p53 and Fas, and peak up-regulation is seen 21 days following exposure to 4 Gy x-ray [136]. The death receptor, DR5, is also a p53 inducible gene that has been shown to be up-regulated along with Puma in spermatogonia enriched cell populations exposed to 1.6 Gy x-ray [134, 137]. Exposure to the toxicant MEHP induces germ cell apoptosis that is dependent on the p53 protein and its downstream activation of Fas and DR5, which localizes them on the germ cell membrane [134].
Fas and DR5

In the testis, the Sertoli cells express FasL, which binds to the Fas receptor expressed by the germ cells to trigger the apoptotic process [119, 138, 139]. The interaction between Trail and its receptor, DR5, triggers a signaling pathway similar to the Fas/FasL system [140]. DR5 is up-regulated in spermatogonia and spermatocytes in a p53 dependent manner following testicular injury with x-ray, as shown in studies with cell populations enriched for spermatogonia through cell sorting [137].

The involvement of Fas in apoptosis was first recognized through its role in immune regulation and the control of immune privileged sites [112, 141, 142]. The regulation of germ cell apoptosis by Fas and FasL expression between the Sertoli and germ cells was a controversial finding for many years, until several studies were performed with mice deficient in Fas, FasL, or both. *gld* (generalized lymphoproliferative disease) mice have a spontaneous mutation in FasL preventing it from being bound and activated by Fas [143]. These mice have spontaneous incidences of germ cell apoptosis and have the same levels of apoptosis as wild-type mice exposed to the germ cell toxicant, x-ray [144]. However, when the *gld* mice are challenged with the Sertoli cell toxicant mono-(2-ethylhexyl) phthalate (MEHP), they have minimal levels of Fas induced apoptosis, highlighting the importance of the Fas system in Sertoli cell mediated germ cell apoptosis [144]. Mice with little to no Fas protein, known as *lpr* (lymphoproliferation) mice, have a transposon insertion in the intron2 region of the Fas gene [145–147]. The overwhelming importance of Fas in testicular homeostasis is demonstrated with these *lpr* mice, because even though they lack the ability to properly transcribe the Fas gene, the testis is still able to maintain normal levels of Fas [119]. Point mutations in both the FasL and Fas genes result in nonfunctional proteins in the *lpr* [148] (lpr complementing gld) mice [112].
*lpr*<sup>c9</sup> mice are exposed to 0.5 Gy or 5 Gy x-ray, they have increased spermatid head counts and a greater number of apoptotic seminiferous tubules, respectively, when compared to wild-type mice [105].

Fas signaling is involved in testis injury following many different insults, including chemical and radiation exposure, cryptorchidism and ischemia reperfusion [105, 119, 138, 148, 149]. Exposure to 2 Gy x-ray increases the number of apoptotic cells 24 hours after exposure, with maximum *Fas* mRNA levels at 3 hours post-exposure, while no changes are seen in *FasL* [98, 150]. Exposure to the Sertoli cell toxicant, MEHP, causes an increase in *Fas* and *FasL*, while exposure to another Sertoli cell toxicant, HD, only induces up-regulation of *Fas* [148]. Fas has also been shown to be involved with heat induced testicular germ cell apoptosis [120].

**Bcl2 Family**

The Bcl2 family is divided into three groups; group I members possess anti-apoptotic activity and groups II and III members promote cell death [110]. The pro-apoptotic groups II and III include Bax, Bak, Bcl-X<sub>s</sub> and Bad [126]. Bcl2, Bcl-X<sub>l</sub>, and Mcl are anti-apoptotic and belong to group I [126]. The anti- and pro-apoptotic roles of the Bcl2 family are important for maintaining the population of germ cells that can be properly supported by the Sertoli cells during normal spermatogenesis. For example, in Bcl2 transgenic mice, the anti-apoptotic gene *Bcl2* is over expressed in spermatogonia, which leads to overpopulation of spermatogonia due to decreased germ cell apoptosis [123, 124]. Bcl2 is localized to late spermatocytes and spermatids and is up-regulated in these cells after 4 Gy x-ray exposure [133]. Bax can be found in all testicular cell types, in both the mouse and the human, but it is not responsive to x-ray exposure [133]. However, the importance of Bax in the testis is seen with Bax
disruption in mice, from the occurrence of spermatogonial hyperplasia and increased early spermatocyte death.  

**Additional Mediators in the Apoptotic Pathway**

There are other genes within the apoptotic pathway that have been shown to play a role in toxicant induced apoptosis in the testis. One of these genes, nuclear factor κB (NFκB) is activated through the oxidative stress pathway, and is pro-apoptotic in germ cell response to both radiation exposure and ischemia reperfusion.  

The NFκB signaling pathway also mediates the initial protection provided by Akt1, following MEHP-induced germ cell apoptosis. The protective effect of Akt1 has also been demonstrated in the testis of WT mice, where Akt1 deficient mice have an earlier onset of germ cell apoptosis and enhanced sensitivity of differentiating spermatogonia, after radiation exposure. Another mechanism of pro-survival used by the testis is the binding of the Sertoli cell stem cell factor to the c-kit ligand expressed by the differentiating spermatogonia.
Table 1.1: Summary of Apoptotic Regulators.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene function</th>
<th>Activation Pathway</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fas/FasL</td>
<td>Pro-apoptotic; death receptor initiator of apoptosis</td>
<td>Extrinsic</td>
</tr>
<tr>
<td>DR4,DR5/TRAIL</td>
<td>Pro-apoptotic; death receptor initiator of apoptosis</td>
<td>Extrinsic</td>
</tr>
<tr>
<td>TNFR1/TNF</td>
<td>Pro-apoptotic; apoptosis initiator</td>
<td>Extrinsic</td>
</tr>
<tr>
<td>FADD</td>
<td>Pro-apoptotic; signaling complex</td>
<td>Extrinsic</td>
</tr>
<tr>
<td>TRADD</td>
<td>Pro-apoptotic; signaling complex</td>
<td>Extrinsic</td>
</tr>
<tr>
<td>Caspase-8, -9, -10</td>
<td>Pro-apoptotic; initiator caspases</td>
<td>Extrinsic/Intrinsic</td>
</tr>
<tr>
<td>Caspase-3, -6, -7</td>
<td>Pro-apoptotic; executioner caspases</td>
<td>Extrinsic/Intrinsic</td>
</tr>
<tr>
<td>p53</td>
<td>Anti-apoptotic/Pro-apoptotic</td>
<td>Extrinsic/Intrinsic</td>
</tr>
<tr>
<td>NFκB</td>
<td>Pro-apoptotic; transcriptional regulator</td>
<td>Intrinsic</td>
</tr>
<tr>
<td>Bax</td>
<td>Pro-apoptotic; increases release of cytochrome c</td>
<td>Intrinsic</td>
</tr>
<tr>
<td>Bid</td>
<td>Pro-apoptotic; increases release of cytochrome c</td>
<td>Intrinsic</td>
</tr>
<tr>
<td>Bak</td>
<td>Pro-apoptotic; increases release of cytochrome c</td>
<td>Intrinsic</td>
</tr>
<tr>
<td>Bad</td>
<td>Pro-apoptotic; inhibits Bcl2 and Bcl-X1 activity</td>
<td>Intrinsic</td>
</tr>
<tr>
<td>Bim</td>
<td>Pro-apoptotic</td>
<td>Intrinsic</td>
</tr>
<tr>
<td>Bcl-X₃</td>
<td>Pro-apoptotic</td>
<td>Intrinsic</td>
</tr>
<tr>
<td>Puma</td>
<td>Pro-apoptotic</td>
<td>Intrinsic</td>
</tr>
<tr>
<td>Bcl2</td>
<td>Anti-apoptotic; inhibits caspase activity through inhibition of cytochrome c release</td>
<td>Intrinsic</td>
</tr>
<tr>
<td>Bcl-X₁</td>
<td>Anti-apoptotic</td>
<td>Intrinsic</td>
</tr>
<tr>
<td>Mcl</td>
<td>Anti-apoptotic</td>
<td>Intrinsic</td>
</tr>
<tr>
<td>Akt1</td>
<td>Anti-apoptotic</td>
<td>Intrinsic</td>
</tr>
</tbody>
</table>
References


98. Campion, S. N., Sandrof, M. A., Yamasaki, H. & Boekelheide, K. Suppression of radiation-induced testicular germ cell apoptosis by 2,5-hexanedione


134. Chandrasekaran, Y. & Richburg, J. H. The p53 protein influences the sensitivity of testicular germ cells to mono-(2-ethylhexyl) phthalate-induced apoptosis by increasing the membrane levels of Fas and DR5 and decreasing the intracellular amount of c-FLIP. *Biol Reprod* 72, 206–13 (2005).


Chapter Two

Identification and Characterization of Adverse Effects in 21st Century Toxicology
Identification and characterization of adverse effects in 21st century toxicology

Douglas A. Keller,* Daland R. Juberg,† Natasha Catlin,‡ William H. Farland,§ Frederick G. Hess,¶ Douglas C. Wolf,|| and Nancy G. Doerrer |||

* Sano US, Bridgewater, New Jersey 08807
† Dow AgroSciences LLC, Indianapolis, Indiana 46268
‡ Brown University, Providence, Rhode Island 02912
§ Colorado State University, Fort Collins, Colorado 80523-2001
¶ BASF Corporation, Research Triangle Park, North Carolina 27709-2000
|| US Environmental Protection Agency, Research Triangle Park, North Carolina 27711
||| ILSI Health and Environmental Sciences Institute, Washington, District of Columbia 20005-1743


2.1 Declaration of Author’s Roles

All authors contributed to the organization and writing of the manuscript equally and approved the final version to be published.
2.2 Abstract

The practice of toxicology is changing rapidly, as demonstrated by the response to the 2007 NRC report on “Toxicity Testing in the 21st Century.” New assays are being developed to replace animal testing; yet the use of data from these assays in decision making is not clear. A Health and Environmental Sciences Institute committee held a May 2011 workshop to discuss approaches to identifying adverse effects in the context of the NRC report. Scientists from industry, government, academia, and NGOs discussed two case studies and explored how information from new, high data content assays developed for screening can be used to differentiate adverse effects from adaptive responses. The terms “adverse effect” and “adaptive response” were defined as well as two new terms, the relevant pathways of toxicological concern (RPTCs) and relevant responses for regulation (RRRs). RPTCs are biochemical pathways associated with adverse events and need to be elucidated before they are used in regulatory decision making. RRRs are endpoints that are the basis for risk assessment and may or may not be at the level of pathways. Workshop participants discussed the criteria for determining whether, at the RPTC level, an effect is potentially adverse or potentially indicative of adaptability, and how the use of prototypical, data-rich compounds could lead to a greater understanding of RPTCs and their use as RRRs. Also discussed was the use of RPTCs in a weight-of-evidence approach to risk assessment. Inclusion of data at this level could decrease uncertainty in risk assessments but will require the use of detailed dosimetry and consideration of exposure context and the time and dose continuum to yield scientifically based decisions. The results of this project point to the need for an extensive effort to characterize RPTCs and their use in risk assessment to make the vision of the 2007 NRC report a reality. 

Key Words: adverse; adaptive; high data content assays; RPTC; RRR; 21st century toxicology
2.3 Introduction

The science supporting regulatory toxicology is undergoing a transformation that will change how toxicology testing, interpretation, and use of data in decision making and public health protection will be performed in the decades ahead. The developing advanced technologies and high-throughput approaches for toxicity testing will reduce animal use, permit legislative action around broader chemical testing needs leading to reform of the U.S. Toxic Substance Control Act (TSCA), and improve efficiency of drug and chemical development. The new testing approaches and techniques are designed to identify markers or endpoints that are a departure from apical endpoints associated with traditional toxicology testing (e.g., cancer, reproductive effects). However, these new early-stage endpoints present a challenge for biological interpretation as well as integration into current risk assessment practices and regulatory decision making. A series of forum articles was published in *Toxicological Sciences* in 2009 – 2010 outlining the challenges and potential solutions to the vision outlined in the 2007 NRC report [1–10]. These articles were useful for setting the stage and identifying many of the issues surrounding full implementation of the goal of the NRC report. The NRC (2007) identified one issue in particular as critical – the need to determine what makes an effect “adverse” [11].

Existing toxicological test designs are based on the identification of an adverse effect at a given dose, which can be used to define a point of departure for subsequent assessment of risk and regulatory decision making. The rapidly expanding availability of *in vitro* predictive tools and technologies will spawn an increasing number of endpoints and potential effects as well as a complex web of biological pathways that will need to be considered as viable markers for safety and risk assessment. An integrative analysis approach will identify new markers of toxicity that will need to be
fully characterized for dose-response, their relation to *in vivo* physiological systems, and their relevance to humans before they can be used appropriately.

Differentiation between an adverse effect and an adaptive response is central to toxicology and is a critical determination in the context of these new toxicity-testing approaches. In anticipation of the need for rigorous scientific input into how new endpoints and markers of biological change may be incorporated into risk assessment, the International Life Sciences Institute (ILSI) Health and Environmental Sciences Institute (HESI) formed a committee in 2009. The committee consisted of scientists from the chemical and pharmaceutical industries, U.S. government agencies, and academic institutions ([http://www.hesiglobal.org/i4a/pages/index.cfm?pageid=3440](http://www.hesiglobal.org/i4a/pages/index.cfm?pageid=3440)). This group first defined the terms adverse and adaptive and then examined the ways in which these terms could be used when high data content information as the sole input. Specific goals included the following: (1) develop an approach to evaluate effects from new *in vitro* toxicity-testing tools for integration into the safety assessment of chemicals; (2) develop criteria to assist in differentiating adverse effects from other types of biological changes; and (3) review and revise the definitions of adverse and adaptive effects based on toxicological and biological considerations relevant to regulatory decision making.

The HESI committee convened a workshop titled “Distinguishing Adverse from Adaptive Effects in the 21st Century” on 10 – 11 May 2011 at the U.S. Environmental Protection Agency (USEPA) facilities in Research Triangle Park, NC. Workshop participants (Supplementary Table A.1) discussed, together and in small groups, the characterization of biological responses; integration of responses within a biological pathway; interpretation of different categories of data for safety assessment; and the potential development of a framework that recognizes, prioritizes, and uses all toxicity testing approaches and data in safety assessment. Two case studies, dimethy-
larsinic acid (DMA) and acetaminophen, were reviewed and discussed in the context of how the data from *in vitro* studies, particularly toxicogenomics and the pathways implicated from those data, related to known outcomes for apical endpoints. Proposals were made for future areas of research.

The following provides an overview of committee discussions prior to and during the workshop. These discussions focused on the use of data and high data content information from *in vitro* studies to inform decisions about adversity and their potential to inform risk assessment and advance regulatory approaches for protection of public health.

### 2.4 Characterization of Biological Response: Defining an Adverse Effect

From the time of Paracelsus, the effects of chemicals on biological systems have been characterized by the apical response, which is the observable outcome in a whole organism such as a clinical sign or pathological state that is indicative of a disease resulting from exposure to a toxicant [1]. This has been used in toxicology as relevant for designing and interpreting studies, comparing compounds, and determining appropriate human exposure limits. As biology continues to develop, computational approaches and *in vitro* studies including high-throughput assays will enable the assessment of alterations of pathways and networks that have been described at the gene, protein, or metabolic level of organization. The present challenge is to determine the value and appropriate use for these data in the context of risk assessment. The adverse effect drives regulatory decisions of chemicals under U.S. legislation, including TSCA and the Federal Insecticide, Fungicide, and Rodenticide Act, and in
Europe under the Registration, Evaluation, Authorisation, and Restriction of Chemicals legislation. Also, many processes that inform risk-based decisions are founded on identifying an adverse effect, including benchmark dose calculations and doses allowed in pharmaceutical clinical trials.

Definitions of adverse effect can be found in many laws, regulations, and in the scientific literature \[11–18\]. From this extensive literature, the HESI committee developed and reached consensus on definitions of adverse effect and adaptive response for the purpose of achieving its specific goals. These working definitions were agreed to in principle by the May 2011 workshop participants:

**Adverse Effect:** A change in morphology, physiology, growth, development, reproduction, or life span of a cell or organism, system, or (sub)population that results in an impairment of functional capacity, an impairment of the capacity to compensate for additional stress, or an increase in susceptibility to other influences.

**Adaptive Response:** In the context of toxicology, the process whereby a cell or organism responds to a xenobiotic so that the cell or organism will survive in the new environment that contains the xenobiotic without impairment of function.

A number of research efforts \[19\] [21] have the potential to change the way toxicity testing is conducted consistent with the NRC vision. The paradigm in which a mode of action (MOA) is determined for a single chemical, followed by development of predictive assays for that MOA, is shifting toward a new paradigm where screening assays will be performed that lead to prediction of an MOA for a compound with confirmation by targeted testing in well-defined assays (Figure 2.1).
Figure 2.1: Present and future testing paradigms for understanding mechanisms of toxicity. Currently, MOAs are postulated followed by determination of intermediate networks and pathways, culminating in screening assays to detect compounds that present this MOA. In the future, screening assays will be used to postulate MOAs by prior understanding of the links between the screen, targets, pathways, networks, and MOAs.
Fulfillment of this paradigm requires sufficient understanding of the intermediate steps between the screening assays that indicate a molecular initiating event and other assays that characterize additional key events in an MOA that will lead to an adverse outcome.

2.5 Placement of the Effect Within a Biological System: Relevant Pathways of Toxicological Concern and the DMA Case Study

To assess the impact of in vitro high data content information on the effectiveness of the working definitions of adverse effects and adaptive responses, the HESI committee used case studies of compounds for which there was good knowledge of MOAs along with detailed mechanistic studies available in the literature. These cases were reviewed in detail by the committee and then discussed at the workshop. For illustration purposes, an overview of the DMA case is highlighted here.

The discussion of DMA began with the assumption that systems biology, including homeostasis based on repair mechanisms and other adaptive responses, will play a major role in the application of new data types. In addition, computational modeling of these pathways and networks will be critical to formulate hypotheses, design targeted tests, and establish a collection of MOAs and adverse outcome pathways associated with toxicity. With this knowledge, risk assessment of drugs and chemicals will have a stronger scientific foundation and can be conducted with greater certainty and applicability to human biology. Although this may not be achievable in the short term [22], efforts such as the USEPA’s computational toxicology pro-
gram and the explosion of bioinformatics tools indicate that the vision will eventually become reality.

The concept of the toxicity pathway is an important part of the NRC report [11]. The pathway concept suggests that toxicity results when a chemical reaches and interacts with an initial key target, beginning a series of biological events that can ultimately result in the development of an adverse outcome. The toxicity pathway is a cellular response pathway that when sufficiently perturbed can result in an adverse effect [11, 13]. At the HESI workshop, participants referred to pathways associated with adverse effects as relevant pathways of toxicological concern (RPTCs). The number of RPTCs is unknown, although it is possible that a relatively small number may describe the majority of toxic responses. At low doses, changes in an RPTC may reflect adaptation, whereas at higher doses the changes may be adverse. A critical near-term need is to determine which pathways are RPTCs and describe them in a quantitative manner so that the changes in these pathways can be used to assess risk. Understanding the key nodes and the dose transition points in the RPTCs will be an important part of this learning. Boekelheide and Andersen (2010) [13] provide an example of how understanding the RPTCs for carcinogenicity could change the way carcinogenicity assessments are conducted. The authors point out the need for understanding pathway dynamics and the role computational methods will play in developing these new approaches. Although some efforts toward defining specific RPTCs have been made, detailed knowledge of RPTCs for use in risk assessment is not yet available [23, 24].

The identification of an adverse outcome after xenobiotic exposure has been a mainstay for assessing risk to inform risk management decisions. Adverse effects used for these decisions tend to be apical outcomes such as tumors, permanent changes in the target tissue, or specific transient changes in the target tissue directly associated
with the ultimate outcome of concern. Another term discussed at the May 2011 HESI workshop was the relevant response for regulation (RRR), that is, denoting the endpoint, which is the basis for a risk assessment. In theory, the RPTC affected at the lowest internal dose could be considered to be the RRR, mitigating the need for in vivo studies. Clearly, for the RPTC to become the RRR, a great deal more must be known about the structure and dose-response of pathways as well as cross talk and redundancy between pathways.

As an example of the current state of the art of an MOA-based risk assessment using the most detailed biological information available, the committee considered the case of DMA. The USEPA established an MOA following the International Programme for Chemical Safety Mode of Action/Human Relevance Framework for DMA, a herbicide and inorganic arsenic metabolite \[25, 26\]. The basis for the DMA MOA was the evaluation of a series of apical endpoints as key events for the ultimate apical endpoint of transitional cell tumors of the urinary bladder in rats. These apical endpoints included transitional cell death, proliferation, and hyperplasia. Experiments characterized the effects in the target cell using transcriptional profiling \[27, 28\]. These studies were designed to identify key molecular changes associated with exposure to DMA and to better characterize the pathways or key events leading to the various apical outcomes. In brief, the authors identified transcriptomic changes at doses below which one sees the apical adverse endpoint of transitional cell death in the target epithelium from exposed rats as well as in vitro \[27, 28\]. The authors suggested that the toxicity observed at the higher doses may add to precursor effects present at the lower doses to drive the development of the apical outcome of a tumor \[27\]. However, although the changes in gene expression indicated potential RPTCs, there was neither enough knowledge of these pathways to quantitatively describe the key events in development of the tumor nor were there
sufficient species-specific descriptions of these pathways that could determine if the rat and human would have different or similar responses. Therefore, the response did not qualify as an RRR.

In the context of the discussion on adaptation and adversity, it may be that the cellular response to DMA sets up a series of activated genes, which allow the transitional cells to survive insults at the lower doses. The adaptive response may impart protection and/or enhanced susceptibility to the toxic effects. As one increases the dose of DMA to the target cell, increasingly severe responses occur, from urothelial toxicity to hyperplasia and ultimately transitional cell tumors. At the lowest doses tested in these studies, only altered gene expression was identified \cite{27, 28}. At intermediate doses, the apical endpoint of cell death and increased cell proliferation occurred but resolved over time, suggesting an additional adaptive response to the continued exposure \cite{26, 27}. After extended treatment with the highest doses of DMA, irreversible tissue responses occurred resulting in the apical endpoints of cellular hyperplasia or tumors \cite{26}. This example illustrates the combined significance of context, amount of exposure, and duration of exposure. The biological significance of various exposure-related effects and the determination of whether they were adverse depended on establishing a relationship among the several key events described for this MOA. Figure \ref{fig:2.2} illustrates the hypothetical dose-response relationship for putative RPTCs and how this information might be used to determine adverse effect levels. This example also illustrates the need to gain a more detailed, quantitative knowledge of molecular initiating events, toxicity pathways, and their interactions to improve the understanding of where transition points occur between adaptive changes and adverse effects. This will aid species extrapolation of effects and decrease uncertainty in risk assessments, potentially reducing reliance on uncertainty factors.
Figure 2.2: Dose transitions for adverse toxicant response with four differentially susceptible nodes. A hypothetical toxicant has functional effects on four network nodes (N1-N4), including four distinct dose transitions detectable at increasing toxicant doses. At the highest cumulative dose, toxicant exposure produces high incidence of an apical endpoint (i.e., cell or organism death). *In silico* studies can link adverse effects to exposure to lower doses of the toxicant.
A similar case can be made for data on hepatotoxicity of acetaminophen \cite{29, 32}; however, this example is not described here.

### 2.6 Characterization of Adversity: Moving From the Science to Risk Assessment Application

At the May 2011 workshop, participants met in breakout groups to discuss issues that the HESI committee had been deliberating for the previous two years, including the following four topics:

- What are the most important criteria to consider in determining whether a system has been perturbed to the point of adversity?

- What criteria should be used to decide if there is sufficient information to identify an effect as adverse?

- How does “context” (e.g., early life exposures) influence the determination of adversity?

- Would a framework approach provide a useful tool for determining adversity/potential adaptability for specific situations?

In addition to characterizing new endpoints that emanate from emerging tools and testing approaches, interpreting these changes demands attention and continued discussion. The data must be interpreted with identification of the most relevant responses considered to be early markers of exposures leading to an adverse effect. An adverse response cannot be ascertained from a single observation. Given the
upstream nature of many reported genomic and other endpoints, it is important to
determine where such changes lie along the continuum of biological response and how
the changes are connected to other levels of biological organization. Additionally,
one would need to characterize the normal background and variability of responses
of the markers and whether these markers of toxicity or exposure can be appropri-
ately extrapolated to humans. If one assumes that RPTCs are involved in adversity,
then for each pathway one would need to determine the dose and time dependence
of the response and the perturbation of the pathway in the context of an affected
network. The critical nodes of the network would need to be identified as well as the
correspondence between affected network nodes and organ function and physiology.
Some HESI workshop participants suggested that there are likely to be a finite num-
ber of MOAs that need to be investigated for the associated RPTC. These MOAs
would include receptor-mediated modes, inflammation, cytotoxicity, and genotoxic-
ity. Some key pathways involved are likely to be stress responses \[23\] and nuclear
receptor-mediated pathways. Although the concept is feasible, pathways have not
been sufficiently elucidated for many MOAs already known to lead to an adverse
outcome, such as genetic alterations leading to cancer \[13\].

The first step toward understanding adversity at the pathway level is to exten-
sively characterize the RPTCs for a few prototypical, data-rich chemicals. As expe-
rience is gained with chemicals with a well-defined MOA, studies could be performed
prospectively with novel compounds or unknowns to determine whether investigation
of the RPTC was sufficient to predict adverse outcomes of apical endpoints. Ulti-
mately, the intermediate steps could be eliminated as additional important RPTCs
are identified, and confidence is gained in the validity of these predictions. A sig-
nificant investment in research describing toxicity pathways and adverse outcome
pathways, with identification of biomarkers associated with adversity, is needed to
achieve this goal. Once RPTCs are sufficiently characterized and shown to be predictive of apical endpoints, chemicals could be tested \textit{in vitro} on the key nodes of RPTCs to determine the potential for causing adverse effects without the need for animal testing.

Our present understanding of adversity is linked to the apical effect, which is typically a phenotypic response. Therefore, phenotypic anchoring of changes in gene or protein expression, or other \textit{in vitro} endpoints, is critical to understanding if changes in a system are adverse or not. As more experience is gained with RPTCs, key transition points in pathways, and other details of biology, the need for phenotypic anchoring with a specific chemical should decrease. How long this paradigm switch takes will be dependent on the quality of data produced to support hypotheses. Studies will need to be designed for this specific purpose.

With the development of new toxicity-testing approaches and identification of biomarkers and signatures of potential adverse effects comes the need for a new framework for how these data are used in risk assessment to inform regulatory decision making. Iteration, revision, and rigorous validation will be needed to ensure that new tools and endpoints are sensitive and accurate, and these new approaches must be accepted by the scientific and regulatory communities as reflective of human biology and relevant for risk assessment. Although all data from screening assays, mechanistic studies, pathway analyses, and other \textit{in vitro} and \textit{in vivo} methods should be considered, a weight-of-evidence (WoE) approach allows for an analysis of the strengths and weaknesses of the data and the relative importance for animals or humans. At the present time, a WoE evaluation requires both classical \textit{in vivo} toxicology and \textit{in vitro} data and relies on animal and human (if available) data. As RPTCs are characterized and better understood, the data used could potentially shift toward pathway analyses, with a decreased reliance on \textit{in vivo} data.
A WoE evaluation for risk assessment also needs to consider variables such as exposure, which will require the use of dosimetry, reverse dosimetry, and biomonitoring. This issue is beyond the scope of this paper but is an important element of the risk assessment process. Consideration of proper context needs to be incorporated when designing studies, as well as during evaluation, to properly interpret the data for informing regulatory decisions. For example, age-related biological differences can impact interpretation of results from exposure to a chemical at various life stages (in utero or early life exposures or for juvenile and adult later life exposures). Toxicological responses resulting from exposure across life stages must be considered along with the time- and dose-response continuum.

Adaptive responses to toxicant exposure may be characterized by reversibility (upon withdrawal of treatment or exposure). Furthermore, adaptive changes are often early homeostatic adjustments, such as metabolism or gene expression/transcriptomic changes [33]. These modulations are typically not considered to be precursors of functional impairment but rather a response that would return to a homeostatic condition (e.g., a return from hormone level variation(s)/cycling or blood pressure increase owing to stress). In some situations, a minor change may be sustained resulting in a “new normal” state where the cell/tissue/organism has adapted without adverse consequences, such as an induction of cytochrome P450 enzymes resulting in hepatocellular hypertrophy and increased liver weight. These adaptive changes, in a different context, may be indicators of a potentially adverse outcome. For example, short-term decrements of circulating thyroid hormone may result in an adaptive response in an adult, nonpregnant female, but the same change in early gestation could be an indicator of a potential adverse outcome on fetal brain development.

During the HESI workshop, participants discussed the usefulness of a formalized framework or a consistent series of questions to be answered for deciding if effects are
adverse or not. This framework was loosely based on previously published decision trees \cite{14,17} and considered factors such as change in tissue or cellular function, reversibility, transition points in pathways, context of exposure, and species differences. Such an approach to interrogating data could prove useful both in the design of additional studies and the assessment of potential risk. It could substitute for currently used tiered-testing schemes that are designed to cover all possibilities rather than to develop targeted perspectives based on knowledge of MOAs of chemicals. Ultimately, the workshop participants agreed that although such an approach for interrogating data would be useful, there is currently not sufficient knowledge to establish a specific framework for decision making. As more data become available on the key RPTCs and critical nodes in these pathways, the development of a framework may become feasible, thus leading to better characterization of the RRR for a specific chemical and exposure scenario.

2.7 Conclusions and Recommendations

The major conclusions of the May 2011 HESI workshop are summarized here:

- Workshop participants agreed that a primary goal for the future is to leverage \textit{in vitro} and \textit{in silico} data to predict later occurring apical endpoints from precursor dose transitions in RPTCs. Therefore, a dose transition considered to be an RRR may not correlate temporally with an observable apical endpoint, but the two should be linked through the pathways in a way that is biologically meaningful.

- All toxicological responses should be viewed and considered within a time- and dose-response continuum. A recurring theme of discussion during the workshop
was the lack of a qualitative distinction between the toxicogenomic profile (and other *in vitro* or *in silico* biomarkers) associated with early- or low-dose exposure (not linked to an adverse apical endpoint) and later or higher dose exposure (potentially or more often linked to an adverse apical endpoint). Because of this, the exact point at which a transition to adversity occurs can appear to be ambiguous or even arbitrary, and the regulatory value of the response for predicting significant biological impact may appear to be questionable.

- Two important concepts that emerged from the workshop were RPTCs and RRR, which are fundamentally different. RPTCs refer to discrete biological mechanisms that are indicators of a toxicopathological response in human cells or organ systems. It is anticipated that a finite number of RPTCs exists and will be identified. An RRR, on the other hand, is a prescribed effect on which regulatory action, designed to protect individuals from unacceptable risk of a specific toxicological outcome, is based. For example, genomic or epigenomic changes that modulate a critical pathway of toxicant metabolism may define an RPTC. If, however, these changes are exceedingly rare in the human population, or if they occur exclusively in experimental models, they might not qualify as an RRR.

- A systematic effort to define and characterize RPTCs is critical. Because the intent is to predict rather than evaluate toxicity, the number and identity of relevant pathways and the most commonly affected RPTCs should be a research priority. For each pathway, it is important to characterize dose transitions, identify the critical nodes, and determine the presence or absence of threshold effects. For each pathway, it is also important to describe specific RRRs. The relationship between dose-response changes in the pathways and in apical endpoints, such as histology, will be a key to having confidence in this approach.
A paradigm needs to be developed and refined that provides an understanding of RPTCs and critical nodes. In addition, links between the pathways into networks must be investigated to understand the dynamics and kinetics of how an organism adapts or proceeds to an adverse effect (Figure 2.3). Model compounds should be used to provide detailed examples of perturbations in the pathways and networks to develop the way the information is used.

- Scientifically informed decision making is essential. This suggests that the emerging risk assessment framework should ultimately promote effective use of rigorous, validated, and standardized *in vitro* and/or *in silico* data that have established relevance to human biology.

- Consideration of context (at the level of organism, tissue, and cell) is critical for determining the point of concern or point of departure. The significance of an *in vitro* or *in silico* response to a putative toxicant can only be determined through a careful and thorough consideration of biological context and a realistic estimate of a relevant exposure to the putative toxicant.

The HESI workshop was held in response to the development of screening assays and high data content information that are produced more rapidly than procedures established for risk assessment. At the present time, the most appropriate use of high data content information is for prioritizing chemicals for additional evaluation and is not yet directly applicable for determining a specific MOA. However, as the science continues to progress, there will be opportunities to use these new methods and types of data to inform risk assessment. It is hoped that the present effort has helped to focus the scientific community’s attention on this important area of research. Understanding the spectrum of adaptation and adversity as it applies to risk assessment will ultimately inform regulatory decisions.
Figure 2.3: Future state of toxicity testing based on knowledge of key toxicity pathways and the critical nodes in the pathways. Boxes in red indicate the areas for research where the most emphasis is needed to allow use of this paradigm.
2.8 Acknowledgements

The authors gratefully acknowledge the USEPA for providing facilities for the May 2011 workshop. The authors also acknowledge Dr. Miriam Sander (Page One Editorial Services) for providing valuable assistance in summarizing workshop discussions and conclusions. This paper does not represent the policies or opinions of the USEPA. The views expressed in this paper are those of the authors and do not necessarily reflect the views of participants in the 10 – 11 May 2011 workshop.
References


Chapter Three

Molecular Alterations Underlying the Enhanced Disruption of Spermatogenesis by 2,5-hexanedione and Carbendazim Co-exposure
Molecular alterations underlying the enhanced disruption of spermatogenesis by 2,5-hexanediione and carbendazim co-exposure

Sarah N. Campion\textsuperscript{a,1}, Natasha Catlin\textsuperscript{a}, E. Andres Houseman\textsuperscript{b}, Janan Hensley\textsuperscript{c}, Yunxia Sui\textsuperscript{b}, Kevin W. Gaido\textsuperscript{c}, Zhijin Wu\textsuperscript{b}, Kim Boekelheide\textsuperscript{a}

\textsuperscript{a} Department of Pathology and Laboratory Medicine, 70 Ship St., Brown University, Providence, RI 02903, USA

\textsuperscript{b} Department of Community Health, 121 South Main St., Brown University, Providence, RI 02912, USA

\textsuperscript{c} The Hamner Institutes for Health Sciences, 6 Davis Drive, P.O. Box 12137, Research Triangle Park, NC 27709, USA

1 Present address: Pfizer Worldwide Research and Development, Eastern Point Road, MS 8274 1260, Groton, CT 06340, USA.


3.1 Declaration of Authors’ Roles

KWG and JH ran the microarrays in their laboratory. NC performed the Loxl1 staining and quantification of Loxl1 expression and contributed to the writing of the manuscript. SNC performed the analysis and contributed to the writing of the manuscript. EAH, ZW and YS designed and directed the analyses methods. KB contributed to the study design and writing of the manuscript.
3.2 Abstract

The current study investigated the co-exposure effects of 2,5-hexanedione (HD) and carbendazim (CBZ) on gene expression underlying the enhanced pathology previously observed. Adult male rats were exposed to HD (0.33 or 1%) followed by CBZ (67 or 200 mg/kg), and testis samples were collected after 3 and 24 h. Microarray analysis at 3 h revealed that CBZ and HD interact in an agonistic, or synergistic, way at the gene level. Further analysis of candidate genes by qRT-PCR at both 3 and 24 h after co-exposure, revealed that Loxl1 and Clca2/Clca4l were both decreased in expression. Immunohistochemical analysis of Loxl1 at 24 h revealed that Loxl1 is localized to the seminiferous tubules, with the most intense staining in the basement membrane, blood vessels, and acrosomes, with the relative intensity reflecting the gene level changes at 3 h. These findings provide candidate genes for further investigation of the testicular response to damage.

3.3 Introduction

The cellular and molecular targets and dose level of each toxicant within a chemical mixture all play a role in determining the biological responses following exposure. There is an emerging need for improved methods for risk assessment and a better understanding of toxicological consequences of mixed exposures, given that most real world exposures involve more than one chemical. Recent work has begun to elucidate how critical the dose and cellular and subcellular targets are during co-exposure to testicular toxicants. This work utilized an established co-exposure paradigm, which involves an 18 day priming exposure of adult male rats to the Sertoli cell toxicant 2,5-hexanedione (HD) followed by acute exposure to either a direct-acting germ
cell toxicant or a second Sertoli cell toxicant. Utilizing this exposure paradigm to study co-exposure responses, it was determined that co-exposure to the Sertoli cell toxicant (HD) and the germ cell toxicant X-radiation (x-ray) results in an attenuation of germ cell toxicity when compared with x-ray exposure alone [1]. In this co-exposure scenario we hypothesize that HD induces an adaptive response of the seminiferous epithelium, which helps render the germ cells resistant to x-ray-induced damage. In contrast to this co-exposure response, co-exposure to two Sertoli cell toxicants, HD and carbendazim (CBZ), results in synergistic effects on testicular injury, much greater than the single toxicant exposures, manifested as enhanced seminiferous tubule diameter, vacuolization, sloughing, and germ cell apoptosis [2].

While HD and CBZ share the same target, Sertoli cell microtubules, these two toxicants have opposing effects on microtubules. HD, a metabolite of the commonly used solvents n-hexane and methyl n-butyl ketone (2-hexanone), causes Sertoli cell dysfunction by promoting rapid assembly and enhanced stability of microtubules [3]. Consequences of HD exposure include spermatid head retention, Sertoli cell vacuolization, and decreased seminiferous tubule fluid, ultimately resulting in germ cell loss and sloughing [3, 4]. HD-induced testicular toxicity requires at least 2 weeks of exposure before the manifestation of pathology [3], which differs from the more rapid onset of pathology following CBZ exposure. A single high dose of CBZ results in testicular alterations within a few hours, including increased testis weights, increased seminiferous tubule diameter, and sloughing of the seminiferous epithelium [2, 4]. CBZ is the toxic metabolite of the benzimidazole fungicide benomyl, which elicits testicular toxicity by inhibiting, rather than promoting microtubule polymerization [5, 6].

Mechanistic investigations into the molecular changes underlying the phenotypic consequences of HD and x-ray co-exposure led to the establishment of a methodol-
ogy for the analysis and interpretation of microarray data from animals co-exposed to multiple dose levels of each toxicant [7]. This same approach was applied in the current study to investigate the dose and co-exposure effects on gene expression following combined HD and CBZ exposure and to determine the molecular mechanisms underlying the pathologic changes. It was hypothesized that HD and CBZ co-exposure would result in synergistic or agonistic effects at the molecular level, reflective of the agonistic phenotypic effects of this co-exposure. The log$_2$-expression values obtained by microarray analysis performed with testis tissue of co-exposed adult male rats were analyzed using LIMMA and summarized across all treatment groups to determine the effect of HD in excess of CBZ. These summarized linear contrasts were examined to identify individual genes and biological pathways where HD modification of CBZ-induced gene alterations was the greatest. Several genes of interest were further analyzed by qRT-PCR and immunohistochemistry to begin to better understand and mechanistically explain the synergistic toxicity elicited by HD and CBZ co-exposure. This is an important area of investigation, which has provided valuable information regarding the molecular profiles of toxicants with synergistic phenotypic effects as compared to the molecular profiles of toxicants with antagonistic phenotypic effects. These are significant pieces of information in the field of mixtures research.

3.4 Materials and methods

3.4.1 Animals

Adult male Fischer 344 rats weighing 200-250 g were purchased from Charles River Laboratories (Wilmington, MA). Upon arrival, rats were acclimated for 1 week prior
to use and maintained in a temperature and humidity controlled environment with a 12 h alternating dark-light cycle. All rats were housed in community cages with free access to water and Purina Rodent Chow 5001 (Farmer’s Exchange, Framingham, MA). The Brown University Institutional Animal Care and Use Committee approved all experimental animal protocols in compliance with National Institute of Health guidelines.

3.4.2 Toxicant exposure

HD was administered in drinking water *ad libitum* for 18 days at concentrations of 0.33% and 1% using a previously established treatment protocol [2]. On day 17, animals (n = 4, for each treatment group) were administered CBZ by gavage, at a dose of 67 mg/kg or 200 mg/kg in corn oil at a dose volume of 2 ml/kg. At either 3 h or 24 h after treatment with CBZ, following continued HD drinking water exposure, rats were euthanized by CO₂ asphyxiation and half of the right testis was homogenized in Tri Reagent (Sigma-Aldrich, St. Louis, MO), snap frozen in liquid nitrogen, and stored at -80°C. The remaining testis tissue was fixed in neutral buffered formalin for histological examination.

3.4.3 RNA isolation and microarray hybridization

Using tissues collected at 3 h after treatment with CBZ, RNA was isolated from testes homogenized in Tri Reagent using the RNeasy Mini Kit (Qiagen, Valencia, CA) following the manufacturer’s protocol. Complementary (cDNA) was synthesized from 2.5 µg total RNA and purified using the Affymetrix One-Cycle Target Labeling and control reagents kit (Affymetrix, Santa Clara, CA) according to man-
ufacturer’s protocol. Equal amounts of purified cDNA per sample were used as the template for subsequent in vitro transcription reactions for complementary RNA (cRNA) amplification and biotin labeling using the Affymetrix GeneChip IVT labeling kit (Affymetrix) included in the One-Cycle Target Labeling kit (Affymetrix). cRNA was purified and fragmented according to the protocol provided with the GeneChip Sample Cleanup module (Affymetrix). All GeneChip arrays (Rat Genome 230 2.0 arrays) were hybridized, washed, stained, and scanned using the Complete GeneChip Instrument System according to the Affymetrix Technical Manual.

3.4.4 Microarray data analysis

Affymetrix CEL files were pre-processed by GCRMA background correction [8], quantile normalization and Robust Microarray summarization, resulting in a single log2-transformed expression measure for each of 31,099 genes. The expression measures were analyzed as previously described [7] to facilitate the detection of non-linear effects of exposure and interactions of co-exposure on mRNA expression. This method of analysis resulted in the generation of a summary statistic with the interpretation of an estimated aggregate HD effect in excess of CBZ, e.g. up-regulation (positive) or down-regulation (negative) by HD. The overall linear trend in CBZ was also summarized by fitting the equivalent saturated model reparameterized using polynomials in exposure dose (i.e. linear and quadratic terms for each exposure together with their interactions), and extracting the linear CBZ term. To control for multiple comparisons, q-values representing false discovery rates (FDR) were computed from the collection of all 31,099 p-values using the qvalue package in R [9].
3.4.5 Quantitative real-time polymerase chain reaction analysis

Using tissues collected at both 3 h and 24 h after treatment with CBZ, Stat-60 reagent (Tel-Test, Friendswood, TX) was used to extract total RNA from whole testis tissue according to the manufacturer’s protocol. RNA concentrations were determined using the NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and RNA quality was determined using the Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA) according to the manufacturer’s instructions. cDNA was synthesized from total RNA isolated from each sample using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA) according to the manufacturer’s protocol. For the detection of MACRO domain containing 1 (Macrod1), dipeptidylpeptidase 7 (Dpp7), SH3 and multiple ankyrin repeat domains 3 (Shank3), chloride channel calcium activated 2/chloride channel calcium activated 4-like (Clca2/Clca4l), lysyl oxidase-like 1 (Loxl1), and tubulin beta 3 (Tubb3) the cDNA templates were amplified using QuantiTect® Primer Assays (Qiagen, Valencia, CA). These primers were pre-optimized and bioinformatically validated. Each sample was run in triplicate in 25 µl reactions. Relative mRNA levels of each target gene were normalized to the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT). Log₂-transformed relative expression ratios were calculated using the ddCt method.
3.4.6 Immunofluorescent staining and densitometric analysis

Rat testes (control, 1% HD, 200 mg/kg CBZ, and 1% HD + 200 mg/kg CBZ; n = 6) were collected 24 h after treatment and cryopreserved in Tissue-Tek OCT compound (Sakura Finetek USA Inc., Torrance, CA). Sections (8 µm) were fixed in acetone for 10 min, air dried for 5 min and then washed in phosphate buffered saline (PBS). Endogenous activity was blocked with 6% goat serum for one hour and then incubated overnight at 4°C with rabbit anti-Loxl1 primary antibody (0.5 µg/ml) (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Alexa Fluor 568 goat anti-rabbit (2.5 µg/ml) (Invitrogen, Carlsbad, CA) was applied to detect the anti-Loxl1 antibody. Sections were counterstained and coverslipped with Vectashield® Hard Set mounting medium with DAPI (Vector Laboratories Inc., CA, USA). Images of Stage IV seminiferous tubules were captured on an Axio Imager.M1 microscope, with an AxioCam MRm camera and Axio Vision 4.8 Software, (Carl Zeiss, Inc, Germany) at 40x magnification and 60 millisecond (ms) exposure for densitometric quantification of the fluorescent staining. The 60 ms exposure time was determined following an exposure time course study that was performed to determine the linear range capabilities of the gray scale.

Images of the anti-Loxl1 staining in Stage IV seminiferous tubules were blinded and uploaded into Image J (NIH, Bethesda, MD) as black and white JPEGs for densitometric quantification of the fluorescent staining. Using the Image J software, a set of intersecting lines was drawn over each seminiferous tubule to separate each cross section into quadrants. Within each quadrant, two circles (with standard areas) were drawn over the basement membrane and over the closest acrosome to the basement membrane. The mean gray value of the area of each circle was measured.
and recorded. The mean gray values for both the basement membrane and the acrosome from the four quadrants were averaged together. The averaged value for the acrosome was then divided by the averaged value for the basement membrane to create a ratio of mean gray value, which acts as a control to compensate for staining differences between sections. The ratios of mean gray value for each stage IV seminiferous tubule were then averaged, resulting in one ratio of mean gray value per animal.

3.4.7 Statistical analysis

qRT-PCR data were analyzed using a one-way analysis of variance (ANOVA) with Bonferroni post hoc analysis. The analyses were performed separately for each gene, comparing the expression data among all treatment groups (Control, HD, CBZ, HD + CBZ) for each individual gene. Immunofluorescent quantification data was also analyzed by one-way ANOVA with Bonferroni post hoc analysis. \( p \)-values < 0.05 were considered significant.

3.5 Results

3.5.1 Estimates of HD effects on gene expression

Initial studies investigating co-exposure to the two Sertoli cell toxicants HD and CBZ revealed that they interact to produce synergistic effects on testicular toxicity, at the phenotypic level. To determine if these toxicants similarly interact at the molecular level to affect how they each contribute to the gene expression profile during co-
exposure, a summary statistic was used, as previously described [7], to estimate the extent to which HD modifies gene expression above and beyond the CBZ-induced gene expression for each gene during co-exposure. This approach facilitates the identification of genes that exhibit enhanced alteration (up- or down-regulation) by HD co-exposure at the molecular level that mirrors the enhanced toxicity with combined HD and CBZ co-exposure as compared to CBZ alone. CBZ linear effects (the summarized linear trend in CBZ) on gene expression were also determined to provide an indication of the expression induced by CBZ during co-exposure so that we can understand if the HD effect on top of CBZ is an attenuation or enhancement of these changes. The time point for microarray analysis was 3 h following CBZ treatment, because this represents a time prior to the manifestation of pathology due to CBZ exposure.

HD exerted a significant modification of CBZ-induced gene expression alterations (FDR < 0.05) for 5 genes: Dpp7, MacroD1, Shank3, Clca2/Clca4l and Loxl1 (Table 3.1 and Figure 3.1). All 5 of these genes were negatively affected by HD, which represented an enhanced down-regulation on top of CBZ, as indicated by the linear CBZ effects and agonistic interactions in Table 3.1. An agonistic vs antagonistic interaction is described by Campion et al. [7]. Genes that are significantly affected by CBZ, defined as genes with FDR < 0.05 for linear CBZ effects, are listed in Table 3.2. There are 16 genes with significant CBZ linear effects, and of interest, Clca2/Clca4l is the only gene that is significantly affected by both HD and CBZ as detected by microarray analysis.
Table 3.1: Genes with significant HD effects ($q < 0.05$).

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene description</th>
<th>HD effect</th>
<th>Linear CBZ effect</th>
<th>Type of interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dpp7</td>
<td>Dipeptidylpeptidase 7</td>
<td>-1.9670</td>
<td>-0.1638</td>
<td>Agonistic</td>
</tr>
<tr>
<td>Macrod1</td>
<td>MACRO domain containing 1</td>
<td>-2.2351</td>
<td>-0.2211</td>
<td>Agonistic</td>
</tr>
<tr>
<td>Shank3</td>
<td>SH3 and multiple ankyrin repeat domains 3</td>
<td>-2.3335</td>
<td>-0.0784</td>
<td>Agonistic</td>
</tr>
<tr>
<td>Clca2/Clca4l</td>
<td>Chloride channel calcium activated 2/chloride channel calcium activated 4-like</td>
<td>-3.1066</td>
<td>-0.7292</td>
<td>Agonistic</td>
</tr>
<tr>
<td>Loxl1</td>
<td>Lysyl oxidase-like 1</td>
<td>-3.2776</td>
<td>-0.2296</td>
<td>Agonistic</td>
</tr>
</tbody>
</table>
Figure 3.1: Co-exposure effects on gene expression. Relative gene expression following exposure to 0% (blue line), 0.33% (green line), or 1% (red line) in combination with 0, 67 or 200 mg/kg CBZ was determined by microarray analysis using tissues obtained at 3 h following CBZ treatment.
Table 3.2: Genes with significant CBZ effects ($q < 0.05$).

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene description</th>
<th>Linear CBZ effect</th>
<th>HD effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDX58</td>
<td>DEAD (Asp-Glu-Ala-Asp) box polypeptide 58</td>
<td>0.7170</td>
<td>1.8503</td>
</tr>
<tr>
<td>GPI</td>
<td>Glucose phosphate isomerase</td>
<td>-0.2068</td>
<td>-0.7908</td>
</tr>
<tr>
<td>AFG3L1</td>
<td>AFG3(ATPase family gene 3)-like 1 ($S. $cerevisiae)</td>
<td>-0.2283</td>
<td>-0.0024</td>
</tr>
<tr>
<td>ADIPOR2</td>
<td>Adiponectin receptor 2</td>
<td>-0.3353</td>
<td>-0.4796</td>
</tr>
<tr>
<td>RECQL5</td>
<td>RecQ protein-like 5</td>
<td>-0.3957</td>
<td>-0.4150</td>
</tr>
<tr>
<td>RGD1564036</td>
<td>Similar to RIKEN cDNA 3010026009</td>
<td>-0.4380</td>
<td>1.2676</td>
</tr>
<tr>
<td>FALZ</td>
<td>Fetal Alzheimer antigen</td>
<td>-0.5206</td>
<td>-0.9260</td>
</tr>
<tr>
<td>DCXR</td>
<td>Dicarbonyl L-xylulose reductase</td>
<td>-0.5393</td>
<td>-0.6166</td>
</tr>
<tr>
<td>XTP3TPA</td>
<td>XTP3-transactivated protein A</td>
<td>-0.5552</td>
<td>-0.8730</td>
</tr>
<tr>
<td>CD164L2</td>
<td>CD164 sialomucin-like 2</td>
<td>-0.5785</td>
<td>-0.7292</td>
</tr>
<tr>
<td>F5BNO46</td>
<td>F-box protein 46</td>
<td>-0.5815</td>
<td>0.2491</td>
</tr>
<tr>
<td>RGD1307155</td>
<td>Similar to CG18661PA</td>
<td>-0.6127</td>
<td>-0.9075</td>
</tr>
<tr>
<td>Clca2/Clca4l</td>
<td>Chloride channel calcium activate 2/chloride channel calcium activated 4-like</td>
<td>-0.7292</td>
<td>-3.1066</td>
</tr>
<tr>
<td>F5BNO15</td>
<td>F-box and leucine-rich repeat protein 15</td>
<td>-0.7509</td>
<td>0.3523</td>
</tr>
<tr>
<td>MMP9</td>
<td>Matrix metallopeptidase 9</td>
<td>-0.7558</td>
<td>-1.3143</td>
</tr>
<tr>
<td>MAFF</td>
<td>v-mad musculoaponeurotic fibrosarcoma oncogene homologue F (avian)</td>
<td>-1.0051</td>
<td>0.4993</td>
</tr>
</tbody>
</table>
3.5.2 qRT-PCR of candidate genes

To confirm the co-exposure effects on gene expression detected by gene array analysis, qRT-PCR analysis was performed focusing on the 5 genes that were significantly altered by HD in excess of CBZ at the high dose levels. The expression of Tubb3 was also investigated since tubulin is anticipated to be affected by HD and CBZ. At 3 h after CBZ exposure (Figure 3.2), there were similar trends in expression for all of the genes, with decreased expression occurring with combined CBZ and HD exposure. Macrod1 gene expression was significantly increased compared to control (1.52-fold change) after HD treatment alone, while expression was significantly decreased as compared to HD alone with co-exposure (0.9x control). Comparing this to the microarray results at the same dose levels, Macrod1 was reduced to 0.6x control with co-exposure, but with no change in expression in the other treatment groups. Dpp7 was significantly reduced with co-exposure (0.8x control) as compared to HD and CBZ alone, similar to the decrease of 0.6x control detected on the array. Reductions in Clca2 expression with co-exposure were 0.7x control (Figure 3.2) and 0.57x control (Figure 3.1) as detected by qRT-PCR and microarray, respectively. No significant changes in Shank3, Loxl1 and Tubb3 expression were detected at the 3 h time point, however large decreases with HD and CBZ combined exposure were observed for Shank3 and Loxl1 (about 0.6x of control for both genes), which reflect the decreases in expression detected by microarray analysis.

This analysis was extended to a later time point, 24 h, to further investigate the expression of Clca2/Clca4l, Loxl1 and Shank3, which all exhibited the greatest degree of alteration with co-exposure and were likely to play a role in the co-exposure response based on their known functions. Focusing on the effect of CBZ and how this is modified by HD co-exposure, there were strong trends for enhanced CBZ effects
on gene expression with the addition of HD treatment (Figure 3.3). HD exposure alone was not investigated at the 24 h time point (18 days of HD exposure) because this was expected to be very similar to the data obtained at the 3 h time point (17 days and 3 h of HD exposure). Both Clca2 and Loxl1 exhibited slight decreases with CBZ exposure alone and much greater decreases with co-exposure (0.35x control and 0.6x control for Clca2 and Loxl1, respectively). The 24 h results with Shank3 were the opposite of the 3 h findings (increase in expression with co-exposure at 24 h compared to a decrease at 3 h) and exhibited the lowest magnitude of gene alteration.
Figure 3.2: Gene expression 3 h after HD and CBZ co-exposure. mRNA was isolated from testis tissue after 1% HD, 200 mg/kg CBZ, or 1% HD + 200 mg/kg CBZ exposure and Macro1, Dpp7, Shank3, Clca2/Clca4l, Loxl1 and Tubb3 expression was measured by qRT-PCR. Gene expression values are expressed as mean relative expression ± SEM (n = 3-4). Values above the results for each gene indicate the p value for the within-gene ANOVAs that were performed. *p < 0.05 compared to control, †p < 0.05 compared to HD, ‡p < 0.05 compared to CBZ.
Figure 3.3: Gene expression 24 h after HD and CBZ co-exposure. mRNA was isolated from testis tissue after 200 mg/kg CBZ or 1% HD + 200 mg/kg CBZ exposure and Shank3, Clca2/Clca4l, and Loxl1 expression was measure by qRT-PCR. Gene expression values are expressed as mean relative expression ± SEM (n = 3-4). Values above the results for each gene indicate the p-value for the within-gene ANOVAs that were performed.
3.5.3 Loxl1 immunostaining

Immunohistochemistry was performed to evaluate the protein expression of Loxl1 after CBZ and HD co-exposure. Although both Clca2/Clca4l and Loxl1 exhibited large changes in gene expression after combined exposure, only Loxl1 was further investigated at the protein level due to the absence of commercially available antibodies that recognize rat Clca2/Clca4l. Representative images of Loxl1-stained seminiferous tubules, at approximately stage IV, are shown in Figure 3.4. Acrosome staining was more easily identifiable in stage IV seminiferous tubules, compared to tubules of other stages; therefore, stage IV seminiferous tubules were the primary focus of the staining analysis. Loxl1 localized to several areas in the seminiferous tubules in all exposure groups, including the acrosomes (where the most intense staining was observed), the tails of immature spermatozoa, the cytoplasm of Sertoli cells, the basement membrane of the seminiferous tubule, and blood vessels. This staining was more intense in the 1% HD (Figure 3.4B) and 200 mg/kg CBZ (Figure 3.4C) exposed testis tissues, in comparison to the control (Figure 3.4A). The staining appeared to decrease overall in the combined 1% HD and 200 mg/kg CBZ exposed testis as seen in Figure 3.4D.

To further explore the alterations in Loxl1 protein levels in these tissues, a densitometric analysis method was developed to quantify the intensity of Loxl1 staining. The quantification of Loxl1 protein levels at 24 h following CBZ exposure, expressed as mean gray value, is shown in Figure 3.5. The 24 h time point was chosen for analysis of protein levels, because the 3 h gene expression time point would not result in immediate alterations of protein levels. There is an apparent trend in Loxl1 protein expression, which increases with the individual exposures of 1% HD or 200 mg/kg CBZ and appears to decrease with the combined exposure of 1% HD and 200
mg/kg CBZ. Although this trend is not statistically significant, it mimics the gene expression data that was obtained for Loxl1 at the 3 h time point.
Figure 3.4: Localization of Loxl1 protein 24 h following toxicant exposure. Rat testis sections were cut on a cryostat at 8 µm thickness and prepared for Loxl1 protein immunofluorescent detection. Representative images of seminiferous tubules for each toxicant exposure: (A) Control, (B) 1% HD, (C) 200 mg/kg CBZ and (D) 1% HD + 200 mg/kg CBZ. The scale bar is 50 µm, A-D.
Figure 3.5: Quantification of Loxl1 staining. Loxl1 protein levels were quantified in immunofluorescent-stained tissue sections 24 h following control or toxicant (1% HD, 200 mg/kg CBZ or 1% HD + 200 mg/kg CBZ) exposure, n = 6. Protein levels were measured as mean gray value, representing the mean gray value of the acrosome divided by the mean gray value of the seminiferous tubule basement membrane. ANOVA $p = 0.35$. 
3.6 Discussion

The cellular and subcellular targeting of the individual toxicants in a mixture determines the toxicological outcome. The current study has demonstrated that CBZ and HD interact in an agonistic way at both the phenotypic level and at the gene level. Combined exposure to HD and CBZ has synergistic effects on the expression of specific genes, including Clca2/Clca4l and Loxl1, whose altered expression may underlie the enhanced testicular toxicity. Clca2/Clca4l and Loxl1 were identified as candidate genes involved in the enhanced co-exposure response through microarray analysis.

Similar to a previous study [7], microarray data were summarized across different dose levels and treatment groups (toxicant combinations) using a statistical method that estimates the extent to which HD modifies gene expression above and beyond the CBZ-induced gene expression for each gene during co-exposure. This allowed for the identification of genes that are more altered in expression by co-exposure as compared to CBZ alone. These genes are likely to be related to the enhanced pathology.

All 5 genes that were significantly altered by HD on top of CBZ exhibited agonistic, or synergistic, gene expression alterations with co-exposure as detected by gene array analysis, which mirrors the synergistic effects of co-exposure on testicular toxicity. Although previous studies have demonstrated that microarray analysis using RNA isolated from whole testis tissue may not provide the most reliable information for hypothesis generation [10], both Clca2 and Loxl1 proved to be promising genes of interest likely to play a role in the enhanced co-exposure toxicity.

Although most of the microarray expression alterations were confirmed by qRT-PCR, there were some discrepancies. Some of these differences may be due to the differences in sensitivity and the different dynamic ranges of the two different technologies. It is also important to remember that the estimated “effects” used to
identify candidate genes for additional analysis were derived from the microarray expression data for all 9 different treatment groups across different dose levels, while the qRT-PCR was performed only on the high doses. As described in the results section, the microarray fold-changes for those specific treatment groups are quite similar to the qRT-PCR-detected gene alterations in those same treatment groups. The only real discrepancy between the 3 h qRT-PCR results and the microarray results (also obtained at the 3 h time point) is the significant increase in Macrod1 detected by qPCR after 1% HD exposure. No significant changes were detected by array for this treatment group. All other significant changes detected by qRT-PCR confirm the microarray results. The expression pattern of Tubb3 (tubulin, beta 3), as measured by qRT-PCR, provides confidence in these data. Tubulin synthesis is controlled by autoregulation, whereby non-polymerized, free tubulin monomers provide feedback, regulating mRNA levels of tubulin \([11]\). Since CBZ and HD inhibit or promote microtubule assembly, respectively, one would expect CBZ to decrease tubulin mRNA levels and HD to increase tubulin mRNA levels. As would be expected, an increase in tubulin mRNA levels after HD exposure is observed in the current study, with only a slight decrease detected after CBZ exposure. These tubulin expression results provide confidence in the quality of the RNA used for microarray analysis and the resulting data.

Loxl1 catalyzes the oxidation of lysine residues of collagen fibrils and elastins in the extracellular matrix, which controls the cross linking and deposition of elastins. Loxl1 is localized specifically to sites of elastogenesis and plays a role in elastin homeostasis \([12]\). Interestingly, Loxl1/- male mice exhibit lower sperm production and reduced fertility, with no apparent histologic differences \([13]\). Loxl1 appears to play a role in male sexual development and fertility, but the specific mechanism(s) are currently unknown. The substantial reduction in Loxl1 mRNA in the present study
suggests that this gene product may also play a role in the toxicological response of the testis. A closely related family member to Loxl1, lysyl oxidase (LO), is able to prevent the activation of NFκB by inhibiting the signaling pathways that lead to its activation [14], so one would anticipate that reduced LO or Loxl1 levels would lead to greater activation of NFκB. Given that NFκB is proapoptotic in the testis [15], the reduction in Loxl1 observed in the current study corresponds with, and may mechanistically explain, the enhanced injury and apoptosis that occurs following HD and CBZ co-exposure. One might also speculate that altered Loxl1 expression during injury may impact germ cell support through alterations in cell-matrix interactions and cell junction dynamics related to elastic fiber homeostasis. Further mechanistic studies are required to better understand the role of Loxl1 in the testis, both under normal conditions and following toxicant exposure.

In addition to the detected changes in Loxl1 expression after co-exposure, altered expression of the chloride channel Clca2/Clca4l in the testis likely has significant consequences, although the specific role and localization of this particular chloride channel in the testis has not yet been investigated. Chloride channels have been demonstrated to play a role in regulating the volume of spermatozoa, the regulation of seminiferous tubule fluid formation, and in controlling the ionic environment in the testis [16, 17]. The survival of germ cells and the proliferation of spermatogonia are dependent on the production of an acidic microenvironment. Lactate secretion by Sertoli cells helps to maintain this acidic microenvironment and the chloride currents in Sertoli cells may be involved in the proton-linked lactate production in Sertoli cells [18, 19]. Other members of the Clca protein family appear to play a role as cell-cell adhesion molecules, which may be important in the testis [20]. The critical role of chloride channels in the testis is observed in the severe degeneration in mouse testes that occurs when chloride channel expression is disrupted [19].
While Clca2/Clca4l is a strong candidate for further analysis, Clca2/Clca4l staining was not performed because there are no commercially available antibodies to detect the rat protein. The quantification of Loxl1 immunostaining reflects the gene expression as measured by qRT-PCR at 3 h, however the changes in protein levels were not large in magnitude or statistically significant. This non-significance may be attributed to the 24 h time point at which protein expression was examined. The greatest change in Clca2/Clca4l gene expression occurs at 24 h, with the magnitude of change for Loxl1 being consistent between 3 and 24 h. This may reflect a sustained alteration in Loxl1 expression over these time points, or may indicate a peak in gene expression alteration between these 2 time points. This may indicate that significant alterations in Loxl1 protein levels would follow at a later time point.

The localization of Loxl1 within the testis has not been previously investigated. Loxl1 was found to be localized to several areas within the seminiferous tubules, including immature spermatozoa tails, the cytoplasm of Sertoli cells, the basement membrane of the seminiferous tubule, blood vessels, and acrosomes. The most intense staining was localized to the acrosomes. Interestingly, the localization of Loxl1 to the acrosome is similar to the localization of clusterin, a glycoprotein that is produced constitutively by Sertoli cells and has been well-studied in the testis [21]. Clusterin has been found to localize to the cytoplasm of Sertoli cells, in the heads and tails of late spermatids and released spermatozoa, and at the acrosome [21, 22]. Recently, Loxl1 and clusterin have been identified as major genetic variants in pseudoexfoliation syndrome, which have been shown to co-localize in ocular tissues of patients with this disease [23, 24]. There is a possibility that these proteins co-localize in the testis as well, given the similar areas of localization. Further research will need to be performed to confirm this and also to determine the potential relationship of these proteins in the testis. The potential interactions of Loxl1 and
clusterin in the testis at the protein level may also extend to the functional level, as clusterin has been implicated in protecting surviving cells after damage.

In summary, these studies have revealed candidate genes underlying the synergistic disruption of spermatogenesis that occurs following HD and CBZ co-exposure. CBZ and HD interact in an agonistic way at the gene level, reflective of the agonistic effects on testicular toxicity. Loxl1 and Clca2/Clca4l are both reduced in expression following co-exposure and appear to play critical roles in the testis. Further investigation is needed to determine the specific roles of Loxl1 and Clca2/Clca4l in the testicular response to damage. The results of the current study have also demonstrated, for the first time, the localization of Loxl1 in the testis. Additional mechanistic studies will reveal the functional significance of this localization in the testis as well as the testicular role of Loxl1, both under normal conditions and following toxicant exposure.

3.7 Acknowledgements

This work was supported by the National Institute of Environmental Health Sciences at the National Institutes of Health [grant numbers P42 ES013660 and T32 ES07272].


Chapter Four

Laser capture microdissection: Improved Methods for Analysis of Rat Spermatogenesis Stage- and Cell-specific Gene Alterations
Laser capture microdissection: improved methods for analysis of rat spermatogenesis stage- and cell-specific gene alterations

Natasha Catlin\textsuperscript{a}, Susan M. Huse \textsuperscript{a}, Kim Boekelheide\textsuperscript{a}

\textsuperscript{a} Department of Pathology and Laboratory Medicine, Box G-E, Brown University, Providence, RI 02912, USA

4.1 Abstract

Over the past decade, laser capture microdissection (LCM) has grown as a tool for gene expression profiling of small numbers of cells from tumor samples and of specific cell populations in complex tissues. LCM can be used to study toxicant effects on selected cell populations within the testis at different stages of spermatogenesis. There are several LCM-related hurdles to overcome, including issues inherent to the method itself, as well as biases that result from amplifying the LCM-isolated RNA. Many of the technical issues associated with the LCM method were addressed, including increasing RNA yield and obtaining more accurate quantification of RNA yields. With the LCM method optimized to generated RNA quantities sufficient for qRT-PCR array analysis without amplification, we were able to directly compare results from the use of the unamplified and amplified RNA from individual samples. The addition of an amplification step for gene expression studies using LCM RNA results in bias, especially for low abundance transcripts. However, for comparisons within the same transcript, the amplification rate is consistent across samples. Given these results, researchers should use caution when comparing results generated from the amplification of LCM RNA, to RNA that has not been amplified.
4.2 Introduction

Laser capture microdissection (LCM) is a useful technique for isolating populations of cells and subsequently their DNA, RNA, and protein \[1\]. It is used in molecular profiling of diseases, isolation of small populations of cells from large tissues (i.e. Kupffer cells from the liver \[2\], and examination of cell-type specific toxicant responses \[3, 4\]. Characteristically, LCM is used to capture small numbers of cells for RNA isolation and subsequent amplification in transcriptomic analyses \[5\]. The amplification of LCM material is generally a necessity due to the extensive amount of time required to perform LCM to obtain ng amounts of RNA for each sample. Optimization of the LCM methods has been the focus of many efforts and has included factors such as the proper selection of consumables and staining reagents, tissue handling, and extraction methods, all to maximize both the quantity and quality of isolated RNA from LCM sections \[4\]. Optimization efforts have extended to the examination of various amplification methods and gene expression platforms, and the different biases these may introduced.

LCM isolation of cell populations is a highly manipulated process that generates additional questions, including whether amplification bias occurs and where it originates \[6–10\]. Researchers have compared the use of pooled LCM RNA with different array platforms (Affymetrix U133 plus 2.0 vs. X3P arrays) and different amplification methods (NuGen vs. Arcturus) and have found minimal differences between the methods \[9\]. Also, unamplified and amplified RNA have been compared using several amplification techniques and varying sub-nanogram input concentrations of intact total RNA, and found that arrays with unamplified RNA had higher Pearson’s correlations than amplified RNA in an unprocessed dataset \[8\]. Utilizing RNA amplification techniques with downstream gene analysis is useful for highly expressed
genes, but decreases the absolute discovery rate \[8\]. Furthermore, amplification techniques introduce biases into downstream applications and reduce the signal-to-noise ratio \[8\], making it difficult to discern subtle changes in gene expression responses. Mazurek et al. identified an amplification dependent bias, where the method used resulted in a large number of probe sets with significant expression differences between LCM derived and whole tissue samples \[7\]. However, none of the studies examining LCM RNA amplification bias have compared the use of the same input LCM RNA, as unamplified and amplified versions.

The testis is a complex organ, with multiple cell types (Sertoli, Leydig and germ cells) and many varying stages of spermatogenesis depending on the organism. This complexity is compounded when the testicular toxic effects of chemical mixtures are studied. The use of model germ and Sertoli cell specific toxicants, X-radiation (x-ray) and 2,5-hexanedione (HD), respectively, whose effects have been well characterized, can be useful in the investigation of co-exposure effects on spermatogenesis. In previous high dose studies, the germ cell apoptosis induced by x-ray exposure appears to be attenuated when there is a priming exposure to HD \[3\], \[11\], \[12\]. Certain stages of spermatogenesis are more susceptible to particular toxicants, as illustrated by Hasegawa et al., who found that a greater number of spermatogonia undergo apoptosis following x-ray exposure during stages I-VI and VII-VIII of spermatogenesis, when compared to other stages \[13\]. This stage-specific effect of toxicant exposure on a few genes involved in the apoptosis of germ cells was studied further through laser capture microdissection (LCM) by Campion et al. \[3\]. They found that when the diluting effect of non-affected cell types was removed, the apoptotic effects of the toxicants on germ cells was much greater had previously been realized. The dilution of gene expression effects in whole tissues vs. LCM derived cells, becomes more apparent when the subtle effects of low-dose exposures are studied. This complexity of
multicellular tissues and multiple exposure scenarios exemplifies the need for LCM in broad gene expression studies.

Here, LCM was applied to study cell types that are targeted by specific toxicants, to overcome the attenuation effect that occurs when studying a small subset of cells within a complex multicellular organ [14, 15]. LCM material was used in conjunction with a qRT-PCR array platform to enhance our ability to detect small gene changes in low-dose exposures across an entire pathway, which would otherwise be lost within the inherent noise of whole genome array studies. Many of the LCM associated technical hurdles were addressed here to ultimately increase RNA yield to maximize the utility of LCM. This optimized and streamlined LCM method generated RNA quantities sufficient for qRT-PCR array analysis without amplification, so that we could directly compare results from the use of both unamplified and amplified RNA. The effect of amplification on LCM RNA was examined within samples and across multiple genes, to identify potential biases caused by RNA input concentrations used in the amplification process. It was found that there are highly variable amplification rates across several different transcripts on the qRT-PCR array used, but the within-gene amplification is consistent across samples. These data indicate that if comparisons are to be made between unamplified and amplified material, they should be made with caution.
4.3 Materials and methods

4.3.1 Animals

Adult male Fischer 344 rats (200 - 250 g) were purchased from Charles River Laboratories (Wilmington, MA). Upon arrival, rats were acclimated for one week and maintained in a temperature and humidity controlled environment with a 12 hr alternating dark-light cycle. All rats were housed in community cages with free access to water and Purina Rodent Chow 5001 (Farmer’s Exchange, Framingham, MA). The Brown University Institutional Animal Care and Use Committee approved all experimental animal protocols in compliance with National Institute of Health guidelines.

4.3.2 Toxicant exposure

Animals were exposed using an established treatment protocol \cite{12, 16} to 2,5-hexanedione (HD) in the drinking water for 18 days as a 0.33% solution. Animals were subsequently exposed to 2 Gy caudal half-body radiation on the 17th day of HD exposure, at a dose rate of 0.31 Gy/min using a RT 250 Philips kVp x-irradiation machine (Philips, Hamburg, Germany). At 3 hrs following irradiation, the rats were euthanized by CO₂ asphyxiation and testes were collected and immediately embedded in optimal cutting temperature (OCT) compound (Sakura Finetek, Torrance, CA) as described previously \cite{12}. Only the testis tissue from four animals (two control and two HD + x-ray) of a larger study were chosen randomly for this study, due to the length of time that is required to generate enough LCM RNA that does not require an amplification step.
4.3.3 Laser capture microdissection (LCM) and RNA isolation

Frozen embedded testis tissue was sectioned at 10 µm and bonded to the slide, by allowing it to briefly melt and then quickly re-freeze. Slides were kept on dry ice at all times until fixation in 75% ethanol for 30 sec. Slides were stained, in groups of 4, for 30 sec in a 0.075% staining solution of Arcturus Histogene Staining Solution (Applied Biosystems, Carlsbad, CA, USA). After a 30 sec wash step with nuclease free water, slides were moved quickly through the following dehydration steps: 75% ethanol for 30 sec, 95% ethanol for 30 sec, 2x 100% ethanol for 1 min and xylene for 1 min. The slides were air dried for 5 min in a hood before use.

The PixCell IIe Laser Microdissection System (Arcturus Bioscience, Inc.) was used to perform LCM according to the manufacturer’s manual. The seminiferous tubules with spermatogenesis stages I-VI were identified and the first few cell layers, which mainly included Sertoli cells, spermatogonia, and early stage spermatocytes, were captured as previously described [3] (Figure 4.1B). These stages were chosen due to the higher percentage of x-irradiation sensitive spermatogonia and because of previous research which has shown that these stages have a higher incidence of apoptosis than other spermatogenesis stages [12]. The cap was removed from the slide and the polymer film was carefully peeled off from the edge with a pair of forceps and placed on ice in 600 µl lysis/binding buffer from the Ambion mirVana kit (Life Technologies, Grand Island, NY). This process was repeated until all 12 slides in the round had been completed and added to the lysis/binding buffer.

RNA was isolated using the manufacturer’s protocol (2011 version) for the mirVana kit, from step D.2 to step F.I.5 (where indicated, for tissue) and RNA was
eluted with nuclease-free water. RNA concentration was determined for each sample in triplicate using the low-range protocol detailed by Invitrogen’s Quant-iT Ribogreen Assay kit (Life Technologies, Grand Island, NY) and exhibited an improvement in quality, as assessed by RIN number from 2100 Bioanalyzer (Agilent, Santa Clara, CA) (Figure 4.1C). To conserve the amount of RNA for each sample, RNA quality was assessed for two randomly selected sample replicates before RNA concentration, using the 2100 Bioanalyzer (Agilent, Santa Clara, CA), according to the manufacturer’s instructions (2010 version).

4.3.4 DNase treatment and RNA concentration

The LCM RNA replicates were individually DNase treated according to manufacturer’s instructions (Qiagen, Valencia, CA, USA), then combined and concentrated during RNA cleanup according to the protocol detailed by Qiagen’s RNeasy MinElute Kit, with a few exceptions. The individual samples were added to the column sequentially, after the addition of buffer RLT and 100% ethanol. RNA was eluted with 14 µl of nuclease-free water. RNA concentration was determined for each sample in duplicate using the high-range protocol detailed by Invitrogen’s Quant-iT Ribogreen Assay kit (Life Technologies, Grand Island, NY).
Figure 4.1: Laser capture microdissection methods schematic. (A) LCM workflow. For 1 sample replicate, about 20 seminiferous tubules from 12 slides were microdissected. The polymer film from each cap was peeled off and placed into 600 µl of mirVana Lysis/Binding buffer until all slides had been microdissected. After the final film had been added, the film/buffer mixture was incubated on ice for 45 min, following which RNA was extracted using the mirVana kit. This process was repeated 7 times and then each replicate is DNase treated, then combined and concentrated using the RNeasy MinElute Kit yielding approximately 350 ng of RNA. (B) Micrograph of an intact rat seminiferous tubule before LCM, the remaining tissue following LCM, and the isolated tissue on the LCM cap. Scale bar is 50 µm. (C) Digital gel and electropherogram results obtained with the Agilent 2100 Bioanalyzer before and after DNase treatment and RNA concentration with RINs of 5.40 and 6.50, respectively.
4.3.5 qRT-PCR arrays and analysis

To assess the utility of LCM-derived RNA, we measured the response of genes across a qRT-PCR array platform, using the RT² Profiler PCR Array System (SABiosciences, Valencia, CA) for apoptosis pathway related genes identified in the rat. Complementary DNA (cDNA) was synthesized from 240 ng of LCM cell-specific RNA using the RT² First Strand kit (Qiagen) using the manufacturer’s protocol. For the amplified samples, the RT² PreAmp cDNA Synthesis Primer Mix (Qiagen) was utilized with 10 ng of input RNA according to the manufacturer’s protocol (2012 version), to perform a PCR based pre-amplification of the genes that are present on the qRT-PCR array. Each sample was run in 10 μl reactions, which were loaded onto the qRT-PCR array (a 4 x 96-well array was used) with an epMotion 5075 robot (Eppendorf, Hamburg, Germany). An ABI-7900HT PCR machine (Applied Biosystems, Grand Island, NY) was used with the following cycling program: 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min, followed by a dissociation step. We selected the three optimal housekeeping genes for the two datasets (amplified and unamplified) from the five measured (\textit{Actb}, \textit{B2m}, \textit{Hprt1}, \textit{Ldha}, and \textit{Rplp1}) and normalized the mRNA expression levels ($\Delta$Ct) using the R package SLqPCR \cite{17} which implements Vandesompele et al. \cite{18}. SABiosciences used the arithmetic mean of all five housekeeping genes in the data they provided.

4.3.6 RNA amplification comparison and statistical analysis

The ratios, correlations, means and standard deviations (SD) were calculated using the R Statistical environment and plotted in both R and Prism (Graphpad Software, Inc., La Jolla, CA). The threshold cycle (Ct) values for differing input concentrations
of RNA for amplification were plotted, and the $r^2$ and $p$-values determined using a linear regression in Prism Graphpad.

### 4.4 Results

The process of LCM, from microdissection to concentration of RNA replicates from one sample, is outlined in Figure 4.1A. This process was repeated seven times to compensate for RNA loss during DNase treatment and concentration to ensure that sufficient RNA could be isolated despite eliminating an amplification step. The process yielded approximately 350 ng of RNA. Seminiferous tubules for microdissection were chosen based on their stage of spermatogenesis (stages I-VI) and the first few cell layers along the basement membrane were captured, which mainly included Sertoli cells, spermatogonia, early stage spermatocytes, and spermatids (Figure 4.1B). An improvement in integrity was seen following RNA combination and concentration, as assessed by RIN number from 2100 Bioanalyzer (Figure 4.1C). The before and after DNase treatment and RNA concentration steps yielded RINs of 5.40 and 6.50, respectively. (Agilent, Santa Clara, CA).

To examine the variability of the ratio of amplified to unamplified Ct values, we compared the ratios of four samples (two controls and two exposed) (Figure 4.2). The mean amplification ratio ranges from about 1 to 2, indicating different amplification rates for each gene. However, the error bars (1 SD) show that the amplification ratio within most of the genes is very consistent between samples, implying consistency of amplification on a gene-by-gene basis.
Figure 4.2: Effect of amplification on measured gene expression. Ratio of mean (and standard deviation) of amplified to unamplified mean cycle ratios across genes using sample data from both our lab and SABiosciences. Genes highlighted in black, *Traf3*, *Tp53*, *FaslG*, *Dffb*, *Bcl2a1d* and *Bcl2l11*, have a high standard deviation.
To explore the potential for amplification bias, the $\Delta$Ct values generated for the LCM derived samples in the pre-amplification step were compared to their unamplified counterparts (Figures 4.3 and 4.4). LCM derived samples ($n = 2$, 0.33% HD) were also sent to SABiosciences for an unamplified versus amplified samples, to verify our methods and to compare the reproducibility of the results. The comparison of biological replicates (controls) between amplified and unamplified $\Delta$Ct values for each gene present on the array, show strong correlations with $r^2$ values of 0.99 and 0.98, respectively (Figure 4.3A and B). The data for the 0.33% + 2 Gy biological replicates are not shown, but demonstrate the same strong correlations. Likewise, the comparison of the $\Delta$Ct values within the amplified and unamplified biological replicates performed by SABiosciences yields the same strong correlations, with $r^2$ values of 0.98 and 0.97, respectively (Figure 4.3C and D).

The data points highlighted in red in Figure 4.3A-D demonstrate the behavior of the nine outliers in Figure 4.4A, that result from the comparison of the $\Delta$Ct values for unamplified material versus amplified material generated by our lab. The unamplified and amplified $\Delta$Ct values generated by our lab correlate with an $r^2$ of 0.94, with the exception of the nine outliers identified in red ($Mapk1$, $Mapk8ip1$, $Birc3$, $Birc5$, $Il10$, $Polb$, $Fadd$, $Casp1$ and $Tp53bp2$). Interestingly, these outliers correlate as well, when compared separately ($r^2 = 0.94$) (Figure 4.4A). The SABiosciences data also correlate well ($r^2 = 0.92$) and the outliers from Figure 4.4A are no longer outliers with their data, with the exception of one data point (Figure 4.4B).
Figure 4.3: Consistency of RNA input method across samples. Unamplified and amplified ΔCt value comparison. Graphs are shown comparing the ΔCt values for amplified (A) and unamplified (B) control biological replicates from our lab and amplified (C) and unamplified (D) 0.33% HD exposed sample data generated by SABiosciences.
Figure 4.4: Consistency of RNA input method within samples. The unamplified and amplified ΔCt values were compared within samples for a control sample from our lab (A) and a 0.33% HD exposed sample from SABiosciences (B). The data points highlighted in red (A) indicate outliers in the correlation and the corresponding genes for the data points are labeled.
The amount of input RNA that goes into an amplification reaction may affect the quality of results obtained. Different amounts of pooled LCM RNA (7, 14, 35, and 98 ng) were used as input into the pre-amplification step to test its role in PCR array results (Figure 4.5). In Figure 4.5A, the correlation between 14 ng of input RNA and 7 ng, 35 ng, and 98 ng was strong, with $r^2$ values of 0.9822, 0.9851 and 0.9482, respectively. The Ct values were compared amongst each gene at each input RNA amount (Figure 4.5B). As expected, increasing the input concentration lowers the number of cycles required to reach the threshold, at a rate consistent within transcripts but not between transcripts. The genes that demonstrated the largest variance in Ct values are labeled in black and these include Tnfrsf11b, Birc5, Bnip2, Dffa, Bak1 and Bcl10.
Figure 4.5: Effect of RNA input concentration on threshold cycle values. (A) The threshold cycles for 14 ng of amplification input RNA compared to input RNA values of 7 ng (blue line), 35 ng (green line) and 98 ng (purple line). (B) The cycle threshold values for each gene at each amplification input RNA concentration, 7 ng (blue line), 14 ng (pink line), 35 ng (green line) and 98 ng (purple line). Highlighted genes, Tnfsf10, Bnip1, Bnip2, Dffb, Bcl2a1d, and Bcl2l11, indicate where the Ct values are not consistent across the different RNA values.
4.5 Discussion

LCM has proven to be a useful technique for isolating not only the toxicant sensitive cell types, but also in isolating spermatogenesis stage-specific cell populations \[19\]. Cells are selected based on their morphology and used in downstream applications such as qRT-PCR, gene microarrays, and qRT-PCR arrays. Although this technique allows for cell-specific analysis, there are associated hurdles that need to be overcome to maximize the utility of LCM. Some of these issues, such as tissue handling, consumables, staining reagents and extraction methods, have been addressed by previous methodology papers \[4\].

In the optimization of LCM as a tool for studying stage-specific effects of testicular toxicants, there were still several issues that needed to be addressed, both within the above-mentioned issues and additional obstacles related to increasing RNA yield to limit the need for amplification that may introduce selective biases. Our streamlined method (Figure 4.1A) addressed hurdles such as the limited concentrations of RNA generated by traditional LCM methods and the accuracy of quantification methods. With the repeated capture of multiple slides from the same sample combined with pooled replicates, we achieved both increased RNA yields (approximately 350 ng) and improved quality following concentration (Figure 4.1). The method detailed here can be used to avoid an amplification step, while still reliably generating enough quality RNA to be used in downstream studies such as qRT-PCR arrays and microarrays.

Using an amplification step introduces bias. The amplification ratio ranges from 1 to 2 across the transcripts, but the standard deviations of the ratios are small (Figure 4.2). These results indicate that there are highly variable amplification rates across
the different transcripts, but the within-transcript amplification is consistent across samples. This argues that comparisons between transcripts should not be made without a correction factor adjusting for the amplification ratio, while the results from single transcripts on the PCR array across multiple samples are comparable. It is possible that the transcripts with highly variable mean cycle amplification ratios (Tnp73, Tnfsf12, Tnfsf10, Dffa, Bak1, Bcl10) (Figure 4.2) are present at very low levels, which would explain the variability observed.

To examine the potential for amplification bias, we used the method outlined in Figure 4.1 to generate enough RNA to compare unamplified and amplified versions of the same sample on a PCR array platform. Two samples were also sent to SABiosciences, so that the pre-amplification experiments we performed could be replicated. From either lab, both the unamplified ∆Ct values and the amplified showed a strong, within-method correlation of log-fold expression (Figure 4.3). There was also a strong correlation in the comparison of unamplified to amplified ∆Ct values from both labs, but the results generated by SABiosciences differed from ours slightly in the lack of outliers (Figure 4.4). This discrepancy is attributed to the amount of input RNA used by each lab for pre-amplification (14 ng vs 100 ng of input RNA). By using a higher amount of input RNA, the SABiosciences amplified samples were more consistent across the range of transcript abundance in the sample (Figure 4.3). Some of the outlying genes, though expressed in the testis, are not highly expressed within the cell types that were microdissected (Figure 4.4). For example, the Mapk1 and Mapk8ip1 transcripts are more abundant within Leydig cells [20, 21] and Il10 is produced by resident testicular macrophages upon inflammation [22]. This demonstrates a necessity to use caution when comparing LCM derived unamplified to pre-amplified results, in particular with transcripts that are known to be of low abundance, and thus may not amplify at the same rate.
To better understand the amplification bias, we examined the effect of low RNA concentration on PCR threshold cycles by varying the input RNA amounts to be amplified. The RNA amounts were chosen based on the RNA concentration that is typically achieved with the LCM method. The respective threshold cycle values for each RNA input amount were compared across the different genes on the PCR array (Figure 4.5B). The amplification RNA input values correlate strongly across all the input values tested: 7ng, 14ng, 35ng, and 98ng (Figure 4.5A). There was more variance with certain low concentration transcripts (Ct > 25), when the amplification input RNA is either very low (7ng) or very high (98ng). In most cases, the choice of input RNA amount makes little difference. However, when comparing the expression of low abundance transcripts combined with low input RNA, the Ct values may fall below detection.

The use of LCM is often necessary because it allows insight into small subsets of a larger cell population, whether these are toxicant sensitive cells in a multicellular tissue, or specific cancerous cells within a tissue. Here, we present an improved and reliable LCM method for generating greater amounts of RNA for down-stream analyses. As amplification is almost always necessary with LCM samples and it can introduce bias in the consistency of amplification across transcripts, unamplified LCM-derived RNA was compared to a PCR-based pre-amplification method. Given the inter-gene variability and the variability of pre-amplified LCM derived RNA, it is important to take low RNA input amounts into consideration when comparing results between unamplified and amplified input RNA experiments. We have determined the genes that are inconsistently amplified across the apoptosis PCR array platform with rat testis tissue. We recommend that a screening procedure be used with future applications of this method to determine the genes that will not respond to amplification due to low abundance of certain transcripts within the LCM selected
cells.

4.6 Acknowledgements

This work was supported by the National Institute of Environmental Health Sciences at the National Institutes of Health (grant numbers P42 ES013660 and 5 T32 ES07272-17).

4.7 Competing Interests

Kim Boekelheide has funding from NIEHS, USEPA, and the American Chemistry Council. He is an occasional expert consultant for chemical and pharmaceutical companies, and owns stock in CytoSolv, an early stage biotechnology company developing a wound healing therapeutic.
References


Chapter Five

The Adaptive Rat Testicular Apoptotic Response to 2,5-hexanedione and X-irradiation

Low Dose Co-exposure
The adaptive rat testicular apoptotic response to 2,5-hexanedione and x-irradiation low dose co-exposure

Natasha Catlin\textsuperscript{a}, Susan M. Huse \textsuperscript{a}, Kim Boekelheide\textsuperscript{a}

\textsuperscript{a} Department of Pathology and Laboratory Medicine, Box G-E, Brown University, Providence, RI 02912, USA

5.1 Abstract

Testicular effects of chemical mixtures may differ from those of the individual chemical constituents. This study assesses the co-exposure effects of the model germ cell- and Sertoli cell-specific toxicants, X-irradiation (x-ray) and 2,5-hexanedione (HD), respectively. X-ray induces germ cell apoptosis and in high dose studies, HD has been shown to attenuate the x-ray effect on germ cells. Here, adult rats were exposed to different levels of x-ray (0.5 Gy, 1 Gy, 2 Gy), and HD in the drinking water for 18 days (0.33\%), either alone or in combination. To assess cell type-specific attenuation of x-ray effects with HD co-exposure, we used laser capture microdissection (LCM) to collect the targeted cell population and examine a panel of apoptosis-related transcripts using PCR arrays. The apoptosis PCR arrays identified significant dose-dependent treatment effects on several genes, with down regulation of $\text{DR5}$, $\text{Naip2}$, $\text{Sphk2}$, $\text{Casp7}$, $\text{Aven}$, $\text{Birc3}$ and upregulation of $\text{Fas}$. The greatest difference in overall spermatogonial transcript response to exposure was seen with 0.5 Gy x-ray exposure, and this low dose response was distinct from either the 1 Gy or 2 Gy x-ray response. Also, the attenuation effect seen with the combined high dose of 5 Gy x-ray and 0.33\% HD, did not persist into the low dose range. These results provide
insight into the testis cell-specific apoptotic response to low dose co-exposures of model testicular toxicants.

5.2 Introduction

There is a need for improved methods to study toxicant exposures in the context of complex mixtures or co-exposures, as exposure to single toxicants is a rare occurrence. Many factors influence the complexity of mixture studies, including the number of cellular and molecular targets, the amount and dose levels of the toxicant mixture components, and the interactions that can occur between these components. To add to this, the study of low-dose effects of toxicant mixtures becomes complicated because many of the common tools for assessing toxicological effects are no longer applicable. Most often, the doses studied produce low-level effects that are difficult to measure analytically as they fall below the limits of detection of lower precision platforms, such as gene microarrays. These low-level effects induced by low dose mixtures of toxicants can still have biological significance, when they are spread across an entire pathway, such as apoptosis.

The use of model toxicants with specific target organs has allowed for the investigation into the underlying mechanisms of mixtures exposures. Recent studies with well-characterized model germ and Sertoli cell specific toxicants, X-irradiation (x-ray) and 2,5-hexanedione (HD), respectively, have been useful in the investigation of co-exposure effects in the testis. HD is a metabolite of n-hexane and methyl-n-butyl ketone, two commonly used solvents, and HD exposure inhibits Sertoli cell function through enhanced microtubule assembly and stability [1]. X-ray induces apoptosis in the actively dividing spermatogonia cell population through DNA damage [2]. Also,
certain stages of spermatogenesis are more susceptible to x-ray exposure [3]. High
dose studies performed with these toxicants, have demonstrated that x-ray induced
germ cell apoptosis is attenuated with a priming exposure to HD [4–6].

Although the above studies begin to reveal mechanistic insights into mixtures
behavior, these studies have largely been performed with whole testis tissues. Whole
testis studies provide some insight into the response to toxicant exposure, but they
are limited in that the actual effect of the sensitive cell population is diluted when
combined with other testicular cell types. The testis is a particularly complex tissue,
with several interacting cell types and germ cells in varying stages of development. To
overcome this issue, we used laser capture microdissection (LCM), which has become
a particularly useful tool in the study of toxicant exposure in the testis. LCM has
many applications, including the molecular profiling of diseases (i.e. tumor cells from
an organ [7, 8]) and the examination of cell-type specific toxicant responses [5, 9]. In
the exploration of stage specific testicular sensitivity to x-ray exposure, it was found
that stages I-VI are the most susceptible and that the greatest increase in germ
cell apoptosis is seen in stages II and III [6]. When applied to the examination of
the stage specific effects of high doses of HD and x-ray in a LCM selected sensitive
cell population, LCM revealed that Fas induction by 5 Gy x-ray is significantly
attenuated by HD co-exposure [5]. This attenuation of Fas with HD and x-ray co-
exposure within the sensitive cell population only begins to uncover what occurs
following exposure in the complex apoptotic pathway.

The apoptosis pathway is useful in the investigation of toxicant mixtures in the
testis, as germ cell apoptosis has been identified as the ultimate adverse effect of
both HD and x-ray exposure. Apoptosis is a complex system of cell death that
can be activated either through intracellular driven “stress sensors” such as p53, or
through extracellular signals such as the cell surface receptors Fas and death receptor
5 (DR5). The activation of the apoptotic pathway through either mechanism results in p53 mediated activation of the caspase cascade through up-regulation of Bcl2-associated X protein (Bax), Fas, and DR5, with simultaneous repression of Bcl2. This cascade ultimately results in the apoptosis of the cell (for a review on male germ cell apoptosis see [10]).

Low doses remain largely unstudied and many risk assessment decisions for low doses have been based on high dose extrapolations, which do not necessarily reflect actual low dose exposures [11]. To enhance our ability to detect small gene changes in low-dose exposures that would otherwise have been lost within the noise inherent in whole genome array studies, LCM derived material was used in conjunction with an apoptosis pathway specific qRT-PCR array platform. It is hypothesized that distinct patterns of the apoptosis pathway response in LCM derived cell populations following exposure to low doses of HD and x-ray, will allow for the identification of a transition point in the time line of adaptive and adverse effects. With the combination of LCM and qRT-PCR arrays, a potential adaptive response of 0.5 Gy x-ray is shown with an overall decrease in expression of both pro- and anti-apoptotic genes. The addition of an HD priming exposure enhances the x-ray induced apoptotic transcript response. This study sheds light on the complex stage-specific apoptotic response of the testis to co-exposures of model toxicants.
5.3 Materials and methods

5.3.1 Animals

Adult male Fischer 344 rats (200-250 g) were purchased from Charles River Laboratories (Wilmington, MA) and allowed to acclimate for one week after arrival. All rats were housed in community cages in a temperature and humidity controlled environment with 12 hr light-dark periods and given Purina Rodent Chow 5001 (Farmer’s Exchange, Framingham, MA) and water ad libitum. All experimental animal protocols were approved as in compliance with National Institute of Health guidelines by the Brown University Institutional Animal Care and Use Committee.

5.3.2 Chemicals

HD (CAS# 110-13-4, ≥ 99% purity), CBZ (CAS# 10605-21-7, ≥ 97% purity), and all other chemicals were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise noted.

5.3.3 Toxicant exposure

An established treatment protocol [6, 12] was used to expose the rats to 2,5-hexanedi-one (HD) in the drinking water for 17 days as a 0.33% solution. For the co-exposure groups, animals were given a subsequent exposure of either 0.5 Gy, 1 Gy or 2 Gy caudal half-body radiation on the 17th day of exposure, at a dose rate of 0.31 Gy/min using a RT 250 Philips kVp x-irradiation machine (Philips, Hamburg, Germany).
The timeline of the dosing paradigm for HD and x-ray co-exposure, in addition to the 3 hr and 12 hr time point for tissue collection is illustrated in Figure 5.1. This resulted in a total of seven treatment groups: control, 0.33% HD, 0.5 Gy x-ray, 1 Gy x-ray, 2 Gy x-ray, 0.33% HD + 0.5 Gy x-ray, 0.33% HD + 1 Gy x-ray, and 0.33% HD + 2 Gy x-ray. At 3 hrs following irradiation, a group of rats was euthanized by CO$_2$ asphyxiation and testes were collected (n = 5; for each of the 7 treatment groups) and immediately embedded in optimal cutting temperature (OCT) compound (Sakura Finetek, Torrance, CA) as described previously [6] for laser capture microdissection. Another group of rats were euthanized at 12 hrs following irradiation for transillumination seminiferous tubule dissection, and the testis were collected (n = 5; control, 0.33% HD, 2 Gy x-ray, 0.33% HD+2 Gy x-ray) and either embedded in OCT compound, fixed in modified Davidson’s fluid for plastic and paraffin embedding, or detunicated, longitudinally bisected, and placed in 35°C collagenase solution (0.25% trypsin (Life Technologies, Grand Island, NY), 0.1% Type III collagenase in phosphate buffered saline (PBS)) for transillumination dissection of spermatogenesis stages I-VI, to mimic the stages captured for transcriptional analysis.
HD was administered to Fischer 344 rats as a 0.33% solution in the drinking water *ad libitum* for 17 days. X-ray was given as a single dose of 0.5 Gy, 1 Gy, or 2 Gy on the 17th day. Animals were divided into two time point groups, depending on downstream endpoints. For the 3 hr time point, the animals were exposed to all doses and dose combinations (n = 5; seven groups: 0.33% HD, 0.5 Gy x-ray, 1 Gy x-ray, 2 Gy x-ray, 0.33% HD + 0.5 Gy x-ray, 0.33% HD + 1 Gy x-ray and 0.33% HD + 2 Gy x-ray) and testes were taken at 3 hrs following x-ray exposure for LCM and qRT-PCR arrays. For the 12 hr time point, a group of animals was dosed only with the highest doses (n = 5; 0.33% HD, 2 Gy x-ray, and 0.33% HD + 2 Gy x-ray and the testes were taken for transillumination dissection and western blotting.

**Figure 5.1: Exposure paradigm.** HD was administered to Fischer 344 rats as a 0.33% solution in the drinking water *ad libitum* for 17 days. X-ray was given as a single dose of 0.5 Gy, 1 Gy, or 2 Gy on the 17th day. Animals were divided into two time point groups, depending on downstream endpoints. For the 3 hr time point, the animals were exposed to all doses and dose combinations (n = 5; seven groups: 0.33% HD, 0.5 Gy x-ray, 1 Gy x-ray, 2 Gy x-ray, 0.33% HD + 0.5 Gy x-ray, 0.33% HD + 1 Gy x-ray and 0.33% HD + 2 Gy x-ray) and testes were taken at 3 hrs following x-ray exposure for LCM and qRT-PCR arrays. For the 12 hr time point, a group of animals was dosed only with the highest doses (n = 5; 0.33% HD, 2 Gy x-ray, and 0.33% HD + 2 Gy x-ray and the testes were taken for transillumination dissection and western blotting.
Given the stage-specific effects of HD and x-ray on apoptosis [5, 6], the seminiferous tubules were staged according to the position and shape of the elongated spermatid nuclei using the standards outlined by [13]. LCM, RNA extraction, and RNA concentration was performed as described previously (Chapter 4). Briefly, 10 µm sections of frozen embedded testis tissue were fixed with 75% ethanol, stained with Arcturus Histogene Staining Solution (Applied Biosystems, Carlsbad, CA, USA), dehydrated and allowed to air dry in a hood before laser capture microdissection (LCM). LCM was performed with the PixCell IIe Laser Microdissection System (Arcturus Bioscience, Inc.) according to the manufacturer’s protocol. The peripheral cell layers of seminiferous tubules at spermatogenesis stages I-VI were captured as previously described (Chapter 4, [5]), because of a higher presence of x-ray sensitive spermatagonia in these stages of spermatogenesis [6]. The LCM samples were processed for RNA as previously described (Chapter 4), with the following exception. The number of LCM replicates for each sample was reduced to three (from seven), since a PCR-based amplification step was used and a decreased amount of starting material was necessary.

The toxicant induced gene response of the LCM-derived sensitive cell population was examined through the use of the RT² Profiler PCR Array System (SABiosciences, Valencia, CA) for apoptosis pathway related genes identified in the rat. Due to the time intensity of LCM and the number of toxicant exposure groups, a pre-amplification step was utilized to amplify the transcripts to be measured with the qRT-PCR array. The LCM derived samples were amplified using the RT² PreAmp cDNA Synthesis Primer Mix (Qiagen) with 14 ng of input RNA according to the manufacturer’s protocol (2012 version), to perform a PCR based pre-amplification
of the genes that are present on the qRT-PCR array. Each sample was run in 10 µl reactions, which were loaded onto the qRT-PCR array (4 x 96-well format) with an epMotion 5075 robot (Eppendorf, Hamburg, Germany). An ABI-7900HT PCR machine (Applied Biosystems, Grand Island, NY) was used to run the arrays with the following cycling program: 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min, followed by a dissociation step.

Raw Ct values were normalized to the geometric mean of five housekeeping genes (Actb, B2m, Hprt1, Ldha and Rplp1) to generate relative mRNA levels of each target gene and the ∆∆Ct method [14] was used to analyze expression for each transcript. The fold changes determined by qRT-PCR analysis for the seven significantly altered transcripts were uploaded into Multi-experiment Viewer (MeV version 10.2) and hierarchical clustering was performed on the transcripts using average linkage clustering for Euclidean Distance.

5.3.5 Transillumination seminiferous tubule dissection

Stage-specific sections of the seminiferous tubules were dissected from rat testes, using transillumination methods adapted from those detailed previously [15]. Following removal from the animal, the collagenase solution immersed testis was immediately placed horizontally in a 35°C incubator (Thermo Hybaid, Franklin, MA) and shaken vigorously for eight minutes. After digestion, the collagenase solution was decanted and the tubules were washed three times with ice cold PBS. The loosely associated seminiferous tubules were then poured into a 100 cm³ glass petri dish and kept on ice during tubule dissection. Using forceps, the seminiferous tubules were transferred onto a petri dish on a Nikon SMZ-U Stereoscopic Microscope (Nikon, Melville, NY) and staged according to the methods and images previously described by [16] [17].
Stages I-VI were isolated for protein extraction, to represent the same stages that were laser captured for the qRT-PCR experiments. The staged sections of tubules were cut away using microscissors and placed in a 1.5 ml tube with 1 ml of PBS on ice, until all stage I-VI tubule segments had been isolated. Following completion of tubule segment isolation, the tubules were spun down at maximum speed for 1 min and the PBS was removed. The tubule segments were then flash frozen and stored at -80°C until protein extraction.

5.3.6 Total protein preparation and western blot analysis

Total protein lysates were prepared through homogenization of both the transillumination dissected seminiferous tubules and whole testis tissue in RIPA buffer (150mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 50mM Tris-HCL pH 8.0). Lysates were homogenized on ice every 15 min for 1 hr and then cleared of cells and tissue debris through centrifugation at maximum speed for 20 min. Protein concentrations were measured using the DC™ protein assay (Bio-Rad, Hercules, CA), with BSA as a standard.

For the western blots, 15 µg of protein was denatured by boiling for 10 min in Laemmli SDS sample buffer and separated on a 12% SDS PAGE gel. Proteins were transferred onto a PVDF membrane (Thermo Scientific, Waltham, MA) and blocked for 1 hr with 5% milk in TBSt (50 mM Tris pH 8.0, 150 mM NaCl, 0.1% Tween 20). Total cellular proteins were detected using primary antibodies against DR5 (1:250; Abcam, Cambridge, MA) and Caspase-7 (Casp7) (1:1000; Cell Signaling, Danvers, MA) coupled with horseradish peroxidase-conjugated secondary antibody (1:750 and 1:2000, respectively). The ECL chemiluminescent substrate (Pierce, Rockford, IL) was used as the detection reagent and GAPDH as the internal control for gel loading.
HeLa cell lysate (StressGen Biotechnologies Corp., Victoria, BC) was used as a positive control for both DR5 and Casp7; MCF7 cells treated for 24 hrs with Cisplatin were used as a positive control for cleaved Casp7. Total protein from rat brain was prepared as a negative control for DR5.

Protein levels for DR5, Pro-Casp7 and cleaved Casp7 were quantified by scanning the films. The band intensities were analyzed using the ImageJ software and the methods detailed at http://www.lukemiller.org/journal/2007/08/quantifying-western-blots-without.html. Percent cleaved Casp7 was calculated by dividing the GAPDH normalized values for cleaved Casp7 by the normalized values for total Casp7.

5.3.7 Statistical analysis

For western blots, the data were graphed in Prism 5 (Graphpad Software, Inc., La Jolla, CA) and are shown as the individual means ± SEM. Statistical differences ($p \leq 0.05$) were calculated with Prism 5 software and using a Student’s unpaired two-tailed $t$-test. Pearson correlations between different exposures were performed using the cor function in the R statistical environment. The incremental effect of HD co-exposure to each x-ray exposure used both paired $t$-tests and paired Wilcoxon rank sum tests were calculated in R (functions t.test and wilcox.test).

5.4 Results

To first examine the effect that low doses have on overall gene expression of the toxicant sensitive cell population, a qRT-PCR array with genes representing the
apoptosis pathway was used. Analysis of the qRT-PCR data found that 7 of the 84 apoptosis related transcripts were significantly altered at one or more of the doses, with fold changes ranging from -6.37 to 3.05 (Figure 5.2 and Supplemental Table A.2). The altered transcripts encompassed both pro- and anti-apoptotic genes, and included *Fas, Casp7, Birc3, DR5, Naip2, Sphk2* and *Aven*. The greatest transcript response was seen following exposure to the lowest dose of 0.5 Gy, where all of the seven transcripts were significantly altered. The number of altered transcripts decreased as the x-ray dose increased (n = 3 and 1; for 1 Gy and 2 Gy x-ray, respectively). The influence of HD on the x-ray induced alterations was examined next. The addition of HD to the x-ray exposure resulted in significantly enhanced transcript alterations at 1 Gy and 2 Gy x-ray plus HD (n = 6 and 3, respectively). No significant response was seen with HD exposure alone. *Fas* was the only transcript that was significantly upregulated across all of the exposures (with the exception of HD), while the rest of the significantly altered transcripts were downregulated. Hierarchical clustering grouped the transcripts according to direction of fold change, with no further apparent separation of transcripts into distinct groups.
Figure 5.2: Heatmap displaying hierarchical clustering of qRT-PCR data. Analysis of qRT-PCR data identified seven significantly altered transcripts, altered at least with one exposure. Expression of the seven transcripts is expressed as fold change (relative to control). Hierarchical clustering separated the transcripts into two groups, those that were upregulated (n = 1; purple) and those that were downregulated (n = 6; green). The intensity of color for each transcript reflects the level of transcript expression, as depicted by the bar to the left of the dendrogram. Significance of a transcript following an exposure is indicated, * p ≤ 0.05, ** p ≤ 0.01, and *** p ≤ 0.001.
In the examination of the targeted germ cell population dose response to x-ray, the greatest difference in overall effect was elicited by exposure to the lowest x-ray dose, 0.5 Gy. To show the difference in response going from 0.5 Gy to 1 or 2 Gy, the log fold changes for 0.5 Gy were plotted against the log fold changes for both 1 and 2 Gy x-ray (Figure 5.3). The blue “x” signs indicate the comparison of 0.5 Gy to 1 Gy x-ray, while the red “+” signs indicate the comparison of 0.5 Gy to 2 Gy. The shift in response of 1 or 2 Gy away from the dashed line shows that fewer genes were responding to these exposures than to the 0.5 Gy exposure. The data are similar with the addition of HD to x-ray exposure (data not shown), and illustrates the lack of overall influence HD has on x-ray induced alterations at low dose exposures.

The transcriptional analysis at 3 hrs was complemented by an investigation at 12 hrs of protein expression of DR5 and Caspase 7 (Casp7), two important members within the apoptotic pathway that were significantly altered at the transcript level. For protein detection by western blotting, both whole testis tissue and stage I-VI seminiferous tubules were probed. The seminiferous tubules were staged through microdissection using a transillumination technique, allowing for stage specific alterations in proteins that were measured. In stage I-VI seminiferous tubules, protein levels of DR5 increased non-significantly with exposure to either x-ray or HD alone (Figure 5.4). However, exposure to the combination of HD and x-ray resulted in a significant increase in DR5 protein at 12 hours following exposure. There was no statistically significant difference in the percentage of cleaved Casp7 following any of the exposures, although there was a slight increase with the x-ray and x-ray plus HD exposures (Figure 5.5). When examined in whole testis tissues, there were no significant alterations in either DR5 or the percent of cleaved Casp7.
Figure 5.3: Dose response comparison of 0.5 Gy x-ray to both 1 and 2 Gy x-ray. The log fold changes for 0.5 Gy were calculated and plotted against the log fold changes for 1 Gy or 2 Gy, represented by a blue “x” or red “+”, respectively. The dotted line represents the correlation where the slope = 1.
Figure 5.4: Western blot analysis of DR5 in staged seminiferous tubules and whole testis. Total cellular proteins from stages I-VI seminiferous tubules (A-B) or whole testis (C-D) were detected using primary antibody against DR5 (1:250). Representative western blot images are shown for each exposure group (control, 2 Gy x-ray, 0.33% and the co-exposure of 2 Gy x-ray with 0.33% HD) (A and C). When quantified, DR5 expression in the staged seminiferous tubules was slightly increased with exposure to either x-ray or HD and was significantly enhanced following co-exposure to HD and x-ray (B). These differences in DR5 expression were not seen with whole testis protein (D). Significant differences between groups was achieved when $p \leq 0.05$. 
Figure 5.5: Western blot analysis of Casp7 in staged seminiferous tubules and whole testis. Total cellular proteins from stages I-VI seminiferous tubules (A-B) or whole testis (C-D) were detected using primary antibody against Casp7 (1:1000). Representative western blot images are shown for each exposure group (control, 2 Gy x-ray, 0.33% and the co-exposure of 2 Gy x-ray with 0.33% HD) (A and C) The antibody detected both Pro-Casp7 and cleaved Casp7, and the percent of cleaved Casp7 was calculated by the division of cleaved Casp7 by total Casp7. When quantified, Casp7 expression exhibited no significant differences between exposures (B and D).
5.5 Discussion

The testis is comprised of several interacting cell types and germ cells at varying stages of development. This complexity proves to be a major hurdle to the study of testicular toxicant mechanisms. Due to the difficulty of separating out individual cells or stages, testis gene expression studies are often performed with RNA isolated from whole testis. In this study, several different approaches were used to examine the spermatogenic stages most susceptible to two testicular toxicants, 2,5-hexanedione (HD) and x-irradiation (x-ray). Optimized laser capture microdissection (LCM) methods were combined with qRT-PCR array approaches to provide mechanistic insight into individual exposures to low doses, and their combination, on target cell populations.

Here we report the application of our improved LCM method, which was optimized to increase RNA yield for the study of a larger number of transcripts on a qRT-PCR array based platform. The apoptotic pathway is useful for toxicity evaluation, because a dose-dependent increase generally indicates a more “toxic” effect. Therefore, with alterations in key aspects of this pathway, conclusions can be drawn on the overall harm that is induced by toxicant exposure. The pathway approach allows for the study of low dose mixtures, which typically do not induce large scale changes, but rather low level changes across an entire pathway. Of the 84 transcripts represented on the qRT-PCR array, only 7 were significantly altered following multiple comparisons correction (Figure ??). Of the 7 altered transcripts, there were both pro- and anti- apoptotic genes represented, illustrating that for cells to undergo apoptosis, there must be simultaneous activation of pro-apoptotic genes with silencing of anti-apoptotic genes. Surprisingly, in this study, the greatest transcript alterations were found with the lowest dose of x-ray (0.5 Gy) predominantly
manifesting as downregulation of transcripts. The downregulation of both pro- and anti-apoptotic genes with the low-dose of x-ray may be a survival attempt of the spermatogonial cell population to avoid the apoptosis signal triggered by downregulation of anti-apoptotic genes. This effect was moderated as the x-ray dose increased, suggesting that the spermatogonial population was no longer evading the apoptosis signal.

The apoptotic pathway is highly dynamic and requires the consecutive, and sometimes simultaneous, turning on and off of pro- and anti-apoptotic factors to induce apoptosis. This simultaneous upregulation and downregulation of apoptotic factors was seen with 0.5 Gy x-ray, where there was simultaneous upregulation of the death receptor Fas and downregulation of both death receptor 5 (DR5) and Caspase 7 (Casp7). The smaller upregulation of Fas, in comparison to the higher doses of x-ray, combined with a strong downregulation of DR5 is quite interesting. Fas and DR5 have been shown to work in concert, interacting with the same downstream targets, and in some cases DR5 can serve as a surrogate mechanism of apoptosis activation when Fas signaling is dysfunctional [18]. At the time point measured, 3 hrs, an adaptive response to 0.5 Gy exposure might be occurring to avoid apoptosis and maintain homeostasis, in part by overriding the downregulation of anti-apoptotic factors that would otherwise lead to activation of apoptosis. At the higher doses, there was less overall downregulation of pro- and anti-apoptotic factors and an increased expression of Fas, ultimately tipping the scales towards more apoptosis. Previous studies have found similar differences in the rate of apoptosis gene expression of several transcripts within staged seminiferous tubules. Bcl2 and p53 levels remained unaltered in stage I-VI seminiferous tubules at 12 hours with qRT-PCR, while significant increases were seen in levels of Fas and Casp3 [5]. In the same study, apoptosis enhancing nuclease (Aen) transcript expression was found to be low at 3 hrs and high
at 12 hrs, with \( p53 \) up-regulated modulator of apoptosis (\( Puma \)) expression having the exact opposite expression profile. \( Fas \) expression peaks between 12 and 24 hours after a much lower dose of 0.075 Gy \([19]\), a much later peak than that seen with doses of 2 and 5 Gy, where peak expression in \( Fas \) was seen between 6 to 12 hrs \([20]\).

Dose may be influencing the timing of apoptosis initiation, illustrated by our multiple dose study at a fixed time. Transcripts were altered in a much different manner with the 0.5 Gy x-ray exposure, compared to either 1 or 2 Gy exposure (Figure 5.3). This was shown in the dose response comparison of 0.5 Gy x-ray to 1 and 2 Gy x-ray, where there was a distinct shift in overall transcript expression. This could be a reflection of the adaptive effect occurring with the lower dose, with downregulation of pro-apoptotic genes to compensate for downregulation of the anti-apoptotic genes. At later time-points, the downregulation of pro-apoptotic transcripts could be overwhelmed by the cell damage response, as it has been shown previously with TUNEL staining that apoptosis eventually occurs with 0.5 Gy x-ray between 12 and 24 hrs in stage I-VI tubules \([3, 21]\). To contrast this with higher doses, peak TUNEL staining with 2 and 5 Gy x-ray was seen earlier between 6 and 12 hrs following exposure in the same stages of tubules \([6]\).

The timing of the apoptosis pathway response is important as it involves the subsequent activation of many genes in a cascade. A transillumination seminiferous tubule dissection technique allowed for the separation of similar stages as those that were captured through LCM, for examination of alterations in protein levels at 12 hrs. DR5 and Casp7 were studied further to see if the transcript level alterations were translated into protein alterations. At 12 hrs following HD and x-ray co-exposure, DR5 protein was significantly upregulated in the staged tubules (Figure 5.4), while no alterations were seen in DR5 protein levels when examined in whole testis. In both staged seminiferous tubules and whole testis, there were no significant differences in
the amount of cleaved Casp7 (Figure 5.5). DR5 plays a role early on in the initiation of apoptosis, while Casp7, an executioner caspase, is activated later in the apoptosis pathway. This time difference in activation could explain the significant increase in protein levels of DR5 seen with the co-exposure, while Casp7 was unchanged. These results illustrate the important role of timing in the regulation of the apoptotic pathway following toxicant exposure.

The combination of HD and x-ray exposures have some influence on gene expression at 3 hrs following exposure. This was illustrated by an increase in the number of significantly altered transcripts when HD was added to x-ray exposure, suggesting an accentuated effect when HD and x-ray are combined (Figure ??). This was not expected as previous studies with higher doses of HD and x-ray have illustrated an attenuation effect with co-exposure [5]. Contrary to the DR5 3 hr gene expression data showing a non-significant downregulation in transcript levels with 2 Gy x-ray co-exposure with HD, DR5 protein was significantly upregulated at 12 hrs following the same co-exposure in the staged tubules (Figure 5.4). These results with HD and x-ray co-exposure indicate that the attenuation effect seen with the combined high dose of 5 Gy x-ray and 0.33% HD, did not persist into the low dose range.
Figure 5.6: Proposed mechanism of testicular apoptotic response to x-ray exposure. The regulation of key apoptotic factors in the testis are shown following exposure to (A) 0.5 Gy x-ray (B) 1 Gy x-ray or (C) 2 Gy x-ray. Following 0.5 Gy exposure (A) the spermatogonial cells attempt adaptation to cell damage by simultaneously downregulating pro- and anti-apoptotic factors, resulting in a delay in apoptosis activation. With 1 Gy (B) and 2 Gy (C) x-ray exposure, the pro-apoptotic signal is stronger, resulting in a more rapid onset of apoptosis.
The results of the current study have been summarized into a working model that illustrates the overall effect of x-ray exposure on the apoptosis pathway gene regulation and outcome (Figure 5.6). With 0.5 Gy x-ray exposure, death receptors DR5 and Fas have an overall decreased expression, that when combined with a significant decrease in Casp7 expression, overrides the decreased expression of anti-apoptotic factors. This is a potential mechanism underlying the delayed execution of cellular apoptosis in low-dose exposed testes, as demonstrated by other studies with TUNEL staining. As dose is increased up to 1 or 2 Gy x-ray the pro-apoptotic signal grows stronger accompanied by a decrease in anti-apoptotic factors, resulting in more rapid onset of apoptosis. It is apparent that the spermatogonial response to 0.5 Gy x-ray is different than that to either 1 Gy or 2 Gy x-ray, indicating an adaptive response.

This is the first study to examine a representative portion of the entire apoptosis pathway with either laser capture microdissected cells or staged seminiferous tubules. The results shown here provide insight into the complex mechanisms of low dose co-exposures and the necessity of examining stage specificity of effect. More studies are needed to investigate a broader range of time points, taking advantage of cell and stage enrichment techniques, to allow for greater insight into the mechanisms underlying apoptosis.

5.6 Acknowledgements

This work was supported by the National Institute of Environmental Health Sciences at the National Institutes of Health (grant numbers P42 ES013660 and 5 T32 ES07272-17). The authors would like to sincerely thank Marguerite Vantangoli for
providing cisplatin treated MCF7 cells as a control for the Caspase-7 western experiments.

5.7 Competing Interests

Kim Boekelheide has funding from NIEHS, USEPA, and the American Chemistry Council. He is an occasional expert consultant for chemical and pharmaceutical companies, and owns stock in CytoSolv, an early stage biotechnology company developing a wound healing therapeutic.
References


Discussion and Conclusions
6.1 Overview

Low doses do not produce the typically measured endpoints in toxicity testing. These include toxicant exposure markers such as changes in hormone levels or organ weights. Since low doses do not typically produce these alterations, studies must dig deeper to unravel the effects that low doses may have, if any, and develop markers for these low doses to be used in risk assessment. Data generated from the increased use of high-throughput techniques to study genome-wide effects of toxicants can be applied to observe the alterations induced by low doses through pathway-based modeling mechanisms. There is a need for the characterization of these relevant pathways of toxicological concern and identification of markers for dose transition points between adaptive and adverse effects, as well as a need for deeper understanding of pathway dynamics. Currently, it is difficult to incorporate these new “markers” into regulatory decision making and risk assessment, because of the lack of knowledge of pathway dynamics and identification of toxicity pathways.

This dissertation is centered around the goal of developing methods for studying low dose pathway effects, and generating data on transition points leading to adaptive or adverse effects through these improved methods. The use of model compounds with well characterized modes-of-action, allowed for the following questions to be addressed:

- What happens within a toxicity pathway when low dose exposures to single toxicants occur?
- Are there differences in the effects that demonstrate characteristic adaptive responses? How about adverse responses? Do these responses overlap?
- What changes occur within that same toxicity pathway when those single tox-
The answers to these questions generated more data for specific toxicity pathways that can help in the understanding of how that toxicity pathway relates to the continuum of adaptive and adverse effects, ultimately informing risk assessment and regulatory decision making.

6.2 Summary of Major Findings

We began the assessment of co-exposure effects on the testis with a series of high dose studies, because although the effects of the individual mixture components are well known, their combined effects are not as well understood. In Chapter 3, the data from the combination of two of the toxicants, 2,5-hexanediione (HD) and carbendazim (CBZ), are presented. HD and CBZ are both Sertoli cell toxicants with opposing effects on microtubules. In theory, since they have opposing effects, the response should be antagonistic with simultaneous exposure. Surprisingly, this is not the case, and instead they interact synergistically to produce greater alterations in transcript expression that is then translated into greater phenotypic effects, such as enhanced apoptosis (Chapter 3, [1]). This result underlines the need for further evaluation of mixtures, since we cannot accurately predict the response when combining toxicants, even when their modes-of-action are well known. These studies with high dose, whole testis effects were an informative starting point, but to gain a fuller understanding of the response, detailed examination of the toxicant sensitive cell population was needed. In general, only a small percentage of the cells within the testis are affected by a particular exposure, so studies evaluating the whole testis response often show muted overall effects. In addition to mixtures, lower doses that fall within the range
of relevant human exposures were examined.

Though the study of low doses as mixtures is highly relevant to environmental exposures, there are many difficulties involved with these studies and there is a great need for the development of sensitive tools and analytical methods to overcome these hurdles. The development of tools to assess the induction of small changes over relevant pathways of toxicological concern (RPTCs), and the determination of the point within the pathway(s) that the cellular response to toxicants transitions from adaptive to adverse, were a primary focus of the studies described in this dissertation. The testis is a highly complex organ with many interacting cell types, including Sertoli cells, Leydig cells, and the developing germ cells, which are present at many different stages of development. This complexity makes the study of the effects of toxicants with specific target cell types very difficult, as the individual cellular populations tend to make up small percentages of the total cell population of the testis. This becomes even more complicated when trying to examine the effects of more than one toxicant that may target the same cell type, or influence the response of that cell type through effects on neighboring/interacting cells.

Before conducting studies to examine low dose mixture effects on specific testicular cell populations, the development of better tools for assessing these effects was required. This resulted in the optimization of laser capture microdissection (LCM) techniques, through scaling up the method to generate greater RNA yields for broader applications, such as use with more sensitive qRT-PCR arrays (Chapter 4). There were many hurdles associated with this technique that needed to be addressed before it could be applied to a large pathway-based study, including managing low RNA yields. A difficulty with low RNA yields is the need for amplification steps to reduce the amount of time the method requires. Part of the optimization of this method involved the investigation of the potential for amplification bias when LCM-
derived RNA is used. Amplification is almost always necessary with LCM samples. Through our comparison of qRT-PCR array data from unamplified and amplified samples, it was found that amplification can introduce bias in the consistency of amplification across transcripts. As a result, it is important to take low RNA input amounts into consideration when comparing results with low abundance transcripts, as the unamplified and amplified input RNA will generate different signals.

With a method optimized for the study of low dose toxicant effects, the cell type-specific low dose extrapolation of x-ray effects with HD co-exposure was evaluated. LCM was performed to collect the targeted cell population and examine a panel of apoptosis-related transcripts using PCR arrays. Apoptosis was chosen as a toxicity pathway both because it is relevant to the phenotypic effects seen with high dose co-exposures to HD and x-ray and because it is a well-studied prototypical pathway. It was anticipated that the transcript alterations underlying HD-induced abrogation of x-ray-induced apoptosis would be revealed through the study of lower doses. It was surprising to find that the attenuation effect on gene expression seen with the combined high dose of 5 Gy x-ray and 0.33% HD, does not persist into the low dose range. Contrary to the adaptive attenuation effect observed with high co-exposure, the addition of HD to low doses of x-ray accentuates pro-apoptotic signaling.

We begin to see the difference between an adaptive and adverse effect in the LCM-derived cell population. The 0.5 Gy x-ray (both with and without HD) exposed germ cells attempt to override the pro-apoptotic signal through down regulation of pro-apoptotic genes. Since this is an adaptation to promote survival in a new environment, this could be viewed as an adaptive effect. This theory is supported in part by the time delay in stage-specific germ cell apoptosis, between the 0.5 Gy and 5 Gy x-ray exposed testis [2][4]. Within the timeframe of x-ray induced apoptosis only one time point was observed, which does not reflect the overall role that time plays.
in apoptotic pathway dynamics (Figure 6.1). With low doses of x-ray the overall number of germ cells undergoing apoptosis is lower, indicating that more germ cells may be adapting to the exposure with only some eventually succumbing to apoptosis. The greater number of cells undergoing apoptosis with higher doses of x-ray may be indicative of an overwhelming amount of damage that the germ cells cannot adapt to. These results indicate that low dose and high dose responses are different, and that the response at one observed time point with low doses is not necessarily an extrapolation of the effects at higher dose levels.
Figure 6.1: Model of x-ray induced germ cell apoptosis over time. The overall number of germ cells undergoing apoptosis is lower as the dose of x-ray decreases. Germ cell apoptosis induced by x-ray is also time dependent, with peak apoptosis occurring with high (red line), medium (orange line), and low (peach line) x-ray doses between 6-12 hrs, 9-12 hrs, and 12-24 hrs, respectively. The blue dashed line indicates the 3 hr time point that was assessed through LCM and qRT-PCR arrays.
Though the studies presented here have some limitations, the results of these studies have generated several important questions, including: What makes an effect adaptive? What transition from adaptation makes it an adverse effect? Is there overlap between these? In Chapter 2, adaptation was defined as the changes a cell or organism makes for its survival in the new environment created by an exposure, while an adverse effect was defined as an effect that results in an impairment of functional capacity or the ability to compensate during additional stress. Based on these definitions, adaptation within the apoptotic pathway would allow the cell to evade apoptosis and maintain normal function when exposed to a toxicant. When the cell can no longer sustain function following toxicant exposure, or has too much cellular damage to repair, this is the transition point where adaptation moves towards an adverse effect. This is demonstrated in Figure 6.2 where the initial response to a low dose of x-ray is an adaptive response, which transitions into an adverse response over time. With a medium x-ray dose, the adaptive effect is not as prominent, whereas with a high dose of x-ray, the adaptive effect is diminished and adverse effects are seen rapidly. Only a small portion of the relationship between adaptive and adverse effects was demonstrated here, as we only observed a single time point (3 hrs) of the apoptosis pathway. More studies delving further into the apoptotic pathway response, through more time points and the doses surrounding 0.5 Gy x-ray, will help in the determination of this “adaptive” response as genuine.
Figure 6.2: Model of the adverse and adaptive response to x-ray dose over time. Exposure to a low dose of x-ray results in an initial adaptive response, that transitions into an adverse response over time. A medium x-ray dose has a more muted adaptive response along with an adverse response. The adaptive response with a high dose exposure is highly diminished, with the adverse response occurring rapidly following exposure. The blue dashed line indicates the 3 hr time point that was assessed through LCM and qRT-PCR arrays.
6.3 Limitations

While this dissertation provides much needed information in the profiling and characterization of the apoptosis pathway response to model testicular toxicants, a relevant candidate pathway of toxicicological concern, there are some limitations to these studies. The biggest limitation of the large scale use of the LCM method is the amount of time required to generate enough RNA for each sample. For the results detailed in Chapter 5, there were eight exposure groups with five biological replicates, resulting in a total of 40 samples. Each sample required approximately nine hours of active microscope time for LCM, and approximately four hours for RNA isolation, concentration and quantification. LCM is a very time intensive process and with 40 samples and the above time estimates, it takes over 500 hours to provide the material for those samples, even with the addition of an amplification step. With time as a limitation, it is also important to be realistic about the number of time points, doses, and toxicants that can be assessed. Here, we only assessed the transcript alterations at 3 hrs, because the addition of another time point would have doubled the number of overall samples and thus the amount of time required to perform LCM on the additional samples.

LCM is used with frozen tissue, where molecular changes can no longer occur, while other methods use unfixed tissues and cells that allow metabolic and molecular processes to go on. These other methods include flow cytometry and transillumination-assisted dissection, which also require time to perform and may cause the dissected cells of interest to change as a result of the method, especially with the latter technique. Although no difference has been found in rat germ cell apoptosis in unfixed testes up to 48 hours, the Type A spermatogonia do become pyknotic over time [5]. Even though the LCM method requires much more time
methodologically than other cell sorting or dissection methods, it allows for the study of sensitive cellular damage transcripts within the toxicant-targeted cell population. Given the time limitation, we optimized the LCM method to require less time overall and yield higher RNA concentrations for qRT-PCR array application. This allowed for the examination of 84 genes within the apoptosis pathway, rather than the two or three genes that are typically studied with LCM material. With a growing number of qRT-PCR arrays available for every biological pathway imaginable, the coupling of LCM with qRT-PCR arrays results in an effective research tool, with the only limitation being the patience of the investigator.

High-throughput sequencing and transcript profiling techniques typically generate long lists of differentially expressed genes and proteins. These long lists can be difficult to interpret, and a common approach is to group them into pathways, based on the knowledge of how the genes and proteins interact. This approach can be useful when the genes encompassing multiple pathways are studied, as it groups altered transcripts within a pathway, rather than a list of individual genes. Here, the difficulty of data interpretation stems from the fact that only one pathway is studied in depth. A focused study within a single pathway generates much needed information on toxicant effects, but renders the typical analytical tools ineffective, such as Ingenuity Pathways Analysis. The generation of new computational analytical methods for interpreting pathway dynamics is still needed to integrate the individual pathway alterations, and from this determine the biological implications of the exposure(s). To assess the components of signaling pathways, the components must be systematically identified for pathway functionality to identify the specific role they play [6]. This is a major limitation to the use of pathways-based PCR arrays as mechanistic tools in toxicology, as useful analytical tools for determining how the individual pathway components interact within a pathway, still need to be
developed.

6.4 Future Directions

Within this dissertation, the application of laser capture microdissection (LCM) was optimized for use with pathways based array studies. The application of this tool has opened up the ability to study the response of the targeted cells of toxicant exposure, without the diminishing effects of unaffected cell types. There are several avenues this research can be taken in, but there are several gaps in the present study that should be filled out before this LCM approach is applied to broader studies.

6.4.1 DR5 and Fas – role for a compensating mechanism?

The most interesting result from the apoptosis qRT-PCR array was the simultaneous opposing gene expression of Fas and death receptor 5 (DR5), with 0.5 Gy x-ray exposure. In comparison to the higher dose exposures of 1 Gy or 2 Gy x-ray, the expression of Fas was less upregulated while DR5 was significantly downregulated. The combined decreased expression of these two death receptors would lead to an overall decrease in apoptosis, when compared to their expression at higher x-ray doses. In addition to these results, the expression of Fas has been shown to be abrogated in LCM-derived germ cells following 5 Gy x-ray and 1% HD co-exposure, when compared to x-ray response alone [7]. Even though co-exposure to HD and x-ray resulted in Fas attenuation in stage I-VI tubules, there was a non-significant difference in the number of TUNEL positive nuclei with co-exposure [4]. Demonstrating that apoptosis occurs at the same level with the co-exposure when compared
to x-ray exposure, despite a significant decrease in Fas expression with the high dose co-exposure. Both of these results indicate a role for the DR5 receptor in the testicular apoptotic response to toxicant exposure. Investigation of the adaptive evasion response of the 0.5 Gy exposed spermatogonia, resulting in delay of apoptosis, could be enhanced with further investigation of the interaction of DR5 and Fas in apoptosis induction in the testis.

The TNF receptor superfamily of death receptors not only includes Fas, but also DR4, DR5, and DR6 [8]. The DR4 and DR5 receptors bind to their ligand, TRAIL, to activate the same downstream apoptosis factors that are activated by Fas and FasL. All of these death receptors have been identified in the testis, and DR4 and DR5 have been implicated as secondary responders to initiate apoptosis when Fas-mediated apoptosis cannot occur, as in gld mice [8, 9]. gld (generalized lymphoproliferative disease) mice, with a dysfunctional form of FasL, as well as lpr<sup>cg</sup> (lymphoproliferation complementing gld) mice, with nonfunctional Fas receptor and FasL, could be very useful in the further investigation of the DR5 and Fas response to combined x-ray and HD exposure. Since gld mice have non-functional FasL, which is normally expressed by the Sertoli cells, and the lpr<sup>cg</sup> mice have non-functional Fas receptor and FasL, both of these mice with spontaneous mutations would be useful in the examination of apoptosis pathway activation in response to HD and x-ray co-exposure. With the Fas-mediated pathway non-functional, the role that DR5 plays in the adaptive mechanism to low doses of x-ray can be further examined.

These experiments would be performed in the same manner as with the rat exposures and LCM methods detailed in Chapters 4 and 5. The investigation would be focused on the role of DR5 and Fas in apoptosis activation following HD and x-ray co-exposures, and qRT-PCR arrays would be performed to observe the effect of non-functional forms of Fas and FasL on the apoptotic pathway. This would allow for
the elucidation of the role that DR5 plays, if any, in the adaptive pathway response observed with exposures to low doses of x-ray.

6.4.2 LCM apoptosis pathway time course

From these studies, it became quickly apparent that the apoptosis pathway is highly dynamic and that examining gene expression pathway results at only 3 hrs and protein expression at 12 hrs, reveals only a small snapshot of the toxicant-induced apoptosis alterations. A small scale LCM-based gene expression study examined the gene expression of a few apoptosis related genes at both the 3 hr and 12 hr time points following 5 Gy x-ray and 1% HD [7]. Campion et al. found that in stages I-VI, the expression of Casp3 was low at 12 hrs, whereas apoptosis enhancing nuclease (Aen) was more highly expressed at 12 hrs in comparison to the 3 hr time point. Both Casp3, an initiator caspase, and Aen, a p53 mediator of apoptosis, are expressed at different time points within the apoptosis pathway, thereby illustrating that their differential expression over time must be a result of their role within the apoptosis pathway [10, 11]. It was also shown that the expression of p53 up-regulated mediator of apoptosis (PUMA) was significantly upregulated similarly with 5 Gy x-ray exposure, and also with the co-exposure of 5 Gy x-ray + 1% HD at 3 hrs in LCM stages I-VI. However, when examined 12 hrs later, the overall expression of Puma is lower with 5 Gy x-ray exposure alone, but is still significantly upregulated with the co-exposure. This enhanced effect on apoptosis related genes with the HD and x-ray co-exposure is similar to the results that are shown in Chapter 5.

The small scale LCM study of HD and x-ray high dose co-exposure effects on apoptosis, in combination with the apoptosis qRT-PCR array based study from Chapter 5, illustrate the complexity of the apoptosis pathway and the need for further
studies examining the effect of HD and x-ray over time. This requires the addition of several time points into what we have already shown, and can be performed using the LCM methods detailed in Chapter 4. Extending the time course may also answer the question of whether these apoptotic alterations persist past a certain time point. It is known that the 0.5 Gy exposed spermatogonia eventually undergo apoptosis, since TUNEL staining is shown to increase significantly around 12-24 hrs, a later time point than what is shown with higher doses [2]. Understanding how the cells transition from an adaptive response to an adverse response, would help in the characterization of this pathway for future toxicity studies.

It would be interesting to examine other doses of x-ray as well, since a potential adaptive mechanism in spermatogonia exposed to 0.5 Gy x-ray is observed, and there is a difference in the 0.5 Gy and 1 Gy response. The addition of 0.1 Gy to the dose response may exhibit an enhanced adaptive response in comparison to 0.5 Gy, since gene expression profile effects were seen in initial low dose extrapolation efforts. By extending the time points and adding in more doses, extensive characterization of the apoptosis pathway as an RPTC could be performed for its application to other toxicants with apoptosis inducing effects. The addition of time points and doses would be easy to perform, as it only requires time for LCM since the method has been optimized for consistency. To take the characterization of this apoptosis pathway a step further, it would also be beneficial to examine other model testicular toxicants, such as 1,3-dinitrobenzene as a Sertoli cell toxicant [12, 13] and 1,2-dibromo-3-chloropropane as a germ cell toxicant that targets the quiescent germ cell population [14, 15].
6.4.3 Differential stage effects of toxicant exposure

Stage specificity of effects from testicular toxicant exposures occurs quite frequently. All three toxicants examined in this dissertation (HD, CBZ and x-ray) have stage specific effects, depending on their mechanism of action (Figure 6.3). Exposure to HD results in differential effects among testicular cell types as well, making the stages with targeted cell types more susceptible to the exposure. After HD exposure germ cell apoptosis occurs most often in the Type A_2 - A_4 spermatogonia in stages I-VI and X-XIV [4,16], Sertoli cell vacuoles appear in stages I and XII-XIV [17,18], and changes in the elongate spermatids are seen in stage II-VIII and IX-X tubules [19,20]. In addition to changes in specific cell types during different spermatogenesis stages, the frequency and duration of stages can also be affected by high doses of HD, with significant decreases in stage VII and small increases in the presence of stage II, V-VI, and IX-X [18]. The stages effected by CBZ exposure have some overlap with those effected by HD exposure, but as CBZ has different effects on Sertoli cell microtubules, the seminiferous tubule effects are not the same. With CBZ exposure there is germ cell sloughing in stages VI-VII and XIII-XIV [21] and premature release of spermatids from the Sertoli cells can be seen in stage I, VII, IX, and XIV tubules [22]. X-ray exposure mainly effects stages of spermatogenesis where mitotic spermatogonia are present, with dose dependent increases in spermatogonial apoptosis occurring within stages I-VI and VII-VIII [2,3,23].
Figure 6.3: Stage specific effects of toxicant exposure. Testicular toxicants have stage specific effects on the testis, depending on their modes-of-action. The different stage specific effects include germ cell apoptosis (green), Sertoli cell vacuole formation (purple), changes to the elongate spermatids (pink), premature release of the spermatids (blue), and germ cell sloughing (yellow). The occurrence of these stage specific alterations as a result of x-ray (A), HD (B) or CBZ (C) exposure is illustrated.
It is important to study toxicant effects within each spermatogenesis stage, or stage group, due to the diverse stage effects that can occur as a result of toxicant exposure. Stage frequency should be studied as well, based on the findings that spermatogenesis stage frequency can be altered depending on toxicant exposure. The influence that a co-exposure, or complex mixture, has on spermatogenesis stages is largely unstudied. However, a recent study demonstrated a significant decrease in the number of Type A spermatogonia in stage VII following arsenic exposure, an effect which was protected by α-tocopherol and ascorbic acid co-supplementation \[24\]. With this in mind, it would be prudent to extend the current study to other stage groups of spermatogenesis in addition to other toxicants. It would also be interesting to look at the effect that the addition of x-ray onto HD exposure would have on stage frequency, in light of the effect of HD exposure has on stage frequency.

### 6.5 Closing Statements

This dissertation provides a foundation for the study of low dose toxicant mixtures. Before these studies were performed, most co-exposure and mixtures studies only examined the effects on the whole testis. It is well known that each male reproductive toxicant has specific cellular and sub-cellular targets within the testis, in addition to stage-specific effects. A great deal of this dissertation involved optimizing the laser capture microdissection method to perform these cell- and stage-specific co-exposure studies. The result is a reliable and effective tool, that when coupled with qRT-PCR arrays, allows for assessment of relevant pathways of toxicological concern. The resulting data from the application of these tools provides much insight into cell-specific toxicant exposure and can be applicable to both risk assessment for low dose co-exposures and future studies.
References


Appendix A

Supplemental Materials
**Table A.1: Supplemental Table 1.** Participants in the May 10-11, 2011, HESI Workshop on Distinguishing Adverse from Adaptive Effects in the 21st Century, at US EPA facilities in Research Triangle Park, NC.

<table>
<thead>
<tr>
<th>Participants</th>
<th>Affiliation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dr. Melvin Anderson</td>
<td>The Hamner Institutes for Health Sciences</td>
</tr>
<tr>
<td>Dr. John Buchner</td>
<td>National Institute of Environmental Health Sciences</td>
</tr>
<tr>
<td>Dr. Daniel Casciano</td>
<td>University of Arkansas at Little Rock</td>
</tr>
<tr>
<td>Ms. Natasha Catlin</td>
<td>Brown University</td>
</tr>
<tr>
<td>Dr. Rory Conolly</td>
<td>US Environmental Protection Agency</td>
</tr>
<tr>
<td>Ms. Nancy Doerrer</td>
<td>ILSI Health and Environmental Sciences Institute</td>
</tr>
<tr>
<td>Dr. William Farland</td>
<td>Colorado State University</td>
</tr>
<tr>
<td>Dr. Amber Goetz</td>
<td>Syngenta Crop Protection</td>
</tr>
<tr>
<td>Dr. Frederick Hess</td>
<td>BASF Corporation</td>
</tr>
<tr>
<td>Dr. Daland Juberg</td>
<td>DOW AgroSciences</td>
</tr>
<tr>
<td>Dr. Douglas Keller</td>
<td>Sanofi US</td>
</tr>
<tr>
<td>Dr. Peter Mann</td>
<td>Experimental Pathology Laboratories</td>
</tr>
<tr>
<td>Dr. Richard Paules</td>
<td>National Institute of Environmental Health Sciences</td>
</tr>
<tr>
<td>Dr. Mathew Pletcher</td>
<td>Pfizer, Inc.</td>
</tr>
<tr>
<td>Dr. Santhini Ramasamy</td>
<td>US Environmental Protection Agency</td>
</tr>
<tr>
<td>Dr. Ivan Rusyn</td>
<td>University of North Carolina</td>
</tr>
<tr>
<td>Dr. David Saltmiras</td>
<td>Monsanto Company</td>
</tr>
<tr>
<td>Dr. Miriam Sander</td>
<td>Page One Editorial Services</td>
</tr>
<tr>
<td>Dr. Paul Snyder</td>
<td>Purdue University</td>
</tr>
<tr>
<td>Dr. Martin Stephens</td>
<td>Humane Society of the United States</td>
</tr>
<tr>
<td>Dr. Douglas Wolf</td>
<td>US Environmental Protection Agency</td>
</tr>
<tr>
<td>Gene symbol</td>
<td>0.33% HD</td>
</tr>
<tr>
<td>---------------</td>
<td>----------</td>
</tr>
<tr>
<td>Fas</td>
<td>1.39</td>
</tr>
<tr>
<td>Caspase 7</td>
<td>-2.47</td>
</tr>
<tr>
<td>Birc3</td>
<td>-1.90</td>
</tr>
<tr>
<td>Aven</td>
<td>-1.41</td>
</tr>
<tr>
<td>Tnfsf10b (DR5)</td>
<td>-1.52</td>
</tr>
<tr>
<td>Naip2</td>
<td>-1.39</td>
</tr>
<tr>
<td>Sphk2</td>
<td>-1.23</td>
</tr>
</tbody>
</table>