EFFECTS OF QUATERNARY AMMONIUM DISINFECTANTS ON MOUSE REPRODUCTIVE FUNCTION

Vanessa Estella Melin

Dissertation submitted to the faculty of Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

IN

BIOMEDICAL & VETERINARY SCIENCES

Terry C. Hrubec, Co-chair

Marion Ehrich, Co-chair

Geraldine C. Magnin-Bissel

William R. Huckle

Mary Renee Prater

May 5th, 2015

Blacksburg, Virginia

Keywords: quaternary ammonium disinfectants, infertility, reproductive toxicology, testis, spermatogenesis, epigenetics

EFFECTS OF QUATERNARY AMMONIUM DISINFECTANTS

ON

MOUSE REPRODUCTIVE FUNCTION

Vanessa Estella Melin

ABSTRACT

Quaternary ammonium compounds (QACs) are antimicrobial disinfectants commonly used in commercial and household settings. While these compounds have been used for decades, reproductive toxicity has not been thoroughly evaluated. Extensive use of QACs results in ubiquitous human exposure to potentially toxic compounds. Reproductive toxicity of two common QACs, alkyl dimethyl benzyl ammonium chloride (ADBAC) and didecyl dimethyl ammonium chloride (DDAC), was investigated to determine gender-specific toxicity with an emphasis on male reproductive function. Breeding pairs of mice exposed for six months to ADBAC+DDAC exhibited decreases in fertility and fecundity, with fewer pregnancies and decreased numbers of pups over a six month period. Females proceeded through significantly fewer estrus cycles, and both ovulation and implantation rates were reduced. Males exhibited declines in both sperm concentration and motility. Male reproductive toxicity was further assessed in a series of in-vitro and in-vivo experiments. ADBAC+DDAC were cytotoxic to testicular Sertoli cells in culture at concentrations greater than or equal to 0.0005%. Changes in blood-testis-barrier integrity (BTB) were observed at 0.01% ADBAC+DDAC using a two-compartment culture system that measures transepithelial electrical resistance (TER). Sertoli cell cytotoxicity correlated with decreased TER at ADBAC+DDAC concentrations above 0.001%. In-vitro fertilization capacity of epididymal sperm was reduced in males given a 10-day rest period following ADBAC+DDAC exposure. Multigenerational changes in sperm parameters and in mRNA expression of enzymes involved with epigenetic

modifications were evaluated across three generations. Sperm concentration and motility were reduced in F_0 males exposed directly to ADBAC+DDAC. In F_1 males, sperm concentration was increased and motility decreased, while there was no change in the F_2 progeny. Genes involved in epigenetic modifications were altered in the exposed F_0, with upregulation of two histone acetyltransferases (*Hat1* and *Kat2b*) and downregulation of one lysine-specific demethylase (*Kdm6b*). F_1 and F_2 generations were not different from controls except for downregulation of the methyltransferase *Dnmt1* in F_1 progeny. The reproductive toxicity of ADBAC+DDAC identified in these studies, particularly to the male, compels further investigation into the potential effects that these compounds may have on human reproduction.

ACKNOWLEDGEMENTS

I would like to express my deepest appreciation to my advisor, Dr. Terry Hrubec. I am extremely grateful to have had the opportunity to work with and learn from her. She has forever changed my understanding of the world. To my committee members, Dr. Marion Ehrich, Dr. William Huckle, Dr. Geraldine Magnin-Bissel, and Dr. Renee Prater, I am profoundly thankful for your guidance and suggestions throughout my project. I would also like to thank Becky Jones, the BMVS Graduate Program Coordinator, for the tremendous amounts of assistance and support that she has given me.

I wish to give special thanks to the individuals who have provided me with their technical expertise. Thank you to Dr. Stephen Werre for his assistance with data analysis and patience throughout all of my queries. I would also like to express my gratitude to Dr. Sherry Clarke for making sperm analysis possible and Dr. Willard Eyestone for enabling me to complete in-vitro fertilization. I am sincerely grateful to VMRCVM faculty, staff, and friends for all of their help and support through the years.

Thank you to Melissa Makris for her guidance and assistance with running my flow cytometric analyses.

I am forever indebted to the Melin family for their loving support and encouragement, without which my achievements would have been unfeasible.

Thank you to the Passport Foundation and the VA-MD Regional College of Veterinary Medicine for providing the financial support that made this research possible.

Finally, I would like to thank my sister Tiffany Bennett for continuing to be my inspiration.

TABLE OF CONTENTS

DECLARATION OF WORKS PERFORMED………………………………………………………. v
LIST OF FIGURES……………………………………………………………………………… vi
LIST OF TABLES……………………………………………………………………………. viii
LIST OF ABBREVIATIONS………………………………………………………………… x
CHAPTER 1…………………………………………………………………………………... 1

Literature review

CHAPTER 2..……………………………………………………………………………… 38

Exposure to Common Quaternary Ammonium Disinfectants Decreases Fertility in Mice

CHAPTER 3...…………………………………………………………………………….. 63

Quaternary Ammonium Disinfectants Cause Subfertility in Mice by Targeting both Male and Female Reproductive Processes

CHAPTER 4…..………………………………………………………………………….. 88

Disinfectant Compounds ADBAC+DDAC Exhibit Concentration and Temporally Dependent Reproductive Toxicity In-Vitro and In-Vivo

CHAPTER 5…..………………………………………………………………………… 113

Direct and In-utero Exposure to Quaternary Ammonium Disinfectants Alters Sperm Parameters and RNA Expression of Epigenetic Enzymes in the Testis of Male Mice

CHAPTER 6……………………………………………………………………………… 135

Conclusions

APPENDIX A: Fold changes in RNA expression of chromatin remodeling enzymes……………… 138

APPENDIX B: Summary of methods completed in Chapters 2-5…………………………………… 142

APPENDIX C: Raw data tables from experiments completed in Chapters 2-5……………………… 163

LIST OF FIGURES

CHAPTER 1

Figure 1. Basic QAC structure and organization………………………………………………………. 4

CHAPTER 2

Figure 1. Comparison of pregnancy rates for timed matings……………………………………….. 47
Figure 2. Breeding colony productivity……………………………………………………………... 47
Figure 3. Results of LC-MS/MS cage extract analysis……………………………………………... 49
Figure 4. Number of females in each ADBAC+DDAC dose group exhibiting clinical signs necessitating euthanasia……………………………………………………………………………... 51
Figure 5. Least squares mean food intake among exposed and control mice…………………………. 51
Figure 6. The effect of ADBAC+DDAC disinfectant on time to first litter…………………………… 53
Figure 7. Average number of pregnancies per dam in the first 100 days of exposure before any dam loss…………………………………………………………………………………………… 53
Figure 8. The effects of ADBAC+DDAC disinfectant on fecundity of breeding pairs………………... 54
Figure 9. Average weight per pup (g) among breeding pairs exposed to ADBAC+DDAC disinfectant for 180-days………………………………………………………………………………………. 55

CHAPTER 3

Figure 1. Control mouse uterus……………………………………………………………………... 72
Figure 2. Numbers of corpora lutea ………………………………………………………………… 72
Figure 3. Percent of corpora lutea implanted……………………………………………………….. 73
Figure 4. Examples of vaginal cytology in control dams during different stages of estrus, labeled with the predominant cell type and estrus stage (40X magnification)………………………………… 74
Figure 5. Average time spent in estrus as determined by vaginal cytology with ADBAC+DDAC exposure……………………………………………………………………………………………... 74
Figure 6. The average number of mid-gestational embryos in control (0 mg/kg/day) and mice treated with a QAC disinfectant at 120 mg ADBAC+DDAC/kg/day for 8 weeks……………………………. 75
Figure 7. Hemocytometer counts of epididymal extracts in males exposed for 8 weeks to 120 mg/kg/day ADBAC+DDAC…………………………………………………………………………... 76
Figure 8. CASA determination of sperm numbers in control mice and mice exposed to 120 mg/kg/day ADBAC+DDAC for 8 weeks (n=4-5)…………………………………………………... 77
Figure 9. Sperm viability in control and ADBAC+DDAC treated males……………………………... 77

CHAPTER 4

Figure 1. TM4 Sertoli cells treated with ADBAC+DDAC for 72 h exhibited a dose-dependent decrease in mitochondrial metabolism indicating cytotoxicity……………………………………….. 99
Figure 2. The cell cycle distribution of TM4 Sertoli cells was significantly altered by 24 h exposure to ADBAC+DDAC compared to controls………………………………………………………… 99
Figure 3. Stabilization of TER after 96 h in culture………………………………………………... 102
Figure 4a-b. Cauda sperm motility and concentration did not differ in males administered 7-day oral gavage of ADBAC+DDAC compared to males provided vehicle control either when assessed directly or when assessed following a 10 day rest period………………………………………….. 103
Figure 5. Significant dose effects were observed in the fertilizing capacity of sperm from males administered ADBAC+DDAC for 7-days and given a 10-day rest period (n=5/treatment; ANOVA, p=0.05)………………………………………………………………………………………... 104
Figure 6. Successful and unsuccessful IVF………………………………………………………… 105

CHAPTER 5

Figure 1. F_0 females were exposed to ambient ADBAC+DDAC for 8 weeks then transferred to a QF facility……………………………………………………………………………………….. 119
Figure 2. Mating index (A) and pregnancy rate (B) after removal from a QAC facility……………... 123
Figure 3A-B. In the F_0 generation, exposure to 120 mg ADBAC+DDAC/kg/day for 8 weeks significantly reduced sperm concentration and motility compared to controls……………………… 125

APPENDIX B

Figure 1. F_0 females were exposed to ambient ADBAC+DDAC for 8 weeks then transferred to a QF facility……………………………………………………………………………………….. 158

LIST OF TABLES

LITERATURE REVIEW

Table I. Categories and examples of common antimicrobial agents used in industrial, medical, and residential environments and their modes of action against microorganisms………………………… 2
Table II. List of active, federally registered QAC pesticides with CAS numerical identifiers, uses, and alternative names they may appear by………………………………………………………… 4
Table III. Documented uses of QACs in consumer products………………………………………. 10
Table IV. Classes and examples of endocrine disrupting compounds……………………………... 19
Table V. List of chromatin remodeling enzyme classes, function, and gene groupings……………… 23

CHAPTER 5

Table I. Genes evaluated using a pathway targeted PCR array………………………………………. 122
Table II. Changes in testis gene expression of chromatin modification enzymes……………………. 126

APPENDIX A

Table I. Fold changes in RNA expression related to chromatin remodeling enzymes in F_0 males exposed for 8 weeks to ADBAC+DDAC and their F_1 and F_2 progeny………………........................ 138

APPENDIX C

Table I. Overall number of pregnancies in mouse breeding pairs exposed to ADBAC+DDAC for 6 months……………………………………………………………………………………………… 163
Table II. Cumulative food consumption in mouse breeding pairs exposed to ADBAC+DDAC for 6 months……………………………………………………………………………………………… 163
Table III. Total pup number in mouse breeding pairs exposed to ADBAC+DDAC for 6 months…... 164
Table IV. Least squares mean (LSM) estimates of total pup number in mouse breeding pairs exposed to ADBAC+DDAC for 6 months……………………………………………………………. 165
Table V. Male mouse weights over 6 month exposure period to ADBAC+DDAC………………….. 165
Table VI. Average weight per pup over 6 month exposure period to ADBAC+DDAC……………... 166
Table VII. Numbers of corpora lutea in mice exposed for 2 and 8 weeks to 120 mg ADBAC+DDAC/kg/day………………………………………………………………………… 166
Table VIII. Numbers of implantation sites in mice exposed for 2 and 8 weeks to 120 mg ADBAC+DDAC/kg/day………………………………………………………………………… 167

Table IX. Estrus cyclicity data collected from mice exposed to 120 mg ADBAC+DDAC/kg/day for 2 weeks………………………………………………………………………………………... 167
Table X. Mid-gestational embryonic losses in mice exposed to 120 mg ADBAC+DDAC/kg/day for 8 weeks………………………………………………………………………………………... 167
Table XI. Sperm concentration in male mice exposed to 120 mg/kg/day ADBAC+DDAC for 8 weeks……………………………………………………………………………………….. 167
Table XII. Percent resazurin reduction in TM4 cells treated with ADBAC+DDAC for 24 hours…… 168
Table XIII. Percent resazurin reduction in TM4 cells treated with ADBAC+DDAC for 72 hours….. 168
Table XIV. Cell cycle distribution in TM4 cells treated with ADBAC+DDAC……………………... 169
Table XV. Transepithelial electrical resistance values in the TM4 two-compartment BTB model following treatment with ADBAC+DDAC (A+D) and MEHP……….. 170
Table XVI. Cauda sperm motility and concentration in rested and unrested male mice administered 7-day oral gavage of ADBAC+DDAC. Sperm were used for IVF…………………………………… 171
Table XVII. Number of oocytes isolated from superovulated female mice and fertilization rates of sperm isolated from unrested and rested male mice exposed for 7 days to ADBAC+DDAC………... 172
Table XVIII. Sperm concentrations in male mice exposed to ADBAC+DDAC determined by CASA………………………………………………………………………………………………. 172
Table XIX. Sperm motility in male mice exposed to ADBAC+DDAC as determined by CASA…... 173

DECLARATION OF WORKS PERFORMED

All work in this dissertation was conducted by the author, Vanessa E. Melin, with the exception of the following:

CHAPTER 2: ***Exposure to Common Quaternary Ammonium Disinfectants Decreases Fertility in Mice***

Dr. Terry C. Hrubec, associate professor and research associate in the Department of Anatomy at the E. Via College of Osteopathic Medicine, advised study design and data collection

Dr. Bill Siems, research professor in the Department of Chemistry at Washington State University, completed liquid-chromatographic mass-spectrometric analyses of cage extracts

Dr. Patricia Hunt, professor in the School of Molecular Biosciences at Washington State University, contributed in-house mouse husbandry data

Jodi Griswold, a research associate at the Center of Reproductive Biology at Washington State University, managed the C57BL/6 mouse colony

Dr. Stephen R. Werre, the Study Design & Statistical Analysis Lab Supervisor within the Office of Research and Graduate Studies at VA-MD Regional College of Veterinary Medicine, completed statistical analyses of fertility and fecundity data

Haritha Potineni, a graduate student of Dr. Terry Hrubec within the Department of Biomedical Sciences and Pathobiology at the VA-MD Regional College of Veterinary Medicine, assisted with mouse husbandry and data collection at Virginia Tech

CHAPTER 3: ***Quaternary Ammonium Disinfectants Cause Subfertility in Mice by Targeting both Male and Female Reproductive Processes***

Dr. Terry C. Hrubec, associate professor and research associate in the Department of Anatomy at the E. Via College of Osteopathic Medicine, advised study design and data collection

Travis E. Melin, Brian J. Dessify, and Christina T. Nguyen, medical students affiliated with E. Via College of Osteopathic Medicine-Virginia Campus, assisted with collection of mouse vaginal cytology and ovulation/implantation data

Melissa Makris, Flow Cytometry Lab Supervisor within the Department of Biomedical Sciences and Pathobiology at the VA-MD Regional College of Veterinary Medicine, operated the flow cytometer

CHAPTER 4: ***Disinfectant Compounds ADBAC+DDAC Exhibit Concentration and Temporally Dependent Reproductive Toxicity In-Vitro and In-Vivo***

Melissa Makris Flow Cytometry Lab Supervisor within the Department of Biomedical Sciences and Pathobiology at the VA-MD Regional College of Veterinary Medicine, operated the flow cytometer

LIST OF ABBREVIATIONS

In alphabetical order:

ADBAC	Alkyl Dimethyl Benzyl Ammonium Chloride
BTB	Blood Testis Barrier
BSA	Bovine Serum Albumin
CASA	Computer-automated Sperm Analysis
CL	Corpora Lutea
CO_2	Carbon Dioxide
COC	Cumulus Oocyte Complexes
D	Day
DDAC	Didecyl Dimethyl Ammonium Chloride
DDT/DDE	Dichloro Diphenyl Trichloroethane
DEHP	Diethylhexyl Phthalate
DMEM	Dulbecco's Modified Eagle's Cell Culture Medium
DNA	Deoxyribonucleic Acid
DNMT	DNA Methyltransferase
EDC	Endocrine Disrupting Chemical
EPA	Environmental Protection Agency
ER	Estrogen Receptor
F12	Ham's F12 Cell Culture Medium
FBS	Fetal Bovine Serum
FHM	Mouse Embryo Culture Medium
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
FSH	Follicle Stimulating Hormone
GnRH	Gonadotropin Releasing Hormone
GD	Gestational Day
H	Hour
HAT	Histone Acetyltransferase
HBCDD	Hexabromocyclododecane
HCB	Hexachlorobenzene

HCG ……………………………………………………………Human Chorionic Gonadotropin
HDM ……………………………………………………………………Histone Demethylase
HMT …………………………………………………………………Histone Methyltransferase
HPG …………………………….............................Hypothalamic-Pituitary-Gonadal (axis)
IVF ……………………………………………………………………...In-vitro Fertilization
KSOM ……………………………………………………………Mouse Embryo Culture Medium
L …………………………………………………………………………………………Liter
LH ……………………………………………………………………Luteinizing Hormone
LSM ………………………………………………………………………Least Squares Mean
MEHP ………………………………………………………………Mono-2-Ethylhexyl Phthalate
MIN ……………………………………………………………………………………Minute
ML ……………………………………………………………………………………..Milliliter
N ………………………………………………………………………………Sample Size
PBB ………………………………………………………………..Polybrominated Biphenyl
PBDE …………………………………………………………...Polybrominated Diphenyl Ethers
PCB …………………………………………………………………Polychlorinated Biphenyls
PCDD/PCDF ……………………………………………..Polychlorinated Dibenzodioxin/Dibenzofuran
PFCA …………………………………………………………Perfluorinated Carboxylic Acid
PFOS ……………………………………………………………..Perfluorooctane sulfonate
PI …………………………………………………………………………...Propidium Iodide
PMSG ……………………………………………………Pregnant Mare Serum Gonadotropin
POP …………………………………………………………….Persistent Organic Pollutant
QAC ……………………………………………………......Quaternary ammonium compounds
SAM ……………………………………………………………………S-Adenosyl Methionine
SCCP …………………………………………………...Short-chained Chlorinated Paraffin
SD ………………………………………………………………………….Standard Deviation
SEM …………………………………………………………………Standard Error Margin
SET ………………………………........*Drosophila* Su(var)3-9 and 'Enhancer of zeste' protein domain
TER ……………………………………………………Transepithelial Electrical Resistance
TM4 ……………………………………………………………Balb/c Mouse Sertoli Cell Line
TSCA …………………………………………………………...Toxic Substances Control Act
WHO …………………………………………………………………World Health Organization

CHAPTER 1:

Literature Review

1. Antimicrobial agents

Antimicrobial agents are compounds used to kill and/or inhibit growth of microorganisms. Antimicrobials intended for use on non-living objects can be classified into two categories: disinfectants or sanitizers. Disinfectants and sanitizers are classified as pesticides under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) and must be registered with the Environmental Protection Agency (EPA). There are currently over 5,000 disinfectant and sanitizer products registered with the EPA.[1,2] Disinfectants kill fungi, viruses, and/or bacteria, while sanitizers reduce the number of microorganisms to levels considered safe by public health safety standards. In hospitals, disinfectants are used to destroy microorganisms on medical equipment, laundry, floors, and walls. Disinfectants are also marketed commercially as antimicrobial cleaners intended for residential use. Sanitizers are essentially less concentrated versions of disinfectants and are considered safe to use on food contact surfaces.[3,4] Table I lists the most common sanitizers and disinfectants and their modes of action.

Sodium hypochlorite (bleach) and hydrogen peroxide are oxidizing agents most commonly used for residential cleaning and sanitation. Household bleach products typically contain a solution of 5.25% sodium hypochlorite and are diluted with water to 500 ppm for residential cleaning use.[14] Industrial strength solutions used for swimming pool disinfection contain up to 20% sodium hypochlorite. Toxic chloramine gas can form when sodium hypochlorite is mixed with common household cleaners containing ammonia. Several fatalities resulting from inhalation exposure to chloramine gas from bleach and ammonia cleaning mixtures have been reported.[15,16] Hydrogen peroxide cleaning solutions are available at a number of concentrations, ranging from 6-15%% for home disinfectant and stain removing

products. Exposure to sodium hypochlorite and hydrogen peroxide solutions can cause respiratory, dermal, and ocular irritation. Both oxidizing agents are chemically unstable and susceptible to inactivation by organic matter. Quaternary ammonium compounds (QACs) have taken favor over traditional oxidizing agents, since they are non-corrosive, leave no odor, and do not decolorize clothing. QACs were introduced in the 1930's as "non-toxic" disinfectants for cleaning eating utensils, medical instruments, laundry, and floors and walls.[17,18] Structurally, QACs are positively charged compounds with amine-functional centers and aliphatic side chains. The cationic portion of the QAC molecule is attracted to negatively charged proteins on bacterial cell membranes. Once proximate, the long alkyl chains of the QAC molecule pierce the bacterial lipid bilayer, causing membrane disruption and leakage of cellular contents. QACs are effective against most bacteria, some fungi, viruses, and protozoa.[7]

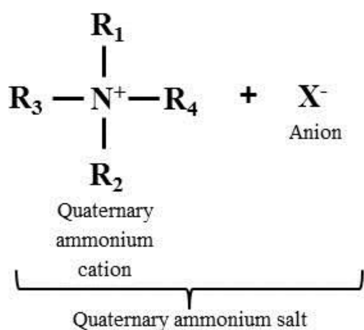
Table I. Categories and examples of common antimicrobial agents used in industrial, medical, and residential environments and their modes of action against microorganisms.

CATEGORY	MECHANISM OF ACTION	LIMITATIONS
QUATERNARY AMMONIUM COMPOUNDS - Alkyl dimethyl benzyl ammonium chloride - Didecyl dimethyl ammonium chloride	Penetration/disruption of the cell wall and leakage of intracellular contents[5-7]	Inactivated by organic material[8]
ALCOHOLS - Ethanol - Isopropanol	Denaturation of proteins[9,10]	Lack sporicidal action[10]
PHENOLS - Triclosan	Penetration/disruption of the cell wall and precipitation of cell proteins[9,11]	Lack sporicidal action[11]
CHLORINES - Sodium hypochlorite - Chlorine dioxide	Combination of factors: loss of intracellular contents; decreased uptake of nutrients; inhibition of DNA and protein synthesis; decreased ATP production; DNA damage[9,12]	Inactivated by organic material[12]
PEROXIDES - Hydrogen peroxide	Concentration dependent generation of hydroxyl free radicals that attack membrane lipids and DNA[9]	Deactivated by catalase producing bacteria such as: *Mycobacterium tuberculosis*, *Legionella pneumophila*, and *Campylobacter jejuni*[13]

1.1. QAC disinfectants

New and existing chemicals are regulated by the Toxic Substances Control Act (TSCA), which was issued in 1976 by the United States Environmental Protection Agency (US EPA). Any chemicals in use before this legislature were "generally recognized as safe" and were not subject to investigation. As a result, QACs were grandfathered in and considered safe.[22] Risk assessments conducted by the EPA typically utilize publicly available information or private submissions of unpublished studies. Chemicals that have been grandfathered in have little toxicity information publicly available and, therefore, are under the radar of EPA's weight-of-the-evidence approach.

QACs are registered as pesticides under FIFRA, where a number of QAC formulations are listed on the federal registry as microbiocides and algaecides (Table II). Fungicides and algaecides are used to kill or prevent fungal and algae growth, respectively. Microbiocides are used to kill or prevent infectious organisms such as viruses or bacteria. Although generally considered safe, the registration status of several dialkyl quaternary products has been cancelled due to the risk of environmental toxicity and many products containing dialkyl quaternaries are not legally eligible for sale in the United States.[23] Each QAC exhibits unique antimicrobial properties and QAC structure is optimized for product-specific functions. Modifications to alkyl chain length have been used to optimize three major properties: antimicrobial, surfactant and antistatic. Substitution of aromatic ring hydrogen with chlorine, methyl, and ethyl groups is used to increase antimicrobial efficiency and improve detergent strength.[24,25] Several generations of QACs have been created and are used in products ranging from algaecides for swimming pools, to antiseptics in candy lozenges and preservatives in eye drop solutions. QACs are organized into four different groups based on the nature of the functional group (R_1-R_4) modifications (Figure 1).



Group I: aliphatic alkyl chain substituents

Group II: non-halogenated benzyl substituents

Group III: di- and trichlorobenzyl substituents

Group IV: unusual substituents, ex: charged heterocyclic compounds

Figure 1. Basic QAC structure and organization. QACs are divided into groups based on the nature of the functional group (R_1-R_4) modifications.

Table II. List of active, federally registered QAC pesticides with CAS numerical identifiers, uses, and alternative names they may appear by. Information adapted from the *National Pesticide Information Retrieval System*.[26]

Name	CAS No.	Uses	Active product pseudonyms
Alkyl* dimethyl benzyl ammonium chloride *(50% C14; 40% C12; 10% C16)	32426-11-2; 5538-94-3; 68424-85-1	Algaecide, Microbiocide	Bardac 205 M; Barquat MB-50; Bioquat 80; BTC 835; BTC 885; Hyamine 3500; Variquat 50MC; Zephiran; C12-16-alkylbenzyldimethylammonium chloride
Alkyl* dimethyl benzyl ammonium chloride *(50% C12; 30% C14; 17% C16; 3% C18)	116958-80-6; 8001-54-5	Algaecide, Microbiocide	Ammonyx; Barquat MB-50; Barquat MB-80; Benzalkonium chloride; alkylbenzyldimethyl chlorides; Roccal II; Zilkonium chloride
Dialkyl* methyl benzyl ammonium chloride *(60% C14; 30% C16; 5% C18; 5% C12)	73049-75-9	Algaecide, Microbiocide	Di-C12-18-alkyl methyl benzyl ammonium chloride; benzyldi-C12-18-alkylmethyl chlorides
Alkyl* dimethyl benzyl ammonium chloride *(60% C14; 25% C12; 15% C16)	68424-85-1	Algaecide, Microbiocide	Barquat OJ-50; FM 65-28 Quat (80%); benzyl-C12-16-alkyldimethyl; chlorides
Alkyl* dimethyl benzyl ammonium chloride *(61% C12; 23% C14; 11% C16; 2.5% C18 2.5% C10 and trace of C8)	61789-71-7	Algaecide, Microbiocide	Benzyl (coconut oil alkyl) dimethylammonim chlorides; Benzylcoco alkyldimethylammonium chlorides; benzylcoco alkyldimethyl; chlorides
Alkyl* dimethyl benzyl ammonium chloride *(58% C14; 28% C16; 14% C12)	68424-85-1	Algaecide, Microbiocide	benzyl-C12-16-alkyldimethyl chloride

Alkyl* dimethyl ethylbenzyl ammonium chloride *(68% C12; 32% C14)	85409-23-0	Algaecide, Microbiocide	BTC 2125M; BX-673; C12-14-alkyl (ethylbenzyl) ///dimethyl ammonium chlorides; C12-14-alkyl ((ethylphenyl)methyl) dimethyl chlorides
Alkyl* dimethyl benzyl ammonium chloride *(65% C12; 25% C14; 10% C16)	68424-85-1	Algaecide, Microbiocide	Benzyl-C12-16-alkyldimethyl chlorides
Alkyl* dimethyl ethylbenzyl ammonium chloride *(60% C14; 30% C16; 5% C12; 5% C18)	68956-79-6	Algaecide, Microbiocide	C12-18-alkyl ((ethylphenyl) methyl) dimethyl chlorides
Alkyl* dimethyl benzyl ammonium saccharinate *(50% C14; 40% C12; 10% C16)	137951-75-8; 68989-01-5	Algaecide, microbiocide	Onyxide 3300; benzyl-C12-18-alkyldimethyl-; salts with 1;2-benzisothiazol-3(2H)-one 1;1-dioxide (1:1)
Oxydiethylenebis (alkyl* dimethyl ammonium chloride) *(as in fatty acids of coconut oil)	68607-28-3	Algaecide, Microbiocide	(Oxydi-2;1-ethanediyl) bis- coco alkyldimethyl dichlorides
Alkyl* dimethyl benzyl ammonium chloride *(67% C12; 25% C14; 7% C16; 1% C18)	68391-01-5	Algaecide, Microbiocide	Maquat LC12S-50%; benzyl-C12-18-alkyldimethyl; chlorides
Alkyl* dimethyl benzyl ammonium chloride * (95% C14; 2.5% C12; 2% C16)	139-08-2; 68391-01-5; 68424-85-1	Algaecide, microbiocide	Barquat MS-100; Benzenemethanaminium; N;N-dimethyl-N-tetradecyl chloride; benzyl-C12-16-alkyldimethyl chlorides
Alkyl* dimethyl benzyl ammonium chloride *(61% C12; 23% C14; 11% C16; 5% C18)	68391-01-5	Algaecide, Microbiocide	MAQUAT LC-125-80%; benzyl-C12-18-alkyldimethyl chlorides
Alkyl* dimethyl benzyl ammonium chloride *(41% C14; 28% C12; 19% C18; 12% C16)	68391-01-5	Algaecide, Microbiocide	Benzyl-C12-18-alkyldimethyl chlorides
Alkyl* dimethyl benzyl ammonium chloride *(60% C12; 30% C14; 5% C16; 5% C18)	68391-01-5	Algaecide, Microbiocide	n-alkyl (60% C12; 30% C14; 5% C16; 5% C18) dimethyl benzyl ammonium chlorides; benzyl-C12-18-alkyldimethyl chlorides

Alkyl (C8-18) dimethyl benzyl ammonium chloride; Alkyl* dimethyl benzyl ammonium chloride *(67% C12; 25% C14; 7% C16; 1% C8; C10; and C18)	63449-41-2	Algaecide, Microbiocide	Arquad B; BTC 65; benzyl-C8-18-alkyldimethyl chlorides; Roccal
Alkyl* trimethyl ammonium chloride *(as in fatty acids of coconut oil)	61789-18-2	Microbiocide	(Coco alkyl) trimethylammonium chloride; (Coconut oil alkyl) trimethylammonium chloride; Arquad C; coco alkyltrimethyl; chlorides; Sinesto-B
n-Alkyl (68% C12; 32% C14) dimethyl dimethylbenzyl ammonium chloride	Not listed	Algaecide, Microbiocide	BTC-190; C12-14-alkyl ((dimethylphenyl) methyl) dimethyl chlorides
Didecyl dimethyl ammonium bromide; Didecyl dimethyl ammonium carbonate and didecyl dimethyl ammonium bicarbonate	148788-55-0, 148812-65-1	Microbiocide	Bardac 22c50; Carboquat cm-50; Carbosan 20; Carbosan 50; Carboserve; Ord-x280; Ord-x372; Ster-bac quat; Sustain 20cq
Didecyl dimethyl ammonium chloride	7173-51-5	Microbiocide, Fungicide	Aeqrx rx-15 industrial water cooling tower algaecide; 10% btc 99 industrial water cooling tower algaecide; Bio-210; Formula 1536; Texcide 499; #800 water treatment microbiocide

The most common QAC ingredients in commercial cleaning and disinfectant solutions are alkyl dimethyl benzyl ammonium chloride (ADBAC) and didecyl dimethyl ammonium chloride (DDAC). ADBAC compounds are broadly classified as group II QACs and represent three structurally unique ADBAC derivatives: alkyl dimethyl benzyl ammonium chloride (ADBAC C12-16), alkyl dimethyl benzyl ammonium chloride (ADBAC C12-18), and benzyl dimethyl octadecyl ammonium chloride (ADBAC C18). DDAC compounds are clustered into group I QACs, and comprise a family of four compounds: didecyl dimethyl ammonium chloride (DDAC C10), dioctyl dimethyl ammonium chloride (DDAC C8), octyl decyl dimethyl ammonium chloride (DDAC C8-10), and alkyl dimethyl ethyl ammonium bromide (DDAB C12-16).

Most toxicity information for ADBAC or DDAC is derived from unpublished reports within private research companies and has not been thoroughly reassessed in academic research. These reports conclude that ADBAC and DDAC are not developmental or reproductive toxicants. Reductions in pup

weight were reported as the main effect in mice, which is indicative of a potential reproductive toxicant.[30-32] Regardless, detailed reproductive endpoints evaluating ADBAC or DDAC toxicity have not been assessed. Additionally, no studies have examined the toxicity of newer combined QAC formulations. Since chemical mixtures can act synergistically to produce greater toxic effects than the sum of the individual components, assessment of common mixtures is essential in the evaluation of chemical risk.[33-35]

1.2. QAC exposure and toxicity

Available data suggests that ADBAC compounds show slight to moderate acute toxicity; however, these toxicity standards are derived from dated studies that have been deemed unacceptable due to insufficient animal numbers, unverified dosing concentrations, and/or the exclusion of a maximum tolerated dose.[27] A rat LD_{50} of 344 mg/kg ADBAC C12-16 was observed following oral administration, while lethality from acute gastrointestinal distress in mice and rats was observed at 500 mg/kg/day.[28] Dermal application of 2848 mg/kg ADBAC C12-16 was lethal to half of the rat test population. [28] A LD_{50} of 850 mg/kg for ADBAC C12-18 was observed following acute oral exposure in rats. [28] Acute dermal application of ADBAC C12-18 in rats identified an LD_{50} of 2300 mg/kg. ADBAC C18 has demonstrated slight acute toxicity in mice and rats; oral administration of ADBAC C18 indicated a LD_{50} of 760 mg/kg in mice and 1250 mg/kg in rats. [28] Toxicity data from individual DDAC compounds are collective and provided solely as “DDAC.” Two studies evaluating acute oral toxicity of DDAC identified LD_{50}’s of 238 mg/kg and 262 mg/kg in rats.[29]

In-vitro toxicity from ADBAC and DDAC has been characterized, but only for a few tissue types. Studies have been limited to ocular, epithelial, and pulmonary cell lines. ADBAC is one of the most common preservatives in eye drop solutions. ADBAC corneal and conjunctival cytotoxicity has been observed in-vitro at concentrations typically present in ophthalmic preparations, ranging from 0.01 to 0.02%.[36-39] ADBAC-induced corneal and conjunctival cytotoxicity in-vitro has been largely attributed to

its surfactant properties and consequent ability to disrupt components of the protective tear film lipid layer.[40] Spermicidal formulations containing ADBAC (labeled as benzalkonium chloride) are available outside of the United States and have demonstrated mucosal toxicity through induction of inflammatory interleukin release in human vaginal epithelial cell lines.[41] In-vitro assessments of DDAC in mouse lung fibroblasts attributed cytotoxicity to pro-inflammatory effects leading to pulmonary fibrosis and disrupted TGF-beta signaling.[42] While these studies identify cytotoxicity from ADBAC and DDAC, they have not been comprehensively validated against in-vivo toxicity studies. This creates data gaps that prevent translation of the risk that these chemicals may pose to humans. Additionally, ADBAC and DDAC are increasingly combined into commercially available products and no in-vitro or in-vivo studies have evaluated the toxicity of combined ADBAC+DDAC.

The extensive use of ADBAC+DDAC QACs in industrial, commercial and residential settings suggests that humans may be chronically exposed. ADBAC is classified by the EPA as a toxicity category II chemical (moderately toxic) when administered orally or through inhalation. Dermal exposure to ADBAC is considered less hazardous, and is classified into toxicity category III (slightly irritating). As a result, the EPA has foregone estimating dermal irritation exposures and risks, and recommends appropriate personal protective equipment to prevent exposure by oral and inhalation routes. Occupational exposure to ADBAC through inhalation routes is very common amongst janitorial staff and healthcare workers.[43-45] Cases of occupational asthma have been reported in healthcare workers using disinfectant solutions containing ADBAC, suggesting that exposure may cause respiratory and mucosal irritation.[44] Instances of ADBAC poisoning through accidental ingestion of household cleaning products have been recorded in infants, children, and elderly adults.[46-48] In one case, ingestion of a 10% QAC cleaning solution was lethal, with corrosive damage to the mucosa of the tongue, pharynx, larynx, esophagus, and stomach.[49] Typical QAC concentration ranges and their uses are listed in (Table III).

Surveys of food production, storage, and preparation facilities indicate that QACs solutions are the most frequently utilized disinfectants. [59] Moreover, the use of QAC solutions in large-scale

agricultural and food processing operations contributes to the global prevalence of these compounds in perishable consumer products. For example, dairy farms frequently use QACs to sanitize the teats of dairy cows' before and after milking to reduce bacterial colonization, contamination, and spoilage; however, this practice leaves detectable QAC residues ranging from 1.0 to 60 μg/mL in the milk supply.[60] Dutch researchers reported that concentrations of QAC residues in commercially prepared food items often exceeded the local legislative limit of 0.5 mg/kg.[61] Regardless, these potential routes of QAC exposure have largely been disregarded, based on the degree to which QAC solutions are diluted before use. Disinfectant solutions containing 200 μg/mL or less of ADBAC active ingredient are not considered significant sources of human exposure and, therefore, do not qualify for food residue tolerance requirements in the United States.[62] As a result, the extent of QAC contamination in consumer food products, and consequently human exposure, remains underestimated.

Extensive QAC use has led to significant environmental contamination. QACs are used in the treatment of municipal sewage. Evaluation of chemical contamination in sewage runoff indicated that QACs occur more frequently than other aquatic contaminants, such as chlorinated pesticides, polychlorinated biphenyls, and polyaromatic hydrocarbons combined.[63] Furthermore, concentrations of QACs identified in wastewaters are genotoxic when applied to mammalian cells, indicated by the aberrant presence of micronuclei in blood cells.[64]

Clearly, QACs are emerging contaminants whose toxicity has not been sufficiently evaluated. The prevalence of QACs as disinfectants in occupational settings, antimicrobials in personal care products, and emerging environmental contaminants underlines the importance of thoroughly assessing their toxicity.

Table III. Documented uses of QACs in consumer products. Abbreviations: alkyl dimethyl benzyl ammonium chloride (ADBAC); dialkyl dimethyl ammonium chloride (DDAC).

QAC name	Products
0.0005% ADBAC	Eyedrop preservative[50]
0.001-0.01% ADBAC	Shampoos for seborrhea capitis and seborrheic dermatitis[50]
0.01% ADBAC	Hard contact disinfection[50]
0.02-0.08% ADBAC	Kitchen cleaner Bathroom cleaner
0.09% ADBAC	Home disinfecting wipes[51]
0.1% ADBAC	Hand sanitizers[52-54]
0.11% ADBAC	Bathroom cleaner[51]
0.1-0.2% ADBAC	Bladder and urethra irrigation and diaper rash creams[50]
1.13% ADBAC	Humidifier bacteriostatic treatment, air freshener[51]
5% ADBAC	Food contact surface sanitizer[51]
ADBAC, 50% by weight	Swimming pool disinfection[55-58]
0.1%	Bathroom cleaner[51]
<0.25% DDAC	Odor eliminator for clothing[51]
0.76% DDAC	Surface disinfectant/decontaminant[51]
1.0-5.0% DDAC	Professional-use cleaner[51]
<5.5% DDAC	Multipurpose disinfectant/deodorizer
10.14% DDAC	Professional-use disinfectant[51]
50% DDAC	Laundry sanitizer[51]

2. The mammalian reproductive system

2.1. Endocrine regulation of the reproductive axis

The reproductive system in both males and females is under primary control of the hypothalamic-pituitary-gonadal (HPG) axis. The hypothalamic component of the HPG axis, typically under central nervous system control, regulates normal pulsatile secretion of gonadotropin releasing hormone (GnRH). GnRH secretion plays a key regulatory role in downstream sex steroid production, which ultimately confers an individual's fertility.[65] Specifically, GnRH stimulates the release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary.[66-70] The frequency and extent of GnRH release differentiates between secretion of FSH or LH. Low frequency release of GnRH stimulates FSH release, while high frequency stimulates LH release.[71] FSH and LH bind to transmembrane receptors FSH-R and LH-R, respectively, which stimulates production of sex steroids in the male and female gonads. FSH and LH regulate the maturation of germ cells in both the male and female; irregular release of these hormones has severe has consequences on reproductive function. A strictly balanced biological milieu between gonadotropin-induced hormone release and sex steroid production is required for germ cell maturation. Exogenous disturbances to this balance would have severe consequences on reproduction and fertility.

2.2. Female reproduction

In females, the FSH-R is localized to granulosa cells of the ovary. Binding of FSH to these receptors stimulates maturation of ovarian follicles. FSH in turn regulates LH-R expression within granulosa cells, thecal cells, luteal cells, and interstitial cells within the ovary and, consequently, female sex steroid production. Binding of FSH to the FSH-R stimulates thecal cell conversion of cholesterol to androgens. Biosynthesis of estrone and estradiol, from the androgens
androstenedione and testosterone respectively, is catalyzed by the cytochrome p450 superfamily monooxygenase enzyme aromatase. Aromatase catalyzed hydroxylations generate estrogen, which

proceeds bi-directionally through cell membranes and targets intra-nuclear estrogen receptors (ER).[72] Most ERs are expressed constitutively in the nucleus instead of existing as unbound, inactive cytoplasmic elements. ER binding induces a conformational change, permitting the ER to serve as a DNA-binding element/transcription factor for target genes. As a result, agonism/antagonism of the ER regulates transcription and subsequent gene expression and, therefore, increases production of estrogens.[73] ERs are not restricted to gonadal tissues; ERs are present in kidney, brain, heart, lung, intestinal, and endothelial cells. As a result, exogenous compounds that bind ERs, such as synthetic estrogens and anti-androgens, can agonize or antagonize ERs and have the potential to cause severe systemic effects in addition to disrupting reproductive function.[74]

Estrogen levels surge near the end of the follicular phase and stimulate anterior pituitary release of FSH and LH. A preovulatory LH surge induces ovulation of mature ovarian follicles. Post-ovulatory cells remain in the ruptured follicle and proliferate to form the corpora lutea (CL), which stimulates secretion of the steroid hormones inhibin, estradiol, and progesterone. Estradiol inhibits further secretion of GnRH's and, consequently, LH and FSH. Progesterone is necessary for decidualization of the endometrium and proper embryonic attachment and maintenance of pregnancy.

2.3. Male reproduction

Spermatogenesis occurs within the seminiferous epithelium of the testis. The seminiferous tubules are lined with Sertoli cells whose specialized tight junctions create a barrier that protects developing sperm. Spermatogonia progress through two meiotic cycles within the Sertoli cell to produce spermatids and a single spermatogonium gives rise to four spermatids. Spermatids remain in the Sertoli cell to undergo spermiogenesis. During spermiogenesis, spermatids mature into sperm and differentiate functionally distinct structures. The acrosome is formed in the head-piece from the Golgi apparatus and contains digestive enzymes that break down the outer layer of the ovum during fertilization. Mitochondria assemble around the mid-piece to serve as an energy source that propels flagellar movement. Spermatid

DNA is re-packaged into protamines that compact sperm genetic material into the head-piece. Following spermiogenesis, sperm are disengaged from the Sertoli cell and deposited into the tubule lumen for further maturation in the epididymis. Sperm within the cauda epididymis are motile; however, sperm are not fully motile or functional until they undergo capacitation within the female oviduct.

2.3.1. Blood-testis-barrier

Germ cells within the gonads express unique antigens from somatic cells. The BTB protects germ cells from immunological attack by the host system and ensures recurrent progression of spermatogenesis. Within the seminiferous epithelium, cell–cell interactions between the Sertoli cells and germ cells support the process of spermatogenesis; however, specific regulation of spermatogenesis is not well understood.[75] Tight junctions between neighboring Sertoli cells form an integral part of the BTB that protects the environment of developing germ cells. Luminal fluids isolated from the rete testis and seminiferous tubules have distinct compositions of hydrophilic compounds, suggesting that a distinct milieu is required for male germ cells.[76] The BTB restricts the movement of substances to the lumen of the seminiferous tubules and is, therefore, responsible for controlling the composition of the tubular luminal fluid.

The outer membrane leaflets of Sertoli cells (spermatogenic supporting cells) express FSH-R, while the LH-R is expressed on steroidogenic Leydig cells within the interstitium of the testis. FSH-R activation stimulates Sertoli cells to release androgen binding protein (ABP). The primary function of ABP within the testis is to control the bioavailability of sex steroids, such as testosterone, through protein binding and stabilization. ABPs concentrate testosterone within the Sertoli cells to levels 20–50 times higher than that in circulating blood and this gradient is maintained by the presence of the blood-testis-barrier (BTB). Disruption of this testosterone gradient through physical disruption of the BTB or endocrine dysregulation prohibits normal progression of spermatogenesis.[78]

2.3.2. Spermatogenesis

Clearly, successful progression of spermatogenesis is dependent on an intact BTB.[79] Junctional complexes of the BTB apportion the seminiferous tubules into basal and adluminal compartments. The basal compartment of the BTB contains spermatogonia and early meiotic preleptotene spermatocytes; it is also where spermatogonial proliferation and renewal occurs. The BTB is restructured during spermatogenesis when preleptotene spermatocytes migrate from the basal into the adluminal compartment; once in the adluminal compartment, germ cells proceed through meiosis and spermiogenesis. In addition to delineating these two compartments, the barrier helps to "lift" pre-meiotic spermatogonia from the basal to the adluminal compartment.[80] Pre-meiotic spermatogonia (2n, 46 single chromosomes) consist of two variants: "dark type" and "pale type," but only pale type spermatogonia traverse to the adluminal compartment to participate in spermatogenesis. Dark type spermatogonia are resting or reserve populations that mitotically divide to maintain the dark type population, while pale type spermatogonia are an independent subpopulation of stem cells that proliferate mitotically and differentiate into B-type spermatogonia. The relationship between type pale and dark type spermatogonia is not well understood and little is known about the regulatory processes that dictate progression of dark spermatogonia into pale type.[81] The differentiation of pale type spermatogonia into B spermatogonia is required for the progression of germ cell development. Pre-meiotic B spermatogonia proceed through interphase in preparation for the first of meiotic division. During interphase, B spermatogonia transition from having 46 single chromosomes to having 46 sister chromatids and become primary spermatocytes. Primary spermatocytes proceed through prophase of the first meiotic division.

After chromosome duplication in interphase, the primary spermatocyte enters into the first meiotic prophase. Prophase I consists of five distinct phase: leptotene, zygotene, pachytene, diplotene, and diakenesis. After chromosomal condensation in leptotene, zygotene chromosomes pair along their length and form synapses to facilitate genetic recombination. During pachytene, non-sister chromatids of homologous chromosomes form chiasmata to facilitate the exchange of genetic information. Homologous

chromosomes separate and decondense during diplotene, but are still connected by chiasmata. The cell progresses into metaphase I, where homologous chromosomes align along the spindle equator. In the ensuing anaphase I stage, homologous chromosomes separate and migrate toward opposite poles and the spindle apparatus degenerates. Sister chromatids, however, do not separate and remain attached throughout telophase I. Chromosome number is halved at the end of first meiotic division, but each chromosome consists of a pair of chromatids.

The first meiotic division is the longest process of spermatogenesis and produces haploid secondary spermatocytes possessing 23 sister chromatids. Secondary spermatocytes proceed through a second meiotic division, yielding haploid spermatids containing 23 single chromosomes which in turn develop into spermatozoa, the mature male germ cells.[82,83]

Dysregulation at the HPG level can alter intra-tubular concentrations of sex steroids, thereby disrupting spermatogenic progression and, reducing male fertility.[84] Testosterone stimulates release of inhibin to support spermatogenic progression. Production of inhibin results in activation of a negative feedback mechanism that decreases FSH biosynthesis. Excessive FSH, or the lack of a normally responsive negative feedback mechanism, typically results in spermatogenic impairment and/or pituitary tumors.[85,86] Environmental toxicants have the ability to disrupt endocrine homeostasis from several different levels along the neuroendocrine hypothalamic-pituitary-gonadal (HPG) axis. Endocrine disrupting compounds (EDCs) are a class of hormonally active compounds that interact with endogenous receptors to disrupt normal hormonal concentrations, and as a result, impair reproductive function. For example, estrogenic compounds competitively bind endogenous estrogen receptors causing estrogen surges and disruption of oocyte maturation and spermatogenesis.[87-90]

2.3.3. Environmental influences on male fertility

Difficulty conceiving can be attributed to male factor infertility in 50% of cases.[91] Over the last 70 years, average sperm numbers have decreased significantly from 113 million to 49.9 million sperm per mL.[92,93] According to World Health Organization (WHO) guidelines, assessment of sperm count is fundamental in detecting male fertility.[94] Multiple factors can cause infertility in males. Decreased sperm counts are the predominant cause of infertility in men; however, sperm immotility is also often related to infertility and is frequently correlated with low sperm concentrations.[95,96] Sperm count and quality decreases with increasing degrees of industrialization. Air pollution contributes significantly to sperm DNA damage indicating that environmental exposures are strongly implicated in causing male reproductive dysfunction.[97] In order to preserve male fertility, more research needs to be directed at identifying the mechanisms by which environmental hazards cause male reproductive dysfunction. A particularly revealing area of study is the Sertoli cell, the primary spermatogenic support cell within the testis. Normal spermatogenesis and reproductive function are contingent on the function of Sertoli cells.

Sertoli cells, have been identified as major targets of environmental toxicants.[79,98-100] For this reason, it is essential to assess Sertoli cell viability and function when evaluating testicular toxicants. Significant declines in male reproductive function have been attributed to environmental contaminants such as exposure to cadmium, mercury, bisphenol-A, and dioxin. These toxic effects are often mediated by disruption of inter-Sertoli tight-junctions, thereby disrupting the BTB and perturbing the growth environment of male germ cells.[101] Disruption of the BTB has been shown to result in damage to germ-cell adhesion, leading to germ-cell loss, reduced sperm count, and male infertility.[98]

While the testis and Sertoli cells are often considered the primary target of male reproductive toxicants, a number of chemicals, such as the common environmental contaminant nonylphenol, act on the epididymis.[102] Common sequelae observed in animals treated with epididymal toxicants include alterations in testosterone biosynthesis, abnormal epididymal fluid reabsorption, decreased motility, and significant reductions in the fertility of cauda epididymal sperm.[103-105] Epididymal toxicants can alter

epididymal sperm number without modifying testicular sperm number. The Leydig-specific toxicant ethane dimethane sulfonate, the anti-androgen hydroxyflutamide, and the organochlorine epichlorohydrin accelerate epididymal sperm transit.[106,107] Spermatozoa enter the caput of the epididymis and require approximately 5.5-14.6 days to reach the cauda epididymis where they are stored for ejaculation.[108] Spermatozoa entering the caput epididymis are immature, lack motility, and are infertile. During their transit to the cauda, spermatozoa mature and become motile. A shorter epididymal transit would, therefore, significantly decrease the number of mature and functional spermatozoa.[109]

3. Endocrine-disrupting compounds

Agonism or antagonism of endogenous hormone receptors by EDCs results in modulation of hormonally active compounds, leading to abnormal production, transport, metabolism, or secretion of endogenous hormones.[110] Hormonal contraceptives, typically a combination of estrogen and progesterone, deliberately disrupt the endocrine axis to inhibit follicular maturation and ovulation. Hormonal contraceptives suppress production of FSH and LH through negative feedback inhibition to prevent ovulation. The efficacy of hormonal contraceptives is contingent on the fact that estrogen-mediated processes are sensitive to perturbation from exposure to exogenous hormones or hormonally active EDCs. Estrus cycle irregularities and decreased ovulatory capacity have been observed in rodents administered common environmental EDCs such as bisphenol A, nonylphenol, genistein, and zearalenone.[111] Documented exposure to EDs through environmental contamination has increased concern over the effects that these compounds may have on human reproductive function.

According to a 2012 WHO report, EDCs pose a sizeable threat to human fertility.[112] Endocrine disturbances can be mediated through direct damage to the structure of cells, disruption of biochemical processes required for normal cell function, and biotransformation to toxic metabolites. Several prevalent compounds, such as bisphenol A and triclosan, in consumer products have recently been identified as EDCs despite having been used for decades.[113]

The pesticide triclosan was first introduced in 1972 as a hospital sanitizing agent, and then became a common ingredient in a number of consumer products such as toothpaste, soaps, and cosmetics. Acute oral administration studies of triclosan suggest it has very low toxicity, with a LD_{50} of 4,350 mg/kg in mice and 3,700 and >5,000 mg/kg in rats. The EPA classifies compounds with an oral LD_{50} between 500 to 5000 mg/kg into toxicity category III, or slightly toxic. Triclosan has recently been reported, however, to have endocrine disrupting effects at significantly lower concentrations. Serum concentrations of thyroxine and estradiol were decreased in rats provided 18.75 mg/kg and 37.5 mg/kg, respectively, of triclosan.[114] There are clearly a number of commercial products that have not been comprehensively evaluated for toxicity and the consequences of their frequent use remain unknown.

Over 800 compounds ranging from pesticides, pharmaceuticals, and personal care products have been identified as EDCs (examples summarized in Table IV). Despite the prevalence of EDCs, the endocrine disrupting effects of most of these compounds have not been investigated in controlled laboratory experiments. Reproduction is dependent on normal endocrine signaling; endocrine disturbances have deleterious effects on ovarian and testis function and, as a result, reproductive outcomes.[115-118] It is crucial, therefore, to evaluate the reproductive effects of EDCs, and their potential relationship to female and male infertility in humans.

Table IV. Classes and examples of endocrine disrupting compounds. Adapted from the 2012 WHO report, *State of the Science of Endocrine Disrupting Chemicals*.[112]

Class of compound	Specific examples of EDCs
PERSISTENT & BIOACCUMULATIVE HALOGENATED CHEMICALS	
Persistent organic pollutants (POPs)	PCDDs/PCDFs, PCBs, HCB, PFOS, PBDEs, PBBs, Chlordane, Mirex, Toxaphene, DDT/DDE, Lindane, Endosulfan
Other persistent and bio-accumulative chemicals	HBCDD, SCCP, PFCAs (ex: PFOA), Octachlorostyrene, PCB methyl sulfones
LESS BIOACCUMULATIVE CHEMICALS	
Plasticizers and other additives in materials and goods	Phthalate Esters (Ex: DEHP), Triphenyl Phosphate, Bis(2-Ethylhexyl) Adipate, N-Butylbenzene, Triclocarban
Polycyclic aromatic chemicals (PACs)	Benzo(*a*)pyrene, Benzo(*a*)anthracene, Pyrene, Anthracene
Halogenated phenolic chemicals (HPCs)	2,4-Dichlorophenol, Pentachlorophenol, Hydroxy-PCBs, Hydroxy PBDEs, Tetrabromobisphenol A, 2,4,6-Tribromophenol, Triclosan
Non-halogenated phenolic chemicals (Non-HPCs)	Bisphenol A, Bisphenol F, Bisphenol S, Nonylphenol, Octylphenol, Resorcinol
PESTICIDES, PHARMACEUTICALS, & PERSONAL CARE PRODUCT INGREDIENTS	
Current-use pesticides	2,4-D, Atrazine, Carbaryl, Malathion, Mancozeb, Vinclozolin, Procloraz, Procymidone, Chloropyrifos, Linuron
Pharmaceuticals, growth promoters, and personal care product ingredients	Endocrine active (ex: Diethylstilbestrol, Ethinylestradiol, Tamoxifen, Levonorgestrel), Selective serotonin reuptake inhibitors (SSRIs; ex: Fluoxetine), Flutamide, 4-Methylbenzyldene camphor, Octyl-methoxycinnamate, Parabens, Cyclic methyl siloxanes (D4, D5, D6), Galaxolide, 3-Benzylidene camphor
OTHER CHEMICALS	
Metals and organometallic chemicals	Arsenic, Cadmium, Lead, Mercury, Methylmercury, Tributylin, Tripherrytin
Natural hormones	17β-Estradiol, Estrone, Testosterone
Phytoestrogens	Isoflavones (ex: Genistein), Coumestans (ex: Coumestrol), Mycotoxins (ex: Zearalenone), Prenylflavonoids (ex: 8-Prenylnaringenin)

4. Epigenetics

Epigenetic modifications induce changes in gene expression without modifying the underlying DNA sequence. DNA modifications and chromatin remodeling enzymes regulate DNA replication, repair, and transcription. Epigenetic modifications enable cells with the same underlying genotype to manifest different phenotypic lineages that support cell-specific functions. The addition or removal of specific chemical groups to DNA or chromatin induces structural changes the 3-dimensional structure of chromatin, thus regulating access to the underlying genetic sequence. There are two main types of epigenetic regulation: DNA methylation and histone modification.

DNA methylation is an epigenetic modification that predominantly occurs on cytosines of CpG sequences.[119] DNA methyltransferases (DNMTs) transfer methyl groups from S-adenosyl methionine (SAM) to the fifth carbon of the cytosine ring to form 5-methyl cytosine. Inactive genes are typically hypermethylated whereas transcriptionally active genes are hypomethylated. DNA methylation is of particular importance during mammalian development, since it directs totipotent cells to a specific anatomical and physiological fate. The embryonic epigenome is demethylated after fertilization to produce totipotent cell precursors and a new (*de novo*) methylation pattern is established after embryo implantation to direct cell fate during gastrulation and establishment of the embryonic germ tissues.[120]

Currently, three DNMTs have been identified in mammals: DNMT1, DNMT3A, and DNMT3B (Table 1). DNMTs are further subdivided into *de novo* DNMTs or maintenance DNMTs. DNMT1 is required for maintenance of already established cell methylation patterns. DNMT3A and B establish new methylation configurations; however, these enzymes work synergistically to establish de novo methylation patterns during embryogenesis.[121] DNMT1 does not participate in prenatal methylation reprogramming of the germline, but is critical for the maintenance of methylation patterns in somatic cells and during replication of DNA.[122] DNMT3, however, is significantly expressed in the prenatal male germ line and establishes de-novo methylation patterns critical for DNA replication in spermatogonia and preleptotene spermatocytes.[123] DNMTs are also considered the key regulators of the epigenetic landscape

within the prenatal and postnatal male testis.[124] The physiological consequences of aberrant DNA methylation are well documented in a number of disorders such as cancer, diabetes, and schizophrenia, indicating the importance of these patterns in disease etiology.[125-129]

In addition to DNA methylation, transcriptional access to DNA is modified through post-translational covalent modifications to histone core and linker proteins. Genetic material is packaged into nucleosome subunits comprised of DNA coiled around an octamer of four core histones (H2A, H2B, H3, and H4). This configuration leaves histone tails exposed and accessible to several post-translational covalent modifications, including methylation, acetylation, phosphorylation, ubiquitination, and sumoylation. The addition of functional groups catalyzes biochemically-induced conformational changes to chromatin structure and, consequently, alters genomic accessibility to transcription factors.

Methylation patterns that regulate histone structure and compaction are established by histone methyltransferases (HMTs). The majority of HMTs possess a conserved protein domain (SET domain) that can detect specific methylation patterns. These SET domain motifs then catalyze the transfer of methyl groups from *S*-adenosyl-L-methionine to lysine and arginine residues present on histone tails. Histone methylation typically induces chromatin compaction and transcriptional silencing; however, specific exceptions have been identified. Specifically, tri- and di-methylation of histone amino acids (lysine 9 on histone H3) is associated with silenced chromatin, and tri- and di-methylation of histone amino acids (lysine 4 on histone H3) is associated with increased transcriptional activity.[130]

Demethylases oppose the activity of DNMTs and HMTs by catalyzing the removal of methyl groups from DNA and histones. Histone demethylases (HDMs) are delineated according to the histone subunits (H1, H2A, H2B, H3, H4) and amino acids on which they act.[131] For example, an HDM that acts on the fourth lysine residue position from the N-terminus of the histone tail of H3 would be abbreviated: H3K4.

Histone acetyltransferases (HATs) transfer acetyl groups derived from acetyl-coA to exposed lysine resides on histone tails. In contrast to methylation, acetylation of histones typically provokes transcriptional upregulation through chromatin decondensation.[132] HATs are subdivided into classes based on their cellular localization. Type A HATs are localized to the nucleus whereas type B HATs reside in the cytoplasm. Type A HATs preferentially bind to poly-acetylated lysine residues since they contain a bromodomain that enables identification of mono-acetylated lysine residues on the N-terminal tails of histones. In contrast, cytoplasmic type B HATs lack a bromodomain and acetylate new (un-acetylated) core histones before they are packed into nucleosome subunits.[133] Histone deacetylases (HDACs) counter the transcriptional activity of HATs by inducing chromatin compaction through removal of acetyl groups from histone lysine residues. Since HDACs are ubiquitous across tissue types and localize to the nucleus and cytoplasm, they are divided into four classes based on genetic sequence homology and conservation: I, II, III, and IV.[134]

HATs are crucial to male reproductive function and histone hyperacetylation is a hallmark of spermatogenesis. Hyperacetylation of core histones is localized to post-meiotic spermatocytes undergoing spermiogenesis, indicating a critical function of HATs in spermatogenesis. During mammalian spermatogenesis, the acetyltransferase Hat1 co-localizes with a germ-cell-specific RNA-binding protein (MVH; DEAD-box type RNA-binding protein). Hat1acetylation of this protein blocks the RNA-binding ability of MVH and is required for spermatid formation, albeit through an unknown mechanism.[135] As a result, variations in histone acetylation may have significant impacts on spermatogenesis. A summary of genes encoding known or predicted chromatin remodeling enzymes and their function are listed in Table V.

Table V. List of chromatin remodeling enzyme classes, function, and gene groupings.

Chromatin Remodeling Enzymes	Description of function	Genes
DNA Methyltransferases DNMTs	- Methylate DNA to regulate gene expression - Regulate X chromosome inactivation, genomic imprinting, and chromatin modifications[136]	Dnmt1, Dnmt3a, Dnmt3b
Histone Acetyltransferases HATs	- Regulate transcription through acetylation of histone proteins - Create binding sites for chromatin remodeling complexes[137]	Atf2, Cdyl, Ciita, Csrp2bp, Esco1, Esco2, Hat1, Kat2a, Kat2b, Kat5, Myst1, Myst2, Myst3, Myst4, Ncoa1, Ncoa3, Ncoa6
Histone Methyltransferases HMTs	- Regulate transcription through methylation of histone proteins - Involved in cell division, gene expression, cell lineage development, and genomic imprinting[136,138]	Carm1 (Prmt4), Dot1l, Ehmt1, Ehmt2, Mll3, Prmt1, Prmt2, Prmt3, Prmt5, Prmt6, Prmt7, Prmt8, Setdb2, Smyd1, Smyd3, Suv39h1
SET Doman Proteins	- Characterized by the presence of a SET domain - Methylate lysine residues to regulate chromatin structure and gene transcription[139]	Ash1l, Kmt2e, Nsd1, Setd1a, Setd1b, Setd2, Setd3, Setd4, Setd5, Setd6, Setd7, Setd8, Setdb1, Suv420h1, Whsc1
Histone Phosphorylation	- Phosphorylate serine, threonine, and tyrosine residues - Serve several cell functions such as cell cycle regulation and damage responses[140,141]	Aurka, Aurkb, Aurkc, Nek6, Pak1, Rps6ka3, Rps6ka5
Histone Ubiquitination	- Catalyze binding of ubiquitin glycine residues to lysine residue on histone tails - Regulate diverse functions such as protein degradation by proteasomes, protein sorting, and endocytosis.[142]	Dzip3, Mysm1, Rnf2, Rnf20, Ube2a, Ube2b, Usp16, Usp21, Usp22
DNA/Histone Demethylases	- Remove methyl groups from DNA and histones to alter chromatin accessibility[131]	Kdm1a, Kdm5b, Kdm5c, Kdm4a, Kdm4c, Kdm6b
Histone Deacetylases HDACs	- Remove acetyl groups from DNA and histones to alter chromatin accessibility[131]	Hdac1, Hdac2, Hdac3, Hdac4, Hdac5, Hdac6, Hdac7, Hdac8, Hdac9, Hdac10, Hdac11

4.1. Epigenetics, the environment, and reproduction

Diet, exercise, stress, and toxicant exposure can induce lasting epigenetic modifications that affect reproductive function.[143] Germ cells forming F_1 progeny are vulnerable to deleterious effects from parental toxicant exposures via direct exposure of the germ line or through epigenetic modifications of DNA or proteins during F_1 gonadal development. Epigenetic changes can be transmissible through gametes and, therefore, between several generations. As a result, the heritability of epigenetic modifications does not follow a normal Mendelian inheritance pattern. For example, the agricultural herbicide vinclozolin is a known anti-androgenic ED. Transgenerational inheritance of vinclozolin was assessed in multiple generations following parental exposure by evaluation of differentially methylated domains of two paternally (H19 and Gtl2) and three maternally (Peg1, Snrpn, and Peg3) imprinted genes. Interestingly, transgenerational effects were observed, but dissipated with each subsequent generation. Male offspring of dams that were administered vinclozolin during critical periods of urogenital formation exhibited significantly decreased sperm concentrations; however, these values gradually normalized to that of controls in succeeding generations. Vinclozolin altered methylation status of paternally-derived H19 andGtl2 and maternally-derived Peg1, Snrpn, and Peg3 in the sperm of the offspring. Stouder et al. concluded that the effects of vinclozolin on male spermatogenesis might be mediated by defective re-methylation of the germline.[144] In another study, decreased spermatogenesis and infertility were observed in adult mice exposed in-utero to the estrogenic insecticide methoxychlor during the period of gonadal differentiation. These reproductive deficiencies persisted into the F_2 generation and were not correlated with tissue abnormalities or altered serum testosterone concentrations.[145] The reproductive effects of methoxychlor are believed to be transmitted through alterations of DNA methylation that lead to re-programming of the male germ-line. The methylated genes responsible for methoxychlor's transgenerational reproductive effects, however, have yet to be identified.[146] These studies demonstrate the importance of multigenerational assessments of environmentally-induced epigenetic modifications in understanding the transmissibility of reproductive dysfunction.

5. Summary

Toxicants can act directly or indirectly to disrupt reproductive homeostasis. Direct-acting toxicants typically possess structural similarity to endogenous molecules, while indirect-acting toxicants induce alterations in metabolism. Regardless, reproductive toxicity is not just the sum of all affected parts, since direct-acting toxicants can also have a number of indirect consequences. Additionally, in-utero exposure to toxicants can have effects that persist for generations. These transgenerational reproductive effects represent a novel mechanism of genetic inheritance and reveal an unexplored territory of chemical toxicity assessments. The magnitude of effects that a toxicant can have at the cellular and molecular level challenges the thoroughness of traditional toxicity paradigms. Safety evaluations need to move beyond acute toxicity studies, as these are no longer sufficient to understand the subtle, yet permanent effects of reproductive toxicants. Exposure to a reproductive toxicant during a critical period, such as formation of the urogenital system, can have profound effects on reproduction that persist for several generations. Additional research needs to focus on evaluating toxicity data of ubiquitous chemical compounds, like QACs. A number of commercial products contain QACs, suggesting that humans are being chronically exposed. Determining the mechanism by which QACs cause reproductive toxicity will aid in assessing the risk that these compounds pose to humans. Additionally, investigating transgenerational germline alterations from in-utero exposure to QACs will determine the possible toxic effects in future generations. Finally, demonstrating reproductive toxicity of QACs in commercial products would highlight the need for more extensive testing of common, readily available compounds.

REFERENCES

[1] Gessert, R. A. Developing data to satisfy drug and pesticide approval requirements of FDA, EPA, and USDA; are cattle, sheep, and poultry minor species? Proceedings, annual meeting of the United States animal health association, 262-265 (1975).

[2] Culleen, L. E. Pesticide registration in the United States: overview and new directions. Quality assurance 3, 291-299 (1994).

[3] Sander, J. E. & Wilson, J. L. Effect of hydrogen peroxide disinfection during incubation of chicken eggs on microbial levels and productivity. Avian diseases 43, 227-233 (1999).

[4] Kingsley, D. H., Vincent, E. M., Meade, G. K., Watson, C. L. & Fan, X. Inactivation of human norovirus using chemical sanitizers. International journal of food microbiology 171, 94-99, doi:10.1016/j.ijfoodmicro.2013.11.018 (2014).

[5] McDonnell, G. & Russell, A. D. Antiseptics and disinfectants: activity, action, and resistance. Clinical microbiology reviews 12, 147-179 (1999).

[6] Ioannou, C. J., Hanlon, G. W. & Denyer, S. P. Action of disinfectant quaternary ammonium compounds against Staphylococcus aureus. Antimicrobial agents and chemotherapy 51, 296-306, doi:10.1128/AAC.00375-06 (2007).

[7] Wessels, S. & Ingmer, H. Modes of action of three disinfectant active substances: a review. Regulatory toxicology and pharmacology 67, 456-467, doi:10.1016/j.yrtph.2013.09.006 (2013).

[8] Christensen, R. P. Antimicrobial activity of environmental surface disinfectants in the absence and presence of bioburden. Journal of the American dental association 119, 493-505 (1989).

[9] Russell AD. Principles of antimicrobial activity and resistance. In: Block SS, ed. Disinfection, sterilization, and preservation. Philadelphia: Lippincott Williams & Wilkins, 2001:31-55.

[10] Ali Y, Dolan MJ, Fendler EJ, Larson EL. Alcohols. In: Block SS, ed. Disinfection, sterilization, and preservation. Philadelphia: Lippincott Williams & Wilkins, 2001:229-54.

[11] Dagley, S., Dawes, E. A. & Morrison, G. A. Inhibition of growth of Aerobacter aerogenes; the mode of action of phenols, alcohols, acetone, and ethyl acetate. Journal of bacteriology 60, 369-379 (1950).

[12] Dychdala GR. Chlorine and chlorine compounds. In: Block SS, ed. Disinfection, sterilization, and preservation. Philadelphia: Lippincott Williams & Wilkins, 2001:135-157.

[13] Srinivasa Rao, P. S., Yamada, Y. & Leung, K. Y. A major catalase (KatB) that is required for resistance to H_2O_2 and phagocyte-mediated killing in Edwardsiella tarda. Microbiology 149, 2635-2644 (2003).

[14] Calabro, M. The Clorox company: a century of integrity and innovation. (Clorox Co., 2013).

[15] Blanc, P. D., Galbo, M., Balmes, J. R. & Olson, K. R. Occupational factors in work-related inhalations: inferences for prevention strategy. American journal of industrial medicine 25, 783-791 (1994).

[16] Pascuzzi, T. A. & Storrow, A. B. Mass casualties from acute inhalation of chloramine gas. Military medicine 163, 102-104 (1998).

[17] Taub Ludwig, H. H., Leuchs Friedrich. Quaternary Ammonium Compounds. (1937).

[18] Mcfarlane, S.B. "Dodecyl-Dimethyl-1, 2, 4, 5-Tetramethyl-Benzyl Ammonium Chloride." Google Patents, 1966.

[19] Pavlikova-Moricka, M., Lacko, I., Devinsky, F., Masarova, L. & Milynarcik, D. Quantitative relationships between structure and antimicrobial activity of new "soft" bisquaternary ammonium salts. Folia microbiologica 39, 176-180 (1994).

[20] Gilbert, P. & McBain, A. J. Potential impact of increased use of biocides in consumer products on prevalence of antibiotic resistance. Clinical microbiology reviews 16, 189-208 (2003).

[21] Ahlstrom, B., Thompson, R. A. & Edebo, L. The effect of hydrocarbon chain length, pH, and temperature on the binding and bactericidal effect of amphiphilic betaine esters on Salmonella Typhimurium. APMIS : acta pathologica, microbiologica, et immunologica Scandinavica 107, 318-324 (1999).

[22] Environmental Protection Agency. Toxic Substances Control Act, < http://www2.epa.gov/laws-regulations/summary-toxic-substances-control-act > (2014).

[23] Purdue University. National Pesticide Information Retrieval System, <http://ppis.ceris.purdue.edu/> (1998-2013).

[24] Carson, R. T., Larson, E., Levy, S. B., Marshall, B. M. & Aiello, A. E. Use of antibacterial consumer products containing quaternary ammonium compounds and drug resistance in the community. The journal of antimicrobial chemotherapy 62, 1160-1162, doi:10.1093/jac/dkn332 (2008).

[25] Hegstad, K., Langsrud S., Lunestad B.T., Scheie A.A., Sunde M., & Yazdankhah S.P. Does the wide use of quaternary ammonium compounds enhance the selection and spread of antimicrobial resistance and thus threaten our health? Microbial drug resistance 16, 91-104, doi:10.1089/mdr.2009.0120 (2010).

[26] Environmental Protection Agency. Toxic Substances Control Act: Section 8b (1976).

[27] EPA, U. S. Vol. Chemical Code # 001846, Tolerance #50238 (Department of pesticide regulation, medical toxicology branch, < http://www.cdpr.ca.gov/docs/risk/toxsums/pdfs/1846.pdf > (1987).

[28] EPA, U. S. Vol. EPA739-R-06-009. Pesticides and toxic substances prevention, United States environmental protection agency http://www.epa.gov/pesticides/reregistration/REDs/adbac_red.pdf (2006).

[29] EPA, U. S. Vol. EPA739-R-06-008. Pesticides and toxic substances prevention, United States environmental protection agency, <http://www.epa.gov/pesticides/reregistration/REDs/ddac_red.pdf> (2006).

[30] Gill, M. W., S. J. Hermansky and C. L. Wagner. Chronic Dietary Oncogenicity Study with Alkyl Dimethyl Benzyl Ammonium Chloride (ADBAC) in Mice (Bushy Run Research Center, Export, PA (1991).

[31] Weaver, E. W. Two-Week Dose Range Finding Screen with Alkyl Dimethyl Benzyl Ammonium Chloride (ADBAC) in Mice. (Bushy Run Research Center, Export, PA (1988).

[32] Van Miller, J. P. Ninety-Day Dose Range-Finding Study with Alkyl Dimethyl Benzyl Ammonium Chloride (ADBAC) in Mice. (Bushy Run Research Center, Export, PA (1988).

[33] Hermens, J., Leeuwangh, P. & Musch, A. Joint toxicity of mixtures of groups of organic aquatic pollutants to the guppy (Poecilia reticulata). Ecotoxicology and environmental safety 9, 321-326 (1985).

[34] Faust, M., Altenburger, R., Backhaus, T., Blanck, H., Boedeker, W., Gramatica, P., Hamer, V., Scholze, M., Vighi, M. and Grimme, L. H. Joint algal toxicity of 16 dissimilarly acting chemicals is predictable by the concept of independent action. Aquatic toxicology 63, 43-63 (2003).

[35] Narotsky, M. G., Best, D. S., McDonald, A., Godin, E. A., Hunter, E. S., 3rd and Simmons, J. E. Pregnancy loss and eye malformations in offspring of F344 rats following gestational exposure to mixtures of regulated trihalomethanes and haloacetic acids. Reproductive toxicology 31, 59-65, doi:10.1016/j.reprotox.2010.08.002 (2011).

[36] Guzman-Aranguez, A., Calvo, P., Ropero, I. & Pintor, J. In vitro effects of preserved and unpreserved anti-allergic drugs on human corneal epithelial cells. Journal of ocular pharmacology and therapeutics : Official journal of the association for ocular pharmacology and therapeutics 30, 790-798, doi:10.1089/jop.2014.0030 (2014).

[37] Kim, E. J., Kim, Y. H., Kang, S. H., Lee, K. W. & Park, Y. J. In vitro effects of preservative-free and preserved prostaglandin analogs on primary cultured human conjunctival fibroblast cells. Korean journal of ophthalmology 27, 446-453, doi:10.3341/kjo.2013.27.6.446 (2013).

[38] Liang, H., Baudouin, C., Daull, P., Garrigue, J. S., Buggage, R. and Brignole-Baudouin, F. In vitro and in vivo evaluation of a preservative-free cationic emulsion of latanoprost in corneal wound healing models. Cornea 31, 1319-1329, doi:10.1097/ICO.0b013e318255a7f8 (2012).

[39] Rosin, L. M. & Bell, N. P. Preservative toxicity in glaucoma medication: clinical evaluation of benzalkonium chloride-free 0.5% timolol eye drops. Clinical ophthalmology 7, 2131-2135, doi:10.2147/OPTH.S41358 (2013).

[40] Kaercher, T., Honig, D. & Barth, W. How the most common preservative affects the Meibomian lipid layer. Orbit 18, 89-97 (1999).

[41] Fichorova, R. N., Bajpai, N., Chandra, N., Hisu, J.G., Spangler, M., Ratnam, V., and Doncel, G.F. Interleukin (IL)-1, IL-6, and IL-8 predict mucosal toxicity of vaginal microbicidal contraceptives. Biology of reproduction 71, 761-769, doi:10.1095/biolreprod.104.029603 (2004).

[42] Ohnuma-Koyama, A., Yoshida, T., Tajima-Horiuchi, H., Takahashi, N., Yamaguchi, S., Ohtsuka, R., Takeuchi-Kashimoto, Y., Kuwahara, M., Takeda, M., Nakashima, N. and Harada, T. Didecyldimethylammonium chloride induces pulmonary fibrosis in association with TGF-beta signaling in mice. Experimental and toxicologic pathology: official journal of the Gesellschaft fur Toxikologische Pathologie 65, 1003-1009, doi:10.1016/j.etp.2013.02.003 (2013).

[43] Nettis, E., Colanardi, M. C., Soccio, A. L., Ferrannini, A. & Tursi, A. Occupational irritant and allergic contact dermatitis among healthcare workers. Contact dermatitis 46, 101-107 (2002).

[44] Purohit, A., Kopferschmitt-Kubler, M. C., Moreau, C., Popin, E., Blaumeiser, M. and Pauli, G. Quaternary ammonium compounds and occupational asthma. International archives of occupational and environmental health 73, 423-427 (2000).

[45] Burge, P. S. & Richardson, M. N. Occupational asthma due to indirect exposure to lauryl dimethyl benzyl ammonium chloride used in a floor cleaner. Thorax 49, 842-843 (1994).

[46] Akutsu, I., Motojima, S., Ogata, H., Fukuda, T., Ikemori, R. and Makino, S. [A case of acute benzalkonium chloride intoxication]. Nihon Naika Gakkai zasshi. The Journal of the Japanese Society of Internal Medicine 78, 1613-1614 (1989).

[47] Wilson, J. T. & Burr, I. M. Benzalkonium chloride poisoning in infant twins. American journal of diseases of children 129, 1208-1209 (1975).

[48] van Berkel, M. & de Wolff, F. A. Survival after acute benzalkonium chloride poisoning. Human toxicology 7, 191-193 (1988).

[49] Hitosugi, M., Maruyama, K. & Takatsu, A. A case of fatal benzalkonium chloride poisoning. International journal of legal medicine 111, 265-266 (1998).

[50] Adam Fraise, J.-Y. M., Syed Sattar. Russell, Hugo and Ayliffe's Principles and Practice of Disinfection, Preservation and Sterilization: Edition 5. (John Wiley & Sons, 2012).

[51] Household Products Database, <http://hpd.nlm.nih.gov/cgi bin/household/list?tbl=TblChemicals&alpha=A> (2014).

[52] Shintre, M. S., Gaonkar, T. A. & Modak, S. M. Efficacy of an alcohol-based healthcare hand rub containing synergistic combination of farnesol and benzethonium chloride. International journal of hygiene and environmental health 209, 477-487, doi:10.1016/j.ijheh.2006.04.006 (2006).

[53] Moadab, A., Rupley, K. F. & Wadhams, P. Effectiveness of a nonrinse, alcohol-free antiseptic hand wash. Journal of the American podiatric medical association 91, 288-293 (2001).

[54] Dyer, D. L., Gerenraich, K. B. & Wadhams, P. S. Testing a new alcohol-free hand sanitizer to combat infection. Association of perioperative registered nurses journal 68, 239-241, 243-234, 247-251 (1998).

[55] Rowhani, T. & Lagalante, A. F. A colorimetric assay for the determination of polyhexamethylene biguanide in pool and spa water using nickel-nioxime. Talanta 71, 964-970, doi:10.1016/j.talanta.2006.07.042 (2007).

[56] Millis, N. F., Eager, E., Hay, A. J., Kasian, P. A., Pickering, W. J. and Tan, M. A. Survey of bacteria in private swimming pools. The medical journal of Australia 1, 573-575 (1981).

[57] Lim, L., Coster, D. J. & Badenoch, P. R. Antimicrobial susceptibility of 19 Australian corneal isolates of Acanthamoeba. Clinical & experimental ophthalmology 28, 119-124 (2000).

[58] Chang, C. Y., Schell, W. A., Perfect, J. R. & Hulka, G. F. Novel use of a swimming pool biocide in the treatment of a rare fungal mastoiditis. The Laryngoscope 115, 1065-1069, doi:10.1097/01.MLG.0000163338.45700.FE (2005).

[59] Holah, J. T., Taylor, J. H., Dawson, D. J. & Hall, K. E. Biocide use in the food industry and the disinfectant resistance of persistent strains of Listeria monocytogenes and Escherichia coli. Journal of applied microbiology 92 Suppl, 111S-120S (2002).

[60] Pankey, J. W. Postmilking teat antisepsis. The Veterinary clinics of North America. Large animal practice 6, 335-348 (1984).

[61] van Bruijnsvoort, M., Rooselaar, J., Stern, A. G. & Jonker, K. M. Determination of residues of quaternary ammonium disinfectants in food products by liquid chromatography-tandem mass spectrometry. Journal of association of official agricultural chemists international 87, 1016-1020 (2004).

[62] Environmental Protection Agency. Vol. EPA-HQ-OPP-2006-0339-0032. Office of Pesticide Programs, Antimicrobials Division, U.S. Environmental Protection Agency (2006).

[63] Li, X. & Brownawell, B. J. Quaternary ammonium compounds in urban estuarine sediment environments--a class of contaminants in need of increased attention? Environmental science & technology 44, 7561-7568, doi:10.1021/es1011669 (2010).

[64] Ferk, F. et al. Benzalkonium chloride (BAC) and dimethyldioctadecyl-ammonium bromide (DDAB), two common quaternary ammonium compounds, cause genotoxic effects in mammalian and plant cells at environmentally relevant concentrations. Mutagenesis 22, 363-370, doi:10.1093/mutage/gem027 (2007).

[65] Eshkol, A., Lunenfeld, B. & Insler, V. The effect of sex steroids on pituitary responsiveness to gonadotropin releasing hormone. Journal of steroid biochemistry 6, 1061-1066 (1975).

[66] Pascoe, R. R., Pascoe, D. R. & Wilson, M. C. Influence of follicular status on twinning rate in mares. Journal of reproduction and fertility. Supplement 35, 183-189 (1987).

[67] Elmazar, M. M., Vogel, R. & Spielmann, H. Maternal factors influencing development of embryos from mice superovulated with gonadotropins. Reproductive toxicology 3, 135-138 (1989).

[68] Renne, U. & Schuler, L. [Selection of laboratory rats for fertility components in synchronized estrus using Suisynchron-Praemix. 2. Results of litter development]. Archiv fur experimentelle Veterinarmedizin 35, 123-130 (1981).

[69] Swenne, I. & Eriksson, U. Diabetes in pregnancy: islet cell proliferation in the fetal rat pancreas. Diabetologia 23, 525-528 (1982).

[70] Grazul-Bilska, A. T., Johnson, M. L., Borowicz, P. P., Minten, M., Bilski, J. J., Wroblewski, R., Velimirovich, M., Coupe, L. R., Redmer, D. A. and Reynolds, L. P. Placental development during early pregnancy in sheep: cell proliferation, global methylation, and angiogenesis in the fetal placenta. Reproduction 141, 529-540, doi:10.1530/REP-10-0505 (2011).

[71] Jayes, F. C., Britt, J. H. & Esbenshade, K. L. Role of gonadotropin-releasing hormone pulse frequency in differential regulation of gonadotropins in the gilt. Biology of reproduction 56, 1012-1019 (1997).

[72] Hunzicker-Dunn, M. & Birnbaumer, L. Adenylyl cyclase activities in ovarian tissues. II. Regulation of responsiveness to LH, FSH, and PGE1 in the rabbit. Endocrinology 99, 185-197, doi:10.1210/endo-99-1-185 (1976).

[73] McInerney, E. M., Weis, K. E., Sun, J., Mosselman, S. & Katzenellenbogen, B. S. Transcription activation by the human estrogen receptor subtype beta (ER beta) studied with ER beta and ER-alpha receptor chimeras. Endocrinology 139, 4513-4522, doi:10.1210/endo.139.11.6298 (1998).

[74] Schreurs, R. H., Sonneveld, E., van der Saag, P. T., van der Burg, B. & Seinen, W. Examination of the in vitro (anti)estrogenic, (anti)androgenic and (anti)dioxin-like activities of tetralin, indane and isochroman derivatives using receptor-specific bioassays. Toxicology letters 156, 261-275, doi:10.1016/j.toxlet.2004.11.014 (2005).

[75] Verhoeven, G. Local control systems within the testis. Bailliere's clinical endocrinology and metabolism 6:2, 313-333 (1992).

[76] Eisenberg, F., Jr. & Parthasarathy, R. Measurement of biosynthesis of myo-inositol from glucose-6-phosphate. Methods in enzymology 141, 127-143 (1987).

[77] Hess, R. A. Oestrogen in fluid transport in efferent ducts of the male reproductive tract. Reviews of reproduction 5, 84-92 (2000).

[78] Wan, H. T., Mruk, D. D., Wong, C. K. & Cheng, C. Y. Targeting testis-specific proteins to inhibit spermatogenesis: lesson from endocrine disrupting chemicals. Expert opinion on therapeutic targets 17, 839-855, doi:10.1517/14728222.2013.791679 (2013).

[79] Cheng, C. Y. & Mruk, D. D. The blood-testis barrier and its implications for male contraception. Pharmacological reviews 64, 16-64, doi:10.1124/pr.110.002790 (2012).

[80] Lui, W. Y., Mruk, D., Lee, W. M. & Cheng, C. Y. Sertoli cell tight junction dynamics: their regulation during spermatogenesis. Biology of reproduction 68, 1087-1097, doi:10.1095/biolreprod.102.010371 (2003).

[81] Nistal, M., Codesal, J., Paniagua, R. & Santamaria, L. Decrease in the number of human Ap and Ad spermatogonia and in the Ap/ Ad ratio with advancing age. New data on the spermatogonial stem cell. Journal of andrology 8, 64-68 (1987).

[82] Hannah-Alava, A. The premeiotic stages of spermatogenesis. Advances in genetics 13, 157-226 (1965).

[83] Steinberger, E., Steinberger, A. & Ficher, M. Study of spermatogenesis and steroid metabolism in cultures of mammalian testes. Recent progress in hormone research 26, 547-588 (1970).

[84] Hansson, V., Weddington, S. C., McLean, W. S., Smith, A. A., Nayfeh, S. N., French, F. S. and Ritzen, E. M. Regulation of seminiferous tubular function by FSH and androgen. Journal of reproduction and fertility 44, 363-375 (1975).

[85] Kovacs, K., Horvath, E., Van Loon, G. R., Rewcastle, N. B., Ezrin, C. and Rosenbloom, A. A. Pituitary adenomas associated with elevated blood follicle-stimulating hormone levels: a histologic, immunocytologic, and electron microscopic study of two cases. Fertility and sterility 29, 622-628 (1978).

[86] Mortimer, C. H., Besser, G. M., McNeilly, A. S., Marshall, J. C., Harsoulis, P., Tunbridge, W. M., Gomez-Pan, A. and Hall, R. Luteinizing hormone and follicle stimulating hormone-releasing hormone test in patients with hypothalamic-pituitary-gonadal dysfunction. British medical journal 4, 73-77 (1973).

[87] Feldman, D. & Krishnan, A. Estrogens in unexpected places: possible implications for researchers and consumers. Environmental health perspectives 103 Suppl 7, 129-133 (1995).

[88] Steinmetz, R., Brown, N. G., Allen, D. L., Bigsby, R. M. & Ben-Jonathan, N. The environmental estrogen bisphenol A stimulates prolactin release in vitro and in vivo. Endocrinology 138, 1780-1786 (1997).

[89] Mlynarcikova, A., Nagyova, E., Fickova, M. & Scsukova, S. Effects of selected endocrine disruptors on meiotic maturation, cumulus expansion, synthesis of hyaluronan and progesterone by porcine oocyte-cumulus complexes. Toxicology in vitro: an international journal published in association with BIBRA 23, 371-377, doi:10.1016/j.tiv.2008.12.017 (2009).

[90] Susiarjo, M., Hassold, T. J., Freeman, E. & Hunt, P. A. Bisphenol A exposure in utero disrupts early oogenesis in the mouse. PLoS genetics 3, e5, doi:10.1371/journal.pgen.0030005 (2007).

[91] McLachlan, R. I. & de Kretser, D. M. Male infertility: the case for continued research. The medical journal of Australia 174, 116-117 (2001).

[92] Giwercman, A., Carlsen, E., Keiding, N. & Skakkebaek, N. E. Evidence for increasing incidence of abnormalities of the human testis: a review. Environmental health perspectives 101 Suppl 2, 65-71 (1993).

[93] Sharpe, R. M. Sperm counts and fertility in men: a rocky road ahead. Science & Society Series on Sex and Science. European molecular biology organization 13, 398-403, doi:10.1038/embor.2012.50 (2012).

[94] Gottardo, F., Kliesch, S. & World Health, O. [Semen analysis: spermiogram according to WHO 2010 criteria]. Der urologe. 50, 101-108, doi:10.1007/s00120-010-2466-6 (2011).

[95] Jouannet, P. & Feneux, D. Sperm analysis. Annales de biologie clinique 45, 335-339 (1987).

[96] Guzick, D. S., Overstreet, J. W., Factor-Litvak, P., Brazil, C. K., Nakajima, S. T., Coutifaris, C., Carson, S. A., Cisneros, P., Steinkampf, M. P., Hill, J. A., Xu, D., Vogel, D. L. and National Cooperative Reproductive Medicine, Network. Sperm morphology, motility, and concentration in fertile and infertile men. The New England journal of medicine 345, 1388-1393, doi:10.1056/NEJMoa003005 (2001).

[97] Somers, C. M. Ambient air pollution exposure and damage to male gametes: human studies and in situ 'sentinel' animal experiments. Systems biology in reproductive medicine 57, 63-71, doi:10.3109/19396368.2010.500440 (2011).

[98] Wong, E. W. & Cheng, C. Y. Impacts of environmental toxicants on male reproductive dysfunction. Trends in pharmacological sciences 32, 290-299, doi:10.1016/j.tips.2011.01.001 (2011).

[99] Espevik, T., Lamvik, M. K., Sunde, A. & Eik-Nes, K. B. Effects of cadmium on survival and morphology of cultured rat Sertoli cells. Journal of reproduction and fertility 65, 489-495 (1982).

[100] Monsees, T. K., Franz, M., Gebhardt, S., Winterstein, U., Schill, W. B. and Hayatpour, J. Sertoli cells as a target for reproductive hazards. Andrologia 32, 239-246 (2000).

[101] Zhang, Y. H., Lin, L., Liu, Z. W., Jiang, X. Z. & Chen, B. H. Disruption effects of monophthalate exposures on inter-Sertoli tight junction in a two-compartment culture model. Environmental toxicology 23, 302-308, doi:10.1002/tox.20343 (2008).

[102] Jayachandra, S. & D'Souza, U. J. Pre- and postnatal toxicity of diazinon induces disruption of spermatogenetic cell line evidenced by increased testicular marker enzymes activities in rat offspring. Journal of environmental pathology, toxicology and oncology: official organ of the International Society for Environmental Toxicology and Cancer 32, 73-90 (2013).

[103] Toth, G. P., Stober, J. A., Zenick, H., Read, E. J., Christ, S. A. and Smith, M. K. Correlation of sperm motion parameters with fertility in rats treated subchronically with epichlorohydrin. Journal of andrology 12, 54-61 (1991).

[104] Klinefelter, G. R., Laskey, J. W., Ferrell, J., Suarez, J. D. & Roberts, N. L. Discriminant analysis indicates a single sperm protein (SP22) is predictive of fertility following exposure to epididymal toxicants. Journal of andrology 18, 139-150 (1997).

[105] de Jager, C., Bornman, M. S. & van der Horst, G. The effect of p-nonylphenol, an environmental toxicant with oestrogenic properties, on fertility potential in adult male rats. Andrologia 31, 99-106 (1999).

[106] Klinefelter, G. R. & Suarez, J. D. Toxicant-induced acceleration of epididymal sperm transit: androgen-dependent proteins may be involved. Reproductive toxicology 11, 511-519 (1997).

[107] Hess, R. A. Effects of environmental toxicants on the efferent ducts, epididymis and fertility. Journal of reproduction and fertility. Supplement 53, 247-259 (1998).

[108] Klaassen, C. D. Casarett & Doull's Toxicology: The Basic Science of Poisons. 6th Edition edn, (McGraw-Hill, 2001).

[109] Fernandez, C. D., Porto, E. M., Arena, A. C. & Kempinas Wde, G. Effects of altered epididymal sperm transit time on sperm quality. International journal of andrology 31, 427-437, doi:10.1111/j.1365-2605.2007.00788.x (2008).

[110] Gadian, T. Toxicology - Basic Science of Poisons. Chem Ind-London, 206-207 (1976).

[111] Olsen, C. M., Meussen-Elholm, E. T., Hongslo, J. K., Stenersen, J. & Tollefsen, K. E. Estrogenic effects of environmental chemicals: an interspecies comparison. Comparative biochemistry and physiology. Toxicology & pharmacology: CBP 141, 267-274, doi:10.1016/j.cca.2005.07.002 (2005).

[112] Hunt, P. A., Susiarjo, M., Rubio, C. & Hassold, T. J. The bisphenol A experience: a primer for the analysis of environmental effects on mammalian reproduction. Biology of reproduction 81, 807-813, doi:10.1095/biolreprod.109.077008 (2009).

[113] LaChapelle, A. M., Ruygrok, M. L., Toomer, M., Oost, J. J., Monnie, M. L., Swenson, J. A., Compton, A. A. and Stebbins-Boaz, B. The hormonal herbicide, 2,4-dichlorophenoxyacetic acid, inhibits Xenopus oocyte maturation by targeting translational and post-translational mechanisms. Reproductive toxicology 23, 20-31, doi:10.1016/j.reprotox.2006.08.013 (2007).

[114] Ambruosi, B., Uranio, M. F., Sardanelli, A. M., Pocar, P., Martino, N. A., Paternoster, M. S., Amati, F. and Dell'Aquila, M. E. In vitro acute exposure to DEHP affects oocyte meiotic maturation, energy and oxidative stress parameters in a large animal model. PloS one 6, e27452, doi:10.1371/journal.pone.0027452 (2011).

[115] Fujimoto, V. Y., Kim, D., vom Saal, F. S., Lamb, J. D., Taylor, J. A. and Bloom, M. S. Serum unconjugated bisphenol A concentrations in women may adversely influence oocyte quality during in vitro fertilization. Fertility and sterility 95, 1816-1819, doi:10.1016/j.fertnstert.2010.11.008 (2011).

[116] Maranghi, F., Rescia, M., Macri, C., Di Consiglio, E., De Angelis, G., Testai, E., Farini, D., De Felici, M., Lorenzetti, S. and Mantovani, A. Lindane may modulate the female reproductive development through the interaction with ER-beta: an in vivo-in vitro approach. Chemico-biological interactions 169, 1-14, doi:10.1016/j.cbi.2007.04.008 (2007).

[117] State of the Science of Endocrine Disrupting Chemicals - 2012. World Health Organization (2015).

[118] Stoker, T. E., Gibson, E. K. & Zorrilla, L. M. Triclosan exposure modulates estrogen-dependent responses in the female wistar rat. Toxicological sciences: an official journal of the Society of Toxicology 117, 45-53, doi:10.1093/toxsci/kfq180 (2010).

[119] Holliday, R. DNA methylation and epigenetic mechanisms. Cell biophysics 15, 15-20 (1989).

[120] Fatemi, M., Hermann, A., Gowher, H. & Jeltsch, A. Dnmt3a and Dnmt1 functionally cooperate during de novo methylation of DNA. European journal of biochemistry/FEBS 269, 4981-4984 (2002).

[121] Baubec, T., Colombo, D. F., Wirbelauer, C., Schmidt, J., Burger, L., Krebs, A. R., Akalin, A. and Schubeler, D. Genomic profiling of DNA methyltransferases reveals a role for DNMT3B in genic methylation. Nature, doi:10.1038/nature14176 (2015).

[122] Xie, S., Wang, Z., Okano, M., Nogami, M., Li, Y., He, W. W., Okumura, K. and Li, E. Cloning, expression and chromosome locations of the human DNMT3 gene family. Gene 236, 87-95 (1999).

[123] La Salle, S., Mertineit, C., Taketo, T., Moens, P. B., Bestor, T. H. and Trasler, J. M. Windows for sex-specific methylation marked by DNA methyltransferase expression profiles in mouse germ cells. Developmental biology 268, 403-415, doi:10.1016/j.ydbio.2003.12.031 (2004).

[124] Grayson, D. R. & Guidotti, A. The dynamics of DNA methylation in schizophrenia and related psychiatric disorders. Neuropsychopharmacology: official publication of the American College of Neuropsychopharmacology 38, 138-166, doi:10.1038/npp.2012.125 (2013).

[125] Costa, E., Dong, E., Grayson, D. R., Guidotti, A., Ruzicka, W. and Veldic, M. Reviewing the role of DNA (cytosine-5) methyltransferase overexpression in the cortical GABAergic dysfunction associated with psychosis vulnerability. Epigenetics: official journal of the DNA Methylation Society 2, 29-36 (2007).

[126] Marumo, T., Yagi, S., Kawarazaki, W., Nishimoto, M., Ayuzawa, N., Watanabe, A., Ueda, K., Hirahashi, J., Hishikawa, K., Sakurai, H., Shiota, K. and Fujita, T. Diabetes Induces Aberrant DNA Methylation in the Proximal Tubules of the Kidney. Journal of the American society of nephrology: JASN, doi:10.1681/ASN.2014070665 (2015).

[127] Li, Q., Wei, W., Jiang, Y. I., Yang, H. & Liu, J. Promoter methylation and expression changes of in cancerous tissues of patients with sporadic breast cancer. Oncology letters 9, 1807-1813, doi:10.3892/ol.2015.2908 (2015).

[128] Charlton, J., Williams, R. D., Sebire, N. J., Popov, S., Vujanic, G., Chagtai, T., Alcaide-German, M., Morris, T., Butcher, L. M., Guilhamon, P., Beck, S. and Pritchard-Jones, K. Comparative methylome analysis identifies new tumour subtypes and biomarkers for transformation of nephrogenic rests into Wilms tumour. Genome medicine 7, 11, doi:10.1186/s13073-015-0136-4 (2015).

[129] Zhang, Y. & Reinberg, D. Transcription regulation by histone methylation: interplay between different covalent modifications of the core histone tails. Genes & development 15, 2343-2360, doi:10.1101/gad.927301 (2001).

[130] Mosammaparast, N. & Shi, Y. Reversal of histone methylation: biochemical and molecular mechanisms of histone demethylases. Annual review of biochemistry 79, 155-179, doi:10.1146/annurev.biochem.78.070907.103946 (2010).

[131] Makowski, A. M., Dutnall, R. N. & Annunziato, A. T. Effects of acetylation of histone H4 at lysines 8 and 16 on activity of the Hat1 histone acetyltransferase. The Journal of biological chemistry 276, 43499-43502, doi:10.1074/jbc.C100549200 (2001).

[132] Dhalluin, C., Carlson, J. E., Zeng, L., He, C., Aggarwal, A. K. and Zhou, M. M. Structure and ligand of a histone acetyltransferase bromodomain. Nature 399, 491-496, doi:10.1038/20974 (1999).

[133] Schreiber, S. L. & Bernstein, B. E. Signaling network model of chromatin. Cell 111, 771-778 (2002).

[134] Nagamori, I., Cruickshank, V. A. & Sassone-Corsi, P. Regulation of an RNA granule during spermatogenesis: acetylation of MVH in the chromatoid body of germ cells. Journal of cell science 124, 4346-4355, doi:10.1242/jcs.096461 (2011).

[135] Reik, W. & Dean, W. DNA methylation and mammalian epigenetics. Electrophoresis 22, 2838-2843, doi:10.1002/1522-2683(200108)22:14<2838::AID-ELPS2838>3.0.CO;2-M (2001).

[136] Sobulo, O. M., Borrow, J., Tomek, R., Reshmi, S., Harden, A., Schlegelberger, B., Housman, D., Doggett, N. A., Rowley, J. D. and Zeleznik-Le, N. J. MLL is fused to CBP, a histone acetyltransferase, in therapy-related acute myeloid leukemia with a t(11;16)(q23;p13.3). Proceedings of the national academy of sciences of the United States of America 94, 8732-8737 (1997).

[137] Williams, K. T. & Schalinske, K. L. Tissue-specific alterations of methyl group metabolism with DNA hypermethylation in the Zucker (type 2) diabetic fatty rat. Diabetes/metabolism research and reviews 28, 123-131, doi:10.1002/dmrr.1281 (2012).

[138] Tresaugues, L., Dehe, P. M., Guerois, R., Rodriguez-Gil, A., Varlet, I., Salah, P., Pamblanco, M., Luciano, P., Quevillon-Cheruel, S., Sollier, J., Leulliot, N., Couprie, J., Tordera, V., Zinn-Justin, S., Chavez, S., van Tilbeurgh, H. and Geli, V. Structural characterization of Set1 RNA recognition motifs and their role in histone H3 lysine 4 methylation. Journal of molecular biology 359, 1170-1181, doi:10.1016/j.jmb.2006.04.050 (2006).

[139] Pascreau, G., Arlot-Bonnemains, Y. & Prigent, C. Phosphorylation of histone and histone-like proteins by aurora kinases during mitosis. Progress in cell cycle research 5, 369-374 (2003).

[140] Pilyugin, M., Demmers, J., Verrijzer, C. P., Karch, F. & Moshkin, Y. M. Phosphorylation-mediated control of histone chaperone ASF1 levels by Tousled-like kinases. PloS one 4, e8328, doi:10.1371/journal.pone.0008328 (2009).

[141] Schnell, J. D. & Hicke, L. Non-traditional functions of ubiquitin and ubiquitin-binding proteins. The Journal of biological chemistry 278, 35857-35860, doi:10.1074/jbc.R300018200 (2003).

[142] Kurian, J. R. & Terasawa, E. Epigenetic control of gonadotropin releasing hormone neurons. Frontiers in endocrinology 4, 61, doi:10.3389/fendo.2013.00061 (2013).

[143] Stouder, C. & Paoloni-Giacobino, A. Transgenerational effects of the endocrine disruptor vinclozolin on the methylation pattern of imprinted genes in the mouse sperm. Reproduction 139, 373-379, doi:10.1530/REP-09-0340 (2010).

[144] Anway, M. D., Cupp, A. S., Uzumcu, M. & Skinner, M. K. Epigenetic transgenerational actions of endocrine disruptors and male fertility. Science 308, 1466-1469, doi:10.1126/science.1108190 (2005).

[145] Skinner, M. K. & Anway, M. D. Seminiferous cord formation and germ-cell programming: epigenetic transgenerational actions of endocrine disruptors. Annals of the New York academy of sciences 1061, 18-32, doi:10.1196/annals.1336.004 (2005).

CHAPTER 2:

Exposure to Common Quaternary Ammonium Disinfectants Decreases Fertility in Mice

Vanessa E. Melin[1], Haritha Potineni[1], Patricia Hunt[2], Jodi Griswold[2], Bill Siems[3], Stephen R. Werre[4], and Terry C. Hrubec[1, 5]

1) Department of Biomedical Sciences and Pathobiology, VA-MD Regional College of Veterinary Medicine, VA Tech, Blacksburg, VA 24061

2) School of Molecular Biosciences, Washington State University, Pullman, WA 99164

3) Department of Chemistry, Washington State University, Pullman, WA 99164

4) Laboratory for Study Design and Statistical Analysis, VA-MD Regional College of Veterinary Medicine, VA Tech, Blacksburg, VA 24061

5) E. Via College of Osteopathic Medicine Virginia Campus, Blacksburg, VA 24060

Reprinted from Reproductive Toxicology, 50.0 (2014): 163-70, with permission from Elsevier, 2015

Licensee: Vanessa E Melin
License Date: Apr 16, 2015
License Number: 3610980677375
Publication: Reproductive Toxicology
Title: Exposure to common quaternary ammonium disinfectants decreases fertility in mice

1. ABSTRACT

Quaternary ammonium compounds (QACs) are antimicrobial disinfectants commonly used in commercial and household settings. Extensive use of QACs results in ubiquitous human exposure, yet reproductive toxicity has not been evaluated. Decreased reproductive performance in laboratory mice coincided with the introduction of a disinfectant containing both alkyl dimethyl benzyl ammonium chloride (ADBAC) and didecyl dimethyl ammonium chloride (DDAC). QACs were detected in caging material over a period of several months following cessation of disinfectant use. Breeding pairs exposed for six months to a QAC disinfectant exhibited decreases in fertility and fecundity: increased time to first litter, longer pregnancy intervals, fewer pups per litter and fewer pregnancies. Significant morbidity in near term dams was also observed. In summary, exposure to a common QAC disinfectant mixture significantly impaired reproductive health in mice. This study illustrates the importance of assessing mixture toxicity of commonly used products whose components have only been evaluated individually.

2. INTRODUCTION

The impetus for this investigation of the effects of quaternary ammonium compounds (QACs) on reproductive performance in the laboratory mouse was changes in breeding performance in mouse colonies used by the Hunt and Hrubec laboratories. Both groups noted abrupt changes in colony productivity and reductions in maternal and fetal health that coincided with the introduction of disinfectants containing QACs, Alkyl (60% C14, 25% C12, 15% C16) dimethyl benzyl ammonium chloride (ADBAC) and didecyl dimethyl ammonium chloride (DDAC).

QACs are commonly found in cleaning solutions used in residential, commercial and medical settings, as well as in restaurants and food production facilities. The ability to adapt and optimize QAC structure for specific functions has increased the utilization of these compounds in consumer products and, as a result, several generations of QACs exist.[1,2] The earliest QACs were benzalkonium chloride compounds that were developed as antimicrobial agents. All QACs are permanently charged ions with four alkyl side chains, and biocidal activity is conferred through alkyl chain length.[3-5] Modifications to alkyl chain length have been used to optimize cleaning and antimicrobial properties. Specifically, through substitution of aromatic ring hydrogen with chlorine, methyl, and ethyl groups to increase antimicrobial efficiency and improve detergent strength, different generations of QAC compounds have been generated. Twin-chain or dialkyl quaternary QACs represent the newest generation and exhibit a wide spectrum of activity. These new synthetic polymeric QACs contain multiple positively charged amine centers that confer antimicrobial, anti-static, and surfactant properties in solution.

QACs are often used in shampoos and laundry products to neutralize negative static charges and in cosmetics to preserve products from microbial contamination.[6-9] The increased reliance on QAC mixtures in consumer products has likely resulted in significant human exposure. For instance, products applied directly to and left on the skin, such as body lotions, often contain QACs.[10] In the health care

industry QACs have replaced many alcohol-based hand sanitizers, being more effective at reducing bacterial contamination and limiting the spread of nosocomial infections.[11] In addition, elementary students are provided instant hand sanitizers containing QACs to decrease the spread of illness and reduce rates of absenteeism.[12] QACs are being increasingly incorporated into contemporary products that are utilized orally, such as mouth wash, applied to the skin or eyes or administered as a nasal spray.[13-20]

QACs have been in use for approximately 50 years and are considered relatively safe. Despite the duration and prevalence of their use in commercial and consumer products, few studies have assessed the toxicity of single QACs. The majority of studies investigating the toxicity of single QACs are unpublished company reports which indicate weight reduction as the main effect in mice.[21-23] Moreover, no peer-reviewed studies have examined the toxicity of newer QAC combinations. Since chemical mixtures can act synergistically to produce greater toxic effects than the sum of the individual components, evaluation of common mixtures is essential in the evaluation of chemical risk.[24-27]

Formulation HWS-256 is a commercial mixture containing a combination of two QACs: Alkyl (60% C14, 25% C12, 15% C16) dimethyl benzyl ammonium chloride (ADBAC, benzalkonium chloride) and didecyl dimethyl ammonium chloride (DDAC). Combinations of ADBAC and DDAC are common in disinfectant and cleaning solutions that are widely used in clinical and residential settings. We proposed that a disinfectant solution containing both ADBAC and DDAC caused severe reproductive defects in exposed mice.[28] This assertion is supported by the results of a six-month breeding study, wherein mice exposed to this QAC mixture demonstrated significant declines in fertility and fecundity. Our results show that the ADBAC+DDAC mixture not only significantly impaired reproduction in breeding pairs, but also contributed to dam morbidity.

3. MATERIALS AND METHODS

3.1. Animals and Experimental Design

3.1.1. Case Western Reserve (CWRU) and Washington State University (WSU)

C57BL/6J (Jackson Laboratory, Bar Harbor, ME) mice were housed in ventilated rack caging (Thorin caging (CWRU) or Lab Products (WSU)) in a pathogen-free facility. Breeding stocks were maintained by brother to sister trio mating of two females with one male. To obtain timed pregnancies, six week-old C57BL/6J female pups born in the new facility were placed with adult males, checked each morning for the presence of a copulation plug, and separated from the male on the morning a plug was found. All animal breeding experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at CWRU and WSU; both institutions are fully accredited by the American Association for Accreditation of Laboratory Animal Care (AAALAC).

3.1.2. Virginia Polytechnic Institute (VPI)

All CD-1 mice (Charles River Labs, Raleigh, NC) were bred for two generations in a room free of QAC disinfectants in order to eliminate any potential effects from previous QAC exposure. Mice were housed in disposable caging (Innovive, San Diego, CA). Exposed mice were maintained in a room utilizing an ADBAC+DDAC disinfectant (HWS-256, Sanitation Strategies, Holt, MI) while control mice were in an adjacent room utilizing ethanol as a disinfectant. Both rooms were climate-controlled with a 12-hour light/dark cycle, 20 – 25 °C, and 30 - 60% relative humidity. Ethanol washes were used to remove ADBAC+DDAC contaminants from equipment and personnel prior to entering the room housing the control mice. Personnel also donned hair bonnets, face masks, disposable gowns, gloves, and dedicated footwear prior to entering the control room to reduce potential ADBAC+DDAC contamination. Mice were dosed by adding HWS-256 into Nutra-gel diet (purified dry mix formula, Bio-Serv, Frenchtown, NJ) which was prepared following manufacturer instructions. Doses of ADBAC+DDAC/kg

body weight/day were calculated based on the sum of active ingredient in the disinfectant (6.76% ADBAC (60% C-14, 25% C-12, 15% C-16) and 10.1% DDAC), with an average daily food consumption of 28% body weight and provided daily in a 25g Nutra-gel diet cube. Fresh gel cubes were added and food consumption monitored each day. In all experiments, mice were acclimated to the gel diet for one week prior to dosing. A short-term dose finding study was performed in unbred mice to identify the lowest observable adverse effect limit (LOAEL). Gel food was dosed with 0, 60, 120, 240, and 480 mg ADBAC+DDAC disinfectant/kg/day and provided to 5 mice per dose group for two weeks. Mice were monitored daily and evaluated against 16 different health parameters for physical appearance, activity, physiology, and body weight loss. Signs of toxicity, such as inappetance, lethargy, and rough haircoat, were observed in animals receiving the 240 and 480 mg doses; thus, the LOAEL was identified as 240 mg ADBAC+DDAC disinfectant/kg/day. Two dose levels below the LOAEL (i.e., 60 and 120 mg ADBAC+DDAC/kg/day) were selected to evaluate the long-term effects of exposure to QACs. At 5 weeks of age, all mice were provided undosed gel diet. At 6 weeks of age, males and females were combined into breeding pairs and randomly assigned to 0 (control), 60, or 120 mg ADBAC+DDAC/kg body weight/day treatment. Ten dedicated breeding pairs per group were subsequently dosed for a total of six months. Mice were monitored daily and evaluated against 16 different health parameters for physical appearance, activity, physiology and feed consumption. Male mouse weight was recorded weekly. Female body weight was not recorded as body weight fluctuated with their stage of pregnancy. At birth, delivered pups were counted, weighed, evaluated for gross malformations, and then euthanized by IP injection of sodium pentobarbital (0.05 mL/g). This experimental design allowed a multi-tier assessment of chronic toxicity from a QAC mixture directly to adult breeding pairs and indirectly to pups. All animal experiments were approved by the IACUC at the College of Veterinary Medicine at VPI, an AAALAC accredited facility.

3.2. Assessing Cage Contamination

To obtain extracts for chemical analysis, five cages were washed with a small volume of methanol using the following procedure: approximately 40 mL of methanol (JT Baker, Phillipsburg, NJ) was used to thoroughly rinse the inside walls of one polysulfone microisolator cage. The methanol was transferred to a second cage, the rinsing procedure was repeated, and the methanol was transferred to the third cage. This procedure was repeated for two additional cages, thus combining the chemical residue from five cages into one methanol sample. The resultant methanol extracts were collected and stored in glass vials that had been previously tested and demonstrated to be free of exogenous chemical residue (i.e., as for cages, test vials were washed with methanol and extracts run to assess contamination). Analysis of cage extracts was performed with an Agilent 1100 Series HPLC system (Agilent Technologies, Waldbronn, Germany) coupled to an API-4000 triple quadrupole mass spectrometer (Applied Biosystems/MDS SCIEX, Ontario, Canada) operated in positive ionization mode. Instrument control, data acquisition, and initial data analyses were performed by Analyst 1.3.1 software. Only the dimethyl didecyl ammonium (DDA) component of the QAC disinfectant was quantitatively analyzed. Five standard concentrations of dimethyl didecyl ammonium bromide (98%, Aldrich, St. Louis MO) ranging from 3.6 nM to 181 nM were analyzed with each sample batch. Chromatographic separation was carried out using a MM-5-C4W-1000 column (Micro-Tech Scientific Inc., Vista, CA). Mobile phase A was 0.1% acetic acid (J.T. Baker, Phillipsburg NJ), and mobile phase B was acetonitrile (EMD Chemicals Inc., Gibbstown NJ). The column was equilibrated with 70% A for 15 min before injection. The gradient was as follows: 70% A for 5 min, followed by a linear decrease to 37.1% A at 16.5 min; 2% A at 17.5 min; 2% A at 27.5 min; 70% A at 30 min. The injection volume of all standards and samples was 5 uL, and the flow rate was 125 uL min^{-1}. Nitrogen was used as the curtain gas (10 psi) and collision gas, and purified air was used as the source gas. The declustering potential was set at 30V and the collision energy at 40V. Quantitative analyses of DDA were monitored as selected ion reaction m/z pair 326/186.

3.3. Statistics

Time effect was normalized to the standard 20-day reproductive intervals in the mouse (total of 180 days). The first 6 days were discarded to control for initial differences in estrus cycle stage and to ensure that each dam was given sufficient time to enter estrus. Pup number and food consumption were measured cumulatively, as is typical of long-term toxicological studies.[29] Normal probability plots showed that cumulative pup numbers, cumulative amount of feed consumed, male mouse weight, and average weight per pup followed a normal distribution. Time to first litter (defined as the time between initial animal pairing/dosing and the appearance of pups) and average number of pregnancies were skewed. Analysis of the average number of pregnancies was performed over the first 100 days prior to any dam loss (n=10). Effects of ADBAC+DDAC treatment on cumulative pup numbers, cumulative amount of feed consumed, male mouse weight, and average weight per pup were assessed using repeated measures analysis of variance (RM-ANOVA). The linear model specified treatment, 20-day intervals, and the interaction between treatment and 20-day intervals as fixed effects with Kenward-Roger as denominator degrees of freedom. The model also specified that the measurements were repeated over dam identification within treatment with an autoregressive order one (AR1) covariance structure. To specifically examine the effect of treatment at each 20-day interval, the slicediff option of the glimmix procedure was applied followed by Tukey's procedure for multiple comparisons. Effects of treatment on total number of pups were assessed using one-way analysis of variance (ANOVA) followed by Tukey's procedure for multiple comparisons. Effects of treatment on time to first litter, average number of pregnancies, and slopes were assessed using the Kruskal-Wallis test (KW) followed by Dunn's procedure for multiple comparisons. Residual plots for the ANOVA models were inspected to verify model adequacy (i.e., errors followed a normal distribution with constant variance). Statistical significance was set to alpha < 0.05. All analyses were performed using SAS version 9.2 (Cary, NC).

4. RESULTS

4.1. Preliminary evidence implicated commercial QAC disinfectant mixture as cause of reproductive toxicity

In 2005, the Hunt laboratory relocated to WSU after 14 years at CWRU. The first experimental study at the new institution required timed pregnancies to obtain late gestation fetuses at specified developmental stages. Although mating performance appeared normal during the first four months of the study, only 5 of 46 (10.9%) mated females became pregnant. This was significantly below the 60-70% pregnancy rate expected based on past experience with this strain (Figure 1). In addition, increased rates of dystocia (prolonged or difficult delivery) were noted in the C57BL/6 breeding colony. As shown in Figure 2, a comparison of breeding performance for comparable breeding cages during the same three-month period suggested a significant reduction in productivity in the new vivarium. A variety of environmental variables, including diet, light, and temperature can affect rodent reproductive performance.[30] To improve breeding, environmental variables were systematically modified to replicate as closely as possible the conditions in the previous facility (e.g., changing mouse feed, adjusting light and ambient temperature, and restricting entry and animal handling). When none of these changes eliminated the problems, WSU researchers began to suspect other environmental effects. Three of the five investigators housing animals in the vivarium were conducting reproductive toxicology studies using a variety of chemical compounds, raising concern that the reproductive abnormalities in WSU animals were the result of inadvertent chemical contamination.

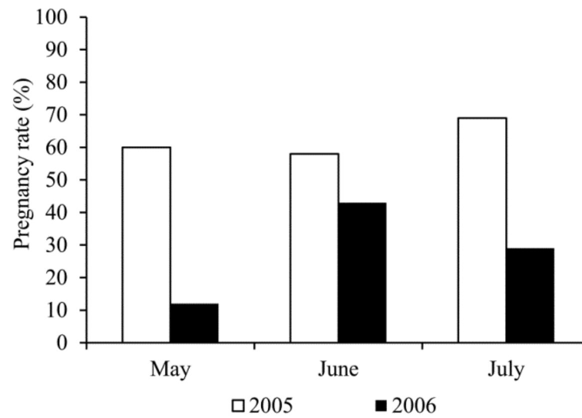


Figure 1. Comparison of pregnancy rates for timed matings. The pregnancy rate for timed matings of C57BL/6 females at CWRU (white bars) and at WSU (black bars). The data represent the pregnancy outcomes during a three-month period of 59 females mated at CWRU in the year preceding the move and 87 females mated at WSU the following year.

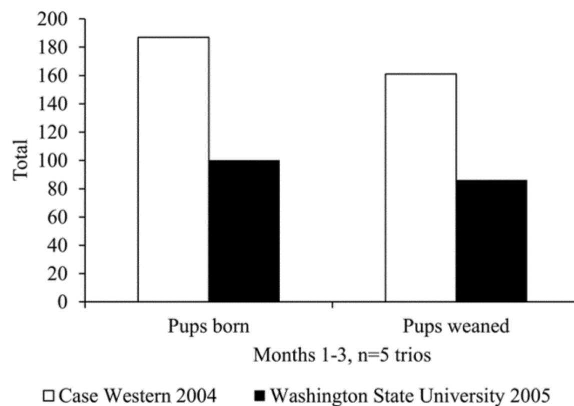


Figure 2. Breeding colony productivity. Total pups born and weaned in the C57BL/6 breeding colony at CWRU (white bars) and at WSU (black bars). The data represent productivity for 5 trio mating's during the same 3-month period at CWRU in the year preceding the relocation of the laboratory and the following year in the new vivarium at WSU.

4.2. WSU caging material analysis identified QAC contamination

To determine if reproductive abnormalities in WSU animals were the result of inadvertent chemical contamination, caging materials were analyzed using gas chromatograph-mass spectrometry (GC-MS). The inside walls of micro-isolator cages were rinsed with methanol (JT Baker, Phillipsburg,

NJ), an effective solvent for organic compounds, and the resultant extract was analyzed via GC-MS. This technique allowed for the detection and potential identification of individual chemicals in a complex mixture. Analysis of the cage extracts revealed a wide array of chemical compounds and, after accounting for components from food and bedding materials, a number of unidentified compounds remained. None of the chemical compounds matched the profiles of the chemicals being used in toxicology studies at WSU; however, a common chemical signature in all extraction experiments was a component of the QAC disinfectant used for sanitation and disinfection in the vivarium.

The QAC disinfectant was suspected as the source of the husbandry issues when a pinworm outbreak elsewhere in the facility required extensive cleaning and fogging of animal rooms with the disinfectant to contain the outbreak. This event coincided with a noticeable reduction in pregnancy rate in the mouse colony. In March 2006, the QAC disinfectant was removed from the facility and replaced with a disinfectant containing sodium chlorite. Levels of QACs were again analyzed in caging materials. To increase precision and accuracy of cage extract analysis, liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used for detection and quantification of a specific ion. As shown in Figure 3, the levels of QACs detected in caging materials uniformly decreased over a period of several months, corresponding with increased breeding performance.

4.3. Exposure experiments at WSU were impeded by QAC contamination

To verify the effect of QAC exposure in a controlled experiment, several breeding cages from WSU were separated from the original colony. These animals were exposed to aerosolized QAC disinfectant three times weekly while an equal number of control breeders were exposed to water aerosolized in the same manner. Breeding performance, dystocia, and gestational day 21 pup weights were monitored. Cages housing QAC-exposed animals were washed and autoclaved separately and washing was followed by an additional purge cycle to remove contaminated water from the cage washer. Within weeks of initiating the experiment, levels of QACs detected during random monitoring of caging

materials increased, suggesting that the limited use of QAC in the exposure study was sufficient to introduce generalized contamination. At this stage, the QAC-exposure study was terminated and all QAC-containing compounds were removed from the facility. Although the inability to obtain control data precluded publication of the findings, these experiences were published in interview format in *Nature*.[28]

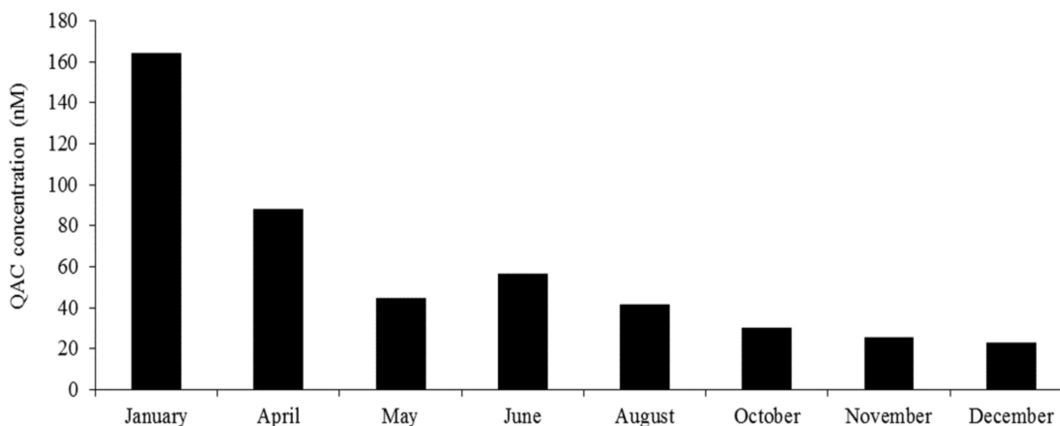


Figure 3. Results of LC-MS/MS cage extract analysis. Bars represent QAC concentration in nMs that was detected in cage extracts. Levels of QACs detected in caging materials uniformly decreased over a period of several months.

4.4. QAC-induced reproductive decline at VPI provided the impetus for further studies

In 2008, the Hrubec laboratory at VPI began to notice a decline in breeding performance in their mouse colony. Around this time, staff began using an ADBAC+DDAC containing disinfectant to clean the floors, walls, and equipment in the animal facility. Additionally, rodent handling procedures often required wetting gloves with disinfectant before touching the animals. The liberal application of disinfectant in the rodent facility suggested it might be responsible for the decline in reproduction. An interview with Dr. Hunt that was published in *Nature* related exposure to the same QAC disinfectant compounds to reproductive defects in mice. In accordance with Dr. Hunt’s observations, VPI colony productivity improved within several generations after discontinuing use of QAC disinfectants in the

facility. To directly test the effects of ADBAC+DDAC disinfectants on reproduction, a controlled breeding study was conducted. In the short-term dose finding study, unbred mice were dosed at 0, 60, 120, 240, and 480 mg ADBAC+DDAC/kg/day for two weeks. Mice in the 240 and 480 mg doses mice became inappetent, lethargic, and developed a rough haircoat. The LOAEL was identified as 240 mg/kg/day and two dose levels below this (i.e., 60 and 120 mg/kg/day) were selected to evaluate the long-term reproductive effects of exposure to QACs. Three groups of breeding pairs were provided 0, 60 or 120 mg ADBAC+DDAC disinfectant/kg/day in a gel food for 180 days. In order to avoid QAC contamination issues experienced by the Hunt lab, mice were housed in disposable caging at VPI and control mice were reared in a separate "QAC- free" room.

4.5. Daily QAC treatment in mice did not affect food consumption but significantly reduced survival

Mice were monitored daily and evaluated against 16 different health parameters for physical appearance, activity, physiology, and body weight loss. Clinical signs initiating euthanasia included: partial closure of eyelids (squinting), significantly reduced activity, ataxia, kyphosis, hypothermia, rapid breathing and dyspnea, and cyanosis. All males and females receiving 0 mg ADBAC+DDAC/kg/day appeared normal and healthy throughout the duration of the study. Clinical signs necessitating euthanasia of pregnant dams were observed in the 60 and 120 dose groups; 1 of the 10 females in the 60 mg/kg/day and 4 of the 10 females in the 120 mg /kg/day dose groups required euthanasia (Figure 4). Clinical signs were only observed in late pregnancy or during delivery and included inappetence, lethargy, ataxia, kyphosis, labored breathing, cyanosis, vaginal hemorrhage, and dystocia. Differences in least squares means for food consumption among treatment groups were not significantly different, indicating that animals were receiving the proper dose of ADBAC+DDAC (RM-ANOVA, $p>0.05$; Figure 5). Male mouse weights did not differ between groups (data not shown, ANOVA, $p=0.81$).

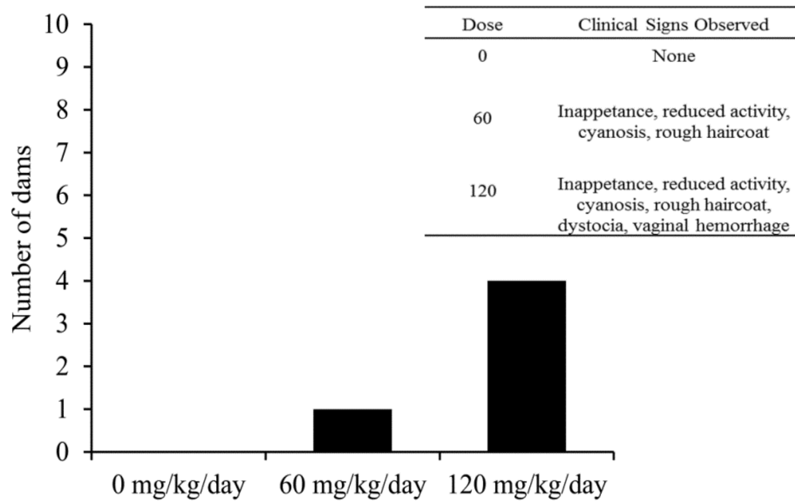


Figure 4. Number of females in each ADBAC+DDAC dose group exhibiting clinical signs necessitating euthanasia. The 120 mg/kg/day dose group experienced the highest female morbidity rate with 4 dams out of 10 euthanized, while in the lower 60 mg/kg/day dose 1 dam of 10 was euthanized. No morbidity was observed in the 0 mg/kg/day group, or in any of the dosed males.

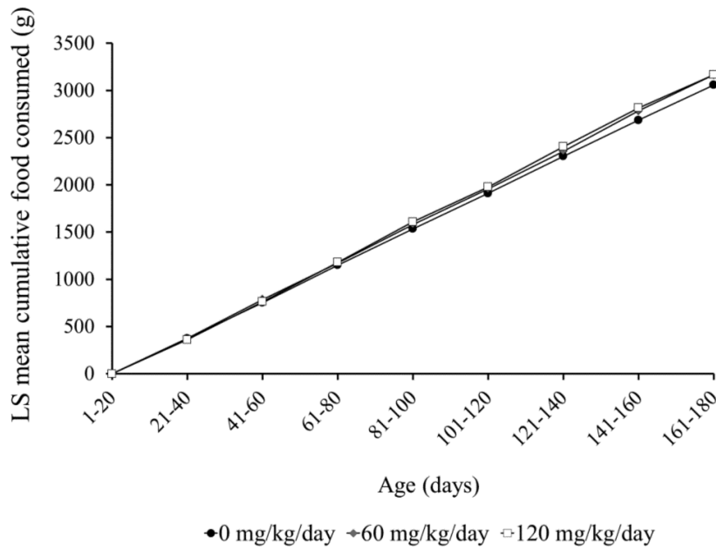


Figure 5. Least squares mean cumulative food intake among in exposed and control mice. Three ADBAC+DDAC disinfectant dose groups of 10 co-housed male and female pairs were provided 25g of dosed gel food daily. Food weights were taken daily and cumulated for each interval. No significant differences were observed in food consumption means among treatment groups (RM-ANOVA, $p>0.05$).

4.6. Daily QAC exposure significantly reduced fertility

Fertility was assessed by measuring time to first litter and the total number of litters per dam for each treatment group over the duration of during the 180 day exposure period. Time to first litter represents the time between the initial pairing and dosing of mice to the appearance of pups. This metric incorporates time to pregnancy and also gestation length and can be affected by either factor. Time to first litter was significantly longer in breeding pairs exposed to 120 mg ADBAC+DDAC disinfectant/kg/day compared to the 0 and 60 treatment groups (p=0.01; Figure 6). First litters were delivered after an average of 20 days of treatment in the 0 and 60 mg/kg/day dose groups, while the 120 dose group delivered after an average of 31 days. Not surprisingly, this contributed to a significant reduction in the average number of pregnancies in the 120 mg/kg/day dose group by comparison with the 0 and 60 treatment groups (p=0.005; Figure 7).

4.7. Daily QAC exposure significantly reduced fecundity

Dam morbidity was a confounding factor that precluded a simple comparison of total pups produced between treatment groups during the 180 day exposure period. An analysis of cumulative pup numbers during the first 100 days of the study prior to loss of any breeding females, demonstrated significantly reduced cumulative pup numbers in the 120 mg/kg/day exposure group compared to 0 and 60 (Figure 8a, RM-ANOVA, p≤0.05). An analysis of pups produced during the entire 180 day treatment using least squares mean estimates to account for dam morbidity, demonstrated a similar reduction of pups in the 120 mg/kg/day group in comparison to 0 and 60 dose groups (p=0.0002 and p=0.004, respectively; Figure 8b, RM-ANOVA, p≤0.05). Starting at days 21-40, significantly fewer pups were delivered in the 120 mg group compared to the 0 and 60 dose groups. Although the number of pups was consistently lower in the 60 dose group than in controls, the difference was not significant during any point of the 180 day exposure period. Finally, a comparison of pup weights at birth revealed no obvious treatment-related pattern, and apparent differences (e.g., during days 1-20 and 41-60 between 0 and 120

mg/kg/day dose groups, p=0.04 and 0.02, respectively; Figure 9) are likely a reflection of other factors such as litter size.

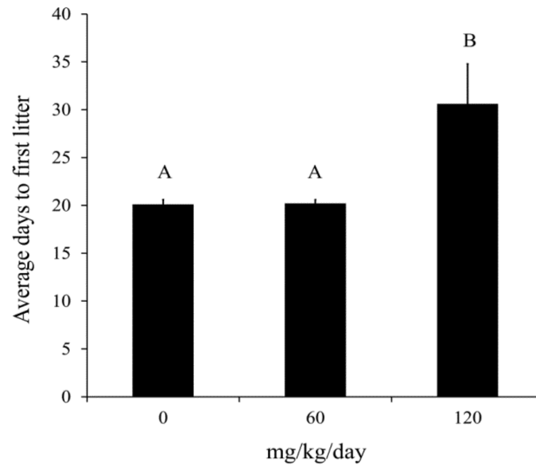
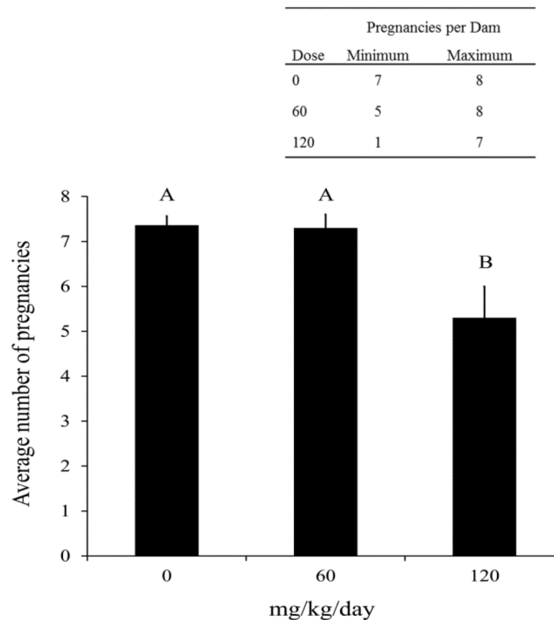


Figure 6. The effect of ADBAC+DDAC disinfectant on time to first litter. Time to first litter includes both time to pregnancy and gestation length and reflects changes in either. Dams in the 120 mg/kg/day treatment group took significantly longer to deliver their first litter compared to 0 (p=0.01) and 60 mg/kg/day (p=0.01) treatment groups (KW followed by Dunn's procedure for multiple comparisons, p≤0.05).



	Pregnancies per Dam	
Dose	Minimum	Maximum
0	7	8
60	5	8
120	1	7

Figure 7. Average number of pregnancies per dam in the first 100 days of exposure before any dam loss. Significantly fewer pregnancies per dam were observed in the 120 mg/kg/day group (p=0.005) compared to 0 and 60 mg/kg/day dose groups (n=10 for all dose groups; KW followed by Dunn's procedure for multiple comparisons, p≤0.05).

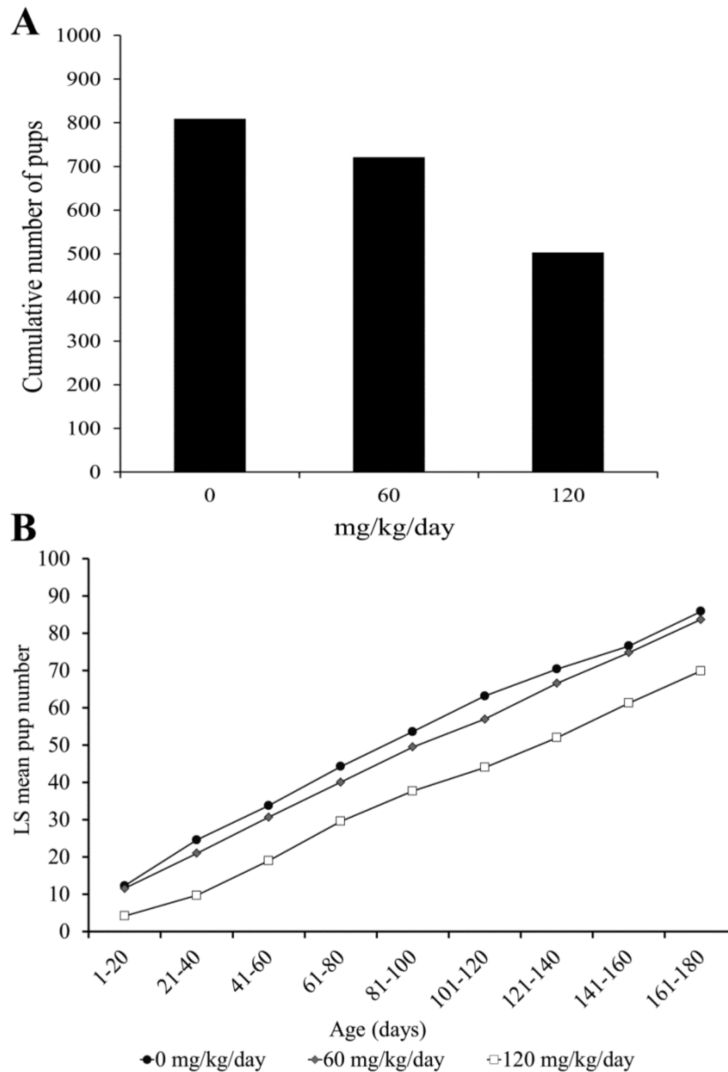


Figure 8. The effects of ADBAC+DDAC disinfectant on fecundity of breeding pairs. A. Cumulative number of pups born per treatment group in the first 100 days of exposure prior to any dam loss. Cumulative pup number was significantly lower in the 120 exposure group even before dam morbidity occurred (RM-ANOVA, $p \leq 0.05$). B. Cumulative least squares means estimate of pup number over the full 180 days. Least squares means were calculated to account for dam morbidity and approximate cumulative pup number over 180 days of treatment. Significantly fewer pups overall were delivered by dams in the 120 group compared to 0 ($p=0.002$) and 60 mg/kg/day ($p=0.008$) treatment groups (RM-ANOVA, $p \leq 0.05$).

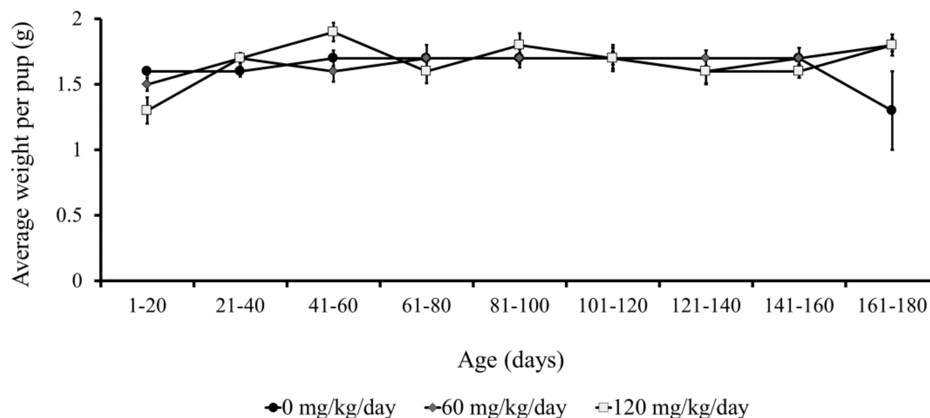


Figure 9. Average weight per pup (g) among breeding pairs exposed to ADBAC+DDAC disinfectant for 180-days. Average weight per pup was significantly different only during days 0-20 between 0 and 120 (p=0.01), days 41-60 between 60 and 120 (p=0.03), and days 171-180 between 0 and 60 (p=0.01) mg/kg/day treatment groups (RM-ANOVA, p≤0.05). No pattern of significance was observed.

5. DISCUSSION

5.1. Impetus and experimental design to test QAC reproductive toxicity

The 1976 Toxic Substances Control Act (TSCA) created a system to oversee the production and use of chemicals in the United States. The original act, however, has never been amended and does not mandate safety evaluations for all chemicals in active commerce. Consequently, it is possible for chemicals to become widely used in the U.S., despite the fact that their potential reproductive effects have not been directly assessed. QAC disinfectants are one such class of chemicals. As disinfectants, they are extremely effective and have several highly desirable attributes: they are odorless, leave no visible residue, and are non-corrosive. As a result, their use and application has grown beyond simple

disinfectants and they have become additives in a wide range of personal care products, including shampoos, cosmetics, and even baby wipes. The only studies of their biological impact have been those conducted by the manufacturers, with the only adverse effect reported being a slight reduction in weight in exposed offspring.[21-23]

The independent realization that the introduction of QAC-containing disinfectants coincided with noticeable declines in fertility in both WSU and VPI mouse breeder colonies prompted the present studies. The first episode correlated with the relocation of the Hunt laboratory to WSU. Although all evidence suggested that the QAC disinfectant was to blame for poor pregnancy rates and increased dystocia due to late fetal demise, efforts to provide experimental evidence were thwarted when a small controlled study resulted in generalized QAC contamination throughout the facility. Nevertheless, the WSU experience provided important preliminary data that was briefly discussed in a published interview.[28] Specifically, data from random cage monitoring during QAC use and in the months after use of QAC disinfectants was discontinued provided evidence that these chemicals persist in the environment (Figure 3). Further, despite careful care and washing of caging materials during an attempted controlled study, the reintroduction of QAC contamination suggested that QACs can be easily spread.

Several years later, the Hrubec laboratory at VPI encountered similar breeding problems suggesting the possibility of an environmental exposure. Because reproductive changes appeared to coincide with the introduction of the QAC disinfectant, attempts to investigate the relationship between QACs and fertility led to the article describing the experience in the Hunt laboratory.[28] Subsequently, the decision to directly test the effects of QACs on reproduction was made. A controlled breeding study was designed and conducted, using disposable cages and oral dosing via food (vs. inhalation) to reduce the possibility of cross contamination. The present results provide compelling evidence that QACs adversely affect rodent fertility and fecundity.

Although preliminary findings from both laboratories suggested that QAC exposure adversely affects reproduction, they did not provide insight to the route of exposure. That is, cage contamination could result in transdermal exposure from contaminated bedding, oral exposure from consumption of contaminated food and water, or even respiratory exposure from aerosolization of the disinfectant used during animal handling and cage change. Because oral dosing via food provided a simple exposure method with little risk of cross contamination, we chose this exposure route for our initial experimental studies. The doses elected were based on toxicity data from a preliminary dose-finding study (see methodology). Mice in the QAC disinfectant room potentially received some additional exposure due to routine husbandry practices. The disinfectant was reconstituted to the manufacturer's suggested concentration and used to disinfect walls, floors, animal racks and caging material; footwear and equipment were also disinfected upon entry and exit of the mouse room. Unfortunately, the influence of this additional exposure cannot be estimated, as disinfectant use and resulting exposure likely varied between individual animal care staff. Any additional exposure, however, would have applied equally to both the 60 and 120 mg/kg/day dose groups. The significant decrease in productivity in the highest exposure group and increased dam loss in both the high and low dose groups confirmed the preliminary fertility findings from both laboratories. Importantly, because no differences in food consumption or male body weight were evident among groups, these differences in reproductive endpoints cannot be ascribed to nutritional deficits.[32]

5.2. QACs decrease murine fertility and fecundity

Even after accounting for maternal morbidity, dams exposed to 120 mg ADBAC+DDAC disinfectant/kg/day produced significantly fewer offspring than dams in the 0 and 60 dose groups. Further, in addition to producing fewer pups per litter (an average of 8/litter for females receiving the 120 treatment compared to 10-12/litter for the 0 and 60 dose groups), high dose females had fewer pregnancies and longer pregnancy intervals; thus, in addition to confirming the preliminary findings from

both laboratories, these results provide the first experimental evidence that QAC exposure decreases fecundity in mice.

5.3. QAC exposure increases dam mortality and may be a teratogen

Although the doses were chosen because they did not elicit signs of toxicity in the initial study, exposure to ADBAC+DDAC disinfectant resulted in an increase in late gestation maternal morbidity in both the 60 and 120 exposure groups. No adverse clinical signs or morbidity were observed in control females or males of any dose group. Both late term abortion and dystocias were observed in dosed females and contributed to dam morbidity. Similarly, late fetal demise and dystocia were observed previously with QAC exposure in the Hunt laboratory (data not shown). Because the endpoint for these studies was live-birth, it could not be determined if fetal death always preceded onset of maternal delivery; therefore, it is not clear from these studies if the maternal distress that necessitated euthanasia was due to maternal or fetal toxicity.

Although these results clearly demonstrate that exposure to a common QAC mixture affects reproduction in the laboratory mouse, the design of this study does not allow the distinction between toxic effects on the dam, sire, fetus or a combination of effects to be made. Nevertheless, consistent with the preliminary findings from both laboratories, the results of prospective studies suggest that QACs affect both the maternal ability to achieve and sustain pregnancy and the developing fetus. An essential next step is to establish dose response relationships, determine the effects of different routes of exposure, and define critical development windows during which exposure elicits effects.

Although QACs are commonly used in combination to enhance bactericidal efficiency of cleaning products, to date, risk assessments have only tested the effects of QACs individually.[33] Indeed, by themselves, ADBAC and DDAC, the two QACs that make up HWS-256, are considered safe.[34] For this reason, it remains possible that these findings are the result of synergistic effects that are greater than

the effects of the individual components. In future studies, assessing the effects of individual QACs as well as QAC mixtures will be essential.

6. CONCLUSIONS

Lastly, although QACs are used in a wide variety of consumer products, current levels of human exposure remain unknown. The relevance of these findings to humans remains to be determined; however, given their widespread use and persistence in the environment, determining the levels of human exposure and whether QACs affect human reproduction is critically important. Indeed, the extensive use of QACs in industrial and clinical settings suggests that some individuals are likely chronically exposed to levels that are similar to those that elicited the preliminary effects in the laboratory rodents. Consequently, epidemiological studies of individuals in specific work environments where QAC disinfects are heavily used (e.g., custodial workers and individuals in clinical settings) may be particularly informative.

Acknowledgements

The authors gratefully acknowledge the technical support of Jodi Jackson. These studies were supported by NIH grant R01 HD37502 (PH) and the Passport Foundation (TH).

REFERENCES

[1] Carson RT, Larson E, Levy SB, Marshall BM, Aiello AE. Use of antibacterial consumer products containing quaternary ammonium compounds and drug resistance in the community. The Journal of antimicrobial chemotherapy 62:1160-2 (2008).

[2] Hegstad K, Langsrud S, Lunestad BT, Scheie AA, Sunde M, Yazdankhah SP. Does the wide use of quaternary ammonium compounds enhance the selection and spread of antimicrobial resistance and thus threaten our health? Microbial drug resistance 16:91-104 (2010).

[3] Pavlikova-Moricka M, Lacko I, Devinsky F, Masarova L, Milynarcik D. Quantitative relationships between structure and antimicrobial activity of new "soft" bisquaternary ammonium salts. Folia microbiologica 39:176-80 (1994).

[4] Ahlstrom B, Thompson RA, Edebo L. The effect of hydrocarbon chain length, pH, and temperature on the binding and bactericidal effect of amphiphilic betaine esters on Salmonella typhimurium. Acta pathologica, microbiologica et immunologica Scandinavica 107:318-24 (1999).

[5] Gilbert P, McBain AJ. Potential impact of increased use of biocides in consumer products on prevalence of antibiotic resistance. Clinical microbiology reviews 16:189-208 (2003).

[6] Farrington JK, Martz EL, Wells SJ, Ennis CC, Holder J, Levchuk JW, et al. Ability of laboratory methods to predict in-use efficacy of antimicrobial preservatives in an experimental cosmetic. Applied and environmental microbiology 60:4553-8 (1994).

[7] Schorr WF. Cosmetic allergy. A comprehensive study of the many groups of chemical antimicrobial agents. Archives of dermatology 104:459-66 (1971).

[8] Gao T. Evaluation of hair humidity resistance/moisturization from hair elasticity. Journal of cosmetic science 58:393-404 (2007).

[9] Blenman TV, Morrison KL, Tsau GJ, Medina AL, Gerlach RW. Practice implications with an alcohol-free, 0.07% cetylpyridinium chloride mouthrinse. American journal of dentistry. 18 Spec No: 29A-34A (2008).

[10] Loretz LJ, Api AM, Barraj LM, Burdick J, Dressler WE, Gettings SD, et al. Exposure data for cosmetic products: lipstick, body lotion, and face cream. Food and chemical toxicology: an international journal published for the British Industrial Biological Research Association 43: 279-91 (2005).

[11] Dyer DL, Gerenraich KB, Wadhams PS. Testing a new alcohol-free hand sanitizer to combat infection. Association of periopertative registered nurses journal 68: 239-41, 43-4, 47-51 (1998).

[12] White CG, Shinder FS, Shinder AL, Dyer DL. Reduction of illness absenteeism in elementary schools using an alcohol-free instant hand sanitizer. The Journal of school nursing: the official publication of the National association of school nurses 17: 258-65 (2001)

[13] Compton FH, Beagrie GS. Inhibitory effect of benzethonium and zinc chloride mouthrinses on human dental plaque and gingivitis. Journal of clinical periodontology 2: 33-43 (1975).

[14] Hallen H, Graf P. Benzalkonium chloride in nasal decongestive sprays has a long-lasting adverse effect on the nasal mucosa of healthy volunteers. Clinical and experimental allergy: journal of the British Society for Allergy and Clinical Immunology 25: 401-5 (1995).

[15] A.C. de Groot & J.P. Nater. Unwanted Effects of Cosmetics and Drugs used in Dermatology. Third Edition ed: Elsevier science B.V (1993).

[16] Baudouin C, Labbe A, Liang H, Pauly A, Brignole-Baudouin F. Preservatives in eyedrops: the good, the bad and the ugly. Progress in retinal and eye research 29: 312-34 (2010).

[17] Flyvholm MA. Preservatives in registered chemical products. Contact dermatitis 53: 27-32 (2005).

[18] Lebe E, Baka M, Yavasoglu A, Aktug H, Ates U, Uyanikgil Y. Effects of preservatives in nasal formulations on the mucosal integrity: an electron microscopic study. Pharmacology 72: 113-20 (2004).

[19] Kamysz W, Turecka K. Antimicrobial preservative effectiveness of natural peptide antibiotics. Acta poloniae pharmaceutica 62: 341-4 (2005).

[20] Muszynski Z, Dlugaszewska J. [Antimicrobial conservation of some cosmetics]. Medycyna doswiadczalna i mikrobiologia 46: 87-90 (1994).

[21] Gill MW, S. J. Hermansky and C. L. Wagner. Chronic Dietary Oncogenicity Study with Alkyl Dimethyl Benzyl Ammonium Chloride (ADBAC) in Mice Unpublished report number 53-515 Bushy Run Research Center, Export, PA (1991).

[22] Weaver E. Two-Week Dose Range Finding Screen with Alkyl Dimethyl Benzyl Ammonium Chloride (ADBAC) in Mice. Unpublished report number 51-514. Bushy Run Research Center, Export, PA (1988).

[23] Van Miller J. Ninety-Day Dose Range-Finding Study with Alkyl Dimethyl Benzyl Ammonium Chloride (ADBAC) in Mice. Unpublished report number 51-504: Bushy Run Research Center, Export, PA (1988).

[24] Hermens J, Leeuwangh P, Musch A. Joint toxicity of mixtures of groups of organic aquatic pollutants to the guppy (Poecilia reticulata). Ecotoxicology and environmental safety 9: 321-6 (1985).

[25] Faust M, Altenburger R, Backhaus T, Blanck H, Boedeker W, Gramatica P, et al. Joint algal toxicity of 16 dissimilarly acting chemicals is predictable by the concept of independent action. Aquatic toxicology 63: 43-63 (2003).

[26] Narotsky MG, Best DS, McDonald A, Godin EA, Hunter ES, 3rd, Simmons JE. Pregnancy loss and eye malformations in offspring of F344 rats following gestational exposure to mixtures of regulated trihalomethanes and haloacetic acids. Reproductive toxicology 31: 59-65 (2011).

[27] Lin GH, Hemming M. Ocular and dermal irritation studies of some quaternary ammonium compounds. Food and chemical toxicology: an international journal published for the British Industrial Biological Research Association 34: 177-82 (1996).

[28] Hunt P. Lab disinfectant harms mouse fertility. Patricia Hunt interviewed by Brendan Maher. Nature 453: 964 (2008).

[29] Tennant DR. Estimating acute dietary intakes of chemicals. Regulatory toxicology and pharmacology 30: S99-102 (1999).

[30] Murray KA, Parker NJ. Breeding genetically modified rodents: tips for tracking and troubleshooting reproductive performance. Lab animal 34: 36-41 (2005).

[31] EPA US. Reregistration Eligibility Decision for Alkyl Dimethyl Benzyl Ammonium Chloride (ADBAC) (2006).

[32] Wade GN, Jones JE. Neuroendocrinology of nutritional infertility. American journal of physiology; regulatory, integrative and comparative physiology 287: R1277-96 (2004).

[33] Jeffrey DJ. Chemicals used as disinfectants: active ingredients and enhancing additives. Revue scientifique et technique 14:57-74 (1995).

[34] EPA US. Reregistration Eligibility Decision for Aliphatic Alkyl Quaternaries (DDAC) (2006).

CHAPTER 3:

Quaternary Ammonium Disinfectants Cause Subfertility in Mice by Targeting both Male and Female Reproductive Processes

Vanessa E. Melin[1], Travis E. Melin[2], Brian J. Dessify[2], Christina T. Nguyen[2], and Terry C. Hrubec[1,2]

1) Department of Biomedical Sciences and Pathobiology, VA-MD Regional College of Veterinary Medicine, VA Tech, Blacksburg, VA 24061

2) E. Via College of Osteopathic Medicine Virginia Campus, Blacksburg, VA 24060

Submitted to Reproductive Toxicology, March 2015.

1. ABSTRACT

Alkyl dimethyl benzyl ammonium chloride (ADBAC) and didecyl dimethyl ammonium chloride (DDAC) are common ingredients in household bathroom and kitchen cleaning sprays. ADBAC+DDAC cause reproductive toxicity in mice. The aim of the present study was to investigate gender-specific reproductive effects from ADBAC+DDAC. Female reproduction was assessed through ovulation, oocyte implantation, and heat cycling. Male reproductive function was assessed by sperm concentration, motility, and viability. Numbers of corpora lutea were not different after 2 weeks, but decreased after 8 weeks of ADBAC+DDAC exposure. Dams exposed for 5 weeks to ADBAC+DDAC spent significantly less time in estrus. ADBAC+DDAC exposed males exhibited declines in both sperm concentration and motility, but not sperm viability. Subfertility in mice from ADBAC+DDAC exposure is, therefore, mediated through reproductive disturbances in both females and males. While the effect of ADBAC+DDAC exposure on human health is unclear, widespread exposure necessitates further consideration of their endocrine disrupting potential.

2. INTRODUCTION

Quaternary ammonium compounds (QACs) are antimicrobial agents commonly found in cleaning solutions used in residential, commercial and medical settings. Mono-alkyl benzalkonium chloride compounds constituted the first generation of antimicrobial QACs, and were routinely used for disinfection of eating utensils and medical instruments, and as active ingredients in cleaning products for floors and walls. The ability to optimize alkyl benzalkonium chloride structures for specific functions has increased the utilization of these compounds and, therefore, several generations exist. Bactericidal action of QACs is mediated through alkyl chain length. The cationic portion of the QAC molecule is attracted to negatively charged proteins on the bacterial cell membrane. Once proximate, the long alkyl chains of the QAC molecule pierce the bacterial lipid bilayer, causing membrane disruption and leakage of cellular contents. Modifications to alkyl chain length have been used to enhance antimicrobial effectiveness of QACs. QACs exhibit a wide spectrum of biocidal activity, and are effective against many bacteria, fungi, and protozoa. Over time, the applications of QACs have gone beyond simple disinfectants. QAC compounds are currently the most common preservatives in ophthalmic solutions and nasal sprays.[1,2]

The addition of QACs in products ranging from algaecides in swimming pools, lumber treatments, anti-static laundry products, to preservatives in cosmetics, has likely resulted in widespread human exposure.[3-8] Incidences of QAC toxicity from consumer products are well documented, and include corneal cytotoxicity, allergic rhinitis, contact dermatitis, and occupational asthma.[9-14] Nevertheless, QACs remain prevalent in consumer and industrial products and are considered relatively safe; however, few peer-reviewed studies have evaluated the toxicity of these compounds.

Between 2001 and 2010 the number of assisted reproductive technology (ART) procedures performed in the United States increased from 107,587 to 147,260 per year.[15] This increased reliance on ART suggests that infertility rates may be increasing. Additionally, global sperm numbers in males have

decreased significantly over the last 70 years, from an estimated 113 million to 49.9 million average sperm per mL.[16,17] Increases in reproductive dysfunction have been attributed to several endocrine disrupting compounds that affect hormone-regulated pathways.

Endocrine disruptors (ED) are a class of hormonally active compounds that imitate or interfere with hormone homeostasis and alter body function including reproduction. Several prevalent compounds in consumer products have been identified as EDs.[18-24] Many of these compounds, such as the plasticizer Bisphenol-A and the common antibacterial triclosan, have been used in consumer products for decades before being identified as EDs.[25] EDs are associated with a number of human health problems, including cancer, metabolic disorders, and infertility.[18-24] For this reason, public concern over potential negative health effects from chronic low-dose exposure to chemicals has risen substantially. Studies aimed at specifically identifying potential endocrine disrupting toxicants in common products are essential.

Alkyl dimethyl benzyl ammonium chloride (ADBAC) and didecyl dimethyl ammonium chloride (DDAC) are among the most common QACs utilized in household bathroom and kitchen cleaners. Additionally, ADBAC+DDAC are routinely used as sanitizers in medical settings, as well as in restaurants and food production facilities. Previously, we reported a decline in breeding performance of mice chronically exposed to 120 mg/kg/day of ADBAC+DDAC.[26] Mice took longer to get pregnant, demonstrated significantly fewer pregnancies and reduced litter sizes. The current study evaluated the gender-specific reproductive toxicity of ADBAC+DDAC.

3. MATERIALS AND METHODS

3.1. Animal husbandry

CD-1 mice were initially purchased from Charles River Laboratories (Raleigh, NC) and were maintained in disposable caging (Innovive, San Diego, CA) on a 12-hour light/dark cycle at 20 – 25 °C

with 30 - 60% relative humidity. Harlan 7013 rodent chow (Harlan Teklad, Madison, WI) and distilled water were provided *ad libitum*. Mice were reared in a facility free of QAC contaminants (QF) for two generations. Control mice were housed in the QF facility, while QAC-exposed mice were transferred prior to dosing and were housed in the facility utilizing the ADBAC+DDAC disinfectant. For breeding, all mice were paired two females to one male and co-housed for a maximum of two heat cycles.

3.1.1. ADBAC+DDAC dosing

Male and female mice were dosed by adding ADBAC+DDAC (Sanitation Strategies, Holt, MI) into Nutra-gel diet (purified dry mix formula, Bio-Serv, Frenchtown, NJ) which was prepared following manufacturer instructions, or dosed by adding the ADBAC+DDAC into distilled water provided for the mice. For experiments dosing in the food, control mice were kept in the QF facility and provided undosed gel food while treated mice were moved to the QAC facility and provided 120 mg/kg/day ADBAC+DDAC in gel food. Food consumption was recorded daily and was not significantly different between treatment groups (data not shown). Doses of ADBAC+DDAC/kg body weight/day were calculated based on the sum of ADBAC+DDAC (6.76% ADBAC (60% C-14, 25% C-12, 15% C-16) and 10.1% DDAC), with an average daily food consumption of 28% body weight and provided daily.[26] For water dosing, ADBAC+DDAC/kg body weight/day were calculated based on the sum of ADBAC+DDAC (6.76% ADBAC (60% C-14, 25% C-12, 15% C-16) and 10.1%), with an average daily water consumption of 10% body weight and provided fresh daily. Control mice were kept in the QF facility and provided undosed distilled water and treated mice were moved to the QAC facility and provided 120 mg/kg/day ADBAC+DDAC in distilled water.

3.2. Pontamine blue assessment of female ovulation and implantation

Percentages of successful implantations were evaluated in mice dosed with ADBAC+DDAC for 2 and 8 weeks in gel food and distilled water, respectively. Both males and females were maintained on treatments for 2 or 8 weeks and throughout breeding. Females were bred to unrelated males and the presence of a copulatory plug designated gestational day (GD) 0. On GD 6, females were given an intravenous injection of Pontamine sky blue dye (Sigma Aldrich, St. Louis, MO) in the tail vein.[27] One minute after injection, females were euthanized using CO_2 inhalation. Gravid uteri were then assessed for blastocyst attachment sites on the uterine epithelium indicated by bands of Pontamine blue staining. The number of pink corpora lutea in the ovary was recorded in 6 females for the 2 week exposure and 9-10 females for the 8 week exposure).

3.3. Quantification of estrus cycling

Mice raised in the QF facility were divided into control and treated groups at 6-8 weeks of age (n=9-10). Control mice were kept in the QF facility and provided undosed gel food. Treated mice were moved to the QAC facility and provided 120 mg/kg/day 6.76% ADBAC+ 10.1% DDAC in gel food for 2 weeks prior to evaluating vaginal cytology and throughout a 20 day evaluation period (5 weeks exposure total). Vaginal cytology was evaluated by inserting a saline moistened cotton tipped applicator into the vagina. Cells were collected and transferred to slides, air dried, and submerged in methanol fixative followed by eosin and methylene blue staining (Harleco Hemacolor, Philadelphia, PA). Cells were then enumerated by counting the differential percentage of each cell type out of 100 cells. Estrus cycle stage was identified according to specifications reported in Byers et al, 2012.[28]

3.4. Evaluation of post-implantation losses

Mice raised in the QF facility were divided into control and treated groups (n=9-10). Control mice were kept in the QF facility and provided undosed gel food. Treated mice were moved to the QAC facility

and provided 120 mg/kg/day 6.76% ADBAC+ 10.1% DDAC in gel food for 8 weeks prior to breeding and throughout gestation. On GD10, females were euthanized by CO_2 inhalation. Mid-gestational embryos were dissected out of the uterus and evaluated. Embryonic resorptions were characterized by the absence of normal embryonic tissue and abnormally sized and shaped decidual tissue. All viable embryos were staged by GD, somite count, branchial arches, extent of heart and limb bud formation, and deepening of the lens pit.

3.5. Sperm collection

Mice raised in the QF facility were divided into control and treated groups (n=10-11). Control mice were kept in the QF facility and provided undosed distilled water. Treated mice were moved to the QAC facility and provided 120 mg/kg/day 6.76% ADBAC+ 10.1% DDAC in distilled water for 8 weeks. Epididymal sperm were collected following Wang et al., 2003.[30] Briefly, males were euthanized by CO_2 inhalation, both cauda epididymes excised and placed in a 35x10 mm Petri dish (Falcon, Oxnard, CA) containing one mL of pre-warmed Dulbecco's Modified Eagle's Medium (DMEM: sterile filtered 1000 mg/L glucose and sodium bicarbonate, pyridoxine HCl with no L-glutamine or phenol red) supplemented with 1 % fetal bovine serum (GIBCO, Grand Island, NY). Cauda epididymes were minced with a number 11 disposable sterile surgical blade (Feather Safety Razor Co., LTD, Osaka, Japan), mixed with a pipette, and incubated at 37°C with 5% CO_2 for 10-15 minutes to release contents. A 500 µL aliquot of cauda epididymal extract was transferred to a 1.5 mL conical tube (Falcon, Oxnard, CA) and diluted to 1.5 mL with pre-warmed DMEM supplemented with 1 % fetal bovine serum.

3.5.1. Hemocytometer sperm counts

A 500 µL aliquot of cauda epididymal extract was diluted 10X with distilled deionized water to inhibit sperm motility. A 10 µl aliquot of the sperm suspension was loaded onto both sides of a Neubauer

hemocytometer (American Optical, Buffalo, NY) and counted twice. Fully intact sperm within five primary squares of the counting chamber were totaled. Duplicate counts were averaged for each sample.

3.5.2. Computer-automated sperm analysis (CASA)

Duplicate 3μL sample extracts were analyzed using 20 μm-deep 4-chamber Leja slides (Minitube of America, Verona, WI). Slides were allowed to settle for 2-3 minutes before analysis. Eight random fields were analyzed with 20X phase-contrast at 30 frames per second (Model BX41, Olympus Optical, Tokyo, Japan) using Sperm Vision 3.5 (Minitube of America, Verona, WI) to calculate sperm concentration and motility. Sperm following a non-linear, linear, or curvilinear swimming pattern were classified as motile, while non-swimming sperm were classified as immotile.

3.5.3. Sperm viability

SYBR-14 and propidium iodide (PI) were used to assess sperm membrane integrity and viability. Cauda epididymal extracts (300 μL) were treated with 2 μL of 2.4 mM PI and 2 μL of 10 μM SYBR-14.[30] Samples were incubated for 15 minutes prior to flow cytometric analysis with a Coulter EPICS XL-MCL benchtop analyzer with a 488 nm excitation source (Indianapolis, IN). Gates were set according to forward and side scatter characteristics of mouse sperm, as published in Garner et al., 1995.[230] Proportions of PI (dead), dual (moribund), and SYBR-14 (live) stained sperm were used to characterize membrane integrity and viability.

3.6. Statistics

Statistical analysis was conducted using Statistix (Tallahassee, FL). Data are expressed as the mean ± SEM. Data normality was determined using normal probability plots and compared using the Student *t*-

test for normally distributed data or two-sample rank testing for data not distributed normally. Significance was set at $p \leq 0.05$).

4. RESULTS

4.1. Ovulation and implantation

The number of CL in females exposed to ADBAC+DDAC for 2 weeks (15.7 ± 1.2) was not significantly different from controls (14.2 ± 1.6; Figure 2A). A subsequent study with increased sample size and a longer 8 weeks dosing period, found decreased numbers of CL in mice exposed to 120 mg/kg/day ADBAC+DDAC. Females exposed to ADBAC+DDAC for 8 weeks had 12.7 ± 0.9 CL while controls had 19 ± 1.2 CL ($p < 0.001$; Figure 2B).

Mice exposed to ADBAC+DDAC for 2 weeks implanted 58.8 ± 19.1% of ovulated oocytes, while 80.1 ± 8.5% of oocytes implanted in the control group. After 8 weeks of exposure, embryos of mice exposed to ADBAC+DDAC implanted at 61.1 ± 13.6%, compared to 77.2 ± 5.9% in controls (Figure 3A-B). There were no significant differences in the percentage of successful implantations between control, and ADBAC+DDAC exposed mice in either the 2, or 8 week exposure periods ($p > 0.05$). Implantations in exposed mice, however, exhibited significant variability compared to controls with the standard error of oocytes that implanted double that of controls. Total implantation failure (percent implantation of 0-30%) was confined to mice in the ADBAC+DDAC exposure groups and contributed to the large variability in implantation rate noted with the 2 and 8 week exposures.

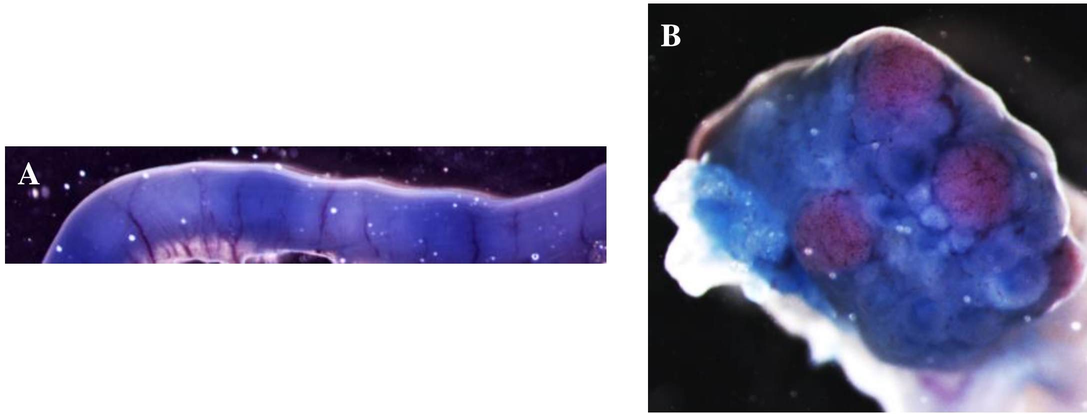


Figure 1. Control mouse uterus. A. Blue bands are indicative of implantation sites, visualized by intravenous Pontamine blue injection. B. Pink follicles represent corpora lutea or the number of oocytes ovulated.

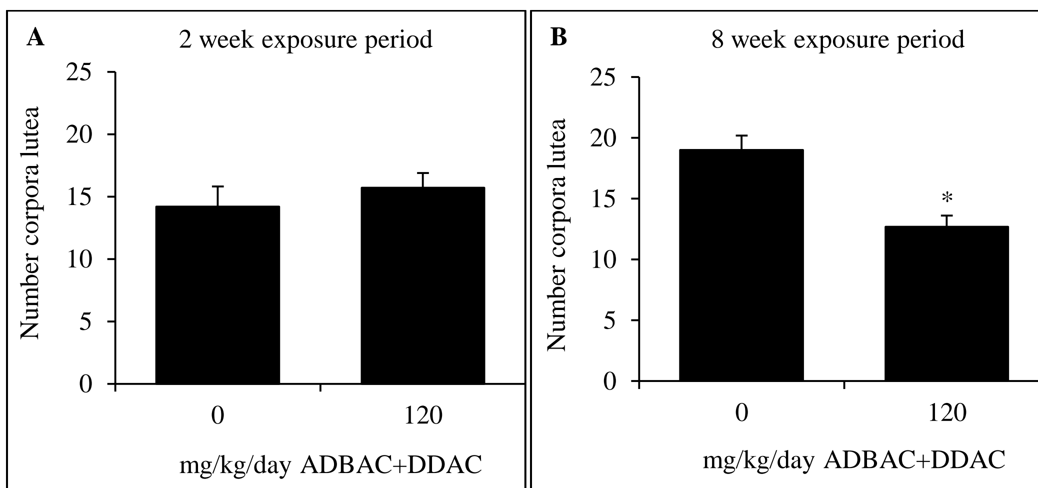


Figure 2. Numbers of corpora lutea. Mice were exposed for 2 and 8 weeks and throughout breeding, up to the day of evaluation on GD6. A. The number of corpora lutea in females exposed to ADBAC+DDAC did not significantly differ from controls after 2 weeks of ADBAC+DDAC exposure ($p>0.05$; t-test). B. After 8 weeks of exposure to ADBAC+DDAC, however, the number of corpora lutea was significantly lower compared to controls ($p<0.001$; t-test).

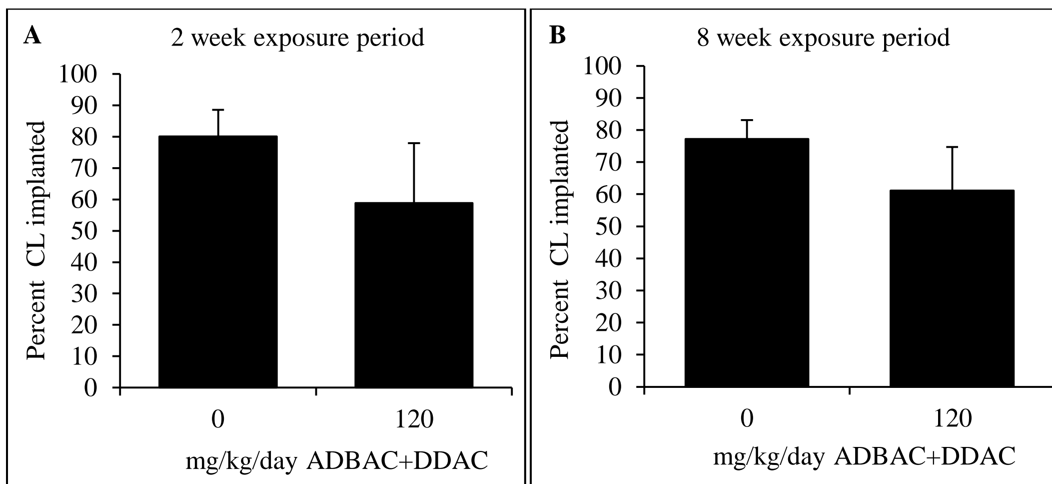


Figure 3. Percent of corpora lutea implanted. Mice were exposed for 2 and 8 weeks and throughout breeding, up to the day of evaluation on GD6. The number of corpora lutea and implantation sites were visualized on gestational day six by intravenous injection of Pontamine blue dye. C. The percent of successful implantations, or proportion of ovulated oocytes that implanted, was not significantly different between controls and females exposed to 120 mg/kg/day ADBAC+DDAC for 2 weeks ($p>0.05$, t-test). D. The percent of successful implantations was not significantly different between controls and females exposed to 120 mg/kg/day ADBAC+DDAC for 8 weeks ($p>0.05$, t-test). Females exposed to ADBAC+DDAC for 2 and 8 weeks exhibited considerable variation in implantation patterns.

4.2. Estrus cycling

Vaginal cytology was evaluated in mice maintained on ADBAC+DDAC for 2 weeks and through the 20 day evaluation period (5 weeks total). The 20 day monitoring period translates to approximately four heat cycles (Figure 4). Females exposed to ADBAC+DDAC progressed through fewer estrus cycles compared to controls ($p=0.009$; Figure 5).

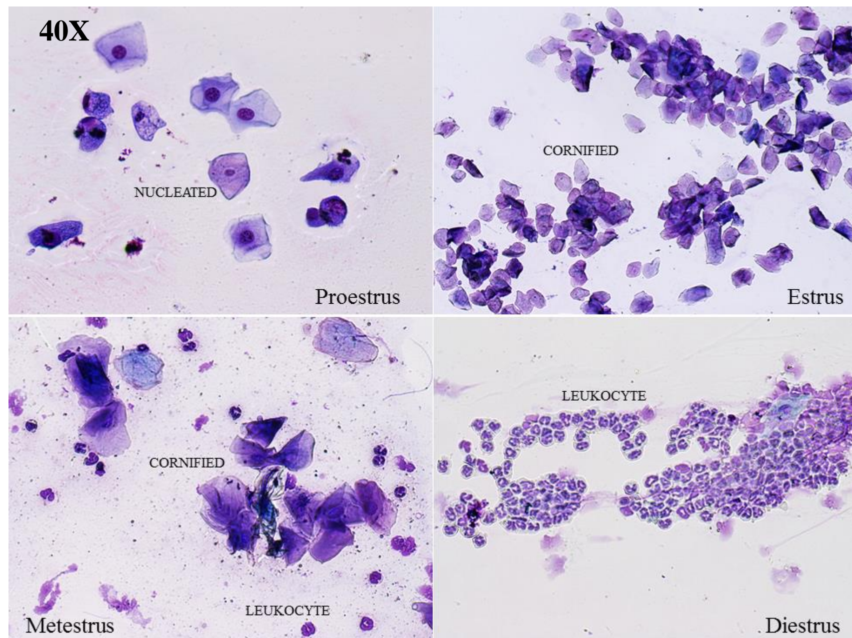


Figure 4. Examples of vaginal cytology in control dams during different stages of estrus, labeled with the predominant cell type and estrus stage (40X magnification). Cells were transferred to a glass slide, fixed with methanol then stained with eosin and methylene blue. Nucleated epithelial cells predominate during proestrus, whereas cornified cells are apparent in estrus. A combination of leukocytes and cornified epithelial cells are present during metestrus, and by diestrus cornified epithelial cells have cleared and leukocytes are the predominant cell type.

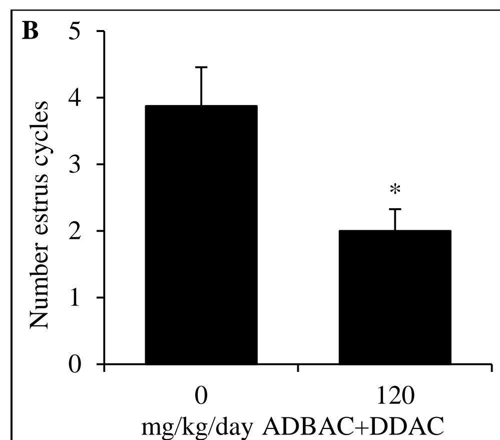


Figure 5. Average time spent in estrus as determined by vaginal cytology with ADBAC+DDAC exposure. Females exposed to ADBAC+DDAC for 2 weeks progressed through fewer estrus cycles compared to controls (p=0.009, t-test; n=8/treatment).

4.3. Post-implantation loss

To determine if pre-natal and in-utero exposure to ADBAC+DDAC induced post-implantation embryonic death, numbers of resorptions and viable embryos were assessed mid-gestation. Significantly fewer viable mid-gestation embryos were observed in mice exposed for 8 weeks to 120 mg/kg/day ADBAC+DDAC compared to controls (p=0.002); however, there was no significant difference between resorptions with ADBAC+DDAC exposure (p=0.6, Figure 6A-B).

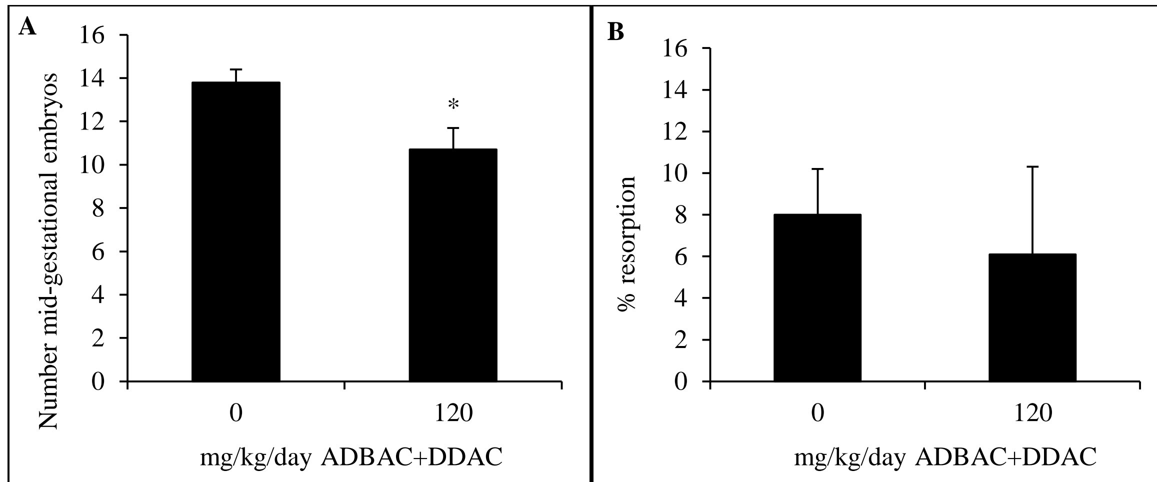


Figure 6. The average number of mid-gestational embryos in control (0 mg/kg/day) and mice treated with a QAC disinfectant at 120 mg ADBAC+DDAC/kg/day for 8 weeks. A. ADBAC+DDAC exposure significantly reduced the number of viable embryos (p=0.002, t-test); B. The average percent per litter of resorbed embryos in control (0 mg/kg/day) and mice treated with a QAC disinfectant at 120 mg ADBAC+DDAC/kg/day for 8 weeks (n=10/group). The number of resorptions was not significantly different between groups (p=0.6, t-test; n=10/treatment).

4.4. Sperm assessment

The effect of ADBAC+DDAC on male reproductive parameters was assessed through sperm counts, motility, and viability analysis.

4.4.1. Hemocytometer counts

Males exposed to 120 mg/kg/day ADBAC+DDAC for 8 weeks had significantly reduced sperm counts (9.7 ±1.0 x 10^6 sperm/mL) compared to control males (17.2 ± 2.2 x 10^6 sperm/mL; p=0.007, Figure 7).

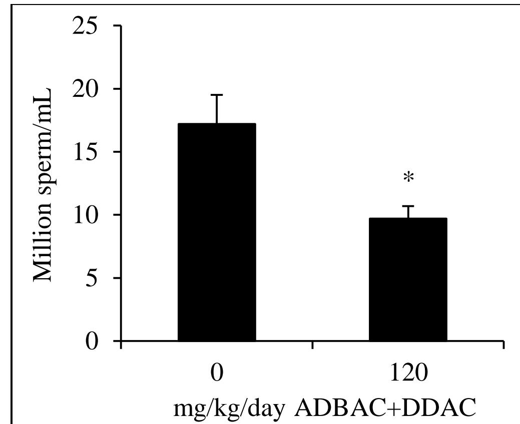


Figure 7. Hemocytometer counts of epididymal extracts in males exposed for 8 weeks to 120 mg/kg/day ADBAC+DDAC. ADBAC+DDAC exposure decreased sperm concentration with hemocytometer counts significantly in exposed males compared to control males , at 9.7 ± 1.0 7 x 10^6 and 17.2 ± 2.2 x 10^6 sperm/mL, respectively (p=0.007, t-test; n=10).

4.4.2. Computer-automated sperm analysis (CASA)

Cauda epididymal sperm extracts were further evaluated using CASA. Control males had significantly higher sperm concentrations (30.1 ±2 x 10^6 sperm/mL) compared to mice exposed to 120 mg/kg/day ADBAC+DDAC for 8 weeks (11.3 ±1.2 x 10^6 sperm/mL; p<0.001, Figure 8A). Sperm motility was also significantly lower in the ADBAC+DDAC exposed mice with 20.9 ± 4.1% motile sperm compared to controls with 41.6 ± 2% motility (p<0.001, Figure 8B).

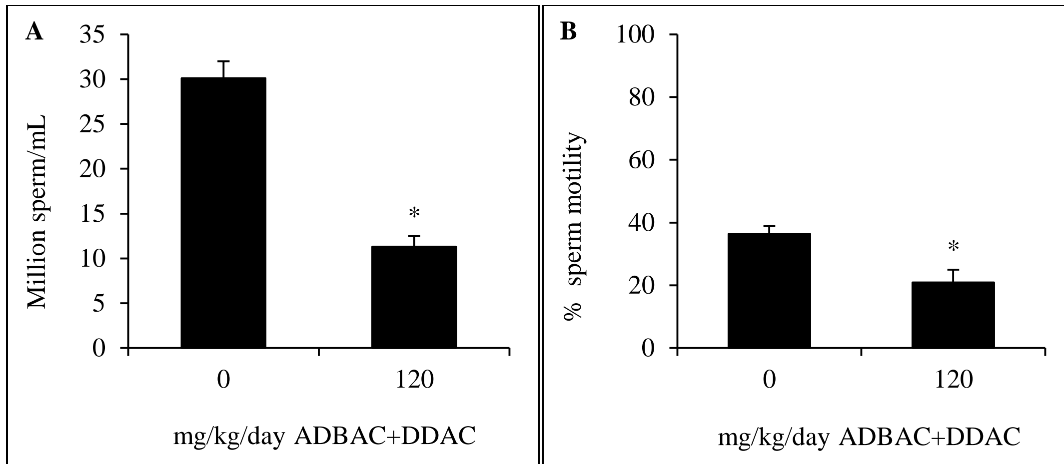


Figure 8. CASA determination of sperm numbers in control mice and mice exposed to 120 mg/kg/day ADBAC+DDAC for 8 weeks (n=4-5). A. Mice exposed to ADBAC+DDAC treatment had the lowest sperm numbers (t-test, $p<0.001$) B. Significantly fewer sperm were motile ($20.9 \pm 4.1\%$) in mice exposed to 120 mg/kg/day ADBAC+DDAC for 8 weeks compared to controls ($41.6 \pm 2\%$; t-test, $p<0.001$).

4.4.3. Sperm viability

Flow cytometric analysis of dual-stained sperm did not identify a significant difference in the proportions of in live, dead and moribund sperm between control and mice dosed with 120 mg ADBAC+DDAC/kg/day for 8 weeks; $p>0.05$, Figure 9A-B).

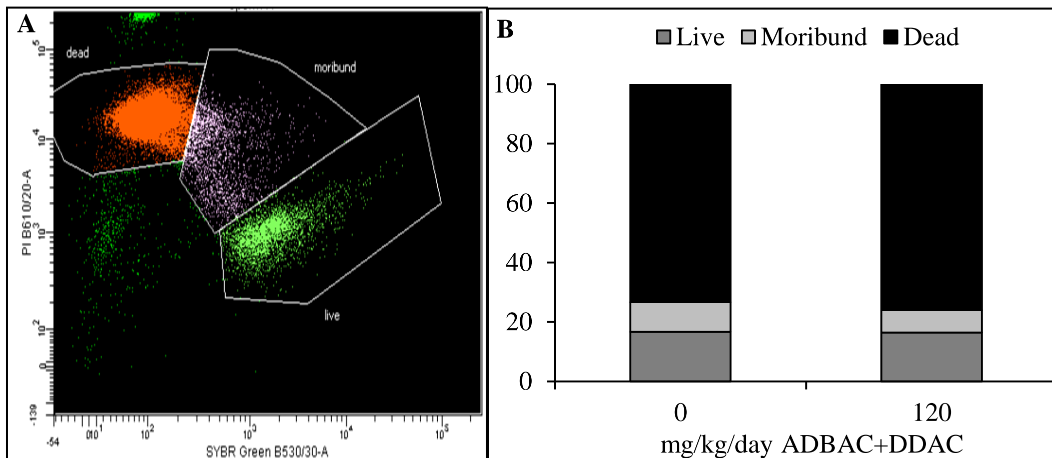


Figure 9. Sperm viability in control and ADBAC+DDAC treated males. A. Flow cytometric gating parameters using dual-staining (PI, SYBR-14) to evaluate sperm viability. B. There were no significant differences in sperm viability between control and mice dosed with 120 mg ADBAC+DDAC/kg/day for 8 weeks ($p< 0.05$, Kruskal-Wallis; n=8-12/treatment).

5. DISCUSSION

QACs are registered as pesticides under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA). As antimicrobials, QACs are extremely effective and are among the most common disinfectants used to prevent microbial growth and contamination in commercial and industrial settings. A survey investigating cleaning activities in commercial food production, storage, and preparation facilities reported that QAC sprays were the most heavily used disinfectants.[31] Additionally, QACs are common preservatives in a wide range of personal care products such as shampoos, cosmetics, and baby wipes. Despite being in use for over 50 years, little is known about QAC toxicity. Mouse breeding pairs exposed over a 6-month period to ADBAC+DDAC exhibited significantly reduced fertility and fecundity; ADBAC+DDAC mice had fewer pregnancies, took longer to get pregnant, and had smaller litter sizes compared to controls.[26] Although these results clearly demonstrate that exposure to ADBAC+DDAC affected reproduction in mouse breeding pairs, the study design did not allow the distinction to be made between toxic effects on the dam, sire, fetus or a combination of effects.

The current study demonstrates that ADBAC+DDAC exposure is toxic to both male and female fertility. Female mice exposed to ADBAC+DDAC exhibited decreased reproductive capacity with decreased ovulation and fewer estrus cycles. Male mice exposed to ADBAC+DDAC exhibited significantly decreased sperm concentration and motility. While the reproductive effects of ADBAC+DDAC have been characterized in this study, the specific mechanism(s) by which ADBAC+DDAC disrupt female and male reproduction remains to be identified.

Female reproduction is dependent on normal ovarian function, since estrus cyclicity and oocyte maturation and release are regulated primarily by hormones secreted by the ovary. Gonadotropic luteinizing hormone (LH) and follicle stimulating hormone (FSH) regulate ovarian prolactin, estradiol, and progesterone secretion and all are required for follicular maturation and subsequent ovulation.

Agonism/antagonism of endogenous hormone receptors by exogenous chemicals results in an excess of hormonally active compounds, leading to abnormal production, transport, metabolism, or secretion of endogenous hormones. Hormonal contraceptives, typically a combination of estrogen and progesterone, deliberately disrupt the endocrine axis to inhibit follicular maturation and ovulation; hormonal contraceptives suppress production of FSH and LH through negative feedback inhibition to prevent ovulation. The efficacy of hormonal contraceptives is dependent on the fact that estrogen-mediated processes are sensitive to perturbation from exposure to exogenous hormones or hormonally active ED chemicals.

A number of environmentally ubiquitous compounds have been identified as estrogenic EDs: the plasticizer bisphenol A, laundry and dish detergent nonylphenol, isoflavone genistein, and mycotoxin zearalenone. Inadvertent exposure to EDs through environmental contamination has increased concern over the effects that these compounds may have on human reproductive function. Estrus cycle irregularities and decreased ovulatory capacity have been observed in rodents administered common environmental ED contaminants bisphenol A, nonylphenol, genistein, and zearalenone.[32-35] In the current study, females exposed to ADBAC+DDAC demonstrated significantly decreased ovulatory capacity, spent less time in estrus, and progressed through fewer estrus cycles compared to controls. It is, therefore, conceivable that ADBAC+DDAC reduced estrus length and frequency through disruption of estrogen-regulated processes. In our study, ADBAC+DDAC treated mice had significantly fewer estrus cycles over the evaluation period. This correlates directly with our 6 month breeding trial which observed significantly fewer litters produced in ADBAC+DDAC treated mice.[26] These two findings support the notion that ADBAC+DDAC disrupt reproductive function in female mice.

Variations in the individual responsiveness to toxicants may be due to dose-time differences, differences in endocrine status, and/or differences in metabolism and excretion of chemicals.[37] Considerable variation in the proportion of implantation sites to corpora lutea was observed in ADBAC+DDAC exposure groups, but not in controls. Both ADBAC+DDAC exposure groups exhibited

standard errors that were double that of controls and this large variance likely contributed to the lack of significance between treated and control pregnancies. This large error was the result of total implantation failure seen only in the ADBAC+DDAC treatment group. Exclusion of dam data exhibiting >90% implantation failure in ADBAC+DDAC exposure groups, indicates that the percent of CLs implanted between control and ADBAC+DDAC exposure groups is not significantly different in the absence of total implantation failure. For this reason, the adverse effects of ADBAC+DDAC on implantation should not be disregarded as biologically insignificant.

Implantation failure is a significant form of infertility in the human population. According to a 2012 report released by the United States Centers for Disease Control and Prevention, utilization of assisted reproductive technologies (ART) has doubled in the last decade, suggesting that rates of human infertility are increasing.[38] Increasing rates of infertility over time suggests an environmental component. For example, implantation failure during ART procedures has been positively correlated with urinary bisphenol-A concentrations. Furthermore, at blood serum concentrations below the populace average, the pesticide hexachlorobenzene was correlated with implantation failure in women undergoing IVF.[39] The relationship between recurrent implantation failure and environmental exposures is complex; however, the effects of environmental exposures on reproduction are important factors to consider in the search for why human rates of infertility have increased over time.

Early embryonic losses can be caused by hormonal disturbances, alterations of the in-utero biochemical environment, and/or by direct embryotoxic effects.[40-43] Mid-gestation embryos are susceptible to post-implantation death, due to the rapid cellular proliferation and growth during gastrulation and neurulation, which occur between GD5-10 in the mouse. Cell injury during these periods would be propagated and likely disrupt further embryonic development and survival, leading to post-implantation embryo losses and resorption. Percent embryonic resorption among ADBAC+DDAC exposed dams were not significantly increased compared to controls. The considerable frequency of total

implantation failure in the ADBAC+DDAC exposed dams, however, precludes observation of the effects of these compounds on embryonic resorption.

Male germ cells are susceptible to damage from environmental exposures. Over the last 70 years, the average sperm concentration in males has decreased by a third from 113 million sperm per mL to 49.9 million.[16,17] More than 90% of male infertility cases are due to low sperm counts and/or poor sperm quality.[39] EDs, such as synthetic estrogenic compounds, are suspected of playing a large role in the rising rates of male infertility. Zearalenone-induced reduction in sperm parameters has been observed in male rodents and included decreased sperm concentration and abnormal sperm morphology.[45] Epidemiological studies evaluating the effects of occupational exposure to bisphenol-A on male reproduction indicated that men with elevated blood/urinary levels of the ED plasticizer had abnormal sperm/semen parameters.[46] Urinary concentrations of genistein have also been correlated with idiopathic male infertility and reduced sperm parameters in Chinese men.[47] In our study, male mice exposed to ADBAC+DDAC had significantly reduced sperm concentrations and motility, but viability was not affected. This suggests that these compounds have a specific target within the male reproductive system. Disruption of estrogen concentrations induces irregular epididymal fluid reabsorption and results in decreased sperm concentrations. Additionally, this process has been shown to decrease sperm concentration and motility, but not sperm viability.[49] Sperm viability was not affected by ADBAC+DDAC exposure. Endocrine dysregulation resulting in viable yet decreased sperm counts, therefore, poses a possible mechanism by which ADBAC+DDAC exerts toxic effects on male mouse fertility.

EDs represent a diverse class of both synthetic and natural compounds such as plasticizers, organochlorine pesticides, flame retardants, industrial solvents, detergents, and phytoestrogens. A molecule's structure can be used to predict its potential to bind androgen and estrogen receptors and estimate its ED behavior; however, EDs are difficult to identify simply based on structure, since EDs disrupt endocrine systems through mechanisms other than receptor binding. It is possible that

ADBAC+DDAC decrease mouse fertility through an endocrine disrupting mechanism; however, other mechanisms can also result in decreased fertility. Chemically reactive compounds or compounds that emulate endogenous molecules are capable of interfering with enzyme systems or signaling pathways which can result in altered cellular homeostasis. Additionally, reproductive toxicants can alter the metabolism or excretion of compounds required for reproductive system maintenance. For example, the plasticizing agent mono-(2-ethylhexyl) phthalate suppresses aromatase action through activation of peroxisome proliferator-activated receptors, ultimately leading to related changes in gene expression in cultured granulosa cells.[49] Reproductive toxicants can also be metabolically activated and change pharmacodynamic and/or pharmacokinetic responsiveness of the exposed individuals. One example is the toxic metabolite of the herbicide molinate which induces severe spermatogenic and testicular damage.[50]

6. CONCLUSIONS

Results of this study support previous observations that QACs ADBAC+DDAC reduce fertility in mice. Exposure to ADBAC+DDAC QACs reduced breeding capacity through disturbances in ovulatory capacity and estrus cyclicity in females and reduction in sperm concentration and motility in male mice. Further experiments are underway to clarify the mechanisms by which ADBAC+DDAC reduce reproductive function in both male and female mice. Given the widespread use of products containing ADBAC+DDAC, the potential risk of endocrine disruption in humans from these compounds should be evaluated further.

Acknowledgments

We wish to thank Caroline Shea for technical assistance. This research was funded in part by the Passport Foundation and the VA-MD Regional College of Veterinary Medicine.

REFERENCES

[1] Kraft, M. & Vogel, E. [Rapid determination of benzalkonium chloride in eye- and nosedrops of the SR86]. Die pharmazie 42, 860-861 (1987).

[2] Yee, R. W., Norcom, E. G. & Zhao, X. C. Comparison of the relative toxicity of travoprost 0.004% without benzalkonium chloride and latanoprost 0.005% in an immortalized human cornea epithelial cell culture system. Advances in therapy 23, 511-519 (2006).

[3] Ford, M. J., Tetler, L. W., White, J. & Rimmer, D. Determination of alkyl benzyl and dialkyl dimethyl quaternary ammonium biocides in occupational hygiene and environmental media by liquid chromatography with electrospray ionisation mass spectrometry and tandem mass spectrometry. Journal of chromatography. A 952, 165-172 (2002).

[4] Pisella, P. J. et al. Conjunctival proinflammatory and proapoptotic effects of latanoprost and preserved and unpreserved timolol: an ex vivo and in vitro study. Investigative ophthalmology & visual science 45, 1360-1368 (2004).

[5] Liang, H., Baudouin, C., Labbe, A., Riancho, L. & Brignole-Baudouin, F. Conjunctiva-associated lymphoid tissue (CALT) reactions to antiglaucoma prostaglandins with or without BAK-preservative in rabbit acute toxicity study. PloS one 7, e33913, doi:10.1371/journal.pone.0033913 (2012).

[6] Tao, W. Field leaching of alkaline copper quaternary-treated red pine lumber over 3 years: long-term dynamics. Water science and technology: a journal of the international association on water pollution research 69, 1475-1481, doi:10.2166/wst.2014.045 (2014).

[7] Miyauchi, T., Mori, M. & Ito, K. Quantitative determination of benzalkonium chloride in treated wood by solid-phase extraction followed by liquid chromatography with ultraviolet detection. Journal of chromatography. A 1095, 74-80, doi:10.1016/j.chroma.2005.07.112 (2005).

[8] Cybulski, J. et al. Long-alkyl-chain quaternary ammonium lactate based ionic liquids. Chemistry 14, 9305-9311, doi:10.1002/chem.200800973 (2008).

[9] Mezger, E., Wendler, O., Mayr, S. & Bozzato, A. Anaphylactic reaction following administration of nose drops containing benzalkonium chloride. Head & face medicine 8, 29, doi:10.1186/1746-160X-8-29 (2012).

[10] Huang, C., Chen, W., Chen, Y. & Liu, Z. [Toxicity research status of benzalkonium chloride on ocular surface]. [Zhonghua yan ke za zhi] Chinese journal of ophthalmology 50, 303-306 (2014).

[11] Berg, O. H., Henriksen, R. N. & Steinsvag, S. K. The effect of a benzalkonium chloride-containing nasal spray on human respiratory mucosa in vitro as a function of concentration and time of action. Pharmacology & toxicology 76, 245-249 (1995).

[12] Corazza, M. & Virgili, A. Airborne allergic contact dermatitis from benzalkonium chloride. Contact dermatitis 28, 195-196 (1993).

[13] Cusano, F. & Luciano, S. Contact allergy to benzalkonium chloride and glutaraldehyde in a dental nurse. Contact dermatitis 28, 127 (1993).

[14] Quirce, S. & Barranco, P. Cleaning agents and asthma. Journal of investigational allergology & clinical immunology 20, 542-550; quiz 542p following 550 (2010).

[15] Centers for Disease Control and Prevention. Statisitics on Reproductive Health of American Women, <http://www.cdc.gov/reproductivehealth/Data_Stats/index.htm> (2012).

[16] Giwercman, A., Carlsen, E., Keiding, N. & Skakkebaek, N. E. Evidence for increasing incidence of abnormalities of the human testis: a review. Environmental health perspectives 101 Suppl 2, 65-71 (1993).

[17] Sharpe, R. M. Sperm counts and fertility in men: a rocky road ahead. Science & Society Series on Sex and Science. European molecular biology organization reports 13, 398-403, doi:10.1038/embor.2012.50 (2012).

[18] Yoon, K., Kwack, S. J., Kim, H. S. & Lee, B. M. Estrogenic endocrine-disrupting chemicals: molecular mechanisms of actions on putative human diseases. Journal of toxicology and environmental health. Part B, Critical reviews 17, 127-174, doi:10.1080/10937404.2014.882194 (2014).

[19] Neltner, T. G., Alger, H. M., Leonard, J. E. & Maffini, M. V. Data gaps in toxicity testing of chemicals allowed in food in the United States. Reproductive toxicology 42, 85-94, doi:10.1016/j.reprotox.2013.07.023 (2013).

[20] Sengupta, P. Environmental and occupational exposure of metals and their role in male reproductive functions. Drug and chemical toxicology 36, 353-368, doi:10.3109/01480545.2012.710631 (2013).

[21] Zhao, Y., Chen, X., Liu, X., Ding, Y., Gao, R., Qiu, Y., Wang, Y. and He, J. Exposure of mice to benzo(a)pyrene impairs endometrial receptivity and reduces the number of implantation sites during early pregnancy. Food and chemical toxicology: an international journal published for the British industrial biological research association 69, 244-251, doi:10.1016/j.fct.2014.04.021 (2014).

[22] Sohoni, P. & Sumpter, J. P. Several environmental oestrogens are also anti-androgens. The Journal of endocrinology 158, 327-339 (1998).

[23] Harrison, P. T., Holmes, P. & Humfrey, C. D. Reproductive health in humans and wildlife: are adverse trends associated with environmental chemical exposure? The Science of the total environment 205, 97-106 (1997).

[24] Reinsberg, J., Wegener-Toper, P., van der Ven, K., van der Ven, H. & Klingmueller, D. Effect of mono-(2-ethylhexyl) phthalate on steroid production of human granulosa cells. Toxicology and applied pharmacology 239, 116-123, doi:10.1016/j.taap.2009.05.022 (2009).

[25] Hunt, P. A., Susiarjo, M., Rubio, C. & Hassold, T. J. The bisphenol A experience: a primer for the analysis of environmental effects on mammalian reproduction. Biology of reproduction 81, 807-813, doi:10.1095/biolreprod.109.077008 (2009).

[26] Melin, V. E., Potineni, H., Hunt, P., Griswold, J., Siems, B., Werre, S. R. and Hrubec, T. C. Exposure to common quaternary ammonium disinfectants decreases fertility in mice. Reproductive toxicology, doi:http://dx.doi.org/10.1016/j.reprotox.2014.07.071.

[27] Dey, S. K. Visualizing early embryo implantation sites by dye injection. CSH protocols 2006, doi:10.1101/pdb.prot4361 (2006).

[28] Byers, S. L., Wiles, M. V., Dunn, S. L. & Taft, R. A. Mouse estrous cycle identification tool and images. PloS one 7, e35538, doi:10.1371/journal.pone.0035538 (2012).

[29] Wang, Y. Epididymal sperm count. Current protocols in toxicology / editorial board, Mahin D. Maines Chapter 16, Unit16 16, doi:10.1002/0471140856.tx1606s14 (2003).

[30] Garner, D. L. & Johnson, L. A. Viability assessment of mammalian sperm using SYBR-14 and propidium iodide. Biology of reproduction 53, 276-284 (1995).

[31] Holah, J. T., Taylor, J. H., Dawson, D. J. & Hall, K. E. Biocide use in the food industry and the disinfectant resistance of persistent strains of Listeria monocytogenes and Escherichia coli. Journal of applied microbiology 92 Suppl, 111S-120S (2002).

[32] Whitten, P. L., Patisaul, H. B. & Young, L. J. Neurobehavioral actions of coumestrol and related isoflavonoids in rodents. Neurotoxicology and teratology 24, 47-54 (2002).

[33] Peretz, J., Gupta, R. K., Singh, J., Hernandez-Ochoa, I. & Flaws, J. A. Bisphenol A impairs follicle growth, inhibits steroidogenesis, and downregulates rate-limiting enzymes in the estradiol biosynthesis pathway. Toxicological sciences: an official journal of the society of toxicology 119, 209-217, doi:10.1093/toxsci/kfq319 (2011).

[34] Willoughby, K. N., Sarkar, A. J., Boyadjieva, N. I. & Sarkar, D. K. Neonatally administered tert-octylphenol affects onset of puberty and reproductive development in female rats. Endocrine 26, 161-168, doi:10.1385/ENDO:26:2:161 (2005).

[35] Nikaido, Y., Yoshizawa, K., Pei, R. J., Yuri, T., Danbara, N., Hatano, T. and Tsubura, A. Prepubertal zearalenone exposure suppresses N-methyl-N-nitrosourea-induced mammary tumorigenesis but causes severe endocrine disruption in female Sprague-Dawley rats. Nutrition and cancer 47, 164-170, doi:10.1207/s15327914nc4702_9 (2003).

[36] Caligioni, C. S. Assessing reproductive status/stages in mice. Current protocols in neuroscience / editorial board, Jacqueline N. Crawley. Appendix 4, Appendix 4I, doi:10.1002/0471142301.nsa04is48 (2009).

[37] Schonfelder, G., Wittfoht, W., Hopp, H., Talsness, C. E., Paul, M. and Chahoud, I. Parent bisphenol A accumulation in the human maternal-fetal-placental unit. Environmental health perspectives 110, A703-707 (2002).

[38] Centers for Disease Control and Prevention. ART Fertility Clinic Success Rates Report. Division of Reproductive Health, National Center for Chronic Disease Prevention and Health Promotion, < http://nccd.cdc.gov/DRH_ART/Apps/FertilityClinicReport.aspx> (2012).

[39] Mahalingaiah, S., Missmer, S. A., Maity, A., Williams, P. L., Meeker, J. D., Berry, K., Ehrlich, S., Perry, M. J., Cramer, D. W. and Hauser, R. Association of hexachlorobenzene (HCB), dichlorodiphenyltrichloroethane (DDT), and dichlorodiphenyldichloroethylene (DDE) with in vitro fertilization (IVF) outcomes. Environmental health perspectives 120, 316-320, doi:10.1289/ehp.1103696 (2012).

[40] Li, B., Liu, H. Y., Dai, L. J., Lu, J. C., Yang, Z. M. and Huang, L. The early embryo loss caused by 2,3,7,8-tetrachlorodibenzo-p-dioxin may be related to the accumulation of this compound in the uterus. Reproductive toxicology 21, 301-306, doi:10.1016/j.reprotox.2005.09.008 (2006).

[41] Tatewaki, R., Otani, H., Tanaka, O. & Kitada, J. A morphological study on the reproductive organs as a possible cause of developmental abnormalities in diabetic NOD mice. Histology and histopathology 4, 343-358 (1989).

[42] Crofts, F., Pino, M., DeLise, B., Guittin, P., Barbellion, S., Brunel, P., Potdevin, S., Bergmann, B., Hofmann, T., Lerman, S. and Clark, R. L. Different embryo-fetal toxicity effects for three VLA-4 antagonists. Birth defects research. Part B, Developmental and reproductive toxicology 71, 55-68, doi:10.1002/bdrb.20001 (2004).

[43] Mahadevaswami, M. P. & Kaliwal, B. B. Evaluation of dimethoate toxicity on pregnancy in albino mice. Journal of basic and clinical physiology and pharmacology 15, 211-221 (2004).

[44] Male Infertility Best Practice Policy Committee of the American Urological, A. & Practice Committee of the American Society for Reproductive, M. Report on optimal evaluation of the infertile male. Fertility and sterility 86, S202-209, doi:10.1016/j.fertnstert.2006.08.029 (2006).

[45] Zatecka, E., Ded, L., Elzeinova, F., Kubatova, A., Dorosh, A., Margaryan, H., Dostalova, P., Korenkova, V., Hoskova, K. and Peknicova, J. Effect of zearalenone on reproductive parameters and expression of selected testicular genes in mice. Reproductive toxicology 45, 20-30, doi:10.1016/j.reprotox.2014.01.003 (2014).

[46] Li, D. K., Zhou, Z., Miao, M., He, Y., Wang, J., Ferber, J., Herrinton, L. J., Gao, E. and Yuan, W. Urine bisphenol-A (BPA) level in relation to semen quality. Fertility and sterility 95, 625-630 e621-624, doi:10.1016/j.fertnstert.2010.09.026 (2011).

[47] Xia, Y., Chen, M., Zhu, P., Lu, C., Fu, G., Zhou, X., Chen, D., Wang, H., Hang, B., Wang, S., Zhou, Z., Sha, J. and Wang, X. Urinary phytoestrogen levels related to idiopathic male infertility in Chinese men. Environment international 59, 161-167, doi:10.1016/j.envint.2013.06.009 (2013).

[48] Hess, R. A., Bunick, D., Lee, K. H., Bahr, J., Taylor, J. A., Korach, K. S. and Lubahn, D. B. A role for oestrogens in the male reproductive system. Nature 390, 509-512, doi:10.1038/37352 (1997).

[49] Lovekamp-Swan, T., Jetten, A. M. & Davis, B. J. Dual activation of PPARalpha and PPARgamma by mono-(2-ethylhexyl) phthalate in rat ovarian granulosa cells. Molecular and cellular endocrinology 201, 133-141 (2003).

[50] Jewell, W. T., Hess, R. A. & Miller, M. G. Testicular toxicity of molinate in the rat: metabolic activation via sulfoxidation. Toxicology and applied pharmacology 149, 159-166, doi:10.1006/taap.1998.8380 (1998).

CHAPTER 4

Disinfectant Compounds ADBAC+DDAC Exhibit Concentration and Temporally Dependent Reproductive Toxicity In-vitro and In-vivo

Vanessa E. Melin[1] and Terry C. Hrubec[1,2]

1) Department of Biomedical Sciences and Pathobiology, VA-MD Regional College of Veterinary Medicine, VA Tech, Blacksburg, VA 24061

2) E. Via College of Osteopathic Medicine Virginia Campus, Blacksburg, VA 24060

To be submitted to *Toxicology in Vitro*

1. ABSTRACT

Quaternary ammonium compounds (QACs) are one of the most common ingredients in industrial and household disinfectant cleaners, and have become additives in a wide range of personal care products, including shampoos, cosmetics, and even baby wipes. The common QAC disinfectant compounds alkyl dimethyl benzyl ammonium chloride (ADBAC) and didecyl dimethyl ammonium chloride (DDAC) cause reproductive disturbances in male and female mice. Male mice exposed to ADBAC+DDAC demonstrated reduced sperm concentration and motility, suggesting that these compounds have a specific target within the male reproductive system. In order to clarify the target site and identify a possible mechanism(s) of action, a series of in-vitro assessments were conducted using mouse Sertoli cells. Sertoli cells constitute the immuno-protective blood-testis-barrier (BTB) that restricts passage of potentially damaging substances into the spermatogenic compartment. Testicular toxicity is often mediated through Sertoli cell damage or disruption of BTB integrity. The effects of ADBAC+DDAC on Sertoli cell metabolism and viability were assessed using resazurin reduction assays. The ability of ADBAC+DDAC to alter BTB permeability, and theoretically enter the spermatogenic compartment, was assessed using a two-compartment culture system. Sertoli cells were seeded onto a support membrane coated with Matrigel extracellular matrix and the effects of ADBAC+DDAC on barrier permeability were assessed by determining changes to transepithelial electrical resistance (TER). Fertilizing capacity of cauda epididymal sperm from male mice exposed to ADBAC+DDAC was also evaluated by in-vitro fertilization. ADBAC+DDAC did not induce significant cytotoxic effects on cell metabolism at 0.0001%; however, significant cytotoxicity was observed at 0.0005, 0.001, 0.005, 0.01, 0.05, and 0.1%. No significant changes in TER were observed at concentrations of 0.001 and 0.0001% ADBAC+DDAC; however significant reductions in TER were observed at 0.01 and 0.1%. In-vitro fertilization assessments indicated that males given a 10-day rest period following ADBAC+DDAC administration demonstrated significantly lower proportions of successful in-vitro fertilization compared to males evaluated

immediately after dosing and to control males. Overall, these results indicate that ADBAC+DDAC cytotoxicity at concentrations above 0.001% correlated with changes in BTB integrity in-vitro. The mechanism by which ADBAC+DDAC affect sperm parameters remains to be elucidated; however, the reduced fertilizing ability of sperm from exposed males given a 10-day rest period may suggest a direct effect on sperm in the testis or indirect effect such as disruption of endocrine function. These findings demonstrate that ADBAC+DDAC target the murine reproductive system and encourage further investigation into the effects of these common compounds on human reproduction.

2. INTRODUCTION

Quaternary ammonium compounds (QACs) are among the most common ingredients in industrial and household disinfectants. QAC toxicity from consumer products are well documented, and include corneal cytotoxicity, allergic rhinitis, contact dermatitis, and occupational asthma.[1-6] Nevertheless, QACs remain prevalent in consumer and industrial products and are considered relatively safe; however, few peer-reviewed studies have evaluated the toxicity of these compounds.

Mouse breeding pairs chronically exposed to 120 mg/kg/day of ADBAC+DDAC demonstrated significantly fewer pregnancies and reduced litter sizes.[7] Subfertility in mice as a result of ADBAC+DDAC exposure was found to be mediated through disturbances in both male and female mouse reproductive function.[8] Specifically, in male mice, ADBAC+DDAC exposure reduced sperm concentration and motility without reducing viability.

Male reproductive disorders have increased over the last 70 years; average sperm numbers have decreased significantly from 113 million to 49.9 million sperm per mL.[9,10] According to World Health Organization guidelines, assessment of sperm count is central to determining male fertility.[11] Normal spermatogenesis and reproductive function are contingent on the function of Sertoli cells, the primary spermatogenic support cells within the testis. It is essential to assess Sertoli cell viability and function when evaluating testicular toxicants. Sertoli cells have been identified as major targets of environmental toxicants such as cadmium, mercury, bisphenol-A, and dioxin.[12,13] Exposure to these toxicants caused Sertoli cell damage and resulted in significant declines in male reproductive function.

Within the seminiferous epithelium of the testis, cell–cell interactions between the Sertoli cells and germ cells manage the process of spermatogenesis. Tight junctions between neighboring Sertoli cells form an integral part of the blood–testis barrier (BTB) that protects developing germ cells from endogenous immunological assault and exogenous toxicants. Toxicants can disrupt inter-Sertoli tight-

junctions which can disrupt the BTB and perturb the germ cell growth environment.[14] Disruption of the BTB results in decreased germ-cell adhesion, leading to germ-cell loss, reduced sperm count, and male infertility.[14] It is, therefore, vital to investigate toxicant-induced changes on Sertoli cell viability and BTB permeability.

An in-vitro two-compartment model using cultured Sertoli cells has been established. This model mimics the BTB as electrical resistance between apical and basal cell compartments can be established and measured.[15-18] Disruption of the BTB is measured by a decrease in resistance when inter-Sertoli junctional integrity is perturbed. The plasticizing agent mono-2-ethylhexyl phthalate (MEHP) has demonstrated significant male reproductive toxicity that is mediated through BTB damage. In-vivo reproductive effects of MEHP have been substantiated in-vitro using the two-compartment BTB model.[16] Decreased sperm parameters resulting from exposure to ADBAC+DDAC in male mice suggest that the BTB may be a target of these toxic effects. The two-compartment in-vitro model can potentially provide useful insight into the effects of ADBAC+DDAC on Sertoli cell function and BTB integrity.

When evaluating male reproductive toxicants, the testis is often considered the primary target; however, a number of chemicals, such as the common environmental contaminant nonylphenol, have been identified as epididymal toxicants.[19] Testicular and epididymal toxicity can be evaluated by histopathology. Post-mortem assessments, however, cannot accurately identify the origin of toxicity to distinguish whether the testis or epididymis was the primary toxicological target. Common endpoints observed in animals treated with epididymal toxicants include alterations in testosterone biosynthesis, abnormal epididymal fluid reabsorption, decreased motility, and significant reductions in the fertility of cauda epididymal sperm.[20-22] Testicular and epididymal processes are androgen-dependent and, therefore, toxicants disrupting Leydig cell metabolism will ultimately have an effect on the function of both organs. Reductions in sperm motility of rats exposed to ethane dimethane sulfonate, a glutathione-dependent alkylating agent, are mediated through Leydig cell toxicity and subsequent reduction of circulating androgen.[23] Exposure to ADBAC+DDAC for 8 weeks decreased motility in cauda epididymal sperm.[8]

The observed decrease in motility may suggest that these QACs target the epididymal maturation process. Epididymal toxicity often manifests secondary to testicular toxicity, making independent identification of epididymal effects difficult. Experimental strategies that focus on epididymal transit time are essential for determining specific toxicant effects. Spermatozoa formed in the testis enter the caput of the epididymis and require approximately 5.5-14.6 days to reach the cauda epididymis where they are stored for ejaculation.[19] Spermatozoa entering the caput epididymis are immature, and since they lack motility, are infertile. During their transit to the cauda, spermatozoa undergo maturation processes, but are not fertile until capacitation within the female reproductive tract. The orderly nature of sperm maturation, therefore, can be utilized to isolate the potential effects of toxicants on the epididymis.

The current study uses a combination of in-vitro and in-vivo experimentation in order to explore the dynamics of ADBAC+DDAC-mediated reproductive toxicity in male mice. Identification of multiple toxicological endpoints related to testicular and/or epididymal function will aid in elucidating the mechanism(s) by which ADBAC+DDAC reduce fertility in male mice. Classifying the effects of ADBAC+DDAC on the male mouse reproductive system in-vitro and in-vivo will aid in identifying the mechanisms by which these chemicals interfere with reproductive processes. These results will help determine if QACS are harmful and potentially contribute to reproductive declines in humans.

3. MATERIALS AND METHODS

3.1. Test Materials

Solutions of ADBAC+DDAC were prepared in sterile culture medium and used for cytotoxicity assessments: benzyl dimethyl hexadecyl ammonium chloride (ADBAC C16; Sigma-Aldrich, St. Louis, MO), benzyl dimethyl tetradecyl ammonium chloride (ADBAC C14; Sigma-Aldrich, St. Louis, MO), benzyl dimethyl dodecyl ammonium chloride (ADBAC C12; Sigma-Aldrich, St. Louis, MO), didecyl

dimethyl ammonium chloride, 80% aqueous solution (DDAC; AK Scientific Inc., Union City, CA). Solutions of ADBAC and DDAC were made based on the ratio of alkyl chain lengths and sum of active ingredients in the QAC disinfectant previously reported by our laboratory which had a composition of 6.76% ADBAC (60% C-14, 25% C-12, 15% C-16) and 10.1% DDAC).[7] A 1% stock solution of ADBAC+DDAC in DMEM-F12 was diluted and used for all cytotoxicity assessments.

3.2. Cell culture reagents

Cell media was prepared in two forms: 1) standard culture KSOM (Millipore, Billerica, MA) supplemented with 1.0 mg/mL bovine serum albumin (BSA) and 2) BSA enhanced KSOM supplemented with 4.0 mg/mL BSA (mKSOM). Two forms of FHM medium (Millipore Corp., Billerica, MA) were also prepared: standard FHM supplemented with 1 mg/ml BSA and mFHM supplemented with 4mg/mL BSA.

3.3. Resazurin reduction assay for cytotoxicity assessments

TM4 mouse Sertoli cells (CRL-1715; ATCC, Manassas, VA) were cultured at 37°C with 5% CO_2. Cells were seeded into 96-well plates at a concentration of 20,000 cells/mL. Plates were incubated for 12 hours (h) at 37°C, with an atmosphere of 5% CO_2 and 95% humidity until all cells evenly adhered. Media was removed and replaced with fresh media or media containing 0.0001%, 0.0005%, 0.001%, 0.005%, 0.01%, 0.05%, or 0.1% ADBAC+DDAC and incubated for 72 h. Mitochondrial metabolism of exposed cells was assessed by adding 20 μL of 0.15 mg/mL resazurin solution (Sigma-Aldrich, St. Louis, MO) to each well.[24] After a four h incubation period, plates were read at 570 and 590 nm using a SpectraMax Plus384 plate reader to determine the extent of resazurin reduction.

3.4. Cell cycle analysis

Cell cycle analysis was completed according to specifications by Pozarowski et al., 2004.[25] Cells were incubated for 24 h (doubling time of TM4 cells is 16 h) with undosed media or media containing cytotoxic concentrations of 0.01% or 0.1% ADBAC+DDAC to determine the phase at which the cell cycle was disturbed. TM4 cell suspensions (10^6 cells/mL) were then pelleted and fixed overnight in cold 70% ethanol. Cells were washed 2X in PBS, and then treated with 100 µg/mL of RNase (Invitrogen, Carlsbad, CA) for 20 min at 37°C. Cells were stained with 200 µL of 50 µg/mL propidium iodide (Invitrogen, Carlsbad, CA) and maintained for 24 h at 4°C before flow cytometric analyses. A FACScan system (Becton Dickinson, Franklin Lakes, NJ) was used to record forward and side scatter characteristics (FSC and SSC, respectively) and fluorescence emissions for a minimum of 1×10^4 cells. Flow cytometric parameters were set to excitation at 488 nm with 15mW argon-laser and emission at 585 nm. All parameters were analyzed using FloJo software (Treestar, Ashland, OR) and the area of each fluorescence peak was used to identify relative number of cell nuclei with respect to DNA copy number.

3.5. Transepithelial electrical resistance (TER) measurements

The two-compartment in-vitro BTB model was established based on Janecki et al., 1992.[17] TM4 cells were plated at a density of 2×10^6 cells/cm^2 into bicameral chambers (12 mm diameter, 0.4 µm pore size, 0.6 cm^2 surface area; Millipore, Bedford, MA) coated with 1:6-1:10 diluted Matrigel mouse extracellular matrix (BD Biosciences, Palo Alto, CA). Cell media was replaced every 2 days (600 and 400 µL of media for the upper and lower chambers, respectively) and maintained to prevent disruption of the cells. Cultures were stabilized at room temperature for 20 minutes before TER measurements. TER measurements were recorded using an EVOM 2 voltmeter equipped with adjustable width double electrodes (World Precision Instruments, Sarasota, FL). Measurements were taken at 24, 48, 72, 96, 120, 144 and 168 h after initial plating to confirm the formation of a complete barrier, indicated by stabilization of TER. After stabilization of TER, media in the upper (apical) chamber was replaced with

media containing 0.0001, 0.001, 0.01, and 0.1% ADBAC+DDAC. TER recordings were done in triplicate at six 24-h intervals (144 h total) to assess the effect of ADBAC+DDAC on barrier permeability. The plasticizing agent phthalic acid mono-2-ethylhexyl ester (MEHP; Sigma-Aldrich, St Louis, MO), a known BTB disruptor, was utilized as a positive control. Final resistance values were calculated by subtracting the mean TER of blank bicameral chambers coated with only Matrigel (no cells), and correcting for the surface area of the bicameral chamber (0.6 cm^2). Values are expressed as Ω*cm^2.

3.6. Husbandry

CD-1 mice were initially purchased from Charles River Laboratories (Raleigh, NC) and bred for at least two generations before being used in experiments. CD-1 mice were maintained in disposable caging (Innovive, San Diego, CA) on a 12-h light/dark cycle at 20 – 25 °C with 30 - 60% relative humidity. Harlan 7013 rodent chow (Harlan Teklad, Madison, WI) and distilled water were provided ad libitum. Control mice were housed in a facility that did not use QAC disinfectants. QAC-exposed mice were housed separately from controls in a facility that utilized QAC disinfectants.

3.7. Sperm collection

Ten male mice were given 30 mg/kg/day ADBAC+DDAC in saline for 7 days via oral gavage, and 10 males received saline vehicle control,. Five males were euthanized after the 7 day exposure and cauda epididymi were collected. Spermatozoa were aspirated from the left and right cauda epididymi in warm FHM medium using a 21-gauge needle. A 10 µL aliquot of the sperm suspension was diluted ten-fold with distilled water to immobilize sperm and counted twice on both sides of a Neubauer hemocytometer (American Optical, Buffalo, NY) for a total of four counts/sample. Only structurally intact sperm within five primary squares of the counting chamber were totaled and duplicate counts were averaged for each sample. Sperm motility was assessed using computer-automated sperm analysis

(CASA). Duplicate 3µL sample extracts were analyzed using 20 µm-deep 4-chamber Leja slides (Minitube of America, Verona, WI). Eight random fields were analyzed with 20X phase-contrast at 30 frames per second (Model BX41, Olympus Optical, Tokyo, Japan) using Sperm Vision 3.5 (Minitube of America, Verona, WI) to calculate sperm motility. Sperm following a non-linear, linear or curvilinear swimming pattern were classified as motile, while non-swimming sperm were classified as immotile.

3.8. In-vitro fertilization

Sperm exhibiting 80-90% motility were adjusted to a concentration of 5x104 sperm and transferred to 250 ul FHM/KSOM medium under 1 mL embryo-tested light mineral oil (Millipore, Bedford, MA) approximately 1 h before the beginning of IVF. Female CD-1 mice were super-ovulated by serial intra-peritoneal injections of pregnant mare serum gonadotropin (PMSG) followed 48 h later by human chorionic gonadotropin (hCG). Approximately 5 IU of each gonadotropin was administered in 100 µL of sterile saline diluent using a 27-gauge needle. Cumulus masses of ovulated eggs (COC) were collected from dam oviducts 12-14 h after hCG injection. After 1-1.5 h of capacitation, 5×10^4 sperm were added to a fertilization dish containing COC and incubated for 4-6 h to fertilize oocytes. All oocytes were then removed from the fertilization dish; any fragmented or irregularly shaped degenerate oocytes were discarded.

3.9. Statistics

Statistical analysis was conducted using Statistix (Tallahassee, FL). Data are expressed as the mean ± SEM. Data normality was determined using normal probability plots. Cell viability and cycle analysis data were evaluated using Kruskal-Wallis. TER measurements were compared using two-way ANOVA to look at the effects of both time and treatment. The effects of ADBAC+DDAC exposure in

sperm concentration, motility, and fertilization rates in unrested and rested male mice relative to controls were evaluated using two-way ANOVA. Significance was set at $p \leq 0.05$.

4. RESULTS

4.1. Resazurin reduction

Sertoli cells treated with vehicle control successfully reduced 96.9 ± 1.9% of the resazurin dye within the 4 h incubation period indicating the cells were metabolically active. TM4 cell metabolism was not affected by 0.0001% ADBAC+DDAC. Significant cytotoxicity was observed when the concentration of ADBAC+DDAC was increased to 0.0005%, with only 62.4 ± 2.6% resazurin reduction. ADBAC+DDAC reduced TM4 cell metabolism in a dose dependent manner. Concentrations of ADBAC+DDAC between 0.01%, 0.05%, and 0.1% exhibited the most significant cytotoxicity, with only 9.9 ± 2.6%, 8.3 ± 2.4%, and 4.6 ± 0.4%, resazurin reduction respectively (Figure 1).

4.2. Cell cycle analysis

Cell cycle distribution was assessed in TM4 cells to assess potential ADBAC+DDAC-induced anomalies in cell division and/or growth. The control group had the greatest proportion of cells, 72.6 ± 1.4%, in the G1 phase of the cell cycle. ADBAC+DDAC at the cytotoxic concentrations of 0.01% and 0.1% significantly reduced the proportion of TM4 cells in the G1 phase of the cell cycle to 57.2 ± 4.4 and 63.2 ± 0.4%, respectively. No significant differences were observed among treatment groups with respect to cell proportions in the S-phase. Cells exposed to 0.1% ADBAC+DDAC demonstrated the greatest proportion of cells in the G2/M phase of the cell cycle at 20.7 ± 1.0%; and was significantly different from controls.

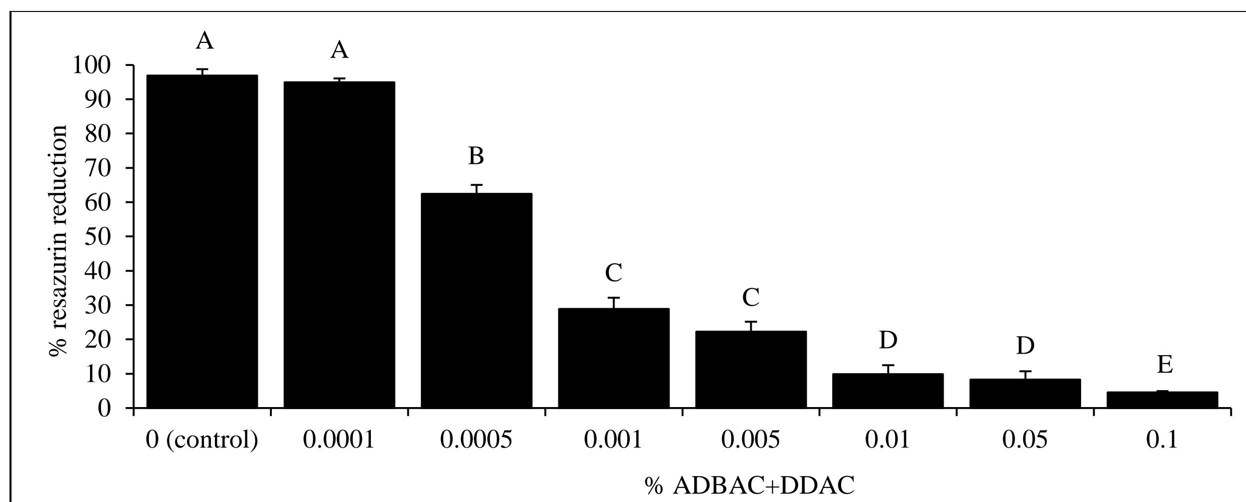


Figure 1. TM4 Sertoli cells treated with ADBAC+DDAC for 72 h exhibited a dose-dependent decrease in mitochondrial metabolism indicating cytotoxicity. Concentrations of ADBAC+DDAC greater than or equal to 0.005% significantly reduced cell metabolism in a dose dependent manner (Kruskal-Wallis, $p<0.05$). Each plate was read at 570 and 590 nm using a SpectraMax Plus384 plate reader to determine the extent of resazurin reduction. Values are means of triplicates ± SEM. Bars with different letters are significantly different (n=6 wells/treatment).

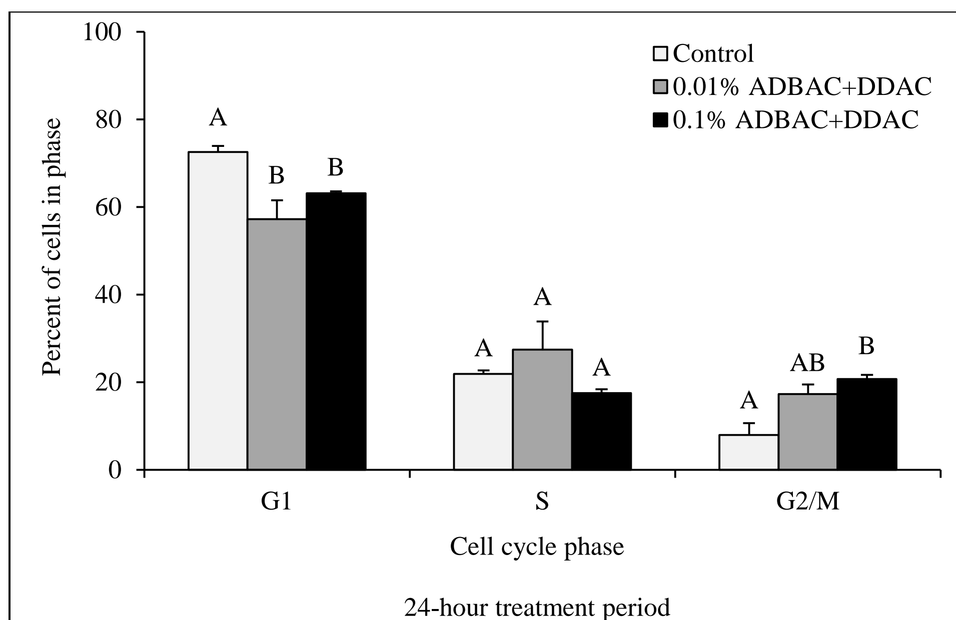


Figure 2. The cell cycle distribution of TM4 Sertoli cells was significantly altered by 24 h exposure to ADBAC+DDAC compared to controls. Cultured cells were staining with propidium iodide and cell cycle distributions determined by flow cytometric analysis. A total of 10,000 events were counted for duplicate samples. Data were analyzed with FlowJo software. Percent of cells in each phase were compared between treatments (Kruskal-Wallis, $p\leq0.05$). Values are means ± SEM. Bars with different letters are significantly different.

4.3. TER measurements

Resistance measurements of TM4 Sertoli cells inoculated onto Matrigel extracellular matrix stabilized after approximately 96 h indicating formation of an intact continuous barrier. There was an overall significant effect of treatment and length of exposure to 1% MEHP on TER, and the interaction between length of exposure and 1% MEHP treatment had significant effects on TER throughout the duration of the experiment (Figure 3A). Treatment with ADBAC+DDAC, length and exposure, and time and treatment interactions each had significant effects on TER overall; the effects of ADBAC+DDAC on TER depended on the concentration of the toxicant as well as the duration of exposure, and the interaction between ADBAC+DDAC concentration and exposure time. Length of exposure significantly affected TER beginning at 24 hours and significant effects of treatment were observed at 48 hours. Significant effects of ADBAC+DDAC treatment and time interaction on TER were observable beginning at 120 hours. Application of ADBAC+DDAC disrupted the BTB, increasing barrier permeability at concentrations above 0.001%. Significant reductions in TER were observed after 96 h treatment with 0.01% and after 24 h with 0.1% ADBAC+DDAC (Figure 3B). ADBAC+DDAC cytotoxicity assessments did not directly correlate with decreases in TER. Cytotoxicity was observed at 0.001% ADBAC+DDAC, but no significant changes in TER were observed at this concentration. Treatment with ADBAC+DDAC at 0.01% and 0.1% was both cytotoxic and caused significant disruption of the BTB.

4.4. Sperm analysis and in-vitro fertilization

Sperm isolated from males treated with 30 mg/kg ADBAC+DDAC for 7 days demonstrated similar concentrations and motility compared to sperm isolated from males given vehicle control. No differences in sperm concentration or motility were observed between sperm isolated immediately following ADBAC+DDAC dosing or those given a 10-day rest period. Additionally, interaction effects

between ADBAC+DDAC and the rest period did not significantly impact sperm parameters (Figure 4A-B).

In order to assess sperm function, in-vitro fertilization rates were calculated for the isolated cauda sperm. Dose effects on in-vitro fertilization rates were not apparent in treated and control mice when sperm was collected immediately after dosing. Only cauda sperm isolated from males given a 10-day rest period following ADBAC+DDAC administration demonstrated significantly lower proportions of successful in-vitro fertilization (Figure 5; ANOVA, $p=0.05$). Figure 6 illustrates that oocytes fertilized with sperm from rested ADBAC+DDAC frequently exhibited unsuccessful fertilization compared to controls.

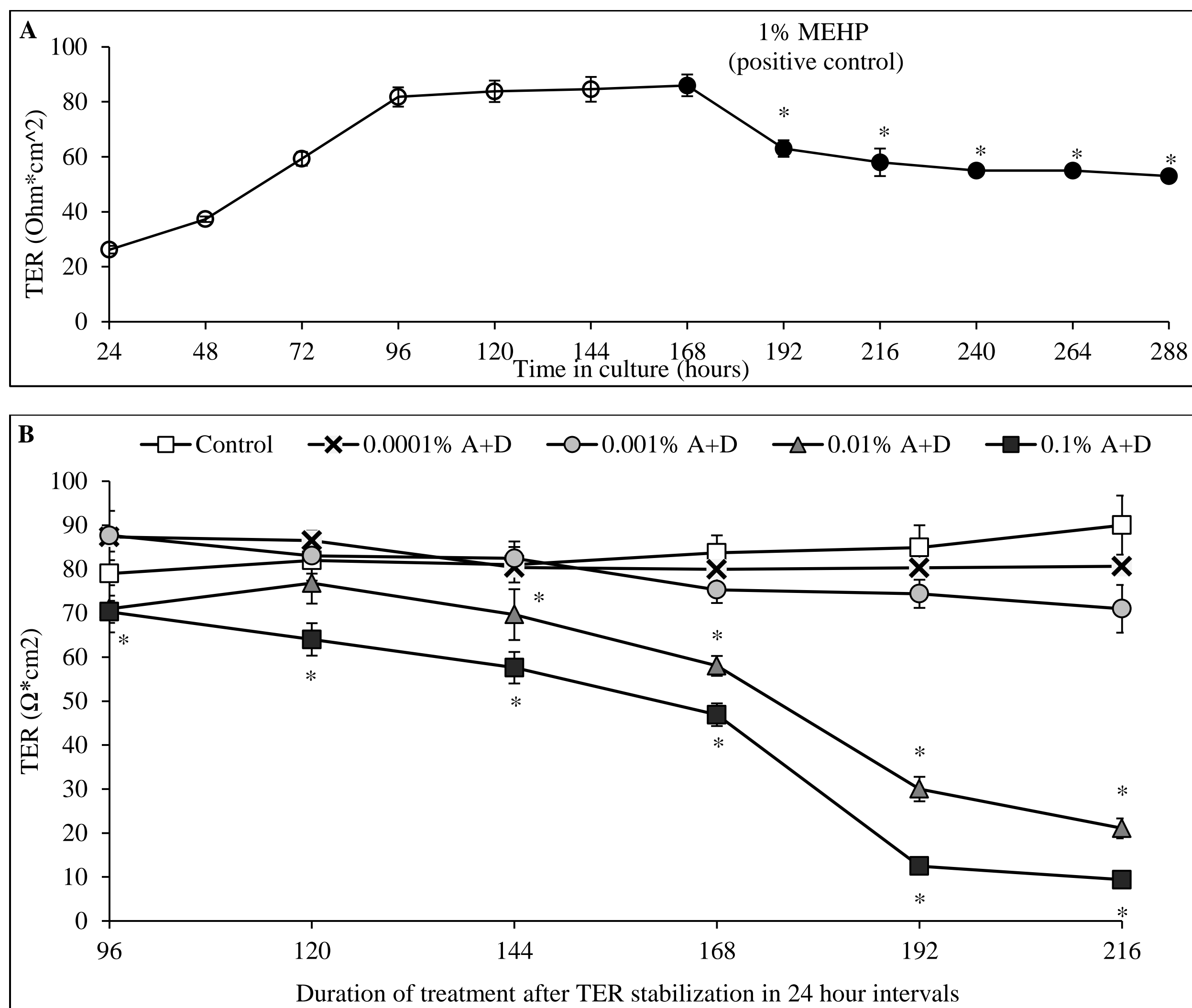


Figure 3. Stabilization of TER after 96 h in culture. TM4 cells were plated at a density of $2{\times}10^6$ cells/cm^2 into bicameral chambers (12 mm diameter, 0.4 µm pore size, 0.6 cm2 surface area; Millipore, Bedford, MA) and coated with 1:6-1:10 diluted Matrigel mouse extracellular matrix. TER measurements were recorded using an EVOM 2 voltmeter equipped with adjustable width double electrodes. Triplicate measurements were taken at 24, 48, 72, 96, 120, 144 and 168 h. Values are means ± SEM. A) Stabilization of TER after 96 h in culture indicated the formation of a continuous barrier. The positive control MEHP reduced TER after 24 h. There was an overall significant effect of treatment and length of exposure to 1% MEHP on TER, and the interaction between length of exposure and 1% MEHP treatment had significant effects on TER throughout the duration of the experiment ($p<0.05$ for treatment, length of treatment, and interaction; Two-way ANOVA). B) The effects of ADBAC+DDAC on TER depended on the concentration of the toxicant as well as the duration of exposure; treatment with ADBAC+DDAC reduced TER in a dose and time-dependent manner. There was an overall significant effect of treatment on TER starting at 24 hours and length of exposure to ADBAC+DDAC significantly affected TER after 96 hours. The interaction between length of exposure and ADBAC+DDAC treatment had significant effects on TER starting at 120 hours ($p<0.05$ for treatment, length of treatment, and interaction; Two-way ANOVA). Significance levels indicate whether TER was significantly altered relative to controls.

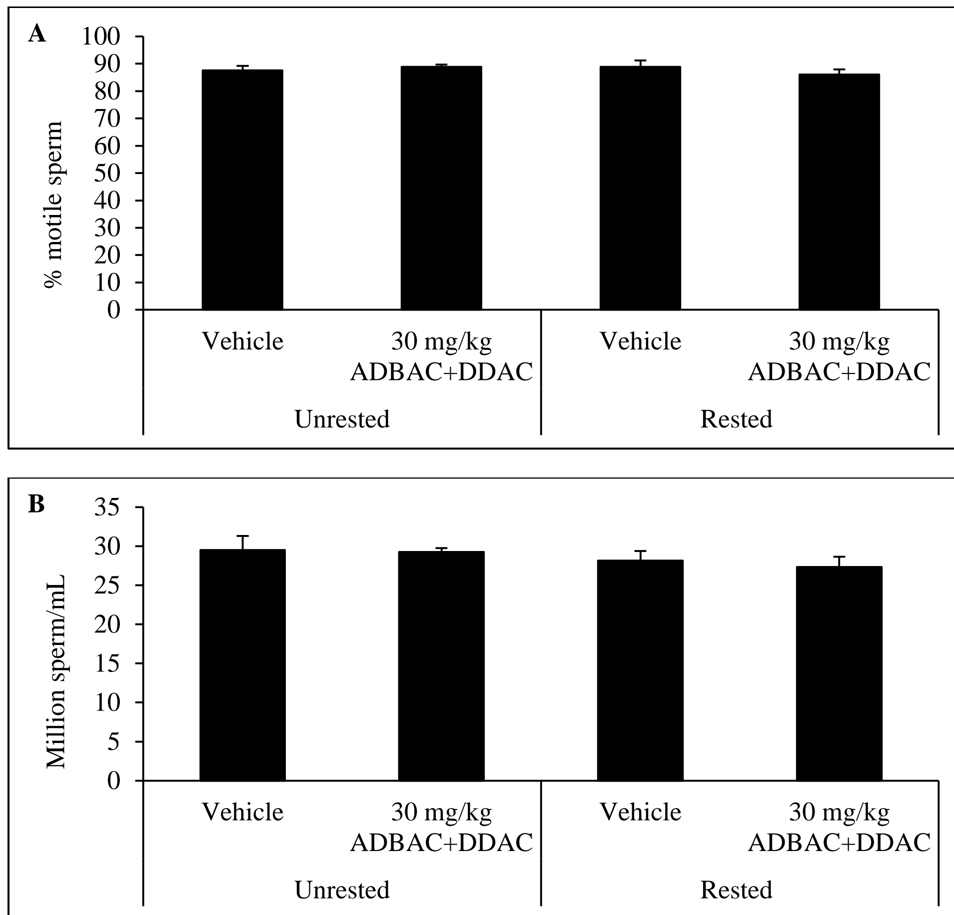
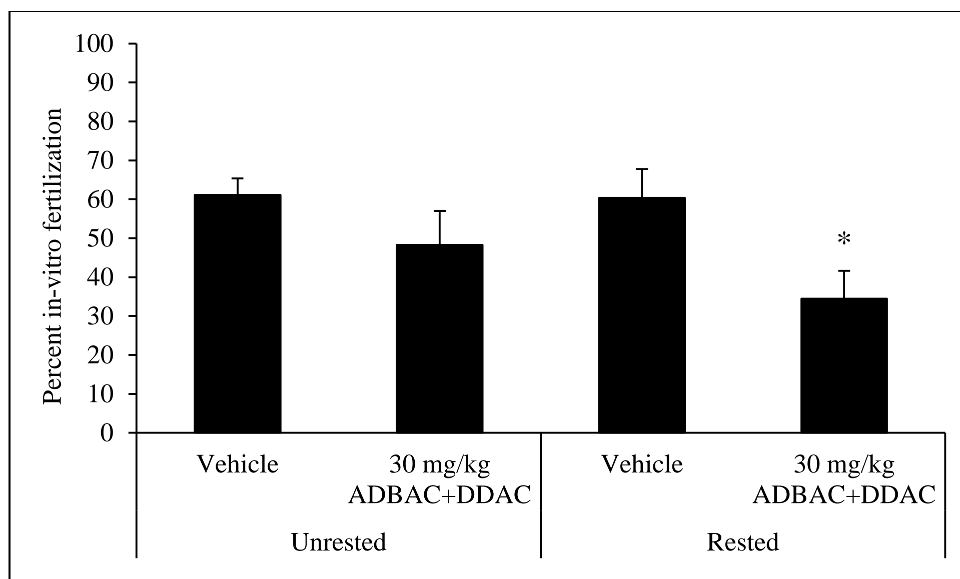


Figure 4a-b. Cauda sperm motility and concentration did not differ in males administered 7-day oral gavage of ADBAC+DDAC compared to males provided vehicle control either when assessed directly or when assessed following a 10 day rest period. Sperm isolated from the cauda epididymis were analyzed using CASA before being used for IVF. No significant effects of treatment, rest period, and treatment*rest period interaction were observed (n=5/treatment; Two-way ANOVA, $p>0.05$). Values are means ± SEM.



Male (n=5/group)	Female (n=5/group)	Total oocytes collected	Total ♂/♀ pronuclei	% IVF (AVG ± SEM)
Unrested control	Control	76	46	61 ± 4
Unrested ADBAC/DDAC	Control	77	37	48 ± 9
Rested control	Control	73	44	60 ± 7
Rested ADBAC/DDAC	Control	70	24	35 ± 7*

Figure 5. Significant dose effects were observed in the fertilizing capacity of sperm from males administered ADBAC+DDAC for 7-days and given a 10-day rest period (n=5/treatment; ANOVA, p=0.05). Cauda epididymal sperm function was assessed in male mice immediately after 7 days of 30 mg/kg ADBAC+DDAC dosing and after a 10-day rest period by in-vitro fertilization. After 4-5 h of exposure to sperm, oocytes demonstrated visible second polar bodies and visible pronuclei formation. By 7-8 h, the fertilized oocytes (zygotes) had clearly formed pronuclei. Values are means ± SEM (n=5/treatment; ANOVA, p>0.05).

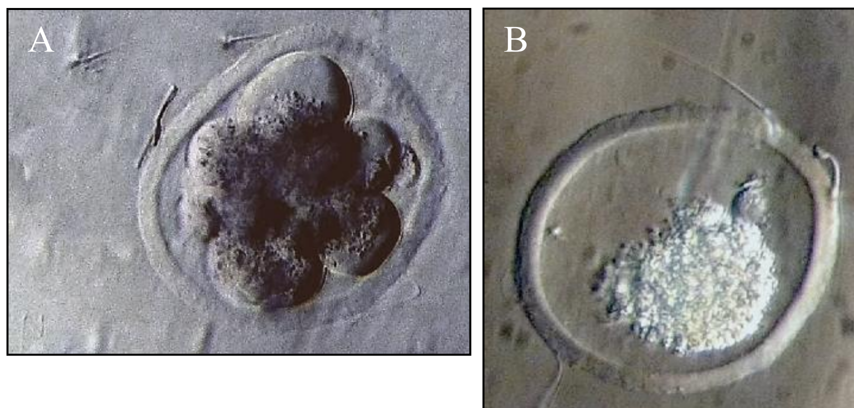


Figure 6. Successful and unsuccessful IVF. A. Morula formation in an oocyte fertilized by rested control sperm. B. Oocyte degeneration following unsuccessful fertilization by rested ADBAC+DDAC sperm.

5. DISCUSSION

ADBAC and DDAC are increasingly combined into commercially available products. Only a few studies have evaluated ADBAC and DDAC toxicity individually and no studies have evaluated the combined effects of ADBAC+DDAC in-vivo even though use of these compounds has resulted in widespread human exposure.[26-31] In-vitro, tissue specific toxicity from ADBAC or DDAC (but not both combined) has been characterized in a few tissues. ADBAC is one of the most common preservatives in eye drop solutions. ADBAC corneal and conjunctival cytotoxicity has been observed in-vitro at concentrations typically present in ophthalmic preparations, ranging from 0.01 to 0.02%.[29-32] ADBAC-induced ocular cytotoxicity has been largely attributed to its surfactant properties and consequent ability to disrupt components of the protective tear film lipid layer at concentrations $> 0.005\%$.[32,33] Spermicidal formulations containing approximately 2% ADBAC (labeled as benzalkonium chloride) are available outside of the United States and have demonstrated mucosal toxicity through induction of inflammatory interleukin release in human vaginal epithelial cell lines.[34] In-vitro assessments of DDAC solution in

mouse lung fibroblasts attributed cytotoxicity to pro-inflammatory effects leading to pulmonary fibrosis and disrupted TGF-beta signaling at concentrations as low as 0.0002%.[35] While these studies identify cytotoxicity from ADBAC and DDAC, many of these effects have not been validated in-vivo. This creates data gaps that prevent translation of chemical risk to exposed human populations.

Testicular toxicity is often mediated through disruption of Sertoli cell and BTB function and/or integrity. Progression of spermatogenesis is dependent on Sertoli-germ cell interactions within an intact BTB.[15] Cadmium chloride, a known testicular toxicant, disrupts reproductive function by targeting the BTB in-vivo and this effect has been confirmed using the in-vitro BTB model.[17] Concentrations of ADBAC+DDAC sufficient to decrease Sertoli cell metabolism were not diametrically associated with modification in BTB permeability. ADBAC+DDAC at a concentration of 0.001% had no effect on BTB permeability, but significantly reduced cell metabolism. This suggests that the Sertoli cells are less susceptible to toxicant damage when they are assembled together into a BTB. Increasing the concentration of ADBAC+DDAC 10-fold to 0.01% significantly reduced cell metabolism and TER. The two-compartment system induces functional polarization of Sertoli cells that coincides with the development of junctional complexes between neighboring cells.[18] It is possible that inter-Sertoli junctions provide a means of protection to the Sertoli cells from the effects of toxicants. ADBAC+DDAC changes in cell metabolism, therefore, may occur in the presence or absence of overt effects on the BTB.

Differences in cell cycle distribution are indicative of toxicant effect on cell growth and division, since damage checkpoints prevent entry into succeeding phases of the cell cycle.[25] Cell cycle analysis indicated that treatment with cytotoxic 0.1% ADBAC+DDAC induced significant increases in the proportions of cells in the G2/M phase and corresponding reductions in the G1 phase, consistent with G2/M cycle arrest and termination of mitosis. Concentrations of ADBAC+DDAC that reduced TER were correlated with G2/M cell cycle arrest, designating that destruction of the BTB is associated with reduced Sertoli cell proliferation. Concentrations of ADBAC as low as 0.00005% have been shown to induce single strand DNA breaks and generation of reactive oxygen species (ROS) in human corneal epithelial

cells.[32] The antioxidant hyaluronic acid, however, reduces the occurrence of ADBAC-induced DNA damage and ROS production in corneal epithelial cells.[33] Antioxidant agents have been shown to reduce the severity of toxicant induced cell cycle arrest, suggesting that oxidative damage plays a major role in halting cell proliferation. Future studies should be directed at determining the extent of oxidative stress induced by the combination of ADBAC+DDAC QACs, in order to clarify a mechanism by which these compounds impede cell cycle progression.

Sperm maturation is reliant on the specialized luminal environment within the epididymis that is maintained by the blood-epididymal-barrier (BEB).[36] The process of spermatogenesis is at the mercy of an intact BTB. BTB and BEB complexes are responsible for regulating bi-directional movement of blood-borne molecules to the lumen of the seminiferous tubule and epididymal duct, and maintain a chemically specialized environment for sperm production and maturation. The presence of analogous junctional proteins such as claudins and connexins in both the BTB and BEB suggests a similar sensitivity to disruption by toxicants.[36] For instance, lanthanum tracers unable to penetrate the BTB also fail to penetrate the BEB.[36] Administration of toxicants according to the spermatogenic cycle evaluates if sperm produced in the testis or deposited into the epididymis are the targets of reproductive toxicants. In order to distinguish between testicular or epididymal toxicity of ADBAC+DDAC, cauda sperm function was assessed by in-vitro fertilization in male mice immediately after dosing to determine epididymal effects and after a 10-day rest period to determine testicular effects. Sperm isolated immediately from males exposed to ADBAC+DDAC for 7-days would have been present within the epididymis throughout dosing and, therefore, would be susceptible to an epididymal toxicant. A rest period of 10-days would be sufficient for testicular and/or caput epididymal sperm to undergo epididymal maturation and arrive at the cauda epididymis. As a result, epididymal toxicity would have been apparent in the sperm population isolated immediately after ADBAC+DDAC dosing. Exposed sperm from rested males, however, were the only sperm to exhibit reduced fertilization capacity in-vitro. Alternatively, the effects seen in rested males could be an indication of endocrine disruption. Delayed manifestations of toxicity are characteristic of

endocrine disrupting compounds (EDC's), since they interfere with the production, distribution, metabolism, and excretion of endogenous hormones responsible for endocrine homeostasis.[37] The acquisition of motility in the epididymis and transit is dependent on androgens. Compounds that inhibit Leydig cell function alter sperm maturational events, such as transit time, and reduce sperm concentration and motility.[37] Sperm concentration and motility in males exposed to ADBAC+DDAC were not significantly different from controls, suggesting that ADBAC+DDAC did not interfere with androgen homeostasis. Rather, these results indicate that fertilization was reduced through an alternate endocrine-dependent mechanism that manifests independent of reductions in sperm concentration and motility. Significant declines in sperm concentration and motility were previously observed in males exposed to 120 mg/kg/day ADBAC+DDAC for 8 weeks[9], indicating that a higher dose and/or longer exposure period may be required to affect sperm concentration and motility; however, shorter exposures to ADBAC+DDAC are evidently sufficient to induce decreases in fertilizing ability of sperm. Uptake and elimination kinetics of ADBAC+DDAC would need to be evaluated further in order to characterize the effects that duration of ADBAC+DDAC exposure has on sperm concentration, motility, and fertilization.

6. CONCLUSION

Concentrations that nearly obliterated TM4 cell metabolism were required to reduce TER. Cytotoxic effects of ADBAC+DDAC on metabolism were largely independent of reductions in TER, indicating that Sertoli cells integrated into the BTB may have differential responses to toxicants compared to their disjointed counterparts. Sperm collected from male mice given a 10-day rest period following ADBAC+DDAC dosing exhibited significantly decreased fertilizing ability, identifying a temporally-dependent mechanism of ADBAC+DDAC reproductive toxicity. These effects on fertilization did not, however, correlate with reductions in sperm concentration and motility. Results from the current study

demonstrate that ADBAC+DDAC reproductive toxicity requires time to manifest and that the ADBAC+DDAC-induced male mouse reproductive toxicity is dependent on the degree and duration of exposure. Determining the mechanisms of ADBAC+DDAC-induced reproductive toxicity will help identify potential risk associated with exposure to these common compounds. These and previous results indicate ADBAC+DDAC are reproductive toxicants in mice; however the risk to humans is currently unknown. It may be possible that populations frequently exposed to ADBAC+DDAC through occupational, residential, or industrial exposure have a greater risk for infertility and reproductive disorders.

Acknowledgements

We wish to thank Dr. Sherrie Clark, Dr. Willard Eyestone, and Dr. Harvey Florman for technical assistance.

REFERENCES

[1] Mezger, E., Wendler, O., Mayr, S. & Bozzato, A. Anaphylactic reaction following administration of nose drops containing benzalkonium chloride. Head & face medicine 8, 29, doi:10.1186/1746-160X-8-29 (2012).

[2] Huang, C., Chen, W., Chen, Y. & Liu, Z. [Toxicity research status of benzalkonium chloride on ocular surface]. [Zhonghua yan ke za zhi] Chinese journal of ophthalmology 50, 303-306 (2014).

[3] Berg, O. H., Henriksen, R. N. & Steinsvag, S. K. The effect of a benzalkonium chloride-containing nasal spray on human respiratory mucosa in vitro as a function of concentration and time of action. Pharmacology & toxicology 76, 245-249 (1995).

[4] Corazza, M. & Virgili, A. Airborne allergic contact dermatitis from benzalkonium chloride. Contact dermatitis 28, 195-196 (1993).

[5] Cusano, F. & Luciano, S. Contact allergy to benzalkonium chloride and glutaraldehyde in a dental nurse. Contact dermatitis 28, 127 (1993).

[6] Quirce, S. & Barranco, P. Cleaning agents and asthma. Journal of investigational allergology & clinical immunology 20, 542-550; quiz 542p following 550 (2010).

[7] Melin, Vanessa E., Potineni, Haritha, Hunt, Patricia, Griswold, Jodi, Siems, Bill, Werre, Stephen R. and Hrubec, Terry C. Exposure to common quaternary ammonium disinfectants decreases fertility in mice. Reproductive toxicology 50, 163-170, doi:10.1016/j.reprotox.2014.07.071 (2014).

[8] Melin, V. E., Melin, T. E., Dessify, B. J., Nguyen, C. T. & Hrubec, T. C. Quaternary ammonium disinfectants cause subfertility in mice by targeting both male and female reproductive processes Reproductive toxicology (pending revisions March 2015).

[9] Giwercman, A., Carlsen, E., Keiding, N. & Skakkebaek, N. E. Evidence for increasing incidence of abnormalities of the human testis: a review. Environmental health perspectives 101 Suppl 2, 65-71 (1993).

[10] Sharpe, R. M. Sperm counts and fertility in men: a rocky road ahead. Science & Society Series on Sex and Science. EMBO reports 13, 398-403, doi:10.1038/embor.2012.50 (2012).

[11] Gottardo, F., Kliesch, S. & World Health Organization. [Semen analysis: spermiogram according to WHO 2010 criteria]. Der Urologe. Ausg. A 50, 101-108, doi:10.1007/s00120-010-2466-6 (2011).

[12] Wong, E. W. & Cheng, C. Y. Impacts of environmental toxicants on male reproductive dysfunction. Trends in pharmacological sciences 32, 290-299, doi:10.1016/j.tips.2011.01.001 (2011).

[13] Espevik, T., Lamvik, M. K., Sunde, A. & Eik-Nes, K. B. Effects of cadmium on survival and morphology of cultured rat Sertoli cells. Journal of reproduction and fertility 65, 489-495 (1982).

[14] Cheng, C. Y. & Mruk, D. D. The blood-testis barrier and its implications for male contraception. Pharmacological reviews 64, 16-64, doi:10.1124/pr.110.002790 (2012).

[15] Monsees, T. K. et al. Sertoli cells as a target for reproductive hazards. Andrologia 32, 239-246 (2000).

[16] Zhang, Y. H., Lin, L., Liu, Z. W., Jiang, X. Z. & Chen, B. H. Disruption effects of monophthalate exposures on inter-Sertoli tight junction in a two-compartment culture model. Environmental toxicology 23, 302-308, doi:10.1002/tox.20343 (2008).

[17] Janecki, A., Jakubowiak, A. & Steinberger, A. Effect of cadmium chloride on transepithelial electrical resistance of Sertoli cell monolayers in two-compartment cultures--a new model for toxicological investigations of the "blood-testis" barrier in vitro. Toxicology and applied pharmacology 112, 51-57 (1992).

[18] Li, M. W., Mruk, D. D., Lee, W. M. & Cheng, C. Y. Disruption of the blood-testis barrier integrity by bisphenol A in vitro: is this a suitable model for studying blood-testis barrier dynamics? The international journal of biochemistry & cell biology 41, 2302-2314, doi:10.1016/j.biocel.2009.05.016 (2009).

[19] de Jager, C., Bornman, M. S. & van der Horst, G. The effect of p-nonylphenol, an environmental toxicant with oestrogenic properties, on fertility potential in adult male rats. Andrologia 31, 99-106 (1999).

[20] Jayachandra, S. & D'Souza, U. J. Pre- and postnatal toxicity of diazinon induces disruption of spermatogenetic cell line evidenced by increased testicular marker enzymes activities in rat offspring. Journal of environmental pathology, toxicology and oncology: official organ of the International Society for Environmental Toxicology and Cancer 32, 73-90 (2013).

[21] Toth, G. P. et al. Correlation of sperm motion parameters with fertility in rats treated subchronically with epichlorohydrin. Journal of andrology 12, 54-61 (1991).

[22] Klinefelter, G. R., Laskey, J. W., Ferrell, J., Suarez, J. D. & Roberts, N. L. Discriminant analysis indicates a single sperm protein (SP22) is predictive of fertility following exposure to epididymal toxicants. Journal of andrology 18, 139-150 (1997).

[23] Klinefelter, G. R., Laskey, J. W., Roberts, N. R., Slott, V. & Suarez, J. D. Multiple effects of ethane dimethanesulfonate on the epididymis of adult rats. Toxicology and applied pharmacology 105, 271-287 (1990).

[24] Emter, R. & Natsch, A. A fast Resazurin-based live viability assay is equivalent to the MTT-test in the KeratinoSens assay. Toxicology in vitro: an international journal 29, 688-693, doi:10.1016/j.tiv.2015.02.003 (2015).

[25] Pozarowski, P. & Darzynkiewicz, Z. Analysis of cell cycle by flow cytometry. Methods in molecular biology 281, 301-311, doi:10.1385/1-59259-811-0:301 (2004).

[26] Ford, M. J., Tetler, L. W., White, J. & Rimmer, D. Determination of alkyl benzyl and dialkyl dimethyl quaternary ammonium biocides in occupational hygiene and environmental media by liquid chromatography with electrospray ionisation mass spectrometry and tandem mass spectrometry. Journal of chromatography. A 952, 165-172 (2002).

[27] Pisella, P. J. et al. Conjunctival proinflammatory and proapoptotic effects of latanoprost and preserved and unpreserved timolol: an ex vivo and in vitro study. Investigative ophthalmology & visual science 45, 1360-1368 (2004).

[28] Liang, H., Baudouin, C., Labbe, A., Riancho, L. & Brignole-Baudouin, F. Conjunctiva-associated lymphoid tissue (CALT) reactions to antiglaucoma prostaglandins with or without BAK-preservative in rabbit acute toxicity study. PloS one 7, e33913, doi:10.1371/journal.pone.0033913 (2012).

[29] Tao, W. Field leaching of alkaline copper quaternary-treated red pine lumber over 3 years: long-term dynamics. Water science and technology: a journal of the international association on water pollution research 69, 1475-1481, doi:10.2166/wst.2014.045 (2014).

[30] Miyauchi, T., Mori, M. & Ito, K. Quantitative determination of benzalkonium chloride in treated wood by solid-phase extraction followed by liquid chromatography with ultraviolet detection. Journal of chromatography. A 1095, 74-80, doi:10.1016/j.chroma.2005.07.112 (2005).

[31] Cybulski, J. et al. Long-alkyl-chain quaternary ammonium lactate based ionic liquids. Chemistry 14, 9305-9311, doi:10.1002/chem.200800973 (2008).

[32] Ye, J., et al. "Role of Benzalkonium Chloride in DNA Strand Breaks in Human Corneal Epithelial Cells." Graefes Arch Clin Exp Ophthalmol 249.11 (2011): 1681-7.

[33] Wu, H., et al. "Genoprotective Effect of Hyaluronic Acid against Benzalkonium Chloride-Induced DNA Damage in Human Corneal Epithelial Cells." Mol Vis 17 (2011): 3364-70.

[34] Fichorova, R. N., Bajpai, M., Chandra, N., Hsiu, J. G., Spangler, M., Ratnam, V. and Doncel, G. F. Interleukin (IL)-1, IL-6, and IL-8 predict mucosal toxicity of vaginal microbicidal contraceptives. Biology of reproduction 71, 761-769, doi:10.1095/biolreprod.104.029603 (2004).

[35] Ohnuma-Koyama, A., Yoshida, T., Tajima-Horiuchi, H., Takahashi, N., Yamaguchi, S., Ohtsuka, R., Takeuchi-Kashimoto, Y., Kuwahara, M., Takeda, M., Nakashima, N. and Harada, T. Didecyldimethylammonium chloride induces pulmonary fibrosis in association with TGF-beta signaling in mice. Experimental and toxicologic pathology: official journal of the gesellschaft fur toxikologische pathologie 65, 1003-1009, doi:10.1016/j.etp.2013.02.003 (2013).

[36] Franca, L. R., Auharek, S. A., Hess, R. A., Dufour, J. M. & Hinton, B. T. Blood-tissue barriers: morphofunctional and immunological aspects of the blood-testis and blood-epididymal barriers. Advances in experimental medicine and biology 763, 237-259 (2012).

[37] Klaassen, C. D. Casarett & Doull's Toxicology: The Basic Science of Poisons. 6th Edition edn, (McGraw-Hill, 2001).

CHAPTER 5:

Direct and In-utero Exposure to Quaternary Ammonium Disinfectants Alters Sperm Parameters and RNA Expression of Epigenetic Enzymes in the Testis of Male Mice

Vanessa E. Melin[1], Travis E. Melin[2], Brian J. Dessify[2], Christina T. Nguyen[2], and Terry C. Hrubec[1,2]

1) Department of Biomedical Sciences and Pathobiology, VA-MD Regional College of Veterinary Medicine, VA Tech, Blacksburg, VA 24061

2) E. Via College of Osteopathic Medicine Virginia Campus, Blacksburg, VA 24060

To be submitted to Reproductive Toxicology

1. ABSTRACT

Quaternary ammonium compounds (QACs) are antimicrobial agents that exhibit a wide spectrum of biocidal activity, and are effective against many bacteria, fungi, and viruses. QACs are also present in a wide variety of consumer products including industrial and household cleaners. It is likely that humans are regularly and repeatedly exposed to QACs in their daily lives. Exposure to the QACs alkyl dimethyl benzyl ammonium chloride (ADBAC) and didecyl dimethyl ammonium chloride (DDAC) reduced reproductive capabilities in both female and male mice. In the male, sperm parameters such as concentration, motility and fertilizing capability were reduced. In order to assess the long-term reproductive impact of ADBAC+DDAC, multi-generational reproductive outcomes were assessed in male and female mice exposed ambient concentrations of to ADBAC+DDAC. Multigenerational effects of ADBAC+DDAC exposure on sperm parameters and epigenetic enzyme mRNA expression were determined by CASA and RT-PCR, respectively. Mice ambiently exposed to ADBAC+DDAC exhibited decreases in breeding performance that persisted in unexposed progeny for two generations. Mouse breeding pairs (F_0) provided 120 mg/kg/day of ADBAC+DDAC for 8 weeks and throughout gestation exhibited significant decreases in sperm concentration and motility; however, the F_1 generation exposed in-utero exhibited a six-fold increase in sperm concentration compared to F_0 ADBAC+DDAC mice and sperm motility comparable to controls. F_2 mice derived from the F_1 generation exhibited sperm parameters similar to controls. Changes in the mRNA expression of chromatin modification enzymes in the testis of directly exposed males in addition to F_1 and F_2 progeny indicated significant upregulation of two histone acetyltransferases (hat1 and kat2b) and significant downregulation of one lysine-specific demethylase (kdm6b) in the testis of F_0 males. The DNA methyltransferase Dnmt1 was downregulated in the testis of F_1 males exposed to ADBAC+DDAC in-utero. These results indicate that the reproductive effects of ADBAC+DDAC on the mouse testis are incurred from direct exposure and do not persist transgenerationally. Timing of ADBAC+DDAC exposure greatly influences the nature of epigenetic

reprogramming in the mouse testis, since in-utero exposure to ADBAC+DDAC caused unique changes to the epigenetic profile compared to adult exposure. The importance of mice as models for human diseases suggests that human male testes may be potential targets of ADBAC+DDAC toxicity.

2. INTRODUCTION

Quaternary ammonium compounds (QACs) are antimicrobial agents that exhibit a wide spectrum of biocidal activity, and are effective against many bacteria, viruses, fungi, and protozoa. Ubiquitous use of QAC cleaning solutions in residential, commercial and medical settings has likely resulted in widespread human exposure.[1] Alkyl dimethyl benzyl ammonium chloride (ADBAC) and didecyl dimethyl ammonium chloride (DDAC) are among the most common QACs utilized in household cleaners. Additionally, ADBAC+DDAC are routinely used as sanitizers and disinfectants in medical settings and in restaurants and food production facilities. Previously we reported a decline in breeding performance of mice chronically exposed to 120 mg/kg/day of ADBAC+DDAC.[2] ADBAC+DDAC reduced breeding capacity through disturbances in ovulatory capacity and estrus cyclicity in females and reduced sperm concentration and motility in males.[2]

Exposure to environmental pollutants can induce epigenetic changes in gene expression.[3] DNA modifications and chromatin remodeling enzymes are responsible for the regulation of DNA replication, repair, and transcription. The addition or removal of specific chemical groups to DNA or chromatin induces structural changes to the 3-dimensional architecture of chromatin that regulate access to the underlying genetic sequence. Genetic material is packaged into nucleosome subunits comprised of DNA coiled around an octamer of four core histones. Remodeling dynamics often regulate access of transcriptional machinery to segments of chromatic DNA through modifications of the histone core and/or linker proteins. Epigenetic alterations are more likely than DNA sequence mutations to dominate phenotypes among progeny.[4] The physiological consequences of aberrant epigenomic remodeling are well documented in a number of disorders such as cancer, diabetes, and schizophrenia, highlighting the importance epigenetics in disease etiology.[5-9]

Diet, exercise, stress, and toxicant exposure can induce lasting epigenetic modifications that affect reproductive function.[3] Changes in direct germ-line methylation patterns are used to re-establish totipotency and renew parental imprinting in germ cells. The genetic contribution of imprints from both parents is determined by differentially methylated regions that regulate allelic expression of imprinted traits. Genomic imprinting ensures that a monoallelic, parental-specific expression pattern is established in germ cells.[10,11] Methylation patterns are erased in primordial germ cells as they migrate across the gonadal ridge and are re-established during gonadal differentiation according to maternal and paternal imprinting. Both cell lineage-specific patterns and germ-line specific patterns of DNA methylation are established by methyltransferases. Cell lineage-specific patterns are established in the early embryo during gastrulation to direct the differentiation of somatic cells. The germ-line specific methylation pattern is sex-specific and is established during gonadal differentiation.[10] Primordial germ cells are directed by these methylated loci to develop into oocytes or sperm, depending on the sex of the embryo. Imprinted genes are established in the parental gametes and, therefore, can reflect the environment of the previous generation's germ cells.[11]

Germ cells are vulnerable to deleterious effects from parental toxicant exposures via direct exposure of the germ line. In-utero exposure to toxicants that interfere with the creation of methylation patterns during gonadal differentiation cause heritable alterations in cell differentiation, proliferation, and ultimately function.[12-14] These changes in epigenetic reprogramming may be transgenerationally inherited, since they possess permanent imprint-like properties that enable heritability across several generations.[13] In-utero exposure to toxicants such as endocrine disrupting compounds (EDCs) during gonadal differentiation can interfere with germ cell reprogramming, resulting in male subfertility that persists for several generations.[14]

Subfertility was observed in a mouse colony exposed to QAC disinfectants ADBAC+DDAC.[2] Declines in sperm quality following ADBAC+DDAC exposure indicate that these compounds target the male reproductive system.[2] The current study investigates multi-generational changes in the mouse testis

epigenome following exposure to ADBAC+DDAC to determine if the reproductive effects of these compounds persist across multiple generations. These transgenerational reproductive effects represent a novel mechanism of genetic inheritance and reveal an unexplored territory of chemical toxicity assessments. The ability of a toxicant to induce reproductive changes in the absence of direct exposure highlights the importance of comprehensively testing common compounds that humans are routinely exposed to. Many of these compounds, like QACs, have been in use for decades but have not undergone thorough testing for reproductive toxicity. Clarification of toxicant-induced changes to the heritable testis epigenome will also identify biomarkers that may be useful in identifying ADBAC+DDAC-induced changes in testis function and could possibly be used to assess toxicity of ADBAC and DDAC in humans.

3. MATERIALS AND METHODS

3.1. Animal husbandry for mating indices and pregnancy rates

CD-1 mice were initially purchased from Charles River Laboratories (Wilmington, MA) and maintained in disposable caging (Innovive, San Diego, CA) on a 12-hour light/dark cycle at 20 – 25 °C with 30 - 60% relative humidity. Harlan 7013 rodent chow (Harlan Teklad, Madison, WI) and distilled water were provided ad libitum. Mice were not dosed, but were ambiently exposed through routine husbandry practices and cleaning to a QAC disinfectant containing 6.76% ADBAC (60% C-14, 25% C-12, 15% C-16) and 10.1% DDAC (Sanitation Strategies). After weaning the F_0 mice were transferred to a QAC-free (QF) facility utilizing a chlorine dioxide disinfectant and held for 8 weeks. Mice were then paired (two females to one male) for breeding at 8-11 weeks of age in the QF facility to derive an F_1 generation. F_1 mice were reared in the QF facility until they were 6-8 weeks old, and then paired for breeding (two females to one male) to derive an F_2 generation which was subsequently bred at 6-8 weeks of age (Figure 1).

3.1.2. Mating indices and pregnancy rates

Mating indices and pregnancy rates assessed in F_0 (n=8) F_1 (n=11), and F_2 (n=10) mated pairs for each generation in the respective facility. Mating index was calculated by assessing the number of females with copulatory plugs, divided by the number of females co-habited with a male. Pregnancy rate was calculated based on the number of females pregnant, divided by the number of females with evidence of breeding (i.e. presence of a copulatory plug).

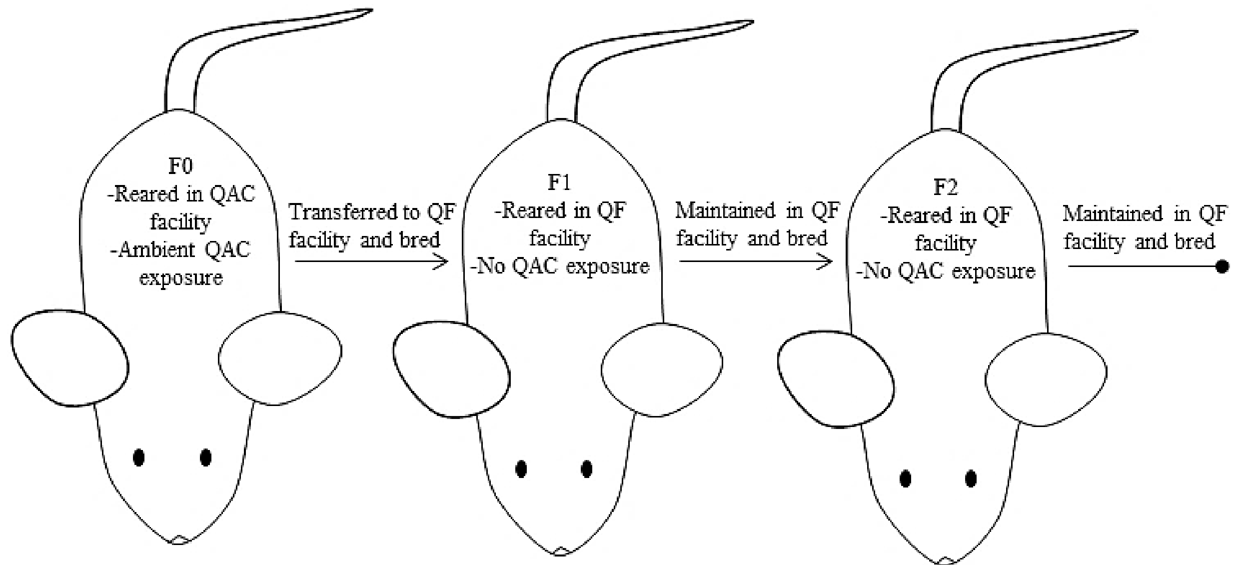


Figure 1. F_0 females were exposed to ambient ADBAC+DDAC for 8 weeks then transferred to a QF facility. F_0 females were bred in the QF facility for one generation to derive F_1 QF mice, which were then bred to produce a F_2 QF population.

3.2. Husbandry for multigenerational assessment of sperm parameters and testis mRNA expression

F_0 male and female mice (n=8-10) were dosed by adding ADBAC+DDAC (Sanitation Strategies, Holt, MI) to distilled water. Doses of ADBAC+DDAC/kg body weight/day were calculated based on the sum of ADBAC+DDAC (6.76% ADBAC (60% C-14, 25% C-12, 15% C-16) and 10.1% DDAC), with an average daily water consumption of 10% body weight and provided daily.[2] Water consumption was not significantly different between treatment groups. F_0 mice (n=10) were exposed for 8 weeks and

throughout breeding and gestation to ADBAC+DDAC or distilled water. At approximately GD 19, F_0 mice were transferred to a QF facility and F_1 progeny were cross-fostered to control dams upon delivery. At 8-10 weeks of age, F_1 males (n=8-10) were bred to untreated control females to derive an F_2 generation (n=8-10). The F_2 generation was euthanized at 8-10 weeks of age for evaluation of sperm and testis.

3.2.1. Sperm collection

For all generations of mice, epididymal sperm were collected following Wang et al., 2003.[15] Briefly, males were euthanized by CO_2 inhalation, both cauda epididymes were excised and placed in one mL of pre-warmed Dulbecco's Modified Eagle's Medium (DMEM: sterile filtered 1000 mg/L glucose and sodium bicarbonate, pyridoxine HCl with no L-glutamine or phenol red) supplemented with 1% fetal bovine serum (GIBCO, Grand Island, NY). Cauda epididymes were cut six times with a sterile surgical blade, mixed with a pipette and incubated at 37°C with 5% CO_2 for 10-15 minutes to release sperm. A 500 µL aliquot of cauda epididymal extract was transferred to a 1.5 mL conical tube and diluted to 1.5 mL with pre-warmed DMEM supplemented with 1% fetal bovine serum.

3.2.2. Computer-automated sperm analysis (CASA)

Sperm concentration and motility was evaluated in F_0 (n=10) F_1 (n=9), and F_2 (n=8) male mice from three different litters per generation. Duplicate 3µL sample extracts were analyzed using 20 µm-deep 4-chamber Leja slides (Minitube of America, Verona, WI). Slides were allowed to settle for 2-3 minutes before analysis. Eight random fields were analyzed with 20X phase-contrast at 30 frames per second (Model BX41, Olympus Optical, Tokyo, Japan) using Sperm Vision 3.5 (Minitube of America, Verona, WI) to calculate sperm concentration and motility. Sperm following a non-linear, linear or

curvilinear swimming pattern were classified as motile, while non-swimming sperm were classified as immotile.

3.2.3. RT-PCR

The left and right testes (epididymis removed) from three unrelated males were used for multi-generational assessment of ADBAC+DDAC on RNA expression. Control testes were collected from one generational matched male for each generation. Testis RNA was isolated using an RNeasy Mini Kit according to manufacturer instructions (Qiagen, Hilden, Germany). RNA concentration and purity was evaluated using a Nanodrop 2000 UV-Vis Spectrophotometer (Thermo Scientific, Waltham, MA). Only RNA with a 260/280 absorbance ratio ≥ 2.0 was used for RT-PCR analysis. RNA starting concentrations were adjusted to 0.5 μg using nuclease-free water before cDNA synthesis using a RT^2 First Strand Kit (SABiosciences, Frederick, MD). RT^2 SYBR Green qPCR Mastermix (SABiosciences, Frederick, MD) was added to cDNA per manufacturer instructions. The Mouse Epigenetic Chromatin Modification Enzymes RT2 Profiler™ PCR Array (SABiosciences, Frederick, MD) was used to detect the RNA expression of enzymes known or predicted to modify genomic DNA and histone structure The PCR Array configuration was a 96-well plate containing primer assays for 84 pathway genes and 5 housekeeping genes to normalize array data (see Table I for total gene listing). One well with a genomic DNA control, three wells with reverse-transcription controls, and three wells with positive PCR controls were used to evaluate inter-well/intra-plate accuracy and precision. PCR amplification was conducted using Bio-Rad iCYCLER iQ5 Real Time PCR instrument with the following cycling conditions: 1 cycle for 10 minutes at 95°C, 40 cycles for 15 seconds at 95°C, and 1 minute at 60°C. Cycle threshold was set at the point of detectable SYBR fluorescence.

Table I. Genes evaluated using a pathway targeted PCR array. The RNA expression was assessed in genes known or suspected to encode or be involved with encoding chromatin remodeling enzymes in the testis of F0 males exposed to ADBAC+DDAC and F1 and F2 progeny.

Functional gene grouping	Genes evaluated
DNA Methyltransferases	Dnmt1, Dnmt3a, Dnmt3b
Histone Acetyltransferase	Atf2, Cdyl, Ciita, Csrp2bp, Esco1, Esco2, Hat1, Kat2a, Kat2b, Kat5, Myst1, Myst2, Myst3, Myst4, Ncoa1, Ncoa3, Ncoa6
Histone Methyltransferases	Carm1 (Prmt4), Dot1l, Ehmt1, Ehmt2, Mll3, Prmt1, Prmt2, Prmt3, Prmt5, Prmt6, Prmt7, Prmt8, Setdb2, Smyd1, Smyd3, Suv39h1
SET Domain Proteins (Histone Methyltransfesase Activity)	Ash1l, Mll5, Nsd1, Setd1a, Setd1b, Setd2, Setd3, Setd4, Setd5, Setd6, Setd7, Setd8, Setdb1, Suv420h1, Whsc1
Histone Phosphorylation	Aurka, Aurkb, Aurkc, Nek6, Pak1, Rps6ka3, Rps6ka5
Histone Ubiquitination	Dzip3, Mysm1, Rnf2, Rnf20, Ube2a, Ube2b, Usp16, Usp21, Usp22
Histone Demethylases	Kdm1a, Kdm5b, Kdm5c, Kdm4a, Kdm4c, Kdm6b
Histone Deacetylases	Hdac1, Hdac2, Hdac3, Hdac4, Hdac5, Hdac6, Hdac7, Hdac8, Hdac9, Hdac10, Hdac11

3.4. Statistics

Statistics were not generated for mating indices and pregnancy rates because the experiment was conducted only once (n=1). Data normality was determined using normal probability plots. Normally distributed sperm parameter data were evaluated using ANOVA. SA Biosciences RT^2 PCR Data Analysis version 3.5 was used to generate the average Ct, $2^{(- Ct)}$, fold change, p-value, and fold regulation for each gene from RT-PCR Ct values. Fold changes were evaluated using t-tests to determine p-values. Changes in gene expression for each generation were determined relative to the generation matched controls. Genes with greater than 2-fold changes in expression and with $p \leq 0.05$ were considered biologically and statistically significant. Statistical analysis was conducted using Statistix (Tallahassee, FL). Significance was set at $p \leq 0.05$. Sperm parameter and gene expression data are expressed as the mean ± SEM.

4. RESULTS

4.1. Mating indices and pregnancy rates

Mating index and pregnancy rate were calculated for each generation as we derived the F_2 generation of unexposed mice. Sexually mature F_0 mice ambiently exposed since birth to an ADBAC+DDAC containing disinfectant had a mating index and pregnancy rate of 75% and 78%, respectively. The next generation, F_1 mice, derived from ambiently exposed F_0 females but born into a QF environment, exhibited a mating index and pregnancy rate of 92%. The F_2 generation maintained and bred in the QF (bred for two generations in a QF environment) demonstrated a mating index and pregnancy rate of 100% (Figure 2A-B).

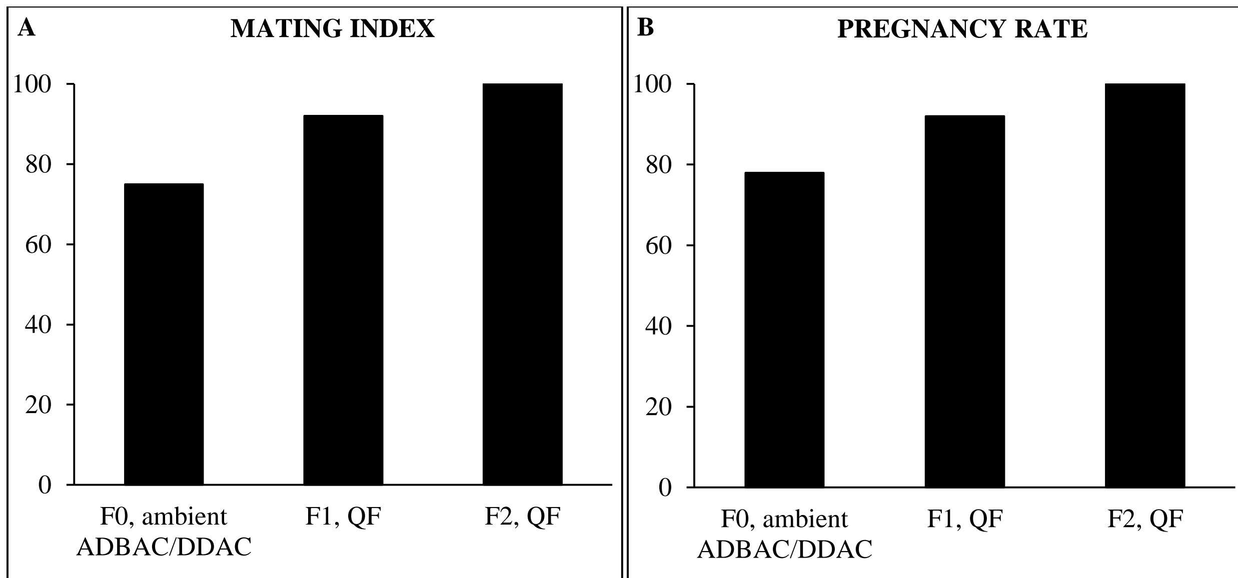


Figure 2. Mating index (A) and pregnancy rate (B) after removal from a QAC facility. F_0 females were exposed to ambient ADBAC+DDAC for 8 weeks then transferred to a QF facility. F_1 and F_2 progeny were born and raised in a QF facility. Mating indices and pregnancy rates were calculated for each generation. Mating index: (number of females with copulatory plug/number of females cohabitated with a male) x 100; Pregnancy rate: (number of females who became pregnant/number of females with a copulatory plug) x 100.

4.2. Sperm concentration and motility

Exposure to 120 mg/kg/day ADBAC+DDAC for 8 weeks significantly reduced sperm parameters in F_0-exposed mice. F_0 control mice exhibited sperm concentrations of 30.1 ± 1.9 x 10^6 sperm/mL, compared to 11.3 ± 1.2 x 10^6 sperm/mL in ADBAC+DDAC directly exposed F_0 mice. Sperm concentration in F_1 males exposed in-utero to ADBAC+DDAC; however, significantly increased to 68.2 ± 5.5 x 10^6 sperm/mL compared to their control counterparts at 32 ± 3.8 x 10^6 sperm/mL (Figure 3A). Sperm motility was significant decreased in directly exposed F_0 and in-utero exposed F_1 generations of males with 44.4 ± 2.5% and 20.9 ± 4.1% respectively (Figure 3B). The increase in sperm concentration observed in the F_1 did not correlate with changes in sperm motility of the F_1 progeny. Sperm parameters of F_2 males derived from exposed F_0 mice ADBAC+DDAC did not differ from controls, indicating that ADBAC+DDAC exposure did not cause changes in sperm parameters that were heritable to the second generation.

4.3. Gene expression of chromatin modifying enzymes

The highest frequency of significant fold changes in mRNA expression relative to controls was observed in F_0 males exposed to ADBAC+DDAC (Table II). F_0 ADBAC+DDAC exposed males had upregulation of Hat1 and Kat2b histone acetyltransferases and downregulation of Kdm6b histone demethylase. In F_1 mice, the only gene that was different from controls was downregulation of Dnmt1 DNA methyltransferase. This suggests that in-utero exposure to ADBAC+DDAC was sufficient to induce post-natal changes to the testicular epigenome.

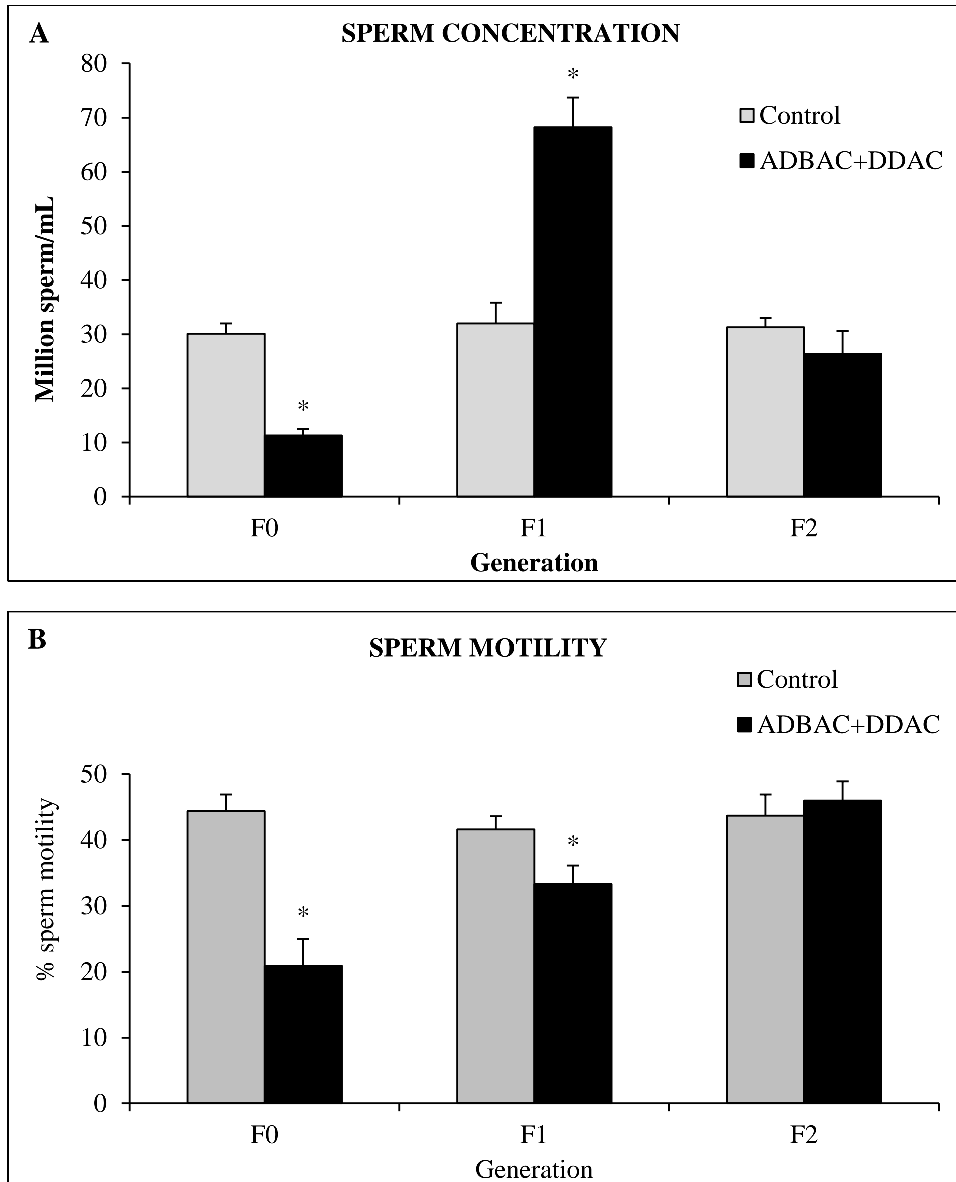


Figure 3A-B. In the F_0 generation, exposure to 120 mg ADBAC+DDAC/kg/day for 8 weeks significantly reduced sperm concentration and motility compared to controls. A. Sperm concentration in F_1-ADBAC+DDAC progeny was significantly higher than F_1 controls. B. Sperm motility was decreased in F_1-ADBAC+DDAC progeny that were exposed in-utero. Sperm parameters in the F_2 generation derived from F_0-exposed were similar to generation matched controls (n=8-10 males/generation, $p \leq 0.05$, ANOVA).

Table II. Changes in testis gene expression of chromatin modification enzymes. Significant changes were predominantly observed in F_0 males that were directly exposed to 120 mg/kg/day ADBAC+DDAC for 8 weeks. In F_0 ADBAC+DDAC males, two histone acetyltransferases were upregulated, while one histone demethylase was downregulated. Testis from F_1 progeny of exposed F0 mice exhibited significant downregulation of one DNA methyltransferase ($p \leq 0.05$, Kruskal-Wallis). Only fold changes greater than 2 with $p \leq 0.05$ were considered biologically and statistically significant. Fold changes are relative to generation-matched controls.

Generation	Upregulated genes	Downregulated genes
F_0	Histone acetyltransferases: **Hat1:** 2.3 **Kat2b:** 2.2	Histone demethylase: **Kdm6b:** 0.40
F_1	---	DNA methyltransferase: **Dnmt1:** 0.33
F_2	---	---

5. DISCUSSION

Epigenetic modifications induce changes in gene expression without modifying the underlying DNA sequence. The addition or removal of specific chemical groups to DNA or chromatin induces structural changes to the 3-dimensional structure of chromatin that regulate access to the underlying genetic sequence. DNA modifications and chromatin remodeling enzymes are responsible for the regulation of DNA replication, repair, and transcription. As a result, the epigenetic state of a cell's genome is indicative of transcriptional mechanisms and reflects the cell's function. Epigenetic modifications enable cell lineages with the same underlying genotype to manifest phenotypic differences that support cell-specific functions. DNA methylation is an epigenetic modification that predominantly occurs on cytosines of CpG sequences.[3] DNA methyltransferases (Dnmts) transfer methyl groups from S-adenosyl methionine (SAM) to the fifth carbon of the cytosine ring to form 5-methyl cytosine. Inactive genes are typically hypermethylated whereas transcriptionally active genes are hypomethylated. DNA

methylation is of particular importance during mammalian development, since the epigenetic program commits totipotent cells to a specific anatomical and physiological fate.

Germ cells forming F_1 progeny are vulnerable to deleterious effects from parental toxicant exposures via direct exposure of the germ line or through epigenetic modifications of DNA or proteins. Germ cells for the F_1 generation are present in the F_0 and are exposed at the same time the mother is exposed. Epigenetic changes are transmissible through gametes and, therefore, between several generations. For example, the agricultural herbicide vinclozolin is a known anti-androgenic endocrine disruptor. Vinclozolin altered methylation status of paternally-derived H19 andGtl2 and maternally-derived Peg1, Snrpn, and Peg3 in the sperm of the offspring. Interestingly, transgenerational effects were observed, but dissipated with each subsequent generation. Male offspring of dams that were administered vinclozolin during critical periods of urogenital formation exhibited significantly decreased sperm concentrations; however, these values gradually normalized to that of controls in succeeding generations. Stouder et al. concluded that the effects of vinclozolin on male spermatogenesis might be mediated by defective re-methylation of the germline.[16] Decreased spermatogenesis and infertility were also observed in adult mice exposed in-utero to the estrogenic insecticide methoxychlor during the period of embryonic sex determination. These reproductive deficiencies persisted into the F_2 generation and were not correlated with tissue abnormalities or altered serum testosterone concentrations.[17] The methylated genes responsible for methoxychlor's transgenerational reproductive effects, however, have yet to be identified.[17] These studies demonstrate the importance of multigenerational assessments of environmentally-induced epigenetic modifications in understanding the transmissibility of reproductive toxicity.[18]

In F_0 ADBAC+DDAC exposed testes, Hat1 expression was upregulated. Histone acetyltransferases (Hats) transfer acetyl groups derived from acetyl-coA to exposed lysine resides on histone tails. In contrast to methylation, acetylation of histones typically provokes transcriptional upregulation through chromatin decondensation.[19] Hats are subdivided into classes based on their cellular

localization; type A Hats are localized to the nucleus whereas type B Hats reside in the cytoplasm. Type A Hats preferentially binds to poly-acetylated lysine residues since they contain a bromodomain that enables identification of mono-acetylated lysine residues on the N-terminal tails of histones. In contrast, cytoplasmic type B Hats lack a bromodomain and thus acetylate new (un-acetylated) core histones before they are packed into nucleosome subunits.[19] Hyperacetylation of core histones is localized to post-meiotic spermatocytes undergoing spermiogenesis, indicating a critical function of Hats in spermatogenesis. The acetyltransferase Hat1 co-localizes with a germ-cell-specific RNA-binding protein during spermatogenesis. This suggests that Hat1 may have a role in RNA processing during spermatogenesis; however, the precise mechanism by which Hat1 acetylation regulates spermatid formation is undetermined.[19] Knockdown of Hat1 function in osteosarcoma cell lines is associated with increased levels of double-strand breaks, suggesting that Hat1 plays a role in repairing DNA damage.[20] Diminished sperm concentration and motility in F_0 ADBAC+DDAC exposed males coincided with upregulation of testis Hat1 RNA. Germ cell differentiation requires chromatin remodeling and double-stranded DNA break repair of sister chromatids, and upregulation of Hat1 could be associated with increased chromatin remodeling and DNA break repair in the testis. While increased DNA damage could explain spermatogenic defects in ADBAC+DDAC exposed mice, double-strand breaks would need to be identified among the spermatogenic populations to determine which stages are susceptible to damage and confirm the association with Hat1.

Kdm6b is a lysine-specific histone demethylase whose expression is primarily localized to undifferentiated spermatogonia. Downregulation of Kdm6b expression is associated with destabilization of germ-cell intercellular bridges.[21] In contrast, male mice enriched with Kdm6b demonstrate larger testicles and stay fertile longer than their unmodified counterparts.[21] This suggests an important role for kdm6b in spermatogonial proliferation and differentiation. In the present study, Kdm6b was downregulated in the testis of F_0 male mice exposed for 8 weeks to 120 mg/kg/day ADBAC+DDAC. Reductions in spermatogenic efficiency due to insufficient Kdm6b expression may represent a mechanism

by which ADBAC+DDAC exposure reduces sperm parameters. F_0 males exposed to ADBAC+DDAC also exhibited significant upregulation of Kat2b in addition to decreased sperm parameters. There are no studies associating Kat2b to changes in reproductive function. Single-nucleotide polymorphisms (SNPs) within Kat2b loci are associated dysregulation of IL-10 inflammatory pathways that lead to the "frail mouse" phenotype.[22] ADBAC (0.001-0.1%) caused induction of the pro-inflammatory cytokine IL-10 in human conjunctival and corneal epithelial cells.[23] Further studies are required to evaluate the inflammatory responses of ADBAC+DDAC-exposed mice and to determine the relationship between Kat2b upregulation and ADBAC+DDAC-induced reproductive dysfunction.

Currently, three Dnmts have been identified in mammals: Dnmt1, Dnmt3A, and Dnmt3B. Dnmts are further subdivided into de novo Dnmts or maintenance Dnmts. Dnmt1 is required for maintenance of already established cell methylation patterns. Dnmt3A and B establish new methylation configurations; however, these enzymes work synergistically to establish de novo methylation patterns during embryogenesis.[24,25] Dnmt1 does not participate in prenatal methylation reprogramming of the germline, but is critical for the maintenance of methylation patterns in somatic cells and during replication of DNA.[10] Dnmt3, however, is significantly expressed in the prenatal male germ line and establishes de-novo methylation patterns critical for DNA replication in spermatogonia and preleptotene spermatocytes.[24] Dnmts are also considered the key regulators of the epigenetic landscape within the prenatal and postnatal male testis.[26] Expression of the maintenance methyltransferase Dnmt1was significantly reduced in F_1 males exposed in-utero to ADBAC+DDAC. These same males demonstrated significant increases in sperm concentration compared to their control counterparts and their F_0 ADBAC+DDAC predecessors. This suggests that downregulation of Dnmt1may be associated with increased rates of spermatogenesis. During normal spermatogenesis, Dnmt1 is localized to the nuclei of spermatogonia. While the exact role of Dnmt1 in spermatogenesis has yet to be clarified, mice with altered Dnmt1expression exhibit abnormal methylation patterns in spermatogenic populations when compared to controls.[27] Germ-cell specific methylation patterns would, therefore, need to be assessed in

mice exposed in-utero to ADBAC+DDAC in order to determine if downregulation of Dnmt1 was associated with a specific spermatogenic population. Identification of altered methylation patterns would clarify if in-utero exposure to ADBAC+DDAC induces epigenetic germline remodeling and determine if in-utero exposure to these compounds poses a significant risk to adult reproductive function.[28] It is interesting to note that mice exposed in-utero to ADBAC+DDAC solely exhibited changes in an enzyme related to DNA methylation and not changes ton enzymes related to histone modifications. These exclusive changes to enzymes related to DNA methylation are, in accordance with toxicant- induced reprogramming of germline methylation patterns.

Modifications to the epigenome were observed in the testis of F_0 males with spermatogenic impairment following exposure to ADBAC+DDAC. Changes in enzyme RNA-expression levels of ADBAC+DDAC males relative to generation-matched controls were not apparent in subsequent generations. Sperm motility and concentration were not decreased in the F_1 progeny of F_0 mice exposed to ADBAC+DDAC in-utero. Sperm concentration of F_1 mice exposed in-utero to ADBAC+DDAC was significantly increased compared to controls. A possible explanation for this is altered endocrine signaling of non-sex hormones resulting from the in-utero exposure. Rats administered goitrogenic compounds as neonates exhibited increased sperm production as adults.[29] This data suggests that neonatal hormonal disturbances can actually increase sperm output, in contrast to most research citing decreased sperm production from endocrine disturbances.[30] Goitrogenic compounds have also been shown to induce DNA fragmentation, which may account for the upregulation of Hat1 DNA repair observed in F_0 ADBAC+DDAC males.[31,32] Further research is needed to determine if ADBAC+DDAC are altering diverse endocrine regulation. Ultimately, this information could help determine whether in-utero exposure to ADBAC+DDAC poses any risk to the human germline. These data show that ADBAC+DDAC alters epigenetic associated enzymes in the testis of directly and in-utero exposed mice and will help identify if these compounds are associated with the increasing rates of testicular abnormalities in humans.

5. CONCLUSION

ADBAC+DDAC are reproductive toxicants in mice that reduce both male and female reproductive capabilities.[2] The current study highlights the importance of assessing the multi-generational toxicity data of chemical compounds, such as QACs. Most commercial cleaning products contain QACs, suggesting that humans are being chronically exposed. ADBAC+DDAC-induced alterations in male reproductive function appear to involve only directly exposed generations of mice and germ cells (F_0 and F_1). Exposure to ADBAC+DDAC, therefore, has multigenerational, but not transgenerational effects that persist beyond the F_2 populace. Investigating the multigenerational alterations to germline methylation patterns from in-utero exposure to ADBAC+DDAC will determine the extent of reproductive risk that these compounds pose to future generations. The dose-responsive reproductive toxicity of ADBAC+DDAC and the identification of multigenerational reproductive and epigenetic effects from ADBAC+DDAC exposure in mice, demonstrates that the effect of ADBAC+DDAC in human populations needs to be determined. Identifying ADBAC+DDAC as endocrine disruptors or reproductive toxicants in humans could explain some of the declines in reproductive function observed over the last 40 years.

REFERENCES

[1] Ford, M. J., Tetler, L. W., White, J. & Rimmer, D. Determination of alkyl benzyl and dialkyl dimethyl quaternary ammonium biocides in occupational hygiene and environmental media by liquid chromatography with electrospray ionisation mass spectrometry and tandem mass spectrometry. Journal of chromatography. A 952, 165-172 (2002).

[2] Melin, V. E., Melin, T. E., Dessify, B. J., Nguyen, C. T. & Hrubec, T. C. Quaternary Ammonium Disinfectants Cause Subfertility in Mice by Targeting Both Male and Female Reproductive Processes Reproductive toxicology (pending revisions March 2015).

[3] Sutherland, J. E. & Costa, M. Epigenetics and the environment. Annals of the New York academy of sciences 983, 151-160 (2003).

[4] Cooper, D. N., Krawczak, M., Polychronakos, C., Tyler-Smith, C. & Kehrer-Sawatzki, H. Where genotype is not predictive of phenotype: towards an understanding of the molecular basis of reduced penetrance in human inherited disease. Human genetics 132, 1077-1130, doi:10.1007/s00439-013-1331-2 (2013).

[5] Grayson, D. R. & Guidotti, A. The dynamics of DNA methylation in schizophrenia and related psychiatric disorders. Neuropsychopharmacology: official publication of the American college of neuropsychopharmacology 38, 138-166, doi:10.1038/npp.2012.125 (2013).

[6] Costa, E., Dong, E., Grayson, D. R., Guidotti, A., Ruzicka, W. and Veldic, M. Reviewing the role of DNA (cytosine-5) methyltransferase overexpression in the cortical GABAergic dysfunction associated with psychosis vulnerability. Epigenetics : official journal of the DNA methylation society 2, 29-36 (2007).

[7] Marumo, T., Yagi, S., Kawarazaki, W., Nishimoto, M., Ayuzawa, N., Watanabe, A., Ueda, K., Hirahashi, J., Hishikawa, K., Sakurai, H., Shiota, K. and Fujita, T. Diabetes Induces Aberrant DNA Methylation in the Proximal Tubules of the Kidney. Journal of the american society of nephrology: doi:10.1681/ASN.2014070665 (2015).

[8] Li, Q., Wei, W., Jiang, Y. I., Yang, H. & Liu, J. Promoter methylation and expression changes of in cancerous tissues of patients with sporadic breast cancer. Oncology letters 9, 1807-1813, doi:10.3892/ol.2015.2908 (2015).

[9] Charlton, J., Williams, R. D., Sebire, N. J., Popov, S., Vujanic, G., Chagtai, T., Alcaide-German, M., Morris, T., Butcher, L. M., Guilhamon, P., Beck, S. and Pritchard-Jones, K. Comparative methylome analysis identifies new tumour subtypes and biomarkers for transformation of nephrogenic rests into Wilms tumour. Genome medicine 7, 11, doi:10.1186/s13073-015-0136-4 (2015).

[10] Weaver, J. R., Susiarjo, M. & Bartolomei, M. S. Imprinting and epigenetic changes in the early embryo. Mammalian genome: official journal of the international mammalian genome society 20, 532-543, doi:10.1007/s00335-009-9225-2 (2009).

[11] Schulz, R., Proudhon, C., Bestor, T. H., Woodfine, K., Lin, C. S., Lin, S. P., Prissette, M., Oakey, R. J. and Bourc'his, D. The parental non-equivalence of imprinting control regions during mammalian development and evolution. PLoS genetics 6, e1001214, doi:10.1371/journal.pgen.1001214 (2010).

[12] Cheng, L. C., Tavazoie, M. & Doetsch, F. Stem cells: from epigenetics to microRNAs. Neuron 46, 363-367, doi:10.1016/j.neuron.2005.04.027 (2005).

[13] Soubry, A., Hoyo, C., Jirtle, R. L. & Murphy, S. K. A paternal environmental legacy: evidence for epigenetic inheritance through the male germ line. BioEssays : news and reviews in molecular, cellular and developmental biology 36, 359-371, doi:10.1002/bies.201300113 (2014).

[14] Skinner, M. K. & Anway, M. D. Seminiferous cord formation and germ-cell programming: epigenetic transgenerational actions of endocrine disruptors. Annals of the New York Academy of Sciences 1061, 18-32, doi:10.1196/annals.1336.004 (2005).

[15] Wang, Y. in Current protocols in toxicology (John Wiley & Sons, Inc., 2001).

[16] Stouder, C. & Paoloni-Giacobino, A. Transgenerational effects of the endocrine disruptor vinclozolin on the methylation pattern of imprinted genes in the mouse sperm. Reproduction 139, 373-379, doi:10.1530/REP-09-0340 (2010).

[17] Aoyama, H. & Chapin, R. E. Reproductive toxicities of methoxychlor based on estrogenic properties of the compound and its estrogenic metabolite, hydroxyphenyltrichloroethane. Vitamins and hormones 94, 193-210, doi:10.1016/B978-0-12-800095-3.00007-9 (2014).

[18] Holliday, R. DNA methylation and epigenetic mechanisms. Cell biophysics 15, 15-20 (1989).

[19] Makowski, A. M., Dutnall, R. N. & Annunziato, A. T. Effects of acetylation of histone H4 at lysines 8 and 16 on activity of the Hat1 histone acetyltransferase. The journal of biological chemistry 276, 43499-43502, doi:10.1074/jbc.C100549200 (2001).

[20] Qin, S., and M. R. Parthun. Histone H3 and the Histone Acetyltransferase Hat1p Contribute to DNA Double-Strand Break Repair. Molecular cell biology 22.23: 8353-65 (2002).

[21] Iwamori, N., T. Iwamori, and M. M. Matzuk. H3k27 Demethylase, Jmjd3, Regulates Fragmentation of Spermatogonial Cysts. PLoS One 8.8 (2013).

[22] Ho, Y. Y., Matteini, A. M., Beamer, B., Fried, L., Xue, Q. L., Arking, D. E., Chakravarti, A., Fallin, M. D. and Walston, J. Exploring Biologically Relevant Pathways in Frailty. Journal of gerontology 66.9: 975-9 (2011).

[23] Pisella, P. J., Debbasch, C., Hamard, P., Creuzot-Garcher, C., Rat, P., Brignole, F. and Baudouin, C. Conjunctival proinflammatory and proapoptotic effects of latanoprost and preserved and unpreserved timolol: an ex vivo and in vitro study. Investigative ophthalmology & visual science 45, 1360-1368 (2004).

[23] Fatemi, M., Hermann, A., Gowher, H. & Jeltsch, A. Dnmt3a and Dnmt1 functionally cooperate during de novo methylation of DNA. European journal of biochemistry / FEBS 269, 4981-4984 (2002).

[24] Baubec, T., Colombo, D. F., Wirbelauer, C., Schmidt, J., Burger, L., Krebs, A. R., Akalin, A. and Schubeler, D. Genomic profiling of DNA methyltransferases reveals a role for DNMT3B in genic methylation. Nature, doi:10.1038/nature14176 (2015).

[25] Xie, S., Wang, Z., Okano, M., Nogami, M., Li, Y., He, W. W., Okumura, K. and Li, E. Cloning, expression and chromosome locations of the human DNMT3 gene family. Gene 236, 87-95 (1999).

[26] Yaman, R., and V. Grandjean. Timing of Entry of Meiosis Depends on a Mark Generated by DNA Methyltransferase 3a in Testis. Molecular reproduction and development 73.3: 390-7 (2006).

[27] Saferali, A., Moussette, S., Chan, D., Trasler, J., Chen, T., Rozen, R. and Naumova, A. K. DNA methyltransferase 1 (Dnmt1) mutation affects Snrpn imprinting in the mouse male germ line. Genome / National Research Council Canada = Genome / Conseil national de recherches Canada 55, 673-682, doi:10.1139/g2012-056 (2012).

[28] Skakkebaek, N. E. "Testicular Dysgenesis Syndrome." Hormone research 60 Suppl 3: 49 (2003).

[29] Cooke, P. S., Hess, R. A., Porcelli, J. & Meisami, E. Increased sperm production in adult rats after transient neonatal hypothyroidism. Endocrinology 129, 244-248, doi:10.1210/endo-129-1-244 (1991).

[30] Kumar, V., Majumdar, C. & Roy, P. Effects of endocrine disrupting chemicals from leather industry effluents on male reproductive system. The journal of steroid biochemistry and molecular biology 111, 208-216, doi:10.1016/j.jsbmb.2008.06.005 (2008).

[31] Bint Akhtar, F., Weinbauer, G. F. & Nieschlag, E. Acute and chronic effects of a gonadotrophin-releasing hormone antagonist on pituitary and testicular function in monkeys. The journal of endocrinology 104, 345-354 (1985).

[32] Barman, H. K., Takami, Y., Ono, T., Nishijima, H., Sanematsu, F., Shibahara, K. and Nakayama, T. Histone acetyltransferase 1 is dispensable for replication-coupled chromatin assembly but contributes to recover DNA damages created following replication blockage in vertebrate cells. Biochemical and biophysical research communications 345, 1547-1557, doi:10.1016/j.bbrc.2006.05.079 (2006).

CHAPTER 6:

Conclusions

Environmental exposures can have profound effects on reproduction. The introduction of QAC-containing disinfectants coincided with noticeable declines in fertility in mouse breeder colonies. Few reproductive studies have evaluated the toxicity of ADBAC+DDAC compounds individually and no studies have evaluated the combined toxicity of ADBAC+DDAC. The ubiquitous nature of ADBAC+DDAC in cleaning solutions and personal care products suggest that many individuals are recurrently exposed to these compounds. Anecdotal evidence that ADBAC+DDAC might decrease reproductive capabilities prompted further research into the effects of QACs on mouse reproduction. A chronic breeding study was conducted for six months. Significant decreases in productivity including smaller litter sizes and longer pregnancy intervals in the 120 mg/kg/day ADBAC+DDAC exposure group and increased dam loss in both the 60 and 120 dose groups were observed. This was the first experimental evidence that QAC exposure decreases fecundity in mice.

These results clearly demonstrate that exposure to a common QAC mixture affects reproduction in the laboratory mouse; however, the design of this study did not distinguish between toxic effects on the dam, sire, and/or fetus. These questions were addressed in a second study which found that female mice exposed to ADBAC+DDAC had decreased ovulation and fewer estrus cycles. Male mice also exhibited reproductive effects from ADBAC+DDAC exposure, with decreased sperm concentration and motility; thus, ADBAC+DDAC target both the female and male mouse reproductive systems. The presence of a biological target in mice suggested that other animals may be susceptible to ADBAC+DDAC-induced reproductive toxicity, including humans. To further evaluate potential reproductive toxicity, additional

studies to identify a possible mechanism of ADBAC+DDAC reproductive toxicity in males were conducted. Reductions in sperm parameters suggested that ADBAC+DDAC interfered with spermatogenesis. Spermatogenesis relies on the normal function Sertoli cells and the blood-testis-barrier (BTB). The BTB is a common target of reproductive toxicants. To test whether ADBAC+DDAC target Sertoli cells and the BTB, in-vitro assessments were conducted to analyze the effects of ADBAC+DDAC on the male reproductive system and identify a potential target in the male reproductive system. Cytotoxic effects of ADBAC+DDAC in the TM4 Sertoli cell line occurred independent of reductions in BTB integrity; however, concentrations of ADBAC+DDAC that nearly obliterated TM4 cell metabolism reduced BTB integrity. ADBAC+DDAC changes in cell metabolism, therefore, may occur before or in the absence of overt effects on the BTB. Furthermore, concentrations of ADBAC+DDAC that reduced TER were also correlated with G2/M cell cycle arrest, suggesting a correlation between mitotic inhibition and BTB integrity.

The primary function of sperm is to successfully fertilize oocytes. The acquisition of fertility is a multi-step process dependent on testis and epididymal environments. As a result, fertilization rates reflect testicular and epididymal function. In order to evaluate the effects of ADBAC+DDAC on sperm fertilizing ability, in-vitro fertilization rates were assessed. Males were dosed with ADBAC+DDAC and fertilizing ability was assessed immediately or after a 10-day waiting period. This time-dependent assessment was elected to investigate whether the testis or epididymis were the primary targets of ADBAC+DDAC-induced reduction in sperm parameters. Sperm collected from male mice given a 10-day rest period following ADBAC+DDAC dosing exhibited significantly decreased fertilizing ability. This finding suggests that fertilization was reduced through a delayed mechanism whose effect(s) manifested independent of reductions in sperm concentration and motility. These findings highlighted the importance of evaluating the effects of frequency and magnitude of ADBAC+DDAC exposure on reproductive function.

Exposure to environmental toxicants not only affects the individual, but also their germline. Several reproductive toxicants have been shown to have transgenerational effects through heritable changes in germline methylation patters. A number of DNA modifications and chromatin remodeling enzymes can be passed to the next generation altering DNA expression in the offspring. F_0 males directly exposed for 8 weeks to 120 mg/kg/day ADBAC+DDAC exhibited the most changes in mRNA expression relative to controls. Direct exposure to ADBAC+DDAC caused upregulation of two histone acetyltransferases and downregulation of one histone demethylase. In-utero (F_1) exposure to ADBAC+DDAC significantly reduced one maintenance methyltransferase. These same males demonstrated significant increases in sperm concentration compared to their control counterparts and their F0 ADBAC+DDAC predecessors; thus, ADBAC+DDAC-induced changes in reproductive function may only occur from direct exposure and did not result in transgenerational effects.

Overall, these studies have demonstrated the significant reproductive toxicity of ADBAC+DDAC in male and female mice. The ability of these compounds to interfere with multiple components of the male and female reproductive system suggests the presence of a prevailing endocrine disrupting mechanism. QACs are used in a wide variety of consumer products. The relevance of these findings to humans remains to be determined. Given their widespread use and persistence in the environment, identifying the extent of human exposure and determining whether QACs affect human reproduction is critically important.

APPENDIX A. Fold changes in RNA expression of chromatin remodeling enzymes

Table I. Fold changes in RNA expression related to chromatin remodeling enzymes in F_0 males exposed for 8 weeks to ADBAC+DDAC and their F_1 and F_2 progeny.

Gene Symbol	Fold Change (comparing to control group)					
	F_0 ADBAC+DDAC		F_1		F_2	
	Fold change	p-value	Fold change	p-value	Fold change	p-value
Ash1l	1.55	0.005	0.64	0.028549	1.52	0.007676
Atf2	1.67	0.169135	0.54	0.062726	1.82	0.004031
Aurka	1.36	0.096549	1.06	0.647978	1.17	0.429265
Aurkb	1.36	0.136522	1.14	0.622237	1.31	0.318537
Aurkc	1.72	0.167688	0.85	0.891687	2.76	0.057991
Carm1	1.24	0.165668	0.53	0.358351	1.37	0.016043
Cdyl	1.61	0.03329	0.61	0.335416	1.22	0.071998
Csrp2bp	0.86	0.260566	0.42	0.177129	0.87	0.085527
Dnmt1	1.89	0.050598	0.33	0.019138	1.02	0.927776
Dnmt3a	0.69	0.046669	0.52	0.462746	1.02	0.787319
Dot1l	0.93	0.303095	1.27	0.330963	1.06	0.595522
Dzip3	1.7	0.000342	1.17	0.449218	1.5	0.001655
Ehmt1	1.74	0.022509	0.89	0.347279	1.86	0.041059
Ehmt2	1.16	0.155356	1.67	0.101875	1.6	0.14335
Esco1	1.87	0.027776	1.14	0.533303	1.74	0.000843
Esco2	1.47	0.041991	1.06	0.370858	1.4	0.018777
Hat1	**2.31**	**0.011212**	0.65	0.331647	1.39	0.040468

Gene Symbol	Fold Change (comparing to control group)					
	F_0 ADBAC+DDAC		F_1		F_2	
	Fold change	p-value	Fold change	p-value	Fold change	p-value
Hdac1	1.4	0.090311	1.43	0.086259	1.41	0.001078
Hdac10	1.02	0.89626	0.64	0.238269	1.28	0.324804
Hdac11	1.47	0.057813	1.07	0.657728	1.4	0.065804
Hdac2	1.67	0.03517	0.58	0.124734	1.28	0.10195
Hdac3	1.15	0.066175	0.76	0.907854	0.61	0.611957
Hdac4	0.99	0.869187	1.21	0.524683	1.32	0.178107
Hdac5	1.33	0.068176	2.47	0.137534	1.73	0.055438
Hdac6	1.89	0.016791	1.24	0.249356	1.77	0.0425
Hdac7	1.5	0.103676	0.95	0.927052	1.17	0.346362
Hdac9	1.53	0.002843	1.03	0.683748	1.76	0.124441
Kat2a	1.43	0.03145	1.03	0.767194	1.27	0.033907
Kat2b	**2.2**	**0.036839**	1.22	0.521163	1.48	0.029279
Kat5	1.5	0.096507	1.54	0.127349	1.09	0.43237
Kdm1a	0.84	0.201724	1.21	0.524691	1.16	0.386275
Kdm4a	0.67	0.163008	0.72	0.231832	1.39	0.355536
Kdm4c	1.42	0.053086	0.65	0.061237	1.22	0.424292
Kdm5b	1.74	0.018498	1.11	0.620657	1.47	0.011425
Kdm5c	0.83	0.09639	1.64	0.313873	1.05	0.585681
Kdm6b	**0.4**	**0.047875**	1.4	0.389798	0.59	0.061504
Kmt2c	1.33	0.061592	1.01	0.691373	1.6	0.011162
Kmt2e	1.4	0.039013	0.98	0.671128	1.39	0.008061
Mysm1	1.78	0.031962	1.14	0.5853	1.64	0.026256

Gene Symbol	Fold Change (comparing to control group)					
	F_0 ADBAC+DDAC		F_1		F_2	
	Fold change	p-value	Fold change	p-value	Fold change	p-value
Kat8	1.29	0.027221	2.71	0.108081	1.33	0.010777
Kat7	1.38	0.024479	1.34	0.468855	1.1	0.068845
Kat6a	1.57	0.1053	0.9	0.767802	1.49	0.015264
Kat6b	1.49	0.100499	0.18	0.937899	1.48	0.012382
Ncoa1	1.12	0.60394	0.76	0.490646	1.05	0.855874
Ncoa3	1.57	0.078596	0.26	0.121825	1.55	0.051893
Ncoa6	0.9	0.848207	0.9	0.7881	0.74	0.226503
Nek6	1.63	0.113279	1.12	0.608105	1.94	0.010521
Nsd1	1.04	0.606966	1.09	0.637009	1.57	0.001963
Pak1	1.31	0.177527	1.55	0.279039	1.24	0.104892
Prmt1	0.97	0.750594	2.34	0.157776	1	0.896074
Prmt2	0.83	0.207666	1.98	0.245837	0.98	0.743734
Prmt3	1.4	0.047184	1.93	0.221185	1.74	0.059185
Prmt5	0.84	0.569088	1.45	0.366741	0.84	0.22447
Prmt6	0.5	0.081487	1.42	0.433494	0.71	0.058298
Prmt7	1.59	0.06997	1.43	0.254232	1.28	0.098236
Rnf20	1.42	0.099711	1.15	0.594059	1.44	0.026051
Rps6ka3	1.54	0.086575	1.35	0.382805	1.5	0.006621
Rps6ka5	1.59	0.048994	1.08	0.682071	1.64	0.014171
Setd1a	1.35	0.195511	0.75	0.659384	1.23	0.226652
Setd1b	1.19	0.186322	0.94	0.869968	1.3	0.004995
Setd2	1.6	0.063145	0.8	0.785625	1.35	0.01449
Setd3	0.92	0.602041	0.95	0.798756	0.83	0.180168

Gene Symbol	Fold Change (comparing to control group)					
	F_0 ADBAC+DDAC		F_1		F_2	
	Fold change	p-value	Fold change	p-value	Fold change	p-value
Setd4	1.38	0.080085	1.44	0.13868	1.54	0.009293
Setd5	0.94	0.756673	0.98	0.831214	1.18	0.1695
Setd7	0.77	0.237198	0.97	0.951681	0.98	0.772787
Setd8	1.59	0.088687	1.15	0.281909	1.5	0.010971
Setdb1	1.31	0.079245	1.04	0.725883	1.24	0.07381
Setdb2	1.11	0.419656	1.12	0.550375	1.24	0.049458
Smyd1	1.16	0.194717	0.99	0.997343	1.25	0.050672
Smyd3	1.21	0.255815	1.01	0.857845	1.4	0.129072
Suv39h1	0.93	0.113491	0.99	0.940146	1.16	0.052062
Suv420h1	1.32	0.235224	0.71	0.11059	1.38	0.033125
Usp16	1.4	0.107729	0.88	0.371782	1.26	0.014968
Usp21	1.23	0.31405	0.78	0.420101	0.93	0.595854
Usp22	1.45	0.156918	1.46	0.09575	1.66	0.00119
Whsc1	1.24	0.180952	0.94	0.73519	0.87	0.713649

APPENDIX B. Summary of methods completed in Chapters 2-5

CHAPTER 2

Animal husbandry and dosing conditions

CD-1 strain mice purchased from Charles River Labs (Raleigh, NC) were housed in disposable caging (Innovive, San Diego, CA) and maintained in a climate-controlled room with a 12-hour light/dark cycle, 20 – 25 °C, and 30 - 60% relative humidity. Soiled bedding from disposable cages was cleaned twice a week and box changes were performed weekly. Ethanol was used for room disinfection and to remove ADBAC+DDAC contaminants from equipment and personnel prior to entering the room housing the mice. Personnel donned hair bonnets, face masks, disposable gowns, gloves, and dedicated footwear prior to entering the control room to reduce potential ADBAC+DDAC contamination. Mice were provided Harlan 7013 rodent chow (Harlan Teklad, Madison, WI) and distilled water in disposable bottles *ad libitum*. At 8 weeks of age, all mice were paired two females to one male and bred for a maximum of two heat cycles (approximately 10 days). Mice were derived for 2 generations (F_2) before being used in QAC exposure studies; F_2 generations of mice were obtained in order to reduce the potential confounding health effects from previous QAC exposure.

At 5 weeks of age, F_2 mice were acclimated for one week to a nutritionally complete and purified Nutra-gel diet (Bio-Serv, Frenchtown, NJ), prepared by mixing purified dry mix formula and 100°C distilled water according to manufacturer instructions. Food consumption was monitored daily and determined by subtracting the weight of food eaten over 24 hours from a 25 g Nutra-gel diet gel cube. An average daily food consumption of 28% body weight in male and female mice was identified and used to calculate food dosing for ADBAC+DDAC exposure studies.

A short-term dose finding study was performed in unbred F_2 mice to identify the lowest observable adverse effect limit (LOAEL) for ADBAC+DDAC. Mice (n=5/dose) were transferred to a room utilizing an ADBAC+DDAC disinfectant (HWS-256, Sanitation Strategies, Holt, MI). Gel food was dosed with 0, 60, 120, 240, and 480 mg ADBAC+DDAC disinfectant/kg/day and provided to mice for two weeks. Mice were monitored daily and evaluated against 16 different health parameters for physical appearance, activity, physiology, and body weight loss. Signs of toxicity, such as inappetance, lethargy, and rough haircoat, were observed in animals receiving the 240 and 480 mg doses; thus, the LOAEL was identified as 240 mg ADBAC+DDAC disinfectant/kg/day. Two dose levels below the LOAEL (i.e., 60 and 120 mg ADBAC+DDAC/kg/day) were selected to evaluate the long-term effects of exposure to QACs.

At 6-8 weeks of age, unbred F_2 males and females were separated into breeding pairs and randomly assigned to 0 (control), 60, or 120 mg ADBAC+DDAC/kg body weight/day treatments. Ten control breeding pairs were isolated in an adjacent QAC-free (QF) room utilizing 70% ethanol as a disinfectant and admitting personnel were required to don personal protective equipment to exclude potential ADBAC+DDAC cross-contamination. Control breeding pairs were provided 25 g of undosed Nutra-gel diet cubes. Twenty breeding pairs were transferred to a room utilizing HWS-256 ADBAC+DDAC disinfectant and separated into 2 groups: 10 breeding pairs were assigned to 60 mg ADBAC+DDAC/kg/body weight/day and 10 breeding pairs were assigned to 120 mg ADBAC+DDAC/kg/body weight/day. Doses of ADBAC+DDAC/kg body weight/day were calculated based on the sum of active ingredient in the disinfectant (6.76% ADBAC (60% C-14, 25% C-12, 15% C-16) and 10.1% DDAC) and an average daily food consumption of 28% body weight. Subsequently, doses of ADBAC+DDAC were provided daily in a 25g Nutra-gel diet cube for a total of 180 days (6 months). Fresh gel cubes were added daily and monitored for food consumption. Mice were monitored daily and evaluated against 16 different health parameters for physical appearance, activity, physiology and feed consumption according to the Ullman-Cullere' and Foltz body condition scoring.[1] Male mouse weight

was recorded weekly. Female body weight was not recorded as body weight fluctuated with their stage of pregnancy.

At birth, delivered pups from all breeding pairs were counted, weighed, evaluated for gross malformations, and then euthanized by IP injection of sodium pentobarbital (0.05 mL/g). All animal experiments were approved by the IACUC at the College of Veterinary Medicine at VPI, an AAALAC accredited facility.

Statistics

Time effect was normalized to the standard 20-day reproductive intervals in the mouse, over total of 180 days. The first 6 days were discarded to control for initial differences in estrus cycle stage and to ensure that each dam was given sufficient time to enter estrus. Pup number and food consumption were measured cumulatively. Normal probability plots showed that cumulative pup numbers, cumulative amount of feed consumed, male mouse weight, and average weight per pup followed a normal distribution. Time to first litter (defined as the time between initial animal pairing/dosing and the appearance of pups) and average number of pregnancies were skewed. Analysis of the average number of pregnancies was performed over the first 100 days prior to any dam loss (n=10). Effects of ADBAC+DDAC treatment on cumulative pup numbers, cumulative amount of feed consumed, male mouse weight, and average weight per pup were assessed using repeated measures analysis of variance (RM-ANOVA). The linear model specified treatment, 20-day intervals, and the interaction between treatment and 20-day intervals as fixed effects with Kenward-Roger as denominator degrees of freedom. The model also specified that the measurements were repeated over dam identification within treatment with an autoregressive order one (AR1) covariance structure. To specifically examine the effect of treatment at each 20-day interval, the slicediff option of the glimmix procedure was applied followed by Tukey's procedure for multiple comparisons. Effects of treatment on total number of pups were assessed using one-way analysis of variance (ANOVA) followed by Tukey's procedure for multiple comparisons.

Effects of treatment on time to first litter, average number of pregnancies, and slopes were assessed using the Kruskal-Wallis test (KW) followed by Dunn's procedure for multiple comparisons. Residual plots for the ANOVA models were inspected to verify model adequacy (i.e., errors followed a normal distribution with constant variance). Statistical significance was set to alpha < 0.05. All analyses were performed using SAS version 9.2 (Cary, NC).

CHAPTER 3

Animal husbandry and dosing conditions

CD-1 strain mice purchased from Charles River Labs (Raleigh, NC) were housed in disposable caging (Innovive, San Diego, CA) and maintained in a climate-controlled room with a 12-hour light/dark cycle, 20 – 25 $^{\circ}$C, and 30 - 60% relative humidity. Soiled bedding from disposable cages was cleaned twice a week and box changes were performed weekly. Ethanol was used for room disinfection and to remove ADBAC+DDAC contaminants from equipment and personnel prior to entering the room housing the mice. Personnel donned hair bonnets, face masks, disposable gowns, gloves, and dedicated footwear prior to entering the control room to reduce potential ADBAC+DDAC contamination. Mice were provided Harlan 7013 rodent chow (Harlan Teklad, Madison, WI) and distilled water in disposable bottles *ad libitum*. At 8 weeks of age mice were paired for breeding, and 2 or 3 females were housed with a male of the same strain. For breeding, all mice were paired two females to one male and co-housed for a maximum of two heat cycles (approximately 10 days). Mice were derived for 2 generations before being used in QAC exposure studies. F_2 mice used as controls were housed in a QF facility, while QAC-exposed mice were transferred prior to dosing and housed in a facility utilizing the ADBAC+DDAC disinfectant.

Male and female mice were dosed by adding ADBAC+DDAC (Sanitation Strategies, Holt, MI) into Nutra-gel diet (purified dry mix formula, Bio-Serv, Frenchtown, NJ) which was prepared following manufacturer instructions, or dosed by adding the ADBAC+DDAC into distilled water provided for the mice. For experiments dosing in the food, control mice were kept in the QF facility and provided undosed gel food while treated mice were moved to the QAC facility and provided 120 mg/kg/day ADBAC+DDAC in gel food. Food consumption was recorded daily and was not significantly different between treatment groups (data not shown). Doses of ADBAC+DDAC/kg body weight/day were calculated based on the sum of ADBAC+DDAC (6.76% ADBAC (60% C-14, 25% C-12, 15% C-16) and 10.1% DDAC), with an average daily food consumption of 28% body weight and provided daily. For water dosing, ADBAC+DDAC/kg body weight/day were calculated based on the sum of ADBAC+DDAC (6.76% ADBAC (60% C-14, 25% C-12, 15% C-16) and 10.1%), with an average daily water consumption of 10% body weight and provided fresh daily. Control mice were kept in the QF facility and provided undosed distilled water and treated mice were moved to the QAC facility and provided 120 mg/kg/day ADBAC+DDAC in distilled water.

Quantification of ovulation and implantation

Percentages of successful implantations were evaluated in mice dosed with ADBAC+DDAC for 2 weeks in gel food and for 8 weeks in distilled water. Both males and females were maintained on treatments for 2 or 8 weeks and throughout breeding. Females were bred to unrelated males and the presence of a copulatory plug in the female mouse vagina designated gestational day (GD) 0. On GD 6, pregnant females were given an intravenous injection of pontamine sky blue dye (Sigma Aldrich, St. Louis, MO) in the tail vein. One minute after injection, females were euthanized using CO_2 inhalation. Gravid uteri were assessed under a stereozoom microscope (Olympus SZX7, Melville, NY) for blastocyst attachment sites on the uterine epithelium (indicated by blue bands of pontamine dye staining) and for pink corpora lutea (CL) in the ovary. Implantation sites and CLs were assessed in 6 females for the 2

week ADBAC+DDAC gel food exposure and 9-10 females for the 8 week ADBAC+DDAC water exposure.

Estrus stage identification

F_2 mice raised in the QF facility were divided into control and treated groups at 6-8 weeks of age (n=9-10). Control mice were kept in the QF facility and provided undosed Nutra-gel cubes. Treated mice were moved to the QAC facility and provided 120 mg/kg/day 6.76% ADBAC+ 10.1% DDAC in Nutra-gel cubes for 2 weeks prior to evaluating vaginal cytology and throughout the 20 day evaluation period (5 weeks exposure total).

Vaginal cytology was collected to determine estrus cycle stage according to specifications reported in Byers et al, 2012.[2] Cells were collected from female mouse vaginas using a 15 cm cotton tipped applicator (Puritan, Guilford, ME) wetted with sterile saline. The applicator tip was inserted superficially into the vagina, to avoid induction of pseudo pregnancy, and the tip was turned 360°. The swab was pressed onto a glass slide to extract cells from the applicator. After the slide was air dried, the three-stage Harleco Hemacolor stain set (contains a methanol fixative, eosin stain and methylene blue stain; Philadelphia, PA) was used to stain the cells, and residual stain was rinsed off the slide with deionized water. After staining, cells were enumerated by counting the differential percentage of each cell type out of 100 cells at the 40X objective using a compound microscope. Nucleated and some immature cornified epithelial cells are prominent in vaginal smears of females in proestrus, although leukocytes may be present during early proestrus. Clusters of cornified epithelial cells are predominant in vaginal smears of females in estrus. A combination of cornified epithelial cells and some polymorphonuclear leukocytes are present during metestrus. Polymorphonuclear leukocytes are the most abundant during diestrus.

Post-implantation losses

F_2 mice raised in the QF facility were divided into control and treated groups (n=9-10 females). Control mice were kept in the QF facility and provided undosed gel food. Treated mice were moved to the QAC facility and provided 120 mg/kg/day 6.76% ADBAC+ 10.1% DDAC in gel food for 8 weeks prior to breeding and throughout gestation. For breeding, 2 or 3 females were housed with a male of the same strain and females were monitored daily for copulatory plugs. The presence of a copulatory plug designated GD 0.

On GD10, females were euthanized by CO_2 inhalation. Mid-gestational embryos were dissected out of the uterus and evaluated under a stereozoom microscope (Olympus SZX7, Melville, NY). Embryonic resorptions were characterized by the absence of normal embryonic tissue and abnormally sized and shaped decidual tissue. Embryos were staged by GD, somite count, branchial arches, extent of heart and limb bud formation and deepening of the lens pit. Embryos exhibiting <25 somites were not included in the analysis; by GD10, most embryos have 30-40 somites at evaluation.

Sperm assessment

Male mice raised in the QF facility were divided into control and treated groups (n=10-11). Control males were kept in the QF facility and provided undosed distilled water. Treated males were moved to the QAC facility and provided 120 mg/kg/day 6.76% ADBAC+ 10.1% DDAC in distilled water for 8 weeks. After the 8 week exposure period, males were euthanized by CO_2 inhalation and both cauda epididymes were excised and placed in a 35x10 mm Petri dish (Falcon, Oxnard, CA) containing one mL of pre-warmed Dulbecco's Modified Eagle's Medium (DMEM: sterile filtered 1000 mg/L glucose and sodium bicarbonate, pyridoxine HCl with no L-glutamine or phenol red) supplemented with 1 % fetal bovine serum (GIBCO, Grand Island, NY). Cauda epididymes were minced with a number 11 disposable sterile surgical blade (Feather Safety Razor Co. LTD, Osaka, Japan), mixed with a pipette, and incubated at 37°C with 5% CO_2 for 10-15 minutes to release contents. A 500 μL aliquot of cauda epididymal extract was transferred to a 1.5

mL conical tube (Falcon, Oxnard, CA) and diluted to 1.5 mL with pre-warmed DMEM supplemented with 1 % fetal bovine serum.

Hemacytometer sperm counts

A 500 µL aliquot of cauda epididymal extract was diluted 10X with distilled deionized water to inhibit sperm motility. A 10 µl aliquot of the sperm suspension was loaded onto both sides of a Neubauer hemocytometer (American Optical, Buffalo, NY) and counted twice. Fully intact sperm (sperm with heads and tails) within five primary squares of the counting chamber were totaled. Sperm counts were averaged, and sperm concentrations were calculated using the following equation: average hemacytometer count x (1 mL of DMEM in Petri dish/500 µL transferred to 15 mL conical tube) x (10 mL of total volume in the 15 mL conical tube / 0.5 µL volume of squares counted). For example, if the average hemacytometer count was 350 sperm, then: 350 x (1 mL/0.5 mL or 2) x (10 mL/0.0005 mL or 20,000) the total sperm count would be 14 x 10^6 sperm/mL.

Computer-automated sperm analysis (CASA)

Duplicate 3µL sample extracts were analyzed using 20 µm-deep 4-chamber Leja slides (Minitube of America, Verona, WI). Slides were allowed to settle for 2-3 minutes before analysis. Eight random fields were analyzed with 20X phase-contrast at 30 frames per second (Model BX41, Olympus Optical, Tokyo, Japan) using Sperm Vision 3.5 (Minitube of America, Verona, WI) to calculate sperm concentration and motility. If the average sperm concentration between fields was not statistically valid (e.g. very high standard error), a new chamber was prepared for analysis. Sperm motility and structure was confirmed used frame overlays and 100X magnification. Sperm following a non-linear, linear, or curvilinear swimming pattern were classified as motile, while non-swimming sperm were classified as immotile.

Flow cytometric assessment of sperm viability

The LIVE/DEAD Sperm Viability Kit (Molecular Probes, Inc. Eugene, OR) containing fluorescent dyes SYBR-14 and propidium iodide (PI) was used for flow cytometric assessment of sperm membrane integrity and viability. SYBR-14 is a membrane permeant dye and was used to stain live sperm with intact membranes, while PI is a membrane impermeant dye and was used to stain dead sperm with damaged membranes. Protected from light, cauda epididymal extracts (300 µL) were treated with 2 µL of 2.4 mM PI and 2 µL of 10 µM SYBR-14. Samples were incubated for 15 minutes prior to flow cytometric analysis with a Coulter EPICS XL-MCL benchtop analyzer with a 488 nm excitation source (Indianapolis, IN) and data were analyzed with FlowJo Software (TreeStar, Ashland, OR). Gates were set to isolate cell singlets and to exclude cellular aggregates and debris. PI positive sperm emitting a red fluorescent signal were detectable at >670 nm using fluorescence emission detector 3. SYBR-14 positive sperm emitting green fluorescence at wavelengths of 515–545 nm was detected by fluorescence detector 1. Non-sperm cellular debris was gated out of cytometric analyses according to scatter properties as detected by the forward-scatter (cell size) and sideways-scatter (cell granularity) characteristics, respectively. For each sample, scatter and fluorescent properties were collected for approximately 1×10^4 double-gated singlet events. A quadrant gate was drawn using single color controls and plots of sideways- and forward-scatter properties as well as SYBR-14 versus PI fluorescence were used to identify populations of live, moribund, and dead sperm.

Statistics

Statistical analysis was conducted using Statistix (Tallahassee, FL). Data are expressed as the mean ± SEM. Data normality was determined using normal probability plots and compared using the Student *t*-test for normally distributed data or two-sample rank testing for data not distributed normally. The dam was specified as the treatment unit to determine differences in ovulation and implantation and post-implantation losses among treatment groups. Significance was set at $p \leq 0.05$.

CHAPTER 4

Preparation of test materials

Solutions of ADBAC+DDAC used for cytotoxicity assessments were prepared in sterile DMEM-F12 (ATCC, Manassas, VA) culture medium. Initially, 1 L of basal media was sterilized using a 0.22 µm Millipore filtration system (Darmstadt, Germany). Solutions of benzyl dimethyl hexadecyl ammonium chloride (ADBAC C16; Sigma-Aldrich, St. Louis, MO), benzyl dimethyl tetradecyl ammonium chloride (ADBAC C14; Sigma-Aldrich, St. Louis, MO), benzyl dimethyl dodecyl ammonium chloride (ADBAC C12; Sigma-Aldrich, St. Louis, MO), didecyl dimethyl ammonium chloride, 80% aqueous solution (DDAC; AK Scientific Inc., Union City, CA) were used to create ADBAC and DDAC stock solutions. Solutions of ADBAC and DDAC were made based on the ratio of alkyl chain lengths and sum of active ingredients in the QAC disinfectant previously reported by our laboratory, which had a composition of 6.76% ADBAC (60% C-14, 25% C-12, 15% C-16) and 10.1% DDAC). A 500 mL stock solution of 1% ADBAC+DDAC in DMEM-F12 was diluted and maintained at 4°C for up to 1 week before being replaced.

TM4 Sertoli cells

TM4 mouse Sertoli cells (CRL-1715; ATCC, Manassas, VA) were cultured in a 1:1 mixture of Ham'S F12 medium (F12) and Dulbecco's modified Eagle's medium (DMEM) with 1.2 g/L sodium bicarbonate and 15 mM HEPES, 5% horse serum, and 2.5% fetal bovine serum (ATCC, Manassas, VA) at 37°C with 5% CO_2. Media was replaced every 72 hours and removed prior to the addition of trypsin-EDTA. Cell monolayers at 80% confluency were dissociated from 75-cm^2 polystyrene tissue culture flasks (Corning, NY) using a solution of 0.25% trypsin-0.53 EDTA (ATCC, Manassas, VA). Monolayers were incubated with 2 mL of trypsin-EDTA solution at 37°C for 4-6 minutes and evaluated under an

inverted microscope to ensure cell dissociation. Following dispersal of the cell layer, 8 mL of serum-supplemented DMEM-F12 was added to collect unattached cells and block further trypsin digestion. The 10 mL cell suspension was transferred to a sterile 15 mL centrifuge tube then centrifuged at 125 x g for 10 minutes to pellet cells. Fresh media (7 mL) was transferred to a new 75-cm^2 polystyrene flask and equilibrated at 37°C and 5% CO_2 during cell centrifugation. After centrifugation, the supernatant was removed and cells were resuspended in 3 mL of fresh serum supplemented DMEM-F12, then transferred to the pre-equilibrated cell culture flask containing 7 mL of fresh media (total volume = 10 mL). A sub-cultivation ratio of 1:10 to 1:20 was used for all passages and cells with passage numbers exceeding 10 were not used in experiments.

Resazurin cytotoxicity assay

Cell-culture grade resazurin sodium salt powder was purchased from Sigma-Aldrich (Carlsbad, CA). A 1 L stock solution of 0.15 mg/mL resazurin was prepared in sterile PBS (pH = 7.4) and sterilized using a 0.22 μm Millipore filtration system. The resazurin solution was stored protected from light at 4°C for no more than 30 days before being replaced.

Using a multichannel pipette, 100 μL aliquots of cell suspension were seeded into the inner 6 x 10 wells of Corning 96-well microtiter plates (Corning, NY) at a concentration of 20,000 cells/mL. Outer wells were filled with 100 μL of culture medium. Plates were incubated for 12 hours (h) at 37°C, with an atmosphere of 5% CO_2 and 95% humidity and evaluated under an inverted microscope to ensure uniform cell attachment. Media was removed and replaced with fresh DMEM-F12 or DMEM-F12 containing 0.0001%, 0.0005%, 0.001%, 0.005%, 0.01%, 0.05%, or 0.1% ADBAC+DDAC made from the 1% ADBAC+DDAC stock solution. A total of 12 wells per plate were reserved for untreated control cells and 6-12 wells were assigned for each ADBAC+DDAC treatment concentration. After a 72 h incubation period at 37°C with an atmosphere of 5% CO_2 and 95% humidity, plates were removed from the incubator and 2X serial dilutions from a starting concentration of 20,000 cells/mL were performed across

12 wells. Mitochondrial metabolism was assessed by adding 20 µL of 0.15 mg/mL resazurin solution to each well containing cells, and also added to 4 wells containing media for background subtraction. After a 4 h incubation period, absorbance was calculated for each well at 570 nm (reduced form) and 590 nm (oxidized form) using a SpectraMax Plus384 plate reader. The absorbance values of the oxidized form of resazurin were calculated by determining the difference in absorbance between resazurin + media and media at both wavelengths. The dye correction factor was calculated by dividing the absorbance of fully oxidized resazurin at 570/590 nm and used to determine the percent difference in resazurin reduction between treated and control cells.

Cell cycle analysis

TM4 cells at 40% confluency were incubated for 24 h (doubling time of TM4 cells is 16 h) with 10 mL of undosed media or 10 mL of media containing cytotoxic concentrations of 0.01% or 0.1% ADBAC+DDAC to determine the phase at which the cell cycle was disturbed. After 24 h, cell monolayers were rinsed with sterile PBS and dissociated using 2 mL of 0.25% trypsin-0.53 mM EDTA. After cell dissociation, 8 mL of sterile PBS supplemented with 2.5% FBS was added to the culture flask containing the cell suspension, gently mixed with a pipette, then transferred to a 15 mL centrifuge tube. Cell suspensions were centrifuged for 10 minutes at 125 x g and the supernatant was discarded. Cell pellets were washed two more times by re-suspension in 10 mL sterile PBS (no serum) and centrifugation. The supernatant was again discarded and the washed cell pellet was fixed by adding 1 mL of cold 70% ethanol (10^6 cells/mL) in a drop-wise fashion while vortexing. Cells were fixed at 4°C for 24 hours. After fixation, cells were washed 2X in PBS, and then treated with 100 µg/mL of RNase (Invitrogen, Carlsbad, CA) for 20 min at 37°C. Cells were stained with 200 µL of 50 µg/mL propidium iodide (Invitrogen, Carlsbad, CA) and maintained for 24 h at 4°C before flow cytometric analyses. A FACScan system (Becton Dickinson, Franklin Lakes, NJ) was used to record forward and side scatter characteristics and fluorescence emissions for a minimum of 1×10^4 cells. Flow cytometric parameters

were set to excitation at 488 nm with 15mW argon-laser and emission at 585 nm and the area of each fluorescence peak was used to identify relative number of cell nuclei with respect to DNA copy number. FloJo software (Treestar, Ashland, OR) was used to determine the fraction of cells in G1, S, and G2, the widths of the G1 and G2 peaks, and the number of cells below G1 and above G2.

Transepithelial electrical resistance (TER) measurements

Matrigel mouse extracellular matrix (BD Biosciences, Palo Alto, CA) was diluted 1:6-1:10 with cold basal DMEM-F12 using sterile, pre-chilled pipette tips. Cold Matrigel is a liquid that rapidly gels at room temperature; therefore, it is essential to use pre-chilled pipette tips to avoid premature gelling. Sterile bicameral chambers (12 mm diameter, 0.4 μm pore size, 0.6 cm^2 surface area; Millipore, Bedford, MA) were placed in each well of a 24-well plate using sterile forceps. Pre-chilled, sterile pipette tips were used to transfer 150 μL of liquid Matrigel to the filter area of each bicameral chamber, avoiding the formation of air bubbles. Plates containing Matrigel-coated chambers were incubated overnight at 37°C until a firm gel formed. Each Matrigel chamber surface was gently rinsed using warm DMEM-F12 prior to plating TM4 cells at a density of 2×10^6 cells/cm^2. DMEM-F12 supplemented with 10% horse serum and 5% FBS was replaced every 2 days (600 and 400 μL of media for the upper and lower chambers, respectively) and maintained to prevent disruption of the cells. Cultures were stabilized at room temperature for 20 minutes before TER measurements. TER measurements were recorded using an EVOM 2 voltmeter equipped with adjustable width double electrodes (World Precision Instruments, Sarasota, FL). Measurements were taken at 24, 48, 72, 96, 120, 144 and 168 h after initial plating to confirm the formation of a complete barrier, indicated by stabilization of TER. After stabilization of TER, media in the upper (apical) chamber was replaced with media containing 0.0001, 0.001, 0.01, and 0.1% ADBAC+DDAC. TER recordings were done in triplicate at six 24-h intervals (144 h total) to assess the effect of ADBAC+DDAC on barrier permeability. The plasticizing agent phthalic acid mono-2-ethylhexyl ester (MEHP; Sigma-Aldrich, St Louis, MO), a known BTB disruptor, was utilized as a

positive control. Final resistance values were calculated by subtracting the mean TER of blank bicameral chambers coated with only Matrigel (no cells), and correcting for the surface area of the bicameral chamber (0.6 cm^2). Values are expressed as $\Omega*cm^2$.

Husbandry

CD-1 mice were initially purchased from Charles River Laboratories (Raleigh, NC) and bred for at least two generations before being used in experiments. CD-1 mice were maintained in disposable caging (Innovive, San Diego, CA) on a 12-h light/dark cycle at 20 – 25 °C with 30 - 60% relative humidity. Harlan 7013 rodent chow (Harlan Teklad, Madison, WI) and distilled water were provided ad libitum. Control mice were housed in a facility that did not use QAC disinfectants. QAC-exposed mice were housed separately from controls in a facility that utilized QAC disinfectants.

In-vitro fertilization (IVF)

Cell media was prepared in two forms: 1) standard culture KSOM (Millipore, Billerica, MA) supplemented with 1.0 mg/mL bovine serum albumin (BSA) and 2) BSA enhanced KSOM supplemented with 4.0 mg/mL BSA (mKSOM). Two forms of FHM medium (Millipore Corp., Billerica, MA) were also prepared: standard FHM supplemented with 1 mg/ml BSA and mFHM supplemented with 4mg/mL BSA.

Ten male mice received 30 mg/kg/day ADBAC+DDAC in saline for 7 days via oral gavage, and 10 males received saline vehicle control. Five males were euthanized by CO_2 inhalation after the 7 day exposure period and both cauda epididymi were collected. Spermatozoa in the sperm capacitation dish were gently aspirated from the left and right cauda epididymi in warm FHM medium by making 5 punctures to the epididymis using a 21-gauge needle. The sperm capacitation dish was incubated for 10 min at 37°C to allow for sperm to swim-out and residual pieces of epididymal tissue were removed from the dish. The sperm solution was gently pipetted into a sterile 600 µL microcentrifuge tube to provide a

swim-up column in which non-motile sperm and cellular debris settle to the bottom of the tube. A 10 µL aliquot of the sperm suspension was diluted ten-fold with distilled water to immobilize sperm and counted twice on both sides of a Neubauer hemocytometer (American Optical, Buffalo, NY) for a total of four counts/sample. Only structurally intact sperm within five primary squares of the counting chamber were totaled and duplicate counts were averaged for each sample. Sperm motility was assessed using computer-automated sperm analysis (CASA). Duplicate 3µL sample aliquots were also analyzed using 20 µm-deep 4-chamber Leja slides (Minitube of America, Verona, WI). Eight random fields were analyzed with 20X phase-contrast at 30 frames per second (Model BX41, Olympus Optical, Tokyo, Japan) using Sperm Vision 3.5 (Minitube of America, Verona, WI) to calculate sperm motility. Sperm following a non-linear, linear or curvilinear swimming pattern were classified as motile, while non-swimming sperm were classified as immotile. Sperm exhibiting 80-90% CASA motility were adjusted to a concentration of $5x10^4$ sperm and transferred to 250 µL FHM/KSOM medium under 1 mL embryo-tested light mineral oil (Millipore, Bedford, MA) approximately 1 h before the beginning of IVF to undergo capacitation. Female CD-1 mice were super-ovulated by serial intra-peritoneal injections of pregnant mare serum gonadotropin (PMSG) followed 48 h later by human chorionic gonadotropin (hCG). Approximately 5 IU of each gonadotropin was administered in 100 µL of sterile saline diluent using a 27-gauge needle. Dams were euthanized by CO_2 inhalation and cumulus masses of ovulated eggs (COC) were collected from dam oviducts 12-14 h after hCG injection. After 1-1.5 h of capacitation, 5×10^4 sperm were added to a fertilization dish containing COC and incubated for 4-6 h to fertilize oocytes. All oocytes were then removed from the fertilization dish; any fragmented or irregularly shaped degenerate oocytes were discarded.

Statistics

Statistical analysis was conducted using Statistix (Tallahassee, FL). Data are expressed as the mean ± SEM. Data normality was determined using normal probability plots. Cell viability and cycle

analysis data were evaluated using Kruskal-Wallis. TER measurements were compared using two-way ANOVA to look at the effects of time, treatment, and the interaction of time and treatment on resistance values. Sperm concentration, motility, and fertilization rates relative to controls were evaluated using ANOVA. Significance was set at $p \leq 0.05$).

CHAPTER 5

Animal husbandry for mating indices and pregnancy rates

CD-1 mice were initially purchased from Charles River Laboratories (Wilmington, MA) and maintained in disposable caging (Innovive, San Diego, CA) on a 12-hour light/dark cycle at 20 – 25 °C with 30 - 60% relative humidity. Harlan 7013 rodent chow (Harlan Teklad, Madison, WI) and distilled water were provided ad libitum. Mice were not dosed, but were ambiently exposed through routine husbandry practices and cleaning to a QAC disinfectant containing 6.76% ADBAC (60% C-14, 25% C-12, 15% C-16) and 10.1% DDAC (Sanitation Strategies). After weaning the F_0 mice were transferred to a QAC-free (QF) facility utilizing a chlorine dioxide disinfectant and held for 8 weeks. Mice were then paired (two females to one male) for breeding at 8-11 weeks of age in the QF facility to derive an F_1 generation. F_1 mice were reared in the QF facility until they were 6-8 weeks old, and then paired for breeding (two females to one male) to derive an F_2 generation which was subsequently bred at 6-8 weeks of age (Figure 1).

Mating indices and pregnancy rates

Mating indices and pregnancy rates assessed in F_0 (n=8) F_1 (n=11), and F_2 (n=10) mated pairs for each generation in the respective facility. Mating index was calculated by assessing the number of

females with copulatory plugs, divided by the number of females co-habited with a male. Pregnancy rate was calculated based on the number of females pregnant, divided by the number of females with evidence of breeding (i.e. presence of a copulatory plug).

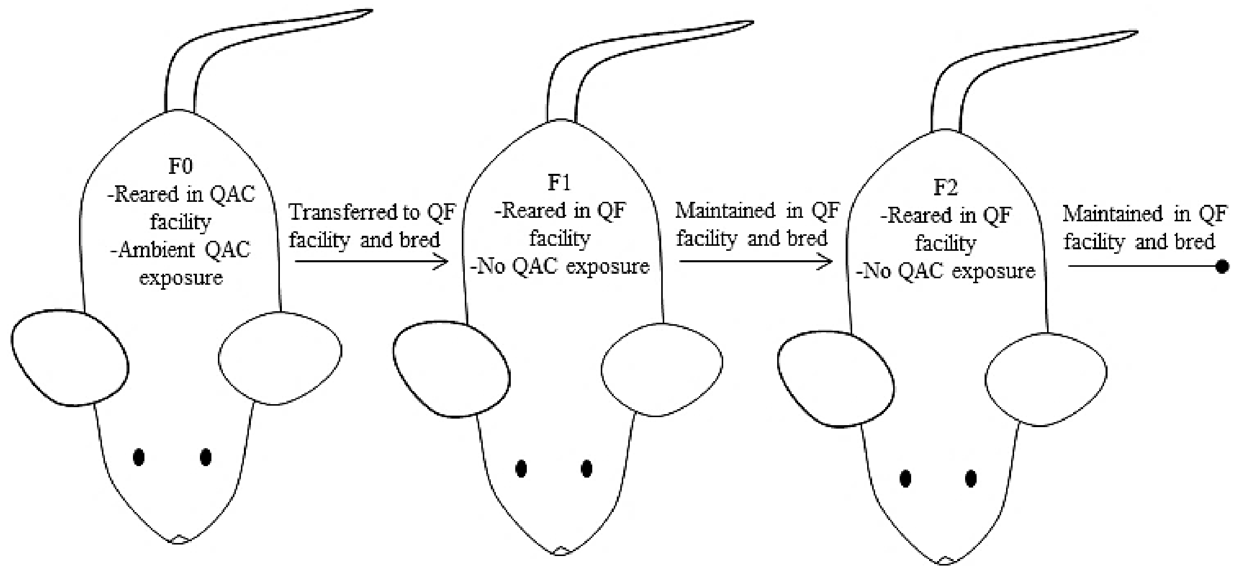


Figure 1. F0 females were exposed to ambient ADBAC+DDAC for 8 weeks then transferred to a QF facility. F0 females were bred in the QF facility for one generation to derive F1 QF mice, which were then bred to produce a F2 QF population.

Husbandry for multigenerational assessment of sperm parameters and testis mRNA expression

F_0 male and female mice (n=8-10) were dosed by adding ADBAC+DDAC (Sanitation Strategies, Holt, MI) to distilled water. Doses of ADBAC+DDAC/kg body weight/day were calculated based on the sum of ADBAC+DDAC (6.76% ADBAC (60% C-14, 25% C-12, 15% C-16) and 10.1% DDAC), with an average daily water consumption of 10% body weight and provided daily.[2] Water consumption was not significantly different between treatment groups. F_0 mice (n=10) were exposed for 8 weeks and throughout breeding and gestation to ADBAC+DDAC or distilled water. At approximately GD 19, F_0 mice were transferred to a QF facility and F_1 progeny were cross-fostered to control dams upon delivery.

At 8-10 weeks of age, F_1 males (n=8-10) were bred to untreated control females to derive an F_2 generation (n=8-10). The F_2 generation was euthanized at 8-10 weeks of age for evaluation of sperm and testis.

Sperm isolation

Epididymal sperm samples were collected from all generations of male mice following euthanasia by CO_2 inhalation. Both cauda epididymes were excised and placed in 1 mL of pre-warmed Dulbecco's Modified Eagle's Medium (DMEM: sterile filtered 1000 mg/L glucose and sodium bicarbonate, pyridoxine HCl with no L-glutamine or phenol red) supplemented with 1% fetal bovine serum (GIBCO, Grand Island, NY). Cauda epididymes were cut six times with a sterile surgical blade, mixed with a pipette and incubated at 37°C with 5% CO_2 for 10-15 minutes to release sperm. A 500 µL aliquot of cauda epididymal extract was transferred to a 1.5 mL conical tube and diluted to 1.5 mL with pre-warmed DMEM supplemented with 1% fetal bovine serum.

CASA

Sperm concentration and motility was evaluated in F_0 (n=10) F_1 (n=9), and F_2 (n=8) male mice from three different litters per generation. Duplicate 3µL sample extracts were analyzed using 20 µm-deep 4-chamber Leja slides (Minitube of America, Verona, WI). Slides were allowed to settle for 2-3 minutes before analysis. Eight random fields were analyzed with 20X phase-contrast at 30 frames per second (Model BX41, Olympus Optical, Tokyo, Japan) using Sperm Vision 3.5 (Minitube of America, Verona, WI) to calculate sperm concentration and motility. Sperm following a non-linear, linear or curvilinear swimming pattern were classified as motile, while non-swimming sperm were classified as immotile.

RT-PCR

The left and right testes (epididymis removed) from three unrelated males were used for multi-generational assessment of ADBAC+DDAC on RNA expression. Control testes were collected from one generational matched male for each generation. Testis RNA was isolated using an RNeasy Mini Kit according to manufacturer instructions (Qiagen, Hilden, Germany). Testes were transferred to a collection tube containing 350 μL of lysis buffer (Buffer RLT in β-meracaptoethanol, 1:100). Testes were homogenized immediately using a tissue homogenizer ensuring no chunks of tissue remained in solution. The homogenate was transferred to a capped test tube. The homogenate was centrifuged for 4 min at 14,000 x g at 20-25°C to pellet cell debris. The supernatant was transferred into a new 2 mL microcentrifuge tube and 1 volume equivalent (~ 350 μL) of 70% ethanol was added to the supernatant and mixed well by pipetting. The supernatant (700 μL) and any precipitate that formed were transferred into an RNeasy Mini Spin Column in a 2 mL collection tube and centrifuged at 10,000 x g for 15 sec. Flow-through was discarded and 350 μL of Buffer RW1 was pipetted into the column and centrifuged at 10,000 x g for 15 sec to wash. The flow-through was discarded and the column was washed with 350 μL Buffer RPE and centrifuged for 15 sec at 10,000 x g. The flow-through and the collection tube were discarded and the column was transferred into a new 2 mL collection tube. Buffer RPE (500 μL) was transferred onto the column and centrifuged for 15 sec at 10,000 x g. The flow-through was discarded. An additional 500 μL of Buffer RPE was added onto the column and centrifuged for 2 min at 10,000 x g to dry the column's silica gel membrane. The column was removed from the collection tube while avoiding contact with the flow-through and placed in a new 2 mL collection tube. The old tube and flow-through were discarded. The column was then centrifuged at 14,000 g for 2 min. The column was then transferred into a new 1.5 mL collection tube and 50 μL RNase-free water was pipetted directly onto the membrane and centrifuged for 1 min at 10,000 x g to elute RNA. The eluted RNA sample was stored at -20°C until cDNA synthesis.

Prior to cDNA synthesis, RNA concentration and purity was evaluated using a Nanodrop 2000 UV-Vis Spectrophotometer (Thermo Scientific, Waltham, MA). Only RNA with a 260/280 absorbance ratio ≥ 2.0 was used for cDNA synthesis and RT-PCR analysis. RNA starting concentrations were adjusted to 0.5 µg using nuclease-free water before cDNA synthesis using a RT^2 First Strand Kit (SABiosciences, Frederick, MD). A 1 µg sample of extracted RNA was mixed with 2 µL of super array 5X genomic DNA elimination mixture to eliminate genomic DNA contamination. RNase-free water was added to makeup the final volume of 10 µL. This mixture was incubated at 42°C for 5 min and immediately chilled on ice. Next, an equal volume of RT cocktail containing 4 µL of 5X super array RT buffer, 1 µL of Primer and External Control Mix, 2 µL of super array RT enzyme mix 3, and 3 µL RNase-free water) was added. The mix was incubated at 42°C for 15 min, and then heated at 95°C for 5 min to inactivate the reverse transcriptase. The mix was then stored at -20°C until analysis.

The Mouse Epigenetic Chromatin Modification Enzymes RT^2 Profiler™ PCR Array (SABiosciences, Frederick, MD) was used to detect the RNA expression of enzymes known or predicted to modify genomic DNA and histone structure The PCR Array configuration was a 96-well plate containing primer assays for 84 pathway genes and 5 housekeeping genes to normalize array data. One well with a genomic DNA control, three wells with reverse-transcription controls, and three wells with positive PCR controls were used to evaluate inter-well/intra-plate accuracy and precision. A total volume of 25 µL of PCR mixture containing 12.5 µL of RT^2 Real-Time SYBR Green PCR master mix from SuperArray Bioscience (containing HotStart DNA Polymerase and SYBR Green dye), 11.5 µL of double-distilled, nuclease-free water, and 1 µL of template cDNA, was loaded in each well of the PCR array. PCR amplification was conducted using Bio-Rad iCYCLER iQ5 Real Time PCR instrument with the following cycling conditions: 1 cycle for 10 minutes at 95°C, 40 cycles for 15 seconds at 95°C, and 1 minute at 60°C. Cycle threshold was set at the point of detectable SYBR fluorescence.

Statistics

Statistics were not generated for mating indices and pregnancy rates because the experiment was conducted only once (n=1). Data normality was determined using normal probability plots. Normally distributed sperm parameter data were evaluated using ANOVA. SA Biosciences RT^2 PCR Data Analysis version 3.5 was used to generate the average Ct, $2^{(- Ct)}$, fold change, p-value, and fold regulation for each gene from RT-PCR Ct values. Fold changes were evaluated using t-tests to determine p-values. Changes in gene expression for each generation were determined relative to the generation matched controls. Genes with greater than 2-fold changes in expression and with $p \leq 0.05$ were considered biologically and statistically significant. Statistical analysis was conducted using Statistix (Tallahassee, FL). Significance was set at $p \leq 0.05$. Sperm parameter and gene expression data are expressed as the mean ± SEM.

REFERENCES

[1] Ullman-Culleré MH and Foltz CJ (1999), Body condition scoring: a rapid and accurate method for assessing health status of mice. Lab Anim Sc 49:319-323.

[2] Byers, S. L., Wiles, M. V., Dunn, S. L. & Taft, R. A. Mouse estrous cycle identification tool and images. PloS one 7, e35538, doi:10.1371/journal.pone.0035538 (2012).

APPENDIX C. Raw data tables from experiments completed in Chapters 2-5

Table I. Overall number of pregnancies in mouse breeding pairs exposed to ADBAC+DDAC for 6 months.

mg ADBAC+DDAC/kg BW/day	Mean	Median	SEM	Min	Max
0	7.4	7	0.2	7	8
60	7.5	7.5	0.3	5	8
120	6	6	0.7	1	7

Table II. Cumulative food consumption in mouse breeding pairs exposed to ADBAC+DDAC for 6 months.

Interval (20 days)	n	mg ADBAC+DDAC/kg/day	Mean	Median	SEM	Min	Max
1	10	0	374.4	378.8	8.7	326.5	412.7
1	10	60	374.4	370.9	13.3	305.5	454.5
1	10	120	361.1	359.1	14.3	286.9	449.1
2	10	0	753.4	761.4	17.7	650.5	835.6
2	10	60	785.8	781.8	19.7	677.2	898
2	10	120	762.7	752.5	17.8	686.9	891.5
3	10	0	1149.8	1162.5	28.4	984.6	1267.5
3	10	60	1171.6	1182.3	25.5	1017.2	1278.6
3	10	120	1181.0	1168.4	22.9	1066.7	1317.3
4	10	0	1535.4	1543.7	37	1320.1	1694.8
4	10	60	1581.2	1597.7	35.3	1363.6	1729.4
4	10	120	1608.7	1600.4	25	1474.2	1741.4
5	10	0	1910.6	1919.2	44.7	1658.8	2109.5
5	10	60	1957.2	1992.9	41.9	1688.8	2137.8
5	8	120	1977.8	1980.7	33	1803.2	2085.7
6	10	0	2303.7	2315.2	56.6	2005.0	2558
6	10	60	2356.1	2403.6	50	2024.0	2592.5
6	6	120	2391.4	2414.8	52.7	2188.7	2564.4
7	10	0	2686.6	2691	65.9	2339.5	3008.1
7	9	60	2819.3	2877.8	41.9	2663.5	3021.9
7	6	120	2801.6	2810.36	60.8	2608.8	3019.5
8	10	0	3058.9	3053.8	73.9	2688.0	3433.1
8	9	60	3200.3	3243.4	50.3	3010.4	3424.3
8	6	120	3153.0	3159.5	70.9	2915.1	3412.2

Table III. Total pup number in mouse breeding pairs exposed to ADBAC+DDAC for 6 months.

Interval (20 days)	n	mg ADBAC+DDAC/kg/day	Mean	Median	SEM	Min	Max
1	10	0	12.0	12.0	0.4	10.0	14.0
1	10	60	11.3	11.0	0.4	9.0	13.0
1	9	120	4.4	0.0	1.8	0.0	12.0
2	10	0	24.2	24.0	0.8	20.0	29.0
2	10	60	20.6	21.0	1.1	12.0	24.0
2	10	120	9.7	8.5	2.3	0.0	23.0
3	10	0	33.4	33.5	1.4	23.0	40.0
3	10	60	30.3	32.5	1.9	21.0	39.0
3	10	120	19.0	16.5	2.6	8.0	34.0
4	9	0	44.3	44.0	1.8	34.0	50.0
4	10	60	39.6	38.0	2.3	31.0	53.0
4	9	120	30.7	28.0	2.3	20.0	41.0
5	10	0	53.8	51.5	1.9	48.0	65.0
5	9	60	50.2	50.0	2.8	38.0	64.0
5	8	120	38.9	39.5	2.8	29.0	52.0
6	10	0	63.3	63.5	2.3	51.0	77.0
6	10	60	57.4	57.5	4.0	38.0	78.0
6	8	120	45.0	47.0	2.4	34.0	52.0
7	9	0	70.8	70.0	2.9	62.0	88.0
7	9	60	66.8	67.0	4.9	49.0	90.0
7	6	120	54.2	56.5	3.3	43.0	64.0
8	9	0	77.6	75.0	3.0	62.0	90.0
8	9	60	75.0	75.0	4.9	55.0	91.0
8	6	120	63.2	62.0	4.4	53.0	78.0
9	5	0	85.6	87.0	4.2	70.0	96.0
9	4	60	82.5	81.5	10.6	62.0	105.0
9	2	120	70.5	70.5	9.5	61.0	80.0

Table IV. Least squares mean (LSM) estimates of total pup number in mouse breeding pairs exposed to ADBAC+DDAC for 6 months.

Interval (20 days)	0 mg/kg/day	60 mg/kg/day	120 mg/kg/day
1	12.3	11.6	4.2
2	24.6	21	9.7
3	33.8	30.7	19
4	44.3	40.1	29.6
5	53.6	49.5	37.7
6	63.2	57	44
7	70.4	66.6	52
8	76.6	74.8	61.3
9	85.9	83.7	69.9

Table V. Male mouse weights over 6 month exposure period to ADBAC+DDAC.

Interval (20 days)	n	mg ADBAC+DDAC/kg/day	Mean	Median	SEM	Min	Max
1	30	0	35.1	34.3	0.6	30.2	41.7
1	30	60	36.6	37	0.4	32	41
1	30	120	34.3	34	0.4	28	39
2	28	0	38.4	37.7	0.7	32	44.5
2	20	60	38.6	39	0.5	33	42
2	19	120	36.8	37	0.5	33	41
3	20	0	39.9	39.3	0.9	32.2	45.9
3	30	60	40	39.8	0.5	34.9	44.1
3	30	120	37.9	38.1	0.4	33.9	41.9
4	18	0	41.5	41	1.1	34.5	49.5
4	20	60	38.9	39.7	0.9	30.5	44.8
4	20	120	36.9	37	0.5	34.1	40.3
6	20	0	44.7	45	1.2	34	52
6	19	60	44.1	45.1	0.8	38.6	50.5
6	14	120	40.3	40.5	0.6	37.5	44.1
7	19	0	44.3	45	1.2	32	52
7	19	60	42.9	42.9	1	34.6	48.1
7	10	120	38.9	38.9	0.7	35	41.6
8	9	0	45.6	45.6	1.6	39	54
8	9	60	46.1	46.1	1.5	37	51
8	5	120	41	41	1	39	45
9	17	0	43.5	43.5	1.1	36.5	50
9	10	60	43.4	43.4	1.4	35.3	48.9
9	8	120	40.7	40.7	0.8	38.4	43.9

Table VI. Average weight per pup over 6 month exposure period to ADBAC+DDAC.

Interval (20 days)	n	mg ADBAC+DDAC/kg/day	Mean	Median	SEM	Min	Max
1	10	0	1.6	1.6	0.03	1.4	1.7
1	10	60	1.5	1.4	0.05	1.3	1.7
1	4	120	1.2	1.3	0.1	0.9	1.4
2	10	0	1.6	1.7	0.04	1.4	1.8
2	9	60	1.7	1.7	0.04	1.5	1.9
2	7	120	1.6	1.6	0.1	1	2.2
3	9	0	1.7	1.7	0.07	1.4	1.9
3	9	60	1.6	1.6	0.08	1.2	2
3	10	120	1.9	1.9	0.07	1.5	2.3
4	9	0	1.7	1.7	0.03	1.5	1.8
4	9	60	1.7	1.7	0.1	1.2	2.4
4	8	120	1.6	1.7	0.09	1.2	1.9
5	9	0	1.7	1.6	0.04	1.5	1.9
5	8	60	1.8	1.7	0.07	1.5	2.1
5	6	120	1.7	1.8	0.09	1.6	2.1
6	9	0	1.7	1.7	0.05	1.4	1.9
6	7	60	1.7	1.7	0.08	1.3	2
6	6	120	1.6	1.7	0.1	1.3	2.3
7	7	0	1.7	1.6	0.09	1.3	2
7	9	60	1.6	1.7	0.06	1.4	1.9
7	5	120	1.7	1.4	0.1	1.3	2
8	6	0	1.7	1.8	0.08	1.3	1.9
8	8	60	1.6	1.7	0.08	1.4	2
8	5	120	1.6	1.5	0.05	1.5	1.7
9	5	0	1.3	1.5	0.3	0.2	1.8
9	4	60	1.8	1.8	0.05	1.7	1.9
9	2	120	1.8	1.8	0.08	1.7	1.9

Table VII. Numbers of corpora lutea in mice exposed for 2 and 8 weeks to 120 mg ADBAC+DDAC/kg/day.

n	mg ADBAC+DDAC/kg/day	Exposure Length	Mean	Median	SEM	Min	Max
6	0	2 wk	14.2	13.0	1.6	10.0	20.0
6	120	2 wk	15.7	16.0	1.2	12.0	19.0
9	0	8 wk	19.0	18.0	1.2	15.0	25.0
9	120	8 wk	12.7	13.0	0.9	6.0	16.0

Table VIII. Numbers of implantation sites in mice exposed for 2 and 8 weeks to 120 mg ADBAC+DDAC/kg/day.

n	mg ADBAC+DDAC/kg/day	Exposure Length	Mean	Median	SEM	Min	Max
6	0	2 wk	10.7	11.0	0.2	10.0	11.0
6	120	2 wk	8.3	11.0	2.7	0.0	14.0
9	0	8 wk	14.2	14	0.6	10	16
10	120	8 wk	8.5	13	1.9	0	14

Table IX. Estrus cyclicity data collected from mice exposed to 120 mg ADBAC+DDAC/kg/day for 2 weeks.

n	mg ADBAC+DDAC/kg/day	Estrus Stage	% Females in Stage	Median	Min	Max
8	0	ESTRUS	37.5	35	25	50
8	0	METESTRUS	2.5	0	0	15
8	0	DIESTRUS	40.6	45	20	55
8	0	PROESTRUS	19.4	15	15	30
8	120	ESTRUS	22.5	17.5	10	40
8	120	METESTRUS	6.25	5	0	20
8	120	DIESTRUS	53.8	55	35	70
8	120	PROESTRUS	16.9	15	5	30

Table X. Mid-gestational embryonic losses in mice exposed to 120 mg ADBAC+DDAC/kg/day for 8 weeks.

n	mg ADBAC+DDAC/kg/day	Mean	Median	SEM	Min	Max
13	0	13.8	13	0.6	12	19
11	120	10.7	12	1	1	13

Table XI. Sperm concentration in male mice exposed to 120 mg/kg/day ADBAC+DDAC for 8 weeks.

Counting Method	n	mg ADBAC+DDAC/kg/day	Mean (x 10^6 sperm/mL)	Median	SEM	Min	Max
Hemocytometer	11	0	17.2	22.0	2.2	8.0	31.3
Hemocytometer	9.7	120	9.7	10.0	1.0	5.1	13.8
CASA	4	0	30.1	29.0	1.9	15.2	45.2
CASA	5	120	11.3	10.0	1.2	4.6	16.4

Table XII. Percent resazurin reduction in TM4 cells treated with ADBAC+DDAC for 24 hours.

n	Concentration of ADBAC+DDAC (%)	Mean	Median	SEM	Min	Max
6	0	96.9	95.1	1.9	92.6	98.6
6	0.0001	94.9	94.0	1.2	90.7	95.6
6	0.0005	62.4	60.2	2.6	58.3	74.4
6	0.001	28.9	24.2	3.3	22.3	35.8
6	0.005	22.3	19.8	2.9	17.1	29.5
6	0.01	9.9	7.0	2.6	5.3	15.6
6	0.05	8.3	5.9	2.4	1.2	12.5
6	0.1	4.6	4.1	0.4	0.0	6.9

Table XIII. Percent resazurin reduction in TM4 cells treated with ADBAC+DDAC for 72 hours.

n	Concentration of ADBAC+DDAC (%)	Mean	Median	SEM	Min	Max
6	0	97.6	96.3	1.4	96.5	99.6
6	0.0001	96.9	95.2	1.1	92.5	97.2
6	0.0005	82.2	81.5	1.2	79.6	81.6
6	0.001	74.2	70.3	2.8	68.5	79.3
6	0.005	69.8	68.5	1.5	66.3	72.9
6	0.01	62.4	61.3	1.2	59.8	64.8
6	0.05	58.3	57.9	2.4	49.7	61.5
6	0.1	46.3	44.1	1.9	42.8	51.6

Table XIV. Cell cycle distribution in TM4 cells treated with ADBAC+DDAC.

Length of treatment (hours)	Concentration of ADBAC+DDAC (%)	Phase of Cell Cycle		
		G1	S	G2M
24	0	73.92	22.72	5.25
24	0	71.18	21.12	10.63
48	0	66.88	14.01	21.08
48	0	67.47	12.87	21.29
72	0	71.06	9.09	21.43
72	0	67.07	13.43	21.38
24	0.01	61.57	20.94	19.49
24	0.01	52.85	33.89	15.1
48	0.01	62.56	19.28	19.47
48	0.01	61.73	22.88	17.22
72	0.01	56.25	28.39	16.84
72	0.01	56.42	32.39	11.09
24	0.1	63.62	18.4	19.78
24	0.1	62.73	16.6	21.71
48	0.1	59.25	21.87	20.61
48	0.1	59.39	16.81	24.87
72	0.1	61.45	14.23	25.59
72	0.1	53.71	15.7	28.2

Table XV. Transepithelial electrical resistance values in the TM4 two-compartment BTB model following treatment with ADBAC+DDAC (A+D) and MEHP.

Length of treatment (hours)	Treatment/Concentration (%)	Mean	SEM	Median	Min	Max
24	0 (control)	79.0	5.0	78.9	56.4	102.6
48	0 (control)	82.0	3.0	83.4	67.2	94.8
72	0 (control)	81.0	4.0	83.7	66.6	97.2
96	0 (control)	83.7	3.9	83.7	66.0	106.2
120	0 (control)	84.9	5.1	89.4	57.0	106.8
144	0 (control)	90.0	6.7	81.0	67.2	129.0
24	0.0001% A+D	87.3	5.8	87.3	56.4	127.2
48	0.0001% A+D	86.5	2.3	86.7	74.4	96.6
72	0.0001% A+D	80.3	4.6	86.1	50.4	94.8
96	0.0001% A+D	79.9	3.7	81.9	51.0	94.2
120	0.0001% A+D	80.3	4.0	83.4	58.2	96.6
144	0.0001% A+D	80.6	5.5	84.9	51.0	108.0
24	0.001% A+D	87.7	5.6	89.7	61.2	123.0
48	0.001% A+D	83.0	5.7	93.0	51.0	99.6
72	0.001% A+D	82.4	3.8	82.5	60.6	102.6
96	0.001% A+D	75.3	3.0	76.2	55.2	87.0
120	0.001% A+D	74.4	3.2	72.6	59.4	91.8
144	0.001% A+D	71.0	5.4	73.2	47.4	90.0
24	0.1% A+D	70.3	2.5	71.4	56.4	81.6
48	0.1% A+D	64.0	3.7	68.1	40.2	78.0
72	0.1% A+D	57.6	3.6	59.1	36.6	70.2
96	0.1% A+D	46.9	2.6	46.5	35.4	60.6
120	0.1% A+D	12.5	1.3	13.5	6.0	19.2
144	0.1% A+D	9.4	1.2	9.0	3.6	15.0
24	0.01% A+D	71.0	5.3	69.9	45.0	100.2
48	0.01% A+D	76.8	4.6	73.8	56.4	105.0
72	0.01% A+D	69.7	5.8	69.6	37.2	111.0
96	0.01% A+D	58.0	2.3	57.9	45.6	69.0
120	0.01% A+D	30.0	2.8	31.5	12.0	42.0
144	0.01% A+D	21.1	2.3	20.4	12.0	30.6
24	1% MEHP	86.0	3.6	87.6	69.6	102.6
48	1% MEHP	62.6	2.8	63.9	46.8	73.2
72	1% MEHP	57.8	4.6	57	42	81
96	1% MEHP	54.5	2.5	55.8	42.6	67.2
120	1% MEHP	55.0	1.3	56.1	48.6	61.8
144	1% MEHP	53.3	1.2	53.4	48.6	58.2

Table XVI. Cauda sperm motility and concentration in rested and unrested male mice administered 7-day oral gavage of ADBAC+DDAC. Sperm were used for IVF.

		Sperm parameters	
	Treatment	Percent motility	Concentration (10^6 sperm/mL)
Unrested Males	0	85	31.2
	0	91	25.4
	0	92	25.3
	0	86	31.7
	0	84	33.9
	30	89	27.6
	30	88	28.7
	30	88	29.5
	30	92	30.5
	30	87	29.9
Rested Males	0	82	25.4
	0	84	32.1
	0	91	29.6
	0	93	26.4
	0	94	27.3
	30	93	26.9
	30	84	31.6
	30	86	23.4
	30	82	28.3
	30	85	26.5

Table XVII. Number of oocytes isolated from superovulated female mice and fertilization rates of sperm isolated from unrested and rested male mice exposed for 7 days to ADBAC+DDAC.

	mg ADBAC+DDAC/kg/day	Percent IVF	Number of oocytes collected from superovulated female	Number of pronuclei
Unrested Males	0	61.5	13	8
	0	66.7	15	10
	0	68.8	16	11
	0	64.3	14	9
	0	44.4	18	8
	30	53.8	13	7
	30	60.0	15	9
	30	23.5	17	4
	30	70.6	17	12
	30	33.3	15	5
Rested Males	0	85.7	14	12
	0	50.0	12	6
	0	68.8	16	11
	0	47.4	19	9
	0	50.0	12	6
	30	50.0	16	8
	30	50.0	12	6
	30	16.7	18	3
	30	20.0	10	2
	30	35.7	14	5

Table XVIII. Sperm concentrations in male mice exposed to ADBAC+DDAC determined by CASA. Two generations of mice (F_1 and F_2) were derived from F_0 males exposed to 120 mg ADBAC+DDAC/kg/day for 8 weeks.

Generation	Treatment	N	Mean (x 10^6 sperm/mL)	SEM	Median	Min	Max
F0	control	8	30.1	1.9	29.4	22.3	36.9
	ADBAC+DDAC	8	11.3	1.2	12.0	7.2	15.4
F1	control	9	32.0	3.8	30.1	17.3	55.6
	ADBAC+DDAC	9	68.2	5.5	74.1	17.9	82.5
F2	control	10	31.3	1.7	28.5	24.9	44.5
	ADBAC+DDAC	10	19.6	0.5	19.6	17.5	21.4

Table XIX. Sperm motility in male mice exposed to ADBAC+DDAC as determined by CASA. Two generations of mice (F_1 and F_2) were derived from F_0 males exposed to 120 mg ADBAC+DDAC/kg/day for 8 weeks.

Generation	Treatment	N	Percent sperm motility	SEM	Median	Min	Max
F0	control	8	46.4	2.5	49.2	32.3	54.1
	ADBAC+DDAC	8	20.9	4.1	34.7	18.3	46.3
F1	control	9	41.6	2.0	42.1	30.5	55.3
	ADBAC+DDAC	9	33.3	2.8	32.9	18.3	46.3
F2	control	10	43.7	3.2	45.4	26.3	56.3
	ADBAC+DDAC	10	36.0	0.9	35.6	30.2	40.9