

Responses to Phthalate Exposure: Biological and Social Perspectives

by

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Preface

The sum of the laboratory work presented in this Masters thesis was conducted in the laboratory of Dr. Mary Hixon. I have executed all of the experiments presented herein with the following exceptions:

In Chapter 2, Assessment of Akt1 kinase activity and tunnel staining were performed by Teresa Rasoulpour and Greg Ouellet and PCR experiments were done by Caitlin Brown.

In Chapter 4, online survey data was acquired with permission of Health Care Without Harm.

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Abbreviations

AHA	American Hospital Association
AMH	anti-Mullerian hormone
ATSDR	Agency for Toxic Substances and Disease Registry
BBzP	butylbenzyl phthalate
cAMP	cyclic adenosine monophosphate
CDH2	N-cadherin
c-kit	receptor tyrosine kinase
CLDN1	claudin-1
CREB	cAMP response element binding protein
DEHP	di-2-ethylhexyl phthalate
DEP	diethyl phthalate
DES	diethylstilbestrol
DiBP	diisobutyl phthalate
DISC	death inducing signal complex
DnBP	di-n-butyl phthalate
ECMO	extracorporeal membrane oxygenation
EGF	epidermal growth factor
EPA	Environmental Protection Agency
F11R	junction adhesion molecule-1
FAK	focal adhesion kinase
FDA	food and drug administration
FGF	fibroblast growth factor

FSH	follicle stimulating hormone
H2E	hospitals for healthy environments
IGF-I	insulin-like growth factor I
IUPAC	International Union of Pure and Applied Chemistry
JAK	janus kinase
JNK	Jun N-terminal kinase
LH	luteinizing hormone
MEHHP	mono-2-(ethyl-5-hydroxyhexyl) phthalate
MEHP	mono-2-ethylhexyl phthalate
MEOHP	mono-2-(ethyl-5-oxohexyl) phthalate
NF- κ B	nuclear factor kappa b
NTP	National Toxicology Program
OCN	occludin
PAK2	p21 activated kinase 2
PI3K/Akt	phosphoinositide-3-kinase/protein kinase B
PKA	protein kinase A
PVC	polyvinyl chloride
PVRL	nectin
SCF	stem cell factor
STAT	signal transducer and activator of transcription
TGF- α	transforming growth factor alpha
TGF- β	transforming growth factor beta
TNF	tumor necrosis factor

CHAPTER 1

Biological Responses to Phthalate Exposure: Background and Significance

Introduction

Normal male reproductive development is the result of a complex series of biological and environmental interactions and is critically important for the maintenance of a healthy and thriving human population. Over the course of the last several decades notable adverse trends in male reproductive health have been observed. Historical data suggests that average sperm count and quality has decreased significantly in the early 20th century and testicular cancer has been shown to be increasing in incidence in the United States (1). In addition, congenital malformations of the reproductive tract, including cryptorchidism (undescended testes) and hypospadias (a developmental abnormality causing the urethral opening to develop somewhere other than the tip of the penis), are becoming increasingly more common (2). In order to understand the reasons behind these alarming trends in reproductive health it is first necessary to be familiar with the intricate biological processes that make up the male reproductive system.

Male reproductive development begins in utero and progresses through a variety of stages. The reproductive system is responsible for producing, maintaining, and transporting sperm as well as producing and secreting a variety of important hormones. This system includes the penis, testes, epididymis, seminal vesicles, vas deferens, and a variety of ducts and glands responsible for transport of spermatazoa and seminal fluid. The testis is made up of a series of round seminiferous tubules which contain germ cells and somatic cells and provides an environment in which spermatogenesis (the development and maturation of the male gamete) can occur (3).

Normal spermatogenesis is essential for the production of healthy, functioning spermatazoa and is susceptible to injury from numerous outside stimuli. In order to

protect the integrity of developing spermatazoa, the body affords the testis a unique structure termed the blood-testis barrier. This barrier is the result of tight junctions between somatic cells in the seminiferous tubules and a metabolic capacity that gives developing spermatazoa some DNA repair capability (4). Advances in industry and technology, while in many ways beneficial, have contaminated the environment with a wide range of chemicals and pollutants that challenge this barrier. It is important to understand the structure and function of the male reproductive system on a cellular and molecular level in order to address the problems that arise as a result of this development.

The Male Reproductive System

The male reproductive system is made up of the scrotum and testes, which contain seminiferous tubules and are responsible for secreting testosterone and generating spermatazoa, the epididymis, which stores and transports mature spermatazoa, the seminal vesicles, prostate gland, and bulbourethral gland which produce fluids that nourish mature spermatazoa, and the penis, vas deferens, and urethra, which transport and expel seminal fluid and mature spermatazoa (5). In addition, the mammalian testis is made up of a series of round seminiferous tubules that contain germ cells and somatic cells (Sertoli cells and Leydig cells). Perhaps the most important function of the male reproductive system is to produce healthy, viable spermatazoa. This process takes place primarily in the testis, which is where this thesis will focus.

The testis is a component of both the reproductive system and the endocrine system, as it functions to produce both mature spermatazoa and male sex hormones (6). The testis is contained by the tunica (a tough outer membrane), and is organized into tightly coiled seminiferous tubules. These tubules (Figure 1) contain germ cells and somatic cells (7). The development of the male gamete through spermatogenesis occurs in the seminiferous tubules and is heavily dependent on the Leydig cells and Sertoli cells. Human Leydig cells develop fully between the 16th and 20th gestational week and are located in the interstitial space of the seminiferous tubules. They remain quiescent until the onset of puberty, at which time, they are triggered by luteinizing hormone (LH) released from the pituitary gland and begin to synthesize and release testosterone, among other androgens. These androgens function as paracrine signals and are used by the Sertoli cells to support spermatazoa production (9).

With a diverse range of tasks to accomplish, Sertoli cells are often referred to as “nurse cells.” Sertoli cells secrete a variety of proteins and growth factors that mediate the unique development of the male reproductive system (Figure 2). Germ cell metabolism is dependent on lactate, transferrin, and androgen binding protein, all secreted by the Sertoli cell. Growth factors including transforming growth factor- α and β (TGF- α and TGF - β), insulin-like growth factor-I (IGF-I), fibroblast growth factor (FGF), and epidermal growth factor (EGF) are also secreted by Sertoli cells. Additionally, Sertoli cell release of anti-Müllerian hormone (AMH) prevents the embryonic development of Müllerian ducts, an important uterine precursor, and recent research indicates that the Sertoli cells generate glial-derived neurotrophic factor which has been found to play an important role in germ cell differentiation (11). Previous research has revealed that germ cell differentiation, proliferation, and death are regulated via multi-directional communication between Sertoli cells and germ cells. Thus, the integrity of the Sertoli cells is critical to spermatogenesis and successful male reproductive development.

Clearly, the male reproductive system is a complex combination of cellular signaling and regulated transport of nutrients, growth factors, and sperm. Understanding the structure and function of this important reproductive organ allows for a more complete picture of the vulnerabilities of this biological system.

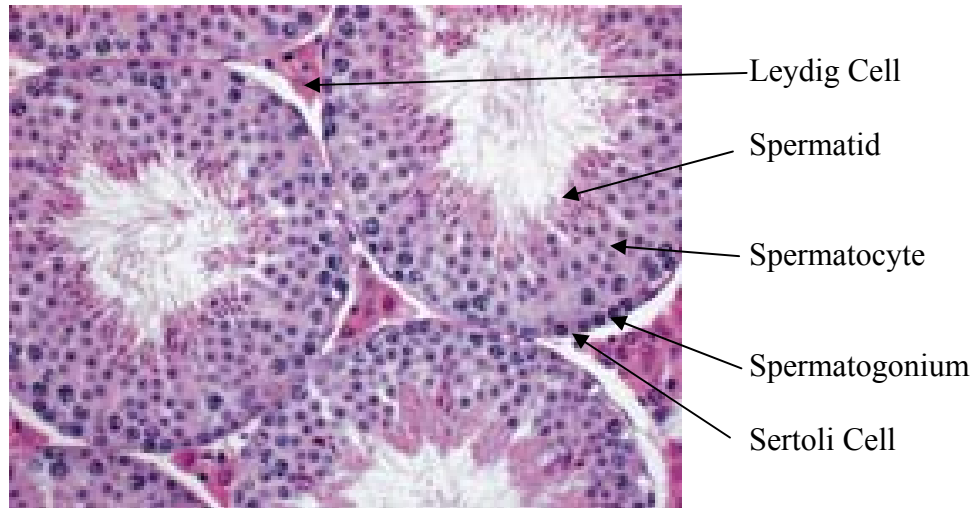


Figure 1. Seminiferous tubule organization. Germ cells and somatic cells can be seen in the seminiferous tubules and interstitial space of the murine testis. Testis cross section adapted from Moira O'Brian, Monarch Institute of Medical Research.

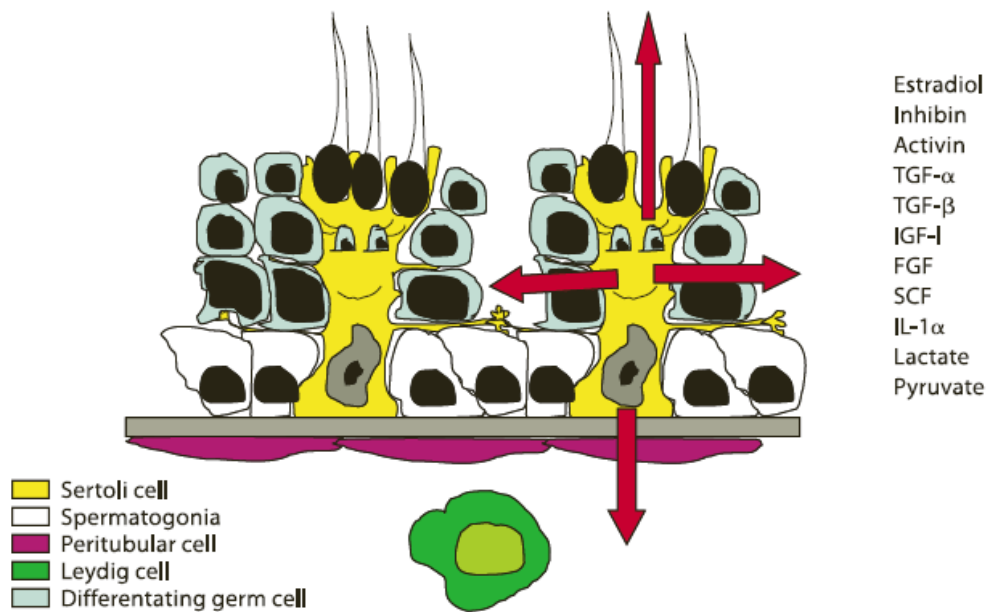


Figure 2. The Paracrine role of the Sertoli cell. Sertoli cells secrete a variety of proteins and growth factors that regulate germ cell growth and survival and mediate successful testicular development. Adapted from Petersen C and Söder O. *Horm Res* 2006;66:153-161 (74).

Spermatogenesis

Spermatogenesis is the process by which male germ cells undergo a series of phases within the seminiferous tubules to eventually become mature spermatozoa. This process begins at puberty and takes place via three general phases. First, in the proliferative phase, male germ stem cells, or spermatogonium, undergo rapid successive divisions and differentiate into spermatocytes. Next, in the meiotic phase, these spermatocytes undergo meiosis during which genetic material is recombined and segregated. Finally, in the spermiogenic phase, spermatids differentiate further into elongated spermatids and are transported to the epididymis to mature into spermatozoa. This process is heavily dependent on the proper regulation of nutrients and growth factors, as well as appropriate levels of germ cell apoptosis (programmed cell death) in order to produce healthy spermatozoa.

In addition to the direct effects of Sertoli cells, several endogenous compounds exert control over spermatogenesis (Figure 2). Sertoli cells express receptors for Follicle Stimulating Hormone (FSH), an important reproductive hormone synthesized in the anterior pituitary gland (12). FSH binding by the Sertoli cell increases the production of the previously mentioned lactate, transferrin, IGF-I (13), and androgen receptors, as well as inhibin, all important for germ cell metabolism. In addition, FSH has been shown to effect testis development via the Cyclic adenosine monophosphate/Protein Kinase A (cAMP/PKA) pathway. Previous research indicates that FSH receptor binding leads to elevation of cAMP, which leads to activation of Protein kinase A (PKA). PKA targets cAMP response element binding protein (CREB) in the Sertoli cell, which plays a role in the ability of the seminiferous tubule to support spermatogenesis (14). FSH has also been

demonstrated to activate the phosphoinositide-3-kinase/protein kinase B (PI3K/Akt) pathway in Sertoli cells, leading to enhanced germ cell survival and proliferation during spermatogenesis (13).

Spermatogenesis begins when the pituitary gland releases LH, which triggers the testicular release of FSH and testosterone. During the proliferative phase, some spermatogonia develop into secondary spermatocytes, while others simply replicate to ensure a constant supply of male germ-line stem cells. In this phase, spermatogonium in the basal membrane of the seminiferous tubules are paired and aligned by open lines of cytoplasm termed intercellular bridges in order to promote synchronous development of all germ cells in a certain phase. These spermatogonium undergo a series of successive divisions in which the number of cells doubles. Germ cells progress from spermatogonial stem cells to proliferative spermatogonium and finally to differentiating spermatogonium. At the end of the differentiating phase mature spermatogonium divide to form primary preleptotene spermatocytes, and the meiotic phase begins (14).

In the meiotic phase, two meiotic divisions take place and the chromosomes of the preleptotene spermatocytes are combined and halved. During the first meiotic division the size and shape of the nuclei increase in size and the germ cell progresses through the leptotene, zygotene, pachytene, and diplotene spermatocyte phases. The completion of this meiotic division produces secondary spermatocytes. The second meiotic division is significantly shorter than the first and produces haploid spermatids. During the meiotic phase germ cell populations are roughly quadrupled and migrate from the basal membrane to an intermediate compartment within the seminiferous tubule (14).

In the third and final phase of spermatogenesis spermatids mature and move to the baso-lateral compartment of the seminiferous tubule to prepare for release to the epididymis. This process involves no cell divisions. Instead spermatids undergo morphological changes to gain the ability to reach and fertilize an egg. They begin to develop an elongated flagellum and unnecessary cytoplasm and organelles (called residual bodies) are phagocytosed by the Sertoli cells. They also develop an acrosome on the spermatid head which contains enzymes necessary for egg penetration. Following these morphological changes, spermatids are released by the Sertoli cells and seminiferous tubules to the epididymis where they gain motility and complete maturation (15). Developing germ cells are highly dependent on Sertoli cells for nutrition as well as metabolic requirements, and as a result, the ultimate number and size of mature germ cells is largely determined by the signaling cross talk of the Sertoli cells (16).

In addition to the progression through these stages, germ cell homeostasis depends on a balance between cell proliferation and apoptosis (programmed cell death). In the adult rodent testis apoptosis usually occurs during stage I and stage XII (14) , and is marked by the fragmentation of the cell's DNA, condensation of chromatin, cell shrinkage, detachment of the plasma membrane, and packaging of the cell contents into apoptotic bodies (18). The Sertoli cell is equipped with a variety of ways to control germ cell proliferation and apoptosis and as a result, is an important regulator of germ cell homeostasis.

Apoptosis is primarily driven by a family of cysteine proteases called caspases (17), Bcl-2 family members (19), and Fas/FasL signaling (20). During apoptosis caspases execute proteolytic cleavage in an ordered and specific manner, causing cellular

disassembly and the activation of other previously inactive caspases (18). Caspase initiated apoptosis causes destruction of the nuclear lamina, disassembly of the cytoskeleton via cleavage of regulatory proteins gelsolin, Focal adhesion kinase (FAK), and p21 activated kinase 2 (PAK2). Caspase cleavage has also been shown to inactivate proteins directly involved in DNA repair and mRNA splicing (18).

The Bcl-2 family mediates the release of cytochrome c and is composed of both pro- and anti-apoptotic members. Bcl-2, Bcl-XL, and Bcl-W are considered anti-apoptotic and facilitate cell survival, while Bax, Bak, Bid, and Bik are considered proapoptotic family members (19). The Fas/FasL apoptotic signaling system has been found to be a significant apoptotic mediator through regulation of Bcl-2 family members (20). When Fas binds to FasL, Fas trimerizes, allowing for the formation of Death inducing signal complex (DISC) (22). This results in either activation of caspases 8 and then 3, or cleavage of Bid, which allows cytochrome c into the cytoplasm. Once in the cytoplasm, cytochrome c is able to activate caspases 3 through 9, resulting in a cascade that eventually leads to cell death. Fas has been localized to rodent germ cells, while FasL has been localized to rodent Sertoli cells and germ cells (73), indicating a significant role for this signaling pathway in apoptotic regulation in rodents.

Controlled germ cell apoptosis is necessary for germ cell homeostasis. In addition to regulation by caspases, Bcl-2 family members, and Fas/FasL signaling, the Sertoli cells also have the ability to regulate germ cell proliferation. One primary method of regulation involves signaling between the stem cell factor (SCF) released from the Sertoli cells, and receptor tyrosine kinase (c-kit). SCF binds to c-kit inducing several different downstream signaling pathways. The Janus kinase/Signal transducer and activator of

transcription (JAK/STAT) pathway, as well as the Mitogen-activated protein kinase (Ras-Raf-MAPK), and PI3K pathways are antiapoptotic downstream targets of c-kit (26). In contrast, the Jun N-terminal kinase (JNK) and Tumor necrosis factor (TNF) pathways are also downstream of c-kit, but are known to be involved in apoptotic signaling in the testis (21). Thus, apoptosis necessary for the maintenance of germ cell homeostasis is accomplished through the activity of Sertoli cells as well as caspases, Bcl-2 family members, and Fas/FasL signaling.

The Blood-Testis Barrier

The blood testis barrier is a protective physical barrier between the blood vessels and seminiferous tubules that prevents the transport of cytotoxic agents into the seminiferous tubules. This barrier is the result of tight junctions between Sertoli cells at the perimeter of every seminiferous tubule (24). In addition, ectoplasmic specializations, desmosome-gap junctions and tubulobulbar complexes exist between Sertoli cells and contribute to the barrier. Stable cell adhesion and function amongst transmembrane junctional proteins occludin (OCLN), claudin-1 (CLDN1), junction adhesion molecule-1 (F11R), N-cadherin (CDH2), and nectin (PVRL) are supported by the Sertoli cell cytoskeleton. These complexes attach to the filamentous and globular actin (G- and F-actin) filaments of the Sertoli cell cytoskeleton via scaffolding proteins in order to achieve this stability (24). The blood-testis barrier is bolstered by the fact the testis contains cytochrome P450 enzymes, which give it the ability to metabolize some cytotoxic agents and allows for the development of spermatazoa with DNA repair capabilities (25). The overall result of this interaction is a barrier that separates blood vessels from seminiferous tubules and also segregates diploid and haploid germ cells (16).

During spermatogenesis germ cells must cross the blood-testis barrier in order to migrate from the basal membrane of the seminiferous tubule to the intermediate and adluminal compartments. This occurs via the precise disassembly, rearrangement, and reassembly of the proteins and cells that make up the barrier. Thus, the integrity of the blood-testis barrier is critical for spermatogenesis and fertility (25). However this barrier is not impenetrable, as toxicants like the phthalate ester mono-2-(ethylhexyl) phthalate

(MEHP) have been shown to have detrimental effects on Sertoli cells and germ cells following ingestion (16).

The Phosphoinositol-3-Kinase (PI3K) / Akt Signaling Pathway

Phosphoinositol-3-Kinase (PI3K) is a lipid kinase that is an upstream regulator of protein kinase B (PKB, also known as Akt). Activation of Akt occurs through a PI3K dependent pathway and has been found to play an important role in cell proliferation and survival (Figure 3) (26). Akt has been found to protect cells from apoptosis induced by a variety of exogenous agents and environmental influences (27, 28) and has been implicated in maintenance of glucose metabolism (29). Additionally, recent research links the PI3K/Akt pathway to testis growth and development and indicates that Akt helps moderate the interaction between FSH and the Sertoli cell (30).

PI3K plays an important role in many biological processes, primarily due to its ability to generate $\text{PtdIns}(3,4,5)\text{P}_3$, a second messenger that acts as a docking site on the plasma membrane (31). This messenger is able to recruit and activate many downstream targets of PI3K, including 3' phosphoinositide-dependent kinase-1 (PDK-1), Tec family kinases, GTPase activating proteins, guanine nucleotide exchange factors, and scaffolding proteins, as well as Akt (31). PI3K has been implicated in survival factor stimulation, oncogene activation (32), and a wide range of T lymphocyte cellular functions (31). PI3K activation of Akt is primarily considered a prosurvival pathway (32).

Akt is hypothesized to inhibit cell death induced by a variety of stimuli and has been shown to be involved in cytoprotective signaling pathways in the testis. This prosurvival protein exists in three isoforms, Akt1, Akt2, and Akt3, which have 85% sequence homology (33). While there has been some evidence in redundancy of function in inhibiting apoptosis (34), the three isoforms have been found to localize differently and

to perform somewhat separate physiological functions (35-37). Additionally, in studies conducted with Akt2-deficient and Akt-3 deficient mice, Akt1 was demonstrated to be sufficient for viability, although the deficient mice did demonstrate impaired glucose homeostasis and growth deficiencies (38).

Perhaps most relevant to this thesis is the role of Akt1 in the testis. While both Akt3 and Akt1 have high testis expression, previous research indicates that Akt1 is the dominant isoform in the testis (39) and plays an important role in the action of FSH on the Sertoli cell (40) as well as the induction of apoptosis (41). *In vitro* studies of Sertoli cells from 20-day old rats indicate that Akt is induced in an FSH dependant manner (13, 40, 42). Additionally, previous reports show that FSH stimulation of transferrin and lactate production were partially blocked in cells incubated with a PI3K inhibitor (42). This suggests that Akt is essential to these two well known Sertoli cell functions.

Akt1 has been localized to both germ cells and Sertoli cells and found to be an important upstream regulator of germ cell homeostasis. This is demonstrated by previous studies with Akt1-deficient mice that show increased rates of apoptosis and attenuation of spermatogenesis (28, 41). Akt1-mediated protective responses may be in part due to activation of Akt1 downstream target nuclear factor- κ B (NF- κ B). NF- κ B is made up of five subunits that are sequestered in the cytoplasm until activation (43). The most commonly characterized subunits, p50 and p65 (44), exist as both homo- and hetero-dimers bound to the inhibitory complex, I κ B α (45). NF- κ B activation triggers the phosphorylation of I κ B α at two specific serine residues which allows for I κ B α polyubiquitination and subsequent proteolytic degradation by the 26S proteasome. This frees NF- κ B subunits to move from the cytoplasm into the nucleus and there transactivate

genes that regulate cell survival, proliferation, differentiation, and apoptosis (46). Thus, Akt1 is able to prevent apoptosis by phosphorylation of downstream anti and/or proapoptotic factors or by inducing signaling cascades that result in the modulation of transcription factors.

Like Akt-deficiency, Akt-hyperactivation can also be problematic in the testis. Over active Akt is known to cause disruption of tissue homeostasis through the induction of tumor growth (47). Tumors that express hyperactive Akt are often found to have defective PTEN, a tumor suppressor that is frequently inactive in malignant human tumors (47). Evidence of this phenomenon can be found in studies using PTEN heterozygous mice which showed that mice with two Akt1 alleles developed significantly higher levels of high-grade prostate intraepithelial neoplasia than mice with just one Akt1 allele (47). Thus, the PI3K/Akt1 pathway is a significant actor in the maintenance of testicular homeostasis and male reproductive development.

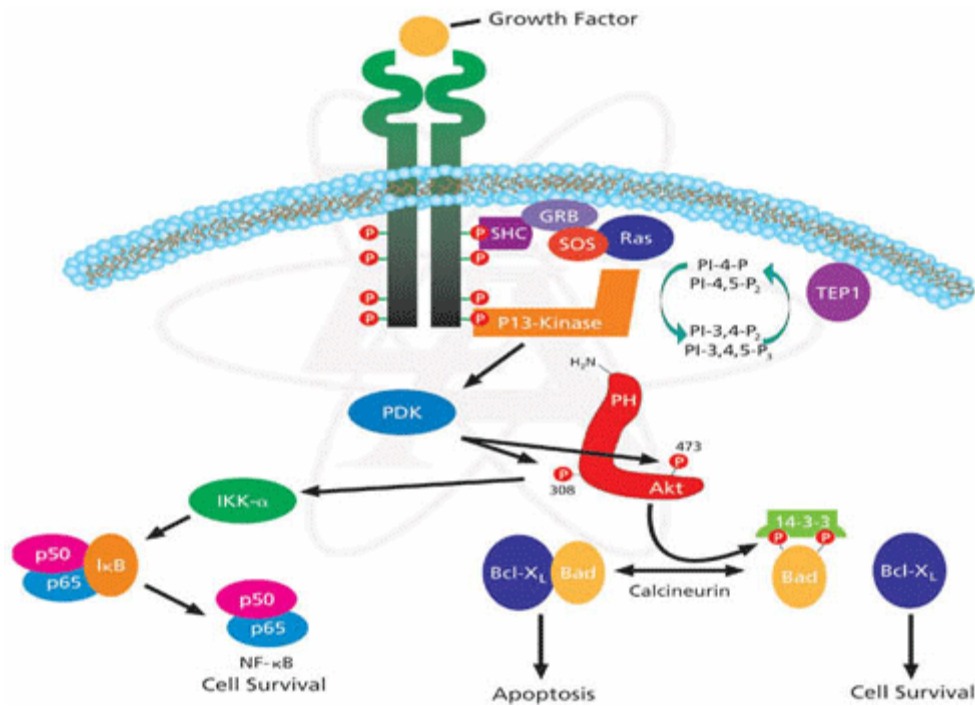


Figure 3. PI3K/Akt Signaling Pathway. PI3K/Akt plays important roles in signaling pathways in response to extracellular growth factors and other extracellular stimuli to regulate several cellular functions including cell growth, apoptosis, differentiation, and cell survival. Adapted from Sigma Aldrich.

Phthalate Toxicity

Male reproductive development is a fragile process that is often influenced by environment-gene interactions that have the potential to result in injury to Sertoli cells and developing germ cells. One such source of these interactions is the ubiquitous use of phthalate esters, a large group of chemical compounds that are frequently used as plasticizers, solvents, and adhesives (48). These chemicals are semi-solid, lipophilic, and have been found to migrate out of the materials that contain them (49). Furthermore, they have been identified as endocrine disruptors and shown to cause developmental and reproductive toxicity in laboratory animals and are associated with developmental reproductive abnormalities in humans and wildlife (50). One specific member of this chemical class, di-2-ethylhexyl phthalate (DEHP), is used in PVC plastics and is commonly found in medical devices (51). The active metabolite of this chemical, mono-2-ethylhexyl phthalate (MEHP) has been identified as a Sertoli cell toxicant and has been found to disrupt spermatogenesis and testis development (51). Thus, it is important to investigate the effects of DEHP exposure and the role that certain genes and biological signaling pathways play in germ cell injury and death.

The endocrine system controls the synthesis, release, transport, metabolism, binding and elimination of hormones in the body. Endocrine disruptors interfere with these processes by mimicking hormones, blocking signaling pathways or altering the way hormonal messages are transmitted (52). Unfortunately, these compounds are difficult to study because their effects are often delayed and are occasionally seen in subsequent generations but not the initially exposed population. Some examples of these types of transgenerational effects include infertility, congenital malformations of the reproductive

tract, cancers of the reproductive organs, and precocious puberty (53). Due to the ubiquitous nature of these compounds and the vulnerability of infants and children, it is important to identify the mechanisms by which endocrine disruptors act on signaling networks and the cell types and organs that they target.

DEHP is an endocrine disruptor that poses a significant threat to the male reproductive tract due to its ability to block or inhibit the effect of androgens in the reproductive tissues (2). This anti-androgenic activity can occur via blockage of androgen receptors, competitive binding to androgen receptors, or inhibition of androgen biosynthesis (54). It is believed that phthalates achieve their anti-androgenic effects largely through the suppression of androgen synthesis (2). The male reproductive tract is especially vulnerable to the anti-androgenic effects of DEHP and MEHP because Leydig cells and Sertoli cells are so dependant on it for the maintenance of spermatogenesis and testicular development.

Neonates, infants, and small children face a greater risk from endocrine disruptors like DEHP and MEHP than do fully developed adults. During *in utero* development and throughout early postnatal development, the nervous and reproductive systems, as well as many important metabolic pathways are still developing (55). As a result, neonates, infants, and children have a decreased ability to eliminate toxicants and an increased vulnerability due to their rapid growth and development. Additionally children have disproportionately high exposure rates due to their small size and increased exposure opportunity due to exploratory hand-to-mouth actions (55). Work associated with The National Children's Study, a prospective epidemiological study that examines the effects of environmental influences on more than 100,000 children from the United States gave

the National Institute of Child Health and Human Development, the National Institute of Environmental Health Sciences, the U.S. Environmental Protection Agency, and Centers for Disease Control and Prevention reason to believe that children are at a significant risk from endocrine disruptors (56). Interestingly, studies in rats show that repeated oral exposure to high doses of DEHP causes lethality in 21-day old animals, but not in adults (65). This indicates that age influences susceptibility to DEHP. Thus, it is important to evaluate the impact of endocrine disruptors on these susceptible populations and to identify possible exposure routes in an attempt to reduce human exposure.

Phthalates, specifically DEHP, are commonly used plasticizers that make the normally rigid and brittle polyvinyl chloride (PVC) flexible. Of the numerous plasticizers available, phthalate esters make up 70% of US consumption (57). DEHP is perhaps the most commonly used in the production of medical devices (57). Studies report that more than 25% of all plastics used in medical devices are PVC. These include intravenous fluid bags and tubing, blood and plasma bags, enteral feeding and dialysis equipment, catheters, and gloves. IV bags made of PVC typically contain between 30 and 40% DEHP by weight, while PVC tubing has been found to contain as much as 80% DEHP by weight. As a result, a common source of DEHP exposure is through medical devices made from PVC (57).

This exposure is especially concerning for infants in neonatal intensive care units, patients on dialysis regimens, and patients receiving blood transfusions. DEHP concentrations in blood products have been measured by many different groups of scientists and found to be 4-650 mg/L (58-62). In drug containing solutions stored and delivered intravenously DEHP concentrations are 3.1-237 mg/L, while sterile water,

electrolytes, and sugar solutions have been measured to have 5 mg DEHP/L (63, 64). These concentrations represent physiologically relevant doses for long term patients and shorter term neonatal patients. The FDA reports a variety of acute toxicity measurements for DEHP and has set the tolerable intake level at 0.6 mg/kg/day (65). Studies from Health Care without Harm report that neonates receiving extracorporeal membrane oxygenation (ECMO), a technique used for patients in pulmonary distress, may receive doses of DEHP as high as 14 mg/kg/day, while those on total peritoneal nutrition (TPN) may receive 2.5 mg/kg/day. Adult trauma patients receive 8.5 mg DEHP/kg/day, while those who undergo coronary artificial bypass grafts receive 1.0 mg/kg/day (65). These doses alone are concerning, and unfortunately often occur in combination with other procedures that expose patients to even more of the toxic chemical. As you will see, DEHP exposure has been linked to physiologically detrimental effects in both animal and epidemiological studies. Thus, hospital settings, especially neonatal intensive care units and long term adult care settings present an important public health concern.

Phthalates are one of the most widely studied classes of testicular toxicants and many animal studies and epidemiological studies have been used to assess the level of concern that exposure to these compounds warrants. In animal studies, DEHP has been found to target Sertoli cells causing the rapid onset of vacuolation, alteration of intercellular contacts, and retraction of important processes (66). Epidemiological studies have shown a correlation between *in utero* exposure to DEHP and decreased anogenital index, a measurement that is androgen dependent and typically much larger in males than in females (67). Based on these studies and others like them, those who study DEHP have reached a relative consensus concerning its toxicity in the male reproductive system.

Toxicity of MEHP in Animal Studies

In vitro and *in vivo* animal studies have been widely used to assess the biochemical and morphological changes in the mammalian testis following exposure to a variety of phthalate esters. One *in vivo* study in Fischer rats sought to explore the role of alterations in Sertoli cell vimentin filament distribution on testicular germ cell apoptosis. 28-day-old rats were gavaged with 2 g/kg MEHP and killed at 0, 3, 6, or 12 hours after exposure (66). Histological examination of testes from these rats revealed a collapse in vimentin filaments as early as three hours after MEHP exposure that progressively increased at six and 12 hours post exposure (66). Correspondingly, terminal deoxynucleotidyl transferase-mediated digoxigenin-dUTP nick end label (TUNEL) staining demonstrated a marked increase in germ cell apoptosis from six to 12 hours after MEHP exposure (66). This study indicates that in rats, MEHP exposure results in Sertoli cell dysfunction and subsequent increase in germ cell apoptosis.

In order to learn more about the cell signaling pathways that are acting in response to MEHP exposure, another lab explored the expression of germ cell-associated death receptors and their downstream targets. This study showed that important death receptor family members Fas, TRAIL-R1 (Death Receptor-4, DR4) and TRAIL-R2 (Death Receptor 5, DR5) were present in the testes of C57BL/6 (B6) mice, gld mice, and Sprague-Dawley rats and were responsive to MEHP exposure (68). While this work cannot conclusively claim that activation of these death receptors is directly responsible for germ cell apoptosis following exposure to MEHP, it does find that MEHP has a deleterious effect on the rodent testis and suggests that the death receptor signaling pathways are significant contributors to this response.

While the actions of MEHP in the Sertoli cell are not completely understood, researchers have attempted to shed light on potential targets of this toxicant. The results of their work indicate that aspects of fuel metabolism, as indicated by lactate production, CO₂ production, pyruvate concentrations and cellular ATP, are important potential targets for MEHP toxicity (69). *In vitro* studies of rat Sertoli cells in primary culture showed that lactate production increased following MEHP exposure, while CO₂ production, pyruvate concentrations, and cellular ATP all decreased (69). This data indicates that the metabolic function of Sertoli cells is altered following MEHP exposure which implicates the mitochondria as a potentially important cellular target.

Toxicity of Phthalates in Epidemiologic Studies

Extensive animal studies concerning the toxicity of phthalate esters are corroborated by a diverse collection of epidemiological studies that examine phthalate exposure in children, industrial workers, and adult men. Well known studies conducted by Shanna Swann and colleagues examine the correlation between anogenital distance and phthalate exposure in order to determine the extent to which phthalates are having a feminizing effect on male infants exposed to DEHP *in utero* (67). Similarly, researchers in China found that workers in plants that produce non-foaming PVC had significantly higher levels of MEHP and other DEHP metabolites in their blood and urine, and significantly lower levels of free testosterone compared to construction workers (70). Finally, a study of semen quality in men recruited from an infertility clinic suggests that there is a positive correlation between sperm DNA damage and the concentration of MEHP in the blood and urine (71). Thus, the study of phthalate exposure and human health seem to reflect similar biological responses in humans to those demonstrated in animal models.

As previously explained, phthalates are ubiquitous and exposure can occur in a variety of settings and at many different life stages. One group of epidemiologists sought to evaluate the effects of *in utero* exposure to five different phthalates. These researchers measured urinary metabolites of di-*n*-butyl phthalate (DnBP), diethyl phthalate (DEP), butylbenzyl phthalate (BBzP), diisobutyl phthalate (DiBP), and DEHP in human male infants and their mothers (67). Each mother's urine was sampled periodically for the duration of her pregnancy in order to estimate the prenatal phthalate exposure of each infant. After birth, each infant's anogenital distance was measured and corrected for

weight. This measurement is dependent on androgen levels and is typically much larger in males than in females, even at birth (71). This study found that male infants with higher *in utero* phthalate exposure were more likely to have a lower than average anogenital distance (67). Some argue that this study is not sound due to the difficulties of maintaining uniform protocols for measuring both the presence of phthalate metabolites and anogenital distance. This is not surprising, as epidemiological studies are often plagued by the impossibility of controlling for all variables in a human population. However, this work is rigorous and supports animal data that suggests that *in utero* exposure to phthalates including DEHP and its metabolites interferes with hormonal signaling.

While this study illustrates the consequences of *in utero* phthalate exposure, other research groups have focused on post-natal exposure. Animal studies indicate that phthalates are Sertoli cell toxicants and as a result, can cause a loss of germ cell integrity. The relationship between phthalate exposure and subsequent decrease in sperm quality has been evaluated in human populations using semen samples collected from male infertility clinics (71). Phthalate exposure was assessed using measurements of phthalate metabolites mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP), mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), and MEHP in urine samples, while sperm quality was assessed via measurement of sperm DNA damage (71). Researchers found that increasing concentrations of MEHP were correlated with an increasing degree of sperm DNA damage (71). This research is particularly interesting because the recorded urinary levels of phthalate metabolites among men in this study were very similar to those

reported by the US population at large (71). Thus, it is possible that exposure to phthalates may affect the fertility of the population at large.

Occupational exposure to phthalate is extremely relevant from a public health perspective, as those who work in industries that either manufacture or handle PVC products are subject to potentially high levels of exposure. A team of researchers in China explored this susceptible population and the effect that nearly constant exposure to phthalates has on their LH, FSH, free testosterone, and estradiol levels (70). Urine and blood samples from adult men working in a factory that produced unfoamed PVC flooring materials were collected and evaluated for MEHP concentrations as well as levels of LH, FSH, free Testosterone (fT), and estradiol (70). These samples were compared to identically evaluated samples from construction workers. The researchers found that the PVC workers had significantly elevated levels of MEHP and other DEHP metabolites and significantly lower levels of fT when compared to the relatively unexposed construction workers (70). This data again corroborates animal studies that suggest that DEHP exposure interferes with endocrine and paracrine signaling and makes a strong case for occupational health advocacy for those who work in these high exposure environments.

Conclusion

Male reproductive development is dependent on the complex interaction between hormonal messaging, cellular metabolism, and gene-environment interactions. This process is crucial for the maintenance of a healthy and self-perpetuating human population. From its pre-natal origins and through every stage of development, the male reproductive system is vulnerable to outside stimuli that have the potential to disrupt the process and starve developing germ cells of vital support and nutrition. Occasionally, we are exposed to these toxicants in environments we expect to be the safest. Phthalate esters, found in PVC and in many medical devices and children's items, illustrate this point. Thus, it is important to evaluate the impact of these compounds and others like them on susceptible populations and to identify exposure routes in an attempt to reduce contact. Furthermore, it is critically important to develop an improved understanding of the mechanisms of action phthalates use to cause toxicity in the fragile male reproductive system. Knowledge about the ways biological systems respond to insult and injury from phthalates allows for the development of therapeutic treatments for exposure, as well as the redesign of a plasticizer that meets modern's society's needs for flexible plastics but does not cause reproductive harm.

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CHAPTER 2

Activation of the Akt/NF- κ B Signaling Pathways Promotes Germ Cell Survival
Following MEHP-Induced Postnatal Injury

This chapter is comprised of a manuscript that will be submitted for publication in a peer-reviewed journal.

Activation of the Akt/NFκB signaling pathways promotes germ cell survival following MEHP-induced postnatal testicular injury

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ABSTRACT

Both the PI3K/Akt and NF κ B signaling pathways have been implicated in germ cell survival in the testis, and NF κ B has been implicated in a transient protective response following MEHP-induced postnatal injury in the rat testis. Here we investigate the mechanism(s) by which Akt1 participates in this response. We found that Akt1 suppresses germ cell apoptosis following exposure to the phthalate metabolite, mono-2-ethylhexyl-phthalate (MEHP), a Sertoli cell toxicant. We found that Akt kinase activity increases in the testes of wild-type mice following MEHP exposure, and that loss of Akt1 results in the premature onset of germ cell apoptosis. To determine if there is crosstalk between the Akt and NF- κ B signaling pathways, we measured levels of total I κ B α , a complex that sequesters NF- κ B subunits in the cytoplasm, phosphorylated I κ B α , the phosphorylated form that is generated when NF- κ B has been activated and examined the expression and subunit localization of the NF- κ B subunits, p50 and p65. We found that MEHP-induced testicular injury resulted in the induction of phosphorylated I κ B α in an Akt1-dependent manner. Expression of the NF- κ B subunits p50 and p65 was elevated in Akt1-deficient testes relative to wild type control testes. By 3 hours post MEHP exposure a significant decrease in the nuclear localization of the p50 subunit was observed in these mice. Akt and NF- κ B downstream target genes, p21 and Bcl-xL were decreased in MEHP-exposed Akt1-deficient testes. Collectively our results indicate that Akt1 plays a role in the initial triggering of a transient germ cell survival response following exposure to MEHP and that this response is mediated, in part, by crosstalk with the NF- κ B signaling pathway.

Introduction

Proper balance of cell survival and programmed cell death (apoptosis) is an essential component of testis development and homeostasis. Previous reports indicate important regulatory pathways of testicular homeostasis to include the p53, NF kappa B (NF- κ B), TNF α , PI3K/Akt, Fas, and FasL signaling systems (1-8). Mutations in some of these target genes such as Fas, FasL, and p53, result in aberrant testicular homeostasis following toxicant exposure (1-3). Given the significance of normal testicular homeostasis and the maintenance of the male germ line, it is important to identify signaling networks that influence apoptosis not only in germ cells but also other testicular cell types following toxicant-induced testicular injury.

Understanding how Akt1 controls testicular homeostasis depends on dissecting the mechanism by which Akt1 and its family members prevent germ cell death. Akt1 has been shown to prevent apoptosis either through direct phosphorylation of proapoptotic factors or by modulating the activity of transcription factors. One potentially relevant target for Akt1-mediated antiapoptotic signaling in the testis is NF- κ B. It is well known that activation of NF- κ B protects different cellular systems from various apoptotic stimuli (9). Importantly, NF- κ B has been implicated in a transient protective response following MEHP-induced postnatal injury of the testis (5).

NF- κ B consists of five members that exist as both homo- and hetero- dimers, the best characterized form being a heterodimer composed of p50 and p65 subunits (10). This heterodimer and others are sequestered in the cytoplasm by association with the inhibitory subunit I κ B α (11). Signals leading to NF- κ B activation trigger I κ B α phosphorylation at two specific serine residues, allowing I κ B α polyubiquitination and

subsequent proteolytic degradation by the 26S proteasome. Newly synthesized I κ B α enters the nucleus and binds NF- κ B, thereby enhancing its dissociation from the DNA causing its re-exportation to the cytoplasm by means of a nuclear export sequence (NES) present on I κ B α (11). NF- κ B is constitutively elevated in hematopoietic cells of I κ B α knockout mice, but not in I κ B α -deficient embryonic fibroblasts. However, sustained activation of NF- κ B was observed in the latter cells after tumor necrosis factor- α (TNF- α) stimulation (29).

Phthalates are testicular toxicants that are ubiquitous environmental pollutants and have been shown to possess endocrine-disrupting activity leading to sexual maldevelopment in male mice exposed *in utero* (13-15). These compounds are used widely to make plastics flexible and are lipophilic, which causes them to leach out of the materials that contain them. MEHP, the toxic metabolite of diethyl-2-hexyl phthalate (DEHP), a phthalate used in PVC, has been shown to cause Sertoli cell damage and subsequent germ cell apoptosis when administered to 28-day-old rats by oral gavage (16). Paracrine signaling between Sertoli and germ cells is one critical component of the germ cell apoptotic response to MEHP (17-18). Importantly, NF- κ B has been implicated in the response to testicular injury following exposure to MEHP. Specifically, NF- κ B subunits p50 and p65 have been shown to translocate to the nucleus of germ cells which is believed to initiate a transient protective effect in the rat seminiferous epithelium (5).

Here we demonstrate that Akt kinase activity increases in the testes of wild-type mice following MEHP exposure, and that loss of Akt1 results in the premature onset of germ cell apoptosis. MEHP-induced postnatal testicular injury resulted in increased phosphorylation of I κ B α and transcription of the NF- κ B target genes Bcl-xL and p21 in

an Akt1-dependent manner, and nuclear localization of p50 was decreased in Akt1-deficient mice following MEHP exposure. Collectively our results indicate that Akt1 and NF- κ B are key targets in the initial triggering of a transient germ cell survival response following postnatal exposure to MEHP.

Materials and Methods

Mice Akt1-heterozygous breeding pairs were obtained from the laboratory of Dr. Morris Birnbaum (University of Pennsylvania, Philadelphia, PA). The animal room climate was kept at a constant temperature (23.3 ± 2 °C) at 30–70% humidity with an alternating 12-h light, 12-h dark cycle. All procedures involving animals were performed in accordance with the guidelines of the institutional animal care and use committee of Brown University in compliance with the guidelines established by the National Institutes of Health.

Primers For genotyping by PCR, the following primers were used in a single reaction:

853, 5'-GTGGATGTGGAATGTGTGCGAG-3'; 854, 5'-GCTCAGTCAGTGAGGCCAGACC-3'; 855, 5'-CACCCCACAAGCTCTTCTTCCA-3'.

The PCR were run with an initial denaturing step of 94 °C for 5 min, 39 cycles of 94 °C for 30 sec, 63 °C for 30 sec, 72 °C for 45 sec, followed by a final extension at 72 °C for 5 min. PCR genotyping of progeny, the wild-type and targeted bands are 310 and 194 bp, respectively.

Exposure Paradigm MEHP was purchased from TCI America (Portland, OR) and certified to be more than 94% pure by gas chromatography. Akt1 wild type, Akt1 heterozygous, and Akt1-deficient mice were gavaged at postnatal day 28. Mice received a single dose (500 mg/kg) of MEHP in corn oil by gavage at a volume equal to 4 ml/kg. Control mice received a similar volume of corn oil vehicle.

Assay of Akt1 kinase activity Mouse testes were lysed in ice-cold lysis buffer (Cell Signaling Technology, Beverly, MA). The extracts were centrifuged to remove cellular debris, and the protein content of the supernatants was determined using the Bio-Rad

protein assay reagent (Bio-Rad, Hercules, CA). A total of 500 μg of protein from the lysate samples were incubated with gentle rocking at 4 $^{\circ}\text{C}$ overnight with immobilized anti-Akt antibody cross-linked to agarose hydrazide beads. After Akt1 was selectively immunoprecipitated from the testes homogenates, the immunoprecipitated products were washed twice in lysis buffer and twice in kinase assay buffer (25 mM Tris, pH 7.5; 10 mM MgCl_2 ; 5 mM β -glycerolphosphate; 0.1 mM sodium orthovanadate; and 2 mM dithiothreitol), and the samples were resuspended in 25 μl of kinase assay buffer containing 200 μM ATP and 1 μg GSK3 α fusion protein (Cell Signaling Technology). The kinase reaction was allowed to proceed at 30 $^{\circ}\text{C}$ for 30 min and was stopped by the addition of 3x SDS sample buffer. Reaction products were resolved by 15% SDS-PAGE followed by Western blotting with an anti-phospho-GSK3 α/β antibody according to the manufacturer's specifications.

TUNEL staining and quantitation For cryosections, unfixed testes were submerged in OCT embedding medium (Sekura Finetek, Inc., Torrance, CA) and snap frozen by immersion in liquid nitrogen. Sections were then cut to 7 μm thickness and mounted on poly-L-lysine-coated glass slides (VWR Scientific, West Chester, PA). Germ cell apoptosis was detected in sections of fresh-frozen testis by the TUNEL labeling method using the ApopTag kit (Chemicon, Temecula, CA). Tissue was counterstained with methyl green. Testis sections were viewed using a Nikon E800 microscope (Melville, NY) using differential interference contrast microscopy. The images were captured with a Kodak DC120 digital camera equipped with a MDS120 adapter (Eastman Kodak Co., Rochester, NY) and processed using Adobe Photoshop 6.0 software (Adobe, San Jose, CA). TUNEL-positive germ cells were quantitated in each tissue section by counting the

number of TUNEL-positive cells in each essentially round seminiferous tubule. For each testis section, approximately 100–200 tubules were counted from at least three different mice. The incidence of apoptosis was then categorized into either of three groups, defined as none, one to three, or more than three TUNEL-positive germ cells per seminiferous tubule cross-section. In the control mouse testis, the percentage of seminiferous tubules with more than three TUNEL-positive cells is less than 10%, so that an increase in apoptosis is easily determined using this data presentation. The data, calculated as a percentage of the total, are expressed as the mean \pm SEM.

RNA isolation Total RNA was isolated from testes of control, 1-, 3-, and 6-hour Akt1 wild-type and Akt1-deficient mice. These testes were detunicated, weighed, and homogenized in TriReagent (Sigma Aldrich, St. Louis, MO) and further RNA isolation was performed according to the TriReagent manufacturer's protocols.

Quantitative RT-PCR Total RNA (1 μ g) was DNase-I (Invitrogen, Carlsbad, CA) treated and reverse-transcribed using iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's protocols, and the cDNA templates were amplified with each of the primer pairs in independent sets of PCR using iQ SYBR Green Supermix (Bio-Rad) on an iCycler iQ Multicolor Real-time PCR Detection System (Bio-Rad). Mouse-specific primers were designed using Molecular Beacon Design 4.0 Software (Bio-Rad). The concentration of Mg^{2+} and the linear range of amplification of cDNAs with each primer pair first were optimized, and cDNAs subsequently were tested. Each sample was run in triplicate, and mRNA levels were analyzed relative to hypoxanthine phosphoribosyltransferase, a housekeeping gene that was not altered in response to MEHP exposure. Log₂-transformed relative expression ratios were calculated as

described using the equation set forth by Pfaffl, in which efficiencies for both the gene of interest and the calibrator hypoxanthine phosphoribosyltransferase were used.

Western blotting Testes from control, 1-, 3-, and 6- hour wild-type and Akt1-deficient mice were detunicated, weighed, and homogenized in three volumes of ice-cold RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, and 0.1% SDS) containing a protease inhibitor cocktail (P2714; Sigma) by 10 strokes in a Dounce homogenizer. Samples were incubated on ice for 30 min. The homogenate was then centrifuged at 13,500 x *g* for 10 min at 4 °C. For Western blotting, 50 µg of testis supernatant was separated by 10% SDS-PAGE unless otherwise specified and transferred to Immobilon-P membrane. Blocking solution (20 mM Tris, pH 7.4; 137 mM NaCl; 10% nonfat dry milk) was added to the membranes for 60 min. Primary antibodies were diluted in blocking solution and added to the membranes at 4 °C overnight. After washing three times with 20 mM Tris (pH 7.4), 137 mM NaCl, and 0.1% Tween 20 (PBS-Tween), horseradish peroxidase-coupled secondary antibody diluted in blocking solution was incubated with the membranes for 1 h at room temperature. Membranes were washed three times with PBS-Tween (0.1%), and secondary antibody was detected by enhanced chemiluminescence according to the manufacturer's instructions (Amersham Pharmacia Biotech). The antibodies used were anti-phospho-Akt (Thr³⁰⁸) (1:1000, Cell Signaling Technology), anti-Akt1 (1:1000, Cell Signaling Technology), Bcl-xL (1:500, Cell Signaling Technology), p21 (1:500, Cell Signaling Technology), phosphorylated IκBα (1:500, Cell Signaling Technology), and β-actin (1:2000, Sigma). Akt kinase activity was measured with an Akt Kinase Assay Kit from Cell Signaling Technology.

Immunostaining Immunostaining was performed based on previous methods. Testes were flash frozen by liquid nitrogen in OCT embedding medium (cat #4583; Sakura Tissue-Tek, Torrance, CA). Testis cross-sections (7 μm) were dried onto polylysine-coated slides and postfixed in -20°C methanol for 3 min. Sections were washed in PBS-Tween (0.1%) and blocked (3% BSA in PBS) for 1 h at room temperature. After blocking, samples were probed with p50 (cat #SC-1190) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:200 dilution overnight at 4°C . Slides were washed and incubated with secondary antibodies: Alexa Fluor 488 conjugated donkey anti-goat or Alexa Fluor 594 conjugated donkey anti-rabbit (Molecular Probes, Eugene, OR) diluted at 1:500. Negative controls were omission of the primary antibody. Fluorescent microscopic images were obtained on a Zeiss Axiovert 35 microscope (Carl Zeiss, New York, NY) connected to a Spot RT camera (Diagnostic Instruments Inc., Sterling Heights, MI). Images were downloaded into Photoshop 6.0 imaging software (Adobe Systems Inc., San Jose, CA) for resizing and fluorescent layering. Final figures were assembled using Canvas 8.0 software (Deneba Systems Inc., Miami, FL)

Statistical analysis The Student's *t* test or one-way ANOVA with Bonferonni *post hoc* analysis were performed using Sigma Stat software (SPSS, Chicago, IL). A *P* value < 0.05 was considered to be statistically significant.

Results

Induction of the Akt signaling pathway following MEHP-induced postnatal testicular injury. To determine whether Akt activation occurs in response to MEHP, Akt kinase activity was assessed by an *in vitro* kinase assay using the known Akt substrate GSK3 $\alpha\beta$. The level of GSK3 $\alpha\beta$ was markedly higher at the 3-hour time point (Fig. 1A), and remained elevated at the 6-hour timepoint. Akt phosphorylation status (Thr 308) was examined by Western analyses in the testis of Akt1-wild type mice 0, 1, 3, and 6 hours after exposure to MEHP. The levels of phosphorylated Akt were elevated at the three and six hour time points. (Fig. 1B), indicating that Akt kinase activity is induced in response to MEHP exposure.

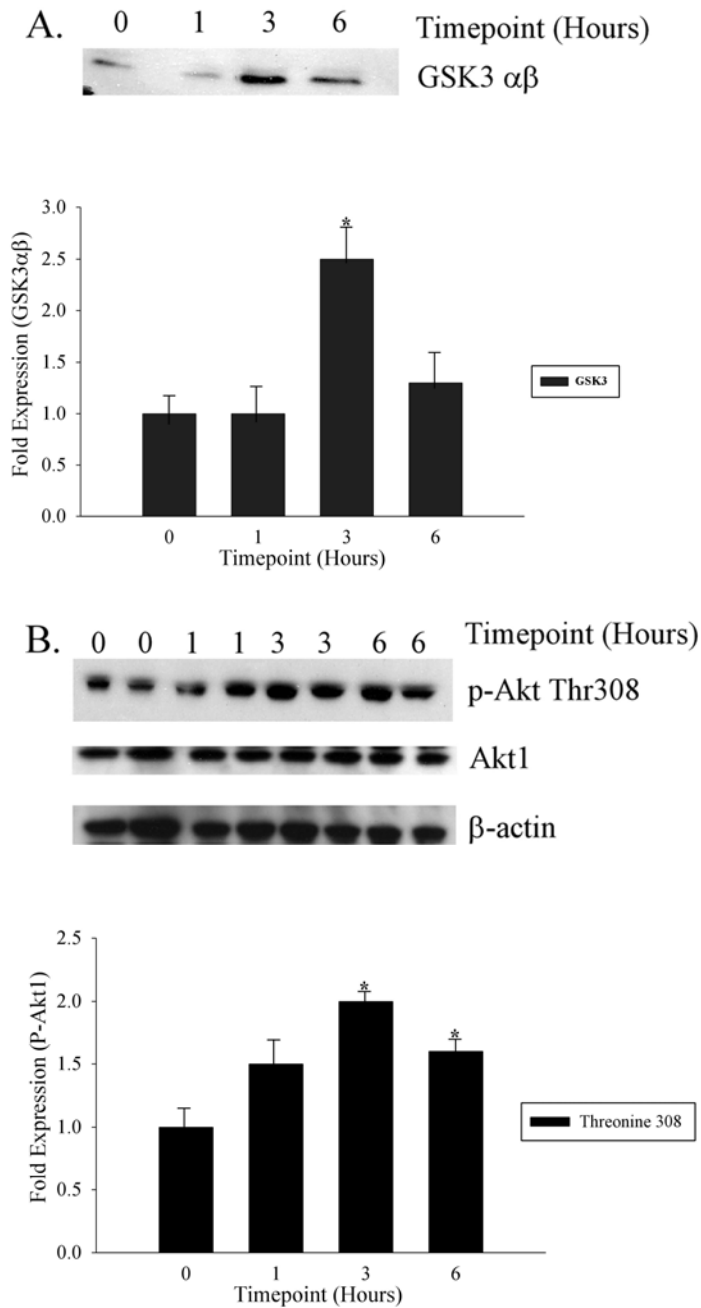


Figure 1. Activation of the Akt kinase after exposure to 500 mg/kg MEHP. A, Akt kinase activity as assessed by in vitro kinase assay using known Akt substrate GSK3 $\alpha\beta$. B, Representative Western blot from two testis samples for each time point are provided. Shown are phosphorylated Thr³⁰⁸ Akt and total Akt1 protein levels. The intensities of phosphorylated Thr³⁰⁸ Akt and total Akt1 were normalized to that of β -actin.

Akt1 suppresses MEHP-induced germ cell apoptosis. We have demonstrated an important role for Akt1 in the suppression of germ cell apoptosis following exposure to 500 mg/kg MEHP. To evaluate the extent of MEHP-induced germ cell apoptosis, apoptosis in the testes of 28-day-old treated Akt1-deficient mice was compared to that of testes of similarly treated Akt1 wild-type and Akt1 heterozygous mice. In wild-type mice a time-dependent increase in TUNEL-positive germ cells was observed 6 hours post MEHP exposure (Fig 2). In Akt1-deficient mice, exposure to MEHP resulted in an increase in the incidence of TUNEL-positive germ cells by 1 and 3 hours post MEHP exposure (Fig.2). To quantitatively evaluate the amount of germ cell apoptosis above baseline induced by MEHP treatment, the percentages of seminiferous tubules displaying greater than three apoptotic germ cells per seminiferous tubule were compared (Fig. 2) After MEHP treatment of Akt1-wild-type and Akt1-heterozygous mice, a time-dependent increase in TUNEL-positive germ cells was evident. A significant increase in apoptosis occurred 6 h after MEHP exposure. In MEHP-treated Akt1-deficient mice a significant increase in TUNEL-positive germ cells was observed as early as 1 h and continued through to the 6 h time point (Fig 2).

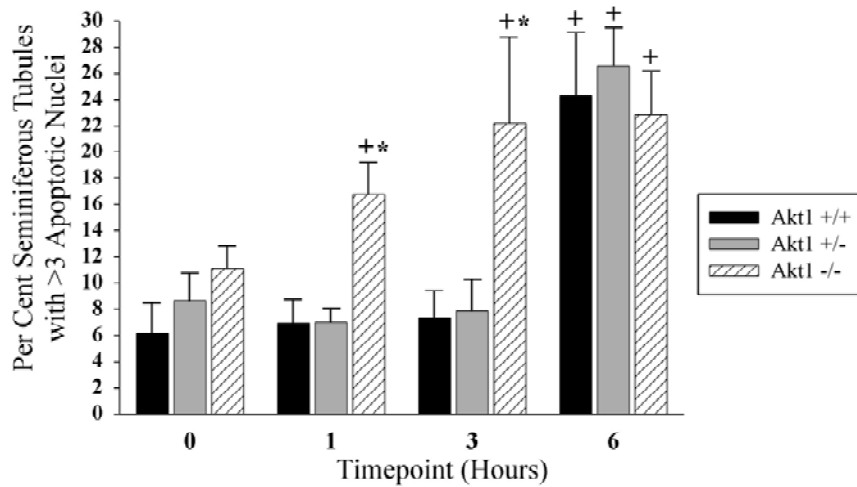


Figure 2. Akt suppresses germ cell apoptosis in mouse seminiferous tubules. Shown is bar graph representing time course of MEHP-induced germ cell apoptosis in Akt1-wild type (*black box*), Akt1-heterozygous (*gray bar*), and Akt1-deficient (*striped bar*) mouse seminiferous tubules. A minimum of three mice per genotype per time point were analyzed. Statistical analyses were conducted using one-way ANOVA ($P < 0.05$); *error bars*, SEM.

MEHP-induced postnatal testicular injury results in the induction of phosphorylated IκBα in an Akt1-dependent manner. We were intrigued by the trend for a basal increase in germ cell apoptosis in the Akt1-deficient mice and postulated that this trend may contribute to increased sensitivity of the Akt1-deficient testes to environmental insults. Therefore, we decided to examine the role of NF-κB signaling. It has been well established that NF-κB activity is regulated by IκB proteins and that phosphorylation and degradation of IκBα results in the activation of NF-κB. Previous studies suggest a link between exposure to phthalates and activation of the NF-κB signaling cascade (5, 19). In order to evaluate whether Akt1 mediated the activation of NF-κB, we examined the phosphorylation of IκBα protein by Western analyses. Figure 3 depicts an approximate 2-fold induction of phosphorylation of the IκBα complex at both 1- and 3-hours following MEHP exposure in Akt1-wild type mouse testes. In contrast, no increase of IκBα phosphorylation was observed in Akt1-deficient mouse testes following exposure to MEHP at any of the time points examined.

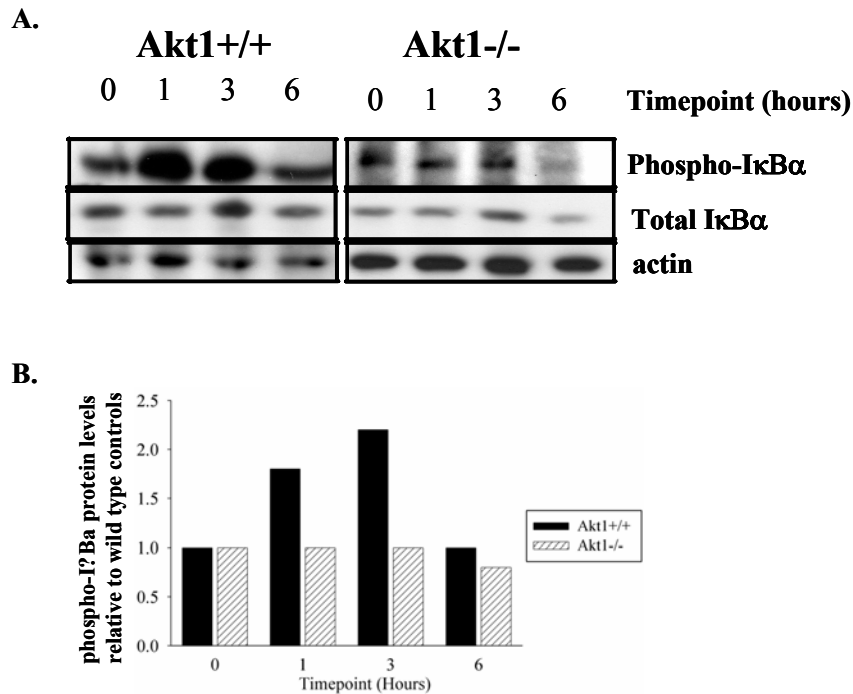


Figure 3. Akt1 increases activation of phosphorylated-IκBα in Akt1-wild type testicular homogenates exposed to 500 mg/kg MEHP. A, Representative Western blot analysis of three testis homogenates for each time point and genotype are provided. Shown are phosphorylated-IκBα and total IκBα protein levels. The intensities of phosphorylated-IκBα and total IκBα are normalized to that of β-actin. B, A Graphical quantitation of phosphorylated-IκBα protein levels in Akt1-deficient and Akt1-wild type mice exposed to 500 mg/kg MEHP. Values represent the mean ± SEM.

The transactivation potential of NF- κ B downstream target Bcl-xL is reduced in an Akt1-dependent manner in the testis following MEHP-induced postnatal testicular injury. In order to confirm the transactivation potential of NF- κ B, the downstream target gene Bcl-xL was examined using RT-PCR and Western analyses. Akt1-wild type testes showed highly elevated levels of Bcl-xL mRNA (Figure 4A) and protein (Figure 4B-C) expression at three and six hours post MEHP exposure while Akt1-deficient testes showed a slight decrease in Bcl-xL mRNA expression and no increase in Bcl-xL protein expression relative to controls (Fig. 4).

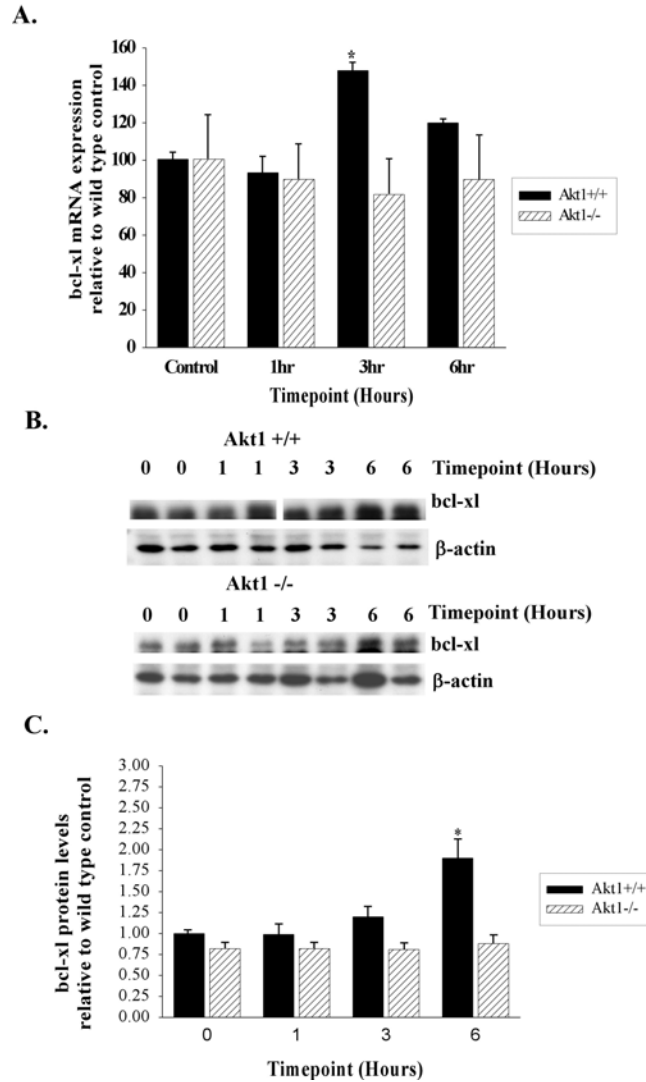


Figure 4. Akt1 increases expression of Bcl-xL protein and mRNA in testicular homogenates exposed to 500 mg/kg MEHP. A, A time course of relative expression of Bcl-xL mRNA in Akt1-wild type and Akt1-deficient mice exposed to 500 mg/kg MEHP. B, Representative Western blots from three testis homogenates for each time point of Akt1-wild type and Akt1-deficient mice after exposure. Shown are Bcl-xL protein levels. C, A graphical quantitation of Bcl-xL protein levels in Akt1-wild type and Akt1-deficient mice after exposure to 500 mg/kg MEHP. The intensities of Bcl-xL were normalized to that of β -actin. Values represent the mean \pm SEM.

The transactivation potential of NF- κ B downstream target p21 is reduced in an Akt1-dependent manner in the testis following MEHP-induced postnatal testicular injury.

RT-PCR analysis indicates that p21 mRNA levels are reduced in Akt1-deficient control testes relative to Akt1-wild type control testes (Fig. 5A). This reduced expression persisted at three and six hours post exposure to MEHP. However, at one hour post-exposure, no significant difference in p21 mRNA was observed between the two genotypes. Protein expression levels of p21 by Western blotting analysis revealed results complementary to RT-PCR data (Fig. 5B-C). Interestingly, mice that received MEHP did not exhibit significantly different p21 mRNA or protein levels compared to control mice of the same genotype. Thus, Akt1 appears to be necessary for normal expression of p21, and p21 does not appear to respond at the transcriptional level to injury by MEHP.

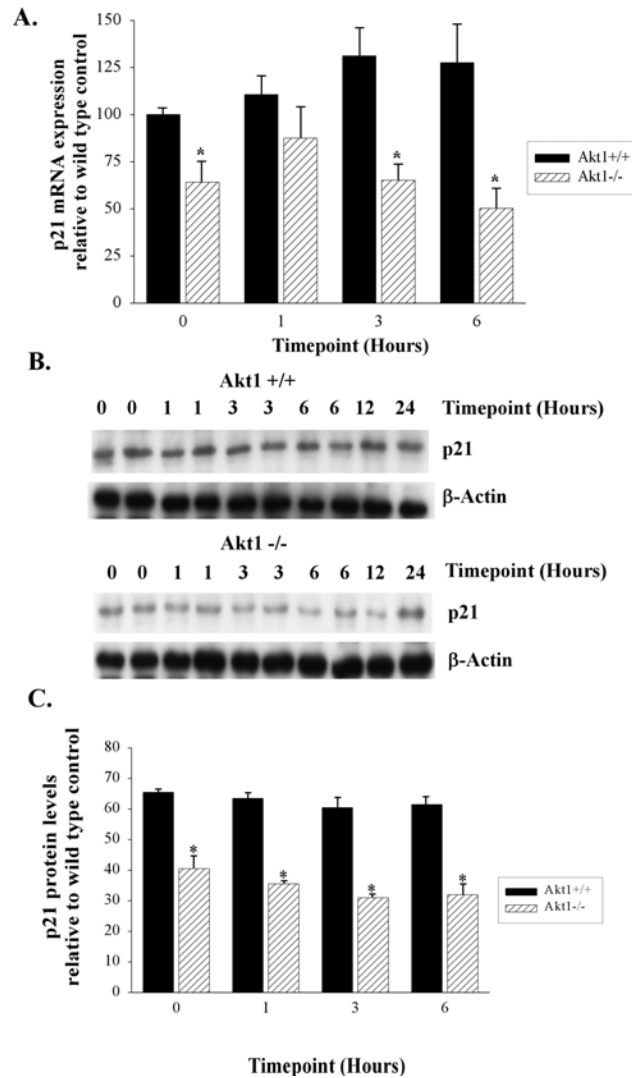


Figure 5. Normal p21 protein levels promote a normal testicular stress response to MEHP via an Akt1-dependent pathway. A, A time course of relative expression of p21 mRNA in Akt1-wild type and Akt1-deficient mice exposed to 500 mg/kg MEHP. B, Representative Western blots from three testis homogenates for each time point of Akt1-wild type and Akt1-deficient mice after exposure. Shown are p21 protein levels. C, A graphical quantitation of p21 protein levels in Akt1-wild type and Akt1-deficient mice after exposure to 500 mg/kg MEHP. The intensities of p21 were normalized to that of β-actin. Values represent the mean ± SEM.

Akt1-wild type mice exhibit nuclear translocation of the NF- κ B subunits p50 following MEHP-induced postnatal testicular injury. As a result of NF- κ B activation and the subsequent phosphorylation of the I κ B α complexes, specific NF- κ B subunits are released and translocate from the cytoplasm into the nucleus (9-12). To determine localization of NF- κ B subunits in Akt1-wild type mice, we used fluorescent immunohistochemistry. Through the evaluation of the nuclear localization of NF- κ B subunits p50 in germ cells we are able to assess NF- κ B activation in the testis following exposure to MEHP (Figure 6). We found that p50 localized to germ cell nuclei in control testes and three hours following MEHP exposure. Staining with DAPI revealed discrete germ cell nuclei in wild-type control testes. Similarly, the staining pattern for p50 was clearly nuclear and mirrored the discrete nuclear localization revealed by DAPI. Wild-type testes examined three hours after MEHP exposure exhibited a less discrete, more diffuse nuclear staining pattern that was also evident in p50 localization. However, these samples display clear nuclear staining for p50 at the control and three hour time points.

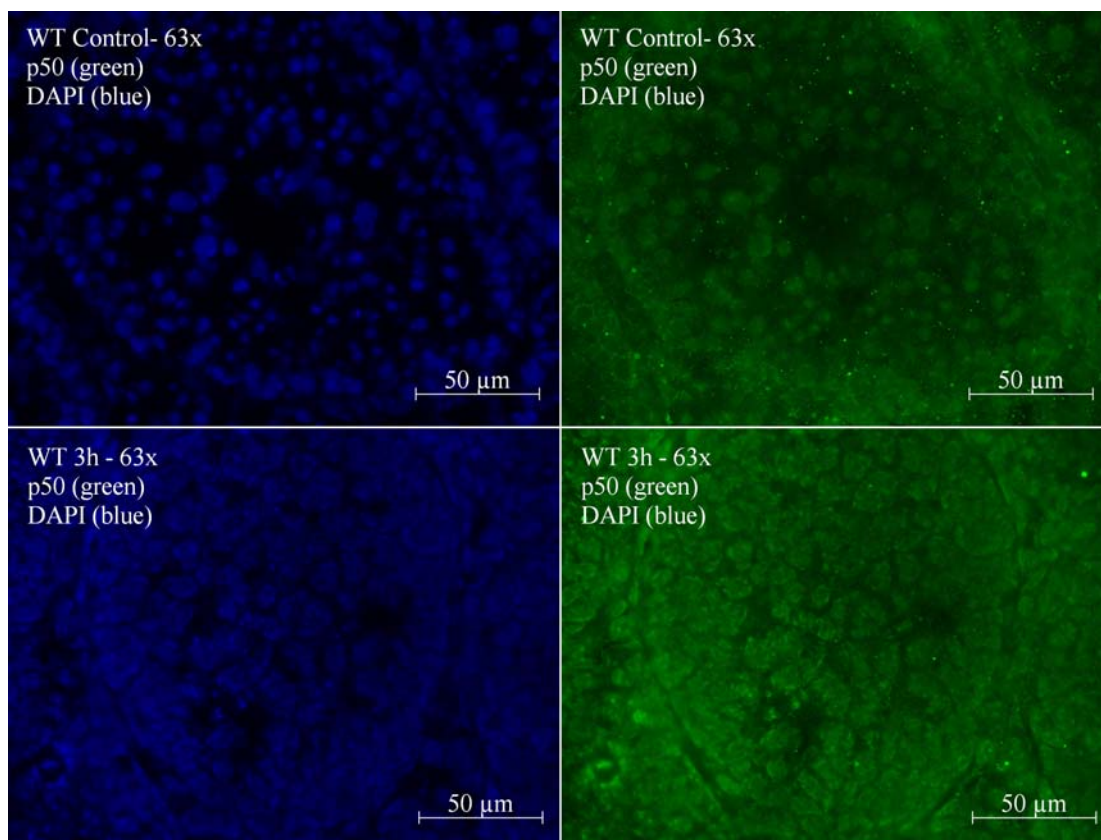


Figure 6. NF- κ B subunit p50 localizes to the nucleus in Akt1-wild type seminiferous tubules. Immunolocalization of NF- κ B subunits p50 (green) in cross-sections of PND-28 Akt1-wild type mouse testes exposed to corn-oil, and at three hours post exposure to MEHP by oral gavage. Scale bar indicates 50 μ m.

Nuclear translocation of NF- κ B subunit p50 is elevated in Akt1-deficient control mice but decreases sharply following MEHP exposure. We again used fluorescent immunohistochemistry to evaluate the nuclear localization of NF- κ B subunit p50 in germ cells as a means of assessing NF- κ B activation in the testis following exposure to MEHP (Figure 7). Interestingly, we found that p50 localized strongly to the nucleus in Akt1-deficient control mice but was negligible at the three hour time point. Assessment of germ cell nuclei using DAPI staining revealed viable germ cell nuclei at both time points, but p50 nuclear localization was seen only in control samples. Both testes were treated identically, thus these images suggest that p50 expression is elevated in control mice and decreases sharply in response to MEHP exposure.

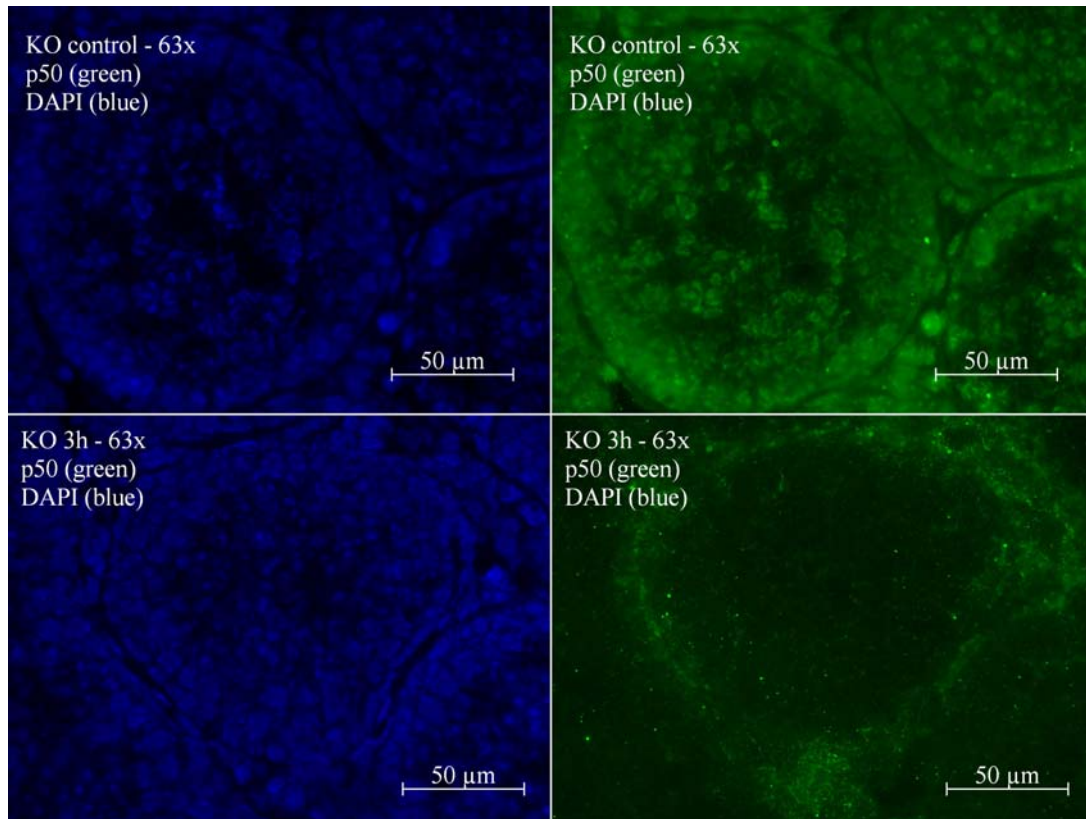


Figure 7. NF- κ B subunit p50 localizes to the nucleus in Akt1-deficient seminiferous tubules at the control time point, but not at the three hour time point. Immunolocalization of NF- κ B subunit p50 (green) in cross-sections of PND-28 Akt1-deficient mouse testes exposed to corn-oil, and at three hours post exposure to MEHP by oral gavage. Scale bar indicates 50 μ m.

Expression of p50 mRNA is significantly elevated in Akt1-deficient mice, but drops sharply in response to exposure to 500 mg/kg MEHP. NF- κ B has been implicated as an important regulator of the production of inflammatory mediators and has been found to play a role in oxidative stress (30, 32-35). In addition NF- κ B has been identified as a having proapoptotic effects in response to injury. In order to assess the extent of p50 activation in Akt1-deficient mice, RT-PCR analyses were performed (Figure 8). Akt1-deficient control mice exhibited a 1.5 fold increase in p50 mRNA expression relative to Akt1-wild type control mice. One hour after MEHP exposure, however, p50 mRNA expression drops significantly but is still elevated relative to Akt1-wild type mice. Three hours after MEHP exposure, p50 mRNA expression increases in Akt1-deficient mice and Akt1-wild type mice. Six hours post exposure, p50 mRNA expression increases nearly two fold in Akt1-wild type mice, but remains low in Akt1-deficient mice.

Expression of p65 mRNA is significantly elevated in Akt1-deficient mice, but drops sharply in response to exposure to 500 mg/kg MEHP. In order to further assess the role played by NF- κ B in Akt1-mediated germ cell apoptosis following MEHP exposure, RT-PCR analysis was used to evaluate mRNA expression of NF- κ B subunit p65 (Figure 8). In Akt1-deficient control mice, p65 mRNA exhibits a four fold increase in expression relative to Akt1-wild type mice. However, MEHP exposure results in a sharp decrease in p65 expression in both Akt1-deficient and Akt1-wild type mice. This trend persists at three and six hours post exposure and p65 mRNA levels do not differ significantly between Akt1-wild type and Akt1-deficient mice.

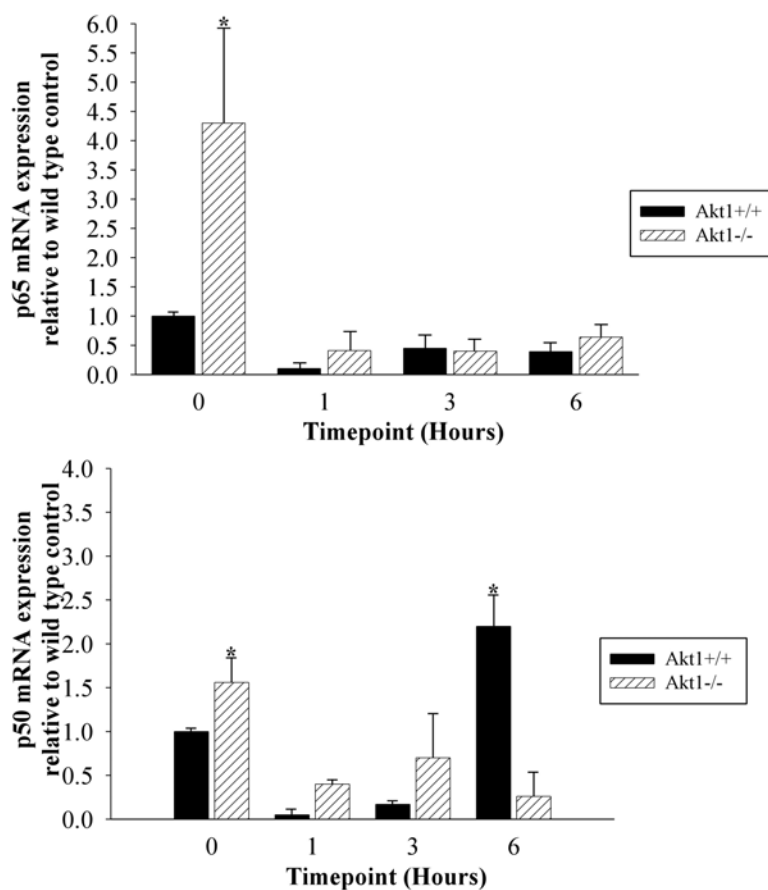


Figure 8. Induction of p65 and p50 expression in Akt1-deficient mice. A time course of relative expression of p65 (upper graph) and p50 mRNA (lower graph) after exposure of Akt1 wild-type and Akt1-deficient mice to 500 mg/kg MEHP at postnatal day 28. Akt1 wild-type (black bar) and Akt1-deficient (striped bar) mice. For all experiments, a minimum of three mice per time point per genotype were analyzed. Statistical analyses were conducted using one-way ANOVA ($P < 0.05$); error bars, SEM. Asterisk (*) indicates significance compared with wild-type control

Akt1-deficient control mice exhibit significantly elevated expression of SMAC/Diablo mRNA. Accumulation of cytosolic SMAC/Diablo has been implicated in the release of cytochrome c and is thought to be in part regulated by continuous oxidative stress in the mitochondria (37). In order to explore the consequences of Akt1 loss and exposure to MEHP, we conducted RT-PCR analyses to assess the expression of SMAC/Diablo in whole testis homogenates exposed to a corn oil vehicle and 1, 3, and 6 hours after exposure to 500 mg/kg MEHP. Importantly, Akt1-deficient control mice were found to have a nearly five fold increase in SMAC/Diablo expression relative to Akt1-wild type controls (Figure 9). Expression of SMAC/Diablo mRNA expression drops significantly in Akt1-wild type and Akt1-deficient mice at one and three hours following MEHP exposure, but increases six hours after MEHP exposure. However, at this time point Akt1-wild type mice exhibit an approximate 1.5 fold higher SMAC/Diablo expression compared to Akt1-deficient samples.

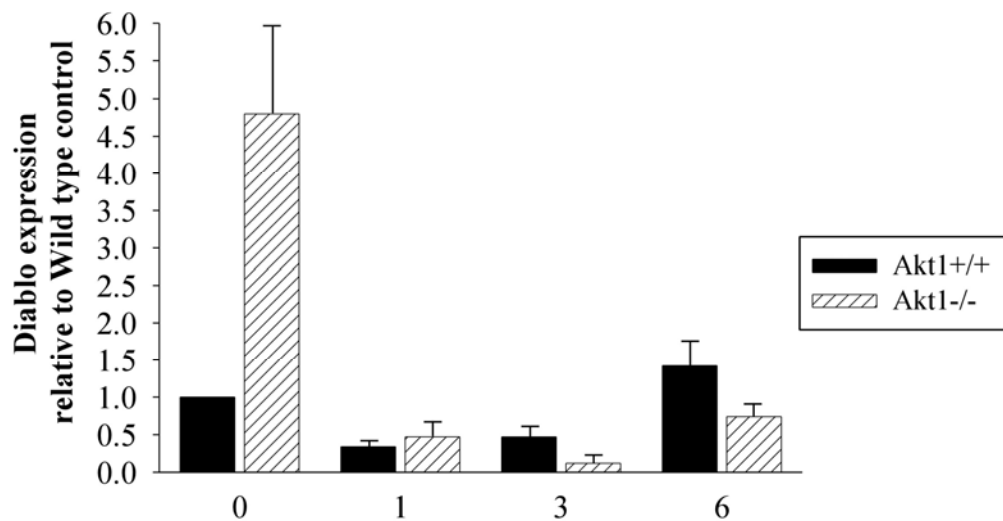


Figure 9. Akt1 deficiency results in increased expression of SMAC/Diablo mRNA in unexposed testicular homogenates. A time course of relative expression of SMAC/Diablo mRNA in Akt1-wild type and Akt1-deficient mice exposed to 500 mg/kg MEHP. Values represent the mean \pm SEM.

Discussion

Accumulating evidence suggests that the PI3 kinase/Akt signaling pathway is a major contributor to germ cell survival and spermatogonial stem cell proliferation (7, 23-25). To investigate the functional consequences of Akt1 in response to MEHP-induced testicular injury, we exposed Akt1-wild type, Akt1 heterozygous, and Akt1-deficient mice to 500mg/kg MEHP and examined germ cell integrity, relative gene expression, and the activity of several important cellular communication pathways. We found that Akt activation occurred within 3 hours following MEHP-induced testicular injury as assessed by phosphorylation of Akt at the threonine 308 site. Increased Akt phosphorylation led to the subsequent increase in phosphorylation of the downstream substrate GSK3 within a similar time frame, indicating a role for Akt activation in the cytoprotective response of germ cells to MEHP-induced postnatal testicular injury. Examination of apoptotic nuclei indicated that MEHP exposure results in an increase in germ cell apoptosis and that Akt1 plays an important role in the suppression of this outcome. Several downstream Akt1 target genes including Bcl-xL, p21, NF- κ B, and SMAC/Diablo have been implicated in the regulation of germ cell apoptosis following various testicular insults (5, 21, 22, 24, 28). With the protective role of Akt1 established, we evaluated these targets and other potential mechanisms that contribute to this protective response.

The NF- κ B pathway has been studied in many different cell types and has been found to upregulate many genes related to apoptosis in response to a wide variety of stimuli. In addition, recent work suggests a testicular NF- κ B response in the rat following exposure to MEHP (5) in which exposure to MEHP resulted in the translocation of NF- κ B subunits to germ cell nuclei and increased nuclear NF- κ B-binding activity after

MEHP exposure (5). Increased activity of NF- κ B was associated with a transient protection of the seminiferous epithelium. Based on these data, we examined subunit localization and transactivation of NF- κ B to determine if the increased sensitivity to MEHP-induced postnatal testicular injury in Akt1-deficient testes was partly due to the aberrant regulation of the NF- κ B signaling pathway. We observed a staining pattern in the Akt1-wild type mouse similar to the pattern observed in the rat. Subunit localization of p50 underwent nuclear translocation following exposure to MEHP in Akt1-wild type mice. In contrast, Akt1-deficient mice exhibited a significantly reduced level of p50 nuclear localization in response to MEHP exposure.

In order to further elucidate the role of NF- κ B subunits in response to the loss of Akt1 and exposure to MEHP, we examined expression of p50 and p65 at the mRNA level. Interestingly, Akt1-deficient control mice were found to have significantly elevated mRNA levels of both p50 and p65. Oxidative stress has been demonstrated to activate signal transduction pathways that involve NF- κ B (32-35) and compelling evidence suggests that NF- κ B can be activated by NADPH oxidase via reactive oxygen species intermediates (30, 31). Thus, we suggest that the observed elevation of NF- κ B in Akt1-deficient control mice may be in part due to the presence of oxidative stress that results from the loss of Akt1. Following MEHP exposure these Akt1-deficient mice experience a significant decrease in NF- κ B activity that corresponds to a significant increase in germ cell death. Here, we contend that NF- κ B activity decreases due to the fact that NF- κ B cannot be active in dead germ cells.

To further characterize NF- κ B activity we examined phosphorylation of the I κ B α complex as a marker of initial NF- κ B activation. I κ B kinase α (IKK α) has an Akt

phosphorylation site at amino acids 18-23, suggesting that the complex is a substrate for Akt (28, 29). Akt has been shown to play a role in the phosphorylation of I κ B, an endogenous inhibitor of NF κ B activity, which culminates in gene regulation (29, 38-39). A similar mechanism may be active in the present model. In further support of an important role of I κ B, phosphorylation of I κ B α was transiently increased following MEHP-induced injury (Fig. 3) although there was no effect on total I κ B protein levels. The ability of MEHP to induce phosphorylation of I κ B α without protein degradation is similar to previous reports (40), and was shown to be sufficient for NF κ B activation. Furthermore, it is possible that the absence of increased I κ B α in Akt1-deficient mice is due to the fact that the degree of baseline I κ B α activation in these mice is sufficient to maintain NF- κ B activity following MEHP exposure. In contrast, Akt1-wild type control mice do not display highly active NF- κ B and therefore must activate the pathway in order to respond to insult from MEHP. This is one possible explanation for increased phosphorylation of I κ B α in Akt1-wild type mice but not in Akt1-deficient mice. Regardless of the precise mechanism, our data suggest at least part of Akt's ability to maintain germ cell viability is dependent on NF κ B activity, likely involving the phosphorylation of I κ B α .

Phosphorylation of I κ B α results in the freeing of NF- κ B subunits and their subsequent translocation into the nucleus. In addition to regulation by the I κ B α complex and the nuclear translocation of NF- κ B subunits, NF- κ B is also controlled by phosphorylation events that increase transactivation potential (26). In order to determine whether an increase in NF- κ B transactivation potential contributed to the observed germ cell survival, we evaluated the mRNA and protein expression of the downstream NF- κ B

target, Bcl-xL, an anti-apoptotic member of the Bcl-2 family that prevents the release of cytochrome c from the mitochondria, and thus prevents the initiation of apoptotic caspase cascades (21-22). MEHP exposure had a pronounced effect on Bcl-xL protein and mRNA expression in Akt1-wild type mice. Similar to the effect observed for phosphorylated-I κ B α , MEHP exposure induces an increase in Bcl-xL mRNA at the three hour time point. Bcl-xL protein expression is also elevated at the three hour time point, although peak expression occurs at six hours. In contrast to the response of wild type testes, no increase in Bcl-xL mRNA or protein was observed in Akt1-deficient testes. This suggests that Akt1-mediated germ cell survival may be occurring through an increase in NF- κ B transactivation potential of Bcl-xL.

I κ B α was the first I κ B family member to be cloned and is still the best characterized. The physiological properties of the other I κ Bs are for the most part poorly understood. Mice lacking Bcl-3 and I κ B α have been generated (reviewed in 3, 6). Bcl-3-deficient mice exhibit specific defects in response to certain immunogenic agents. I κ B α -deficient mice die 7–10 days after birth and exhibit a variety of inflammatory conditions consistent with increased NF- κ B activity (9, 10). NF κ B is constitutively elevated in hematopoietic cells of I κ B α -deficient mice, but not in I κ B α -deficient embryonic fibroblasts. However, sustained activation of NF κ B was observed in the latter cells after tumor necrosis factor- α (TNF- α) stimulation (9, 10). Although differences between the I κ Bs exist in their structures and apparent preferences for specific forms of NF- κ B, I κ B β is the only species whose expression is not regulated by NF- κ B. Consequently, although I κ B β does not compensate for the lack of I κ B α in I κ B α ^{-/-} mice, when placed under control of the NF- κ B-responsive I κ B α promoter, I κ B β is able to effectively substitute for

I κ B α (11). Interestingly, Akt1-deficient mice exhibit reduced levels of total I κ B α relative to Akt1-wild type mice, which may play a role in the observed elevation of apoptosis in these mice.

In addition to Bcl-xL induction, our data indicate that p21 plays an important role in testicular homeostasis. P21 mRNA and protein levels are stable in Akt1-wild type mice; whereas, Akt1-deficient testes exhibit reduced levels of p21 at the mRNA and protein level. Previous data indicates that lamin-1 ligated α 6 β 4 integrin exerts a protective effect in mammary epithelial cells by increasing the activity of Rac and p21-activated kinase 1 which enhances stress induced NF- κ B activity (36). Thus, it is possible that elevated levels of p21 in Akt1-wild type mice promotes NF- κ B-dependent resistance to apoptosis. While it is difficult to elucidate whether p21 is directly regulated via Akt1/NF- κ B/p21 signaling pathway *in vivo*, previous *in vitro* research also indicates that this protein plays an important role NF- κ B-dependent antiapoptotic effects (27). Thus, it is possible that p21 is a key player in the cross talk between Akt1 and NF- κ B transactivation potential and protection following MEHP-induced testicular injury.

In order to corroborate our data suggesting a preemptive stress response in Akt1-deficient mice we examined the mRNA expression of SMAC/Diablo, an important marker of oxidative stress and damage. Similar to mRNA expression of p50 and p65, SMAC/Diablo was highly elevated in Akt1-deficient control mice relative to wild type controls. Previous work suggests that super oxide dismutase (SOD) affects the early release of cytochrome c and SMAC/Diablo (37). Thus we contend that elevated SMAC/Diablo expression indicate a stress response due to loss of Akt1 and supports the conclusion that Akt1-deficient mice are more sensitive to MEHP exposure.

In conclusion, we report that Akt1 suppresses germ cell apoptosis following MEHP-induced testicular injury, and that this effect is mediated in part by Akt1-dependent phosphorylation of I κ B- α and through mechanisms that regulate the transactivation potential of NF- κ B subunits.

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CHAPTER 3

Medical Responses to Phthalate Exposure: Background and Significance

Introduction

The design, construction, and operation of health care facilities can have profound impacts on human and environmental health. Green healthcare, a relatively recent field that developed out of green building philosophies (1), incorporates environmentally friendly practices into medical delivery and seeks to identify potential sources of environmental and public health problems in hospital settings and find ways of removing them (2). A critical pillar in the foundation of green healthcare is the precautionary principle, the idea that policies that protect human health should be evoked in the face of uncertain risks (3). While the green healthcare movement is relatively new, this philosophy is not new to healthcare. Currently, the implementation of this green healthcare philosophy can be seen in the architectural and aesthetic design of hospital buildings, the equipment that is used to deliver life saving medications, and even the cleaning supplies found in janitorial closets (4).

There are many obstacles that must be overcome in order to develop a truly healthy medical environment. Phthalates, for example, present a significant number of persistent challenges to the medical community and the green healthcare initiative (5). While the Food and Drug Administration (FDA), the Environmental Protection Agency (EPA) and Agency for Toxic Substances and Disease Registry (ATSDR) have all released statements regarding the health risks posed by DEHP in PVC plastics used to make medical devices (6-8), the vast majority of hospitals continue to use materials that expose patients to this compound (9). Viable alternative materials exist for nearly every PVC item found in hospitals (10), but the task of replacement is often too intimidating

and too expensive for those aware of the risks. Ultimately, green healthcare addresses and find remedies for serious public health problems that the medical community must acknowledge. By embracing the precautionary principle and the green healthcare philosophy it is possible to find solutions for problems presented by PVC and DEHP.

Green Healthcare

The growth and development of the environmental movement in the late 20th and early 21st centuries has resulted in many efforts to relay the importance of sustainability and environmental protection to governments at the local, state, and federal level, industries in many different sectors, as well as the public at large. Green health care is one such effort and is a relatively new mode of thinking that has origins in green building and environmental health initiatives. The green healthcare philosophy is deeply rooted in the definition of green building. According to the Office of the Federal Environmental Executive, green building is the “practice of designing, constructing, operating, maintaining, and removing buildings in ways that conserve natural resources and prevent pollution (1).” As an environmental initiative, green healthcare is defined as the incorporation of environmentally friendly practices in medical settings in order to facilitate environmental and public health (12). This movement is based on a set of principles that espouse the idea that healthcare practitioners have an obligation to eliminate and avoid practices that are unhealthy for people and the environment (13).

Green healthcare originates in a vast number of grass roots efforts, but gained solidarity with the emergence of two important organizations. In 1996, the campaign for environmentally responsible health care was founded and eventually resulted in Healthcare Without Harm, an organization that today has over 440 members in 55 countries working together to revolutionize the health care industry (12). In addition, 1998 marked the launch of Hospitals for Healthy Environments (H2E), a joint venture between the EPA and the American Hospital Association (AHA) that counts waste

reduction and pollution prevention in medical settings amongst its mission statements (13). In 2001, the two organizations entered into a partnership that also included the American Nurses Association. Together, these organizations have advanced green healthcare and as of January 2005, had grown to include 962 partners representing 1,017 hospitals, 1,789 clinics, and 527 nursing homes across the United States that are incorporating green initiatives in their daily operations (13).

These founding organizations and the ventures they support achieve the goals of the environmental healthcare movement via implementation of a variety of initiatives at the institutional level as well as in the larger operating environment. Implementation of green healthcare initiatives requires the synergy of a unique set of circumstances that, if achieved, has a profound positive impact on public health. First, there must be a demand for improvement and change (14). This is often a product of the emergence of new data that clearly indicates a need for altered operations. In addition, environmentally friendly health care is largely dependent on shifts in the way that health issues are thought about and the way that problems are framed. An understanding of the factors that drive change in medical settings is bolstered by the presence of passionate and effectual advocates championing change from an environmental perspective.

Like any complex system, evolution and change in medical settings is never quick or easy. In the case of toxic exposures in hospitals, change is often an eventual result of new data on the health consequences of compounds that are used in these settings. This new data generates a demand for change. For example, when toxicological testing revealed a strong correlation between asbestos exposure and increased incidence of lung cancers like mesothelioma, regulations were put into place and eventually, the use of

asbestos in the construction of new hospitals ceased. However, emerging data is often controversial and easily refuted. Change is inevitably an intimidating process and typically requires significant up front expense. Thus, policy change in response to emerging data also requires hospital administrators and policy makers that are progressive thinkers and are capable of evaluating the merit of scientific research independent of financial consequences.

In addition to scientific research, hospital demand, and visionary leadership, implementation of green healthcare initiatives also requires the availability of a supply that meets the needs of the transition (15). This includes a wide range of goods and services and, depending on the specifics of the initiative, may consist of architects willing to design hospitals that emphasize energy efficiency, suppliers that produce cleaning supplies that are both effectual and safe, or manufacturers that make plastics that are free of DEHP plasticizers. The existence of such goods and services are occasionally a result of “environmental supply chain dynamics,” a phenomenon where environmental innovations result from relationships between customers and suppliers (15). Research in this field surrounding the British and Japanese food retail sector and the British aerospace industry demonstrates that environmental supply chain dynamics which favor environmentally friendly innovations will result in situations where industry leaders have power over a suppliers and are under sufficient environmental pressure (15). While this dynamic has not been specifically researched in the health care sector, it is surely possible that the medical industry has, and will continue to work together to respond to environmental pressure from advocacy organizations and exert pressure on hospital suppliers to make environmentally friendly materials available.

The development of environmental supply chain dynamics brings up the interesting issue of the role of advocates in the implementation of green health care initiatives. Ever since Florence Nightingale insisted on a clean medical environment for soldiers in the Crimean war, healthcare advocacy has had a significant impact on the ultimate health outcomes of patients in medical facilities (16). Healthcare advocates are critical to the implementation of green health care initiatives not only due to the requisite interest in public health issues, but also because these people have an intimate understanding of the institutional dynamics that dominate hospital policy making. Health care advocates must be able to take advantages of the unique opportunities presented by specific hospitals and also work effectively around the various weaknesses that hospitals possess. With this knowledge and ability, health care advocates serve a critical role in the successful transition to environmentally friendly practices.

The Precautionary Principle and Green Chemistry

Everyday actions are taken, new chemicals are synthesized, and decisions are made that present hazards to the public and to the environment. To be sure, these hazards possess an intrinsic and quantifiable risk. Unfortunately, this risk is usually defined publicly, in the media and in courtrooms, and is commonly the product of a complex mixture of interpretations (17). As a result, there will generally always exist some degree of uncertainty surrounding the risk assessments of environmental decisions. The precautionary principle suggests that if an action or chemical compound is thought to present a threat to human health or the environment the burden of proof shifts to those who advocate taking the action or using the chemical rather than those who advocate precautionary measures (3). The precautionary principle emphasizes planning, safety assessment, and anticipatory action and advocates preventative measures aimed at avoiding environmental disaster even in the absence of conclusive scientific evidence. Essentially, the precautionary principle warns that “the absence of evidence of harm is not the same thing as evidence of the absence of harm (3).” In the case of the use of DEHP-containing materials and PVC, advocates of the precautionary principle would contend that phthalate manufacturers and advocates of PVC use must prove that these materials present no threat to human health before they are used.

The precautionary principle has gained strength in recent years as it has been incorporated into a variety of burgeoning environmental efforts. Past environmental legislation and attempts at environmental protection have emphasized command-and-control type enforcement, seeking to address environmental health issues with demands and regulations. In 1990, with the passage of the Pollution Prevention Act, an important

transition (18). The passage of this act identified pollution source reduction as a significant means of solving environmental problems and acknowledged the need to involve a variety of disciplines in the attempt to reduce toxic production (19). As a direct result, 1991 saw the inception of Green Chemistry as a part of the EPA's Office of Pollution Prevention and Toxics "Alternative Synthetic Pathways for Pollution Prevention" research initiative. In 1993 the green chemistry movement solidified as the US Environmental Protection Agency (EPA) officially began the US Green Chemistry Program which includes incentive programs such as the Presidential Green Chemistry Challenge Awards, a series of awards designed to recognize individuals and businesses for innovations in green chemistry (19). This legislative conversion placed emphasis on precautionary philosophies and resulted in a handful of significant efforts to address environmental health concerns from the beginning of the toxic life cycle rather than the end.

The green chemistry movement's commitment to the precautionary principle has resulted in many new innovations that allow for a healthy environment. Many of these discoveries are directly applicable to the healthcare sector. For example, petrochemicals are a fossil resource used in plastics, resins, detergents, solvents, and lubricants, all of which find uses in hospital and medical settings. A significant portion of the petrochemical industry is dedicated to manufacturing these chemical and industrial materials (20). In an effort to reduce the use of petrochemicals and their toxic byproducts, green chemistry has developed alternative means of building plant-based materials that meet society's needs. Techniques include the chemical modification of naturally made structures via fermentation, hydrothermolysis, and gasification, all of which are

environmentally benign (20). Green chemistry has the potential to significantly alter the way that plastics are made and as a result, holds the power to remove the toxic threats that currently linger in our hospitals and homes.

Green chemistry approaches the challenge of sustainability through the creation of new technologies that meet the needs of society in an environmentally responsible way (19). One part of this philosophy is the idea of the “triple bottom line,” an economic accounting framework that evaluates sustainability based on environmental protection, societal benefit, and market advantage (21). The International Union of Pure and Applied Chemistry (IUPAC), a non-governmental organization widely recognized for establishing international consistency in chemical research, reflects this commitment to sustainability in its Strategic Plan, which states “IUPAC will assist chemistry-related industry in its contributions to sustainable development, wealth creation, and improvement in the quality of life (22).” Green chemistry clearly links the consideration of hazards with production viability and considers the presence of toxic materials at all stages of the synthetic process, not just the waste. This philosophy coupled with a commitment to the precautionary principle results in careful thought about the consequences of the chemicals we use and holds an impressive power to shift the way medical professionals think about healthcare.

Despite documented successes, green chemistry faces criticism from the scientific community. Green chemistry sets lofty goals for chemical production, but these changes do not alter the fact that green chemistry is at its roots, a form of synthetic chemistry. This presents a significant challenge for the movement because synthetic chemistry does not have a very good track record. ‘Wonder’ chemicals of past decades like

polychlorinated biphenyls (PCBs), dichloro-diphenol-trichloro-ethane (DDT), and teflon have turned out to have significant adverse health effects for the population as a whole and especially for communities located near industries that produce and use these compounds (23). Occasionally, chemicals appear benign to those immediately exposed but have severe consequences for later generations. This has been demonstrated repeatedly, most notably with pregnancy pharmaceuticals like thalidomide and diethylstilbestrol (DES) (24). While the negative ramifications of these compounds and others have not always been known at the time of production and use, there has been a demonstrated lack of foresight and environmental responsibility underlying synthetic chemistry. In addition, current toxicology testing depends heavily on animal data, which does not always correlate with human data, as is the case with some phthalate exposures (25).

While synthetic chemistry does have a discouraging history, green chemistry separates itself from its synthetic roots via its strong commitment to the precautionary principle. Green chemists believe that no chemical is intrinsically good or bad, and that a complete understanding of toxicity allows green chemists to remove and avoid those characteristics that make certain chemicals hazardous. Green chemistry is different from the synthetic chemistry of the 1950's because it requires that explicit energy and material consumption, safety, toxicity, and environmental degradability criteria are met (26). Green chemists realize the potential unknowns associated with synthetic chemistry. This acknowledgement coupled with the precautionary principle allows green chemistry to address the scientific criticism that accompanies all forms of synthetic chemistry. Green chemists like those currently working to redesign plasticizers and find alternatives for

compounds like DEHP must acknowledge and internalize these criticisms in order to find viable options and avoid the mistakes of the past.

Phthalates in Medical Settings

Polyvinyl chloride was discovered by two separate teams of scientists in the 19th century. In 1926, this material was made more useful as researchers Waldo Semon and B.F. Goodrich discovered that the incorporation of certain additives made PVC more flexible and more easily processed (27). Plasticized PVC is cheap to produce and highly durable. As a result, this material quickly found a relatively stable place in the daily operations of hospitals and clinics around the world. In recent years, the health effects of the phthalate plasticizers have been reviewed and the safety of their use subsequently questioned. Several regulatory agencies in the United States and in Europe have concluded that the exposure to DEHP that occurs as a result of certain medical procedures that require PVC equipment could pose a risk to patients. In the United States, the Food and Drug Administration (FDA), National Toxicology Program (NTP), and the Agency for Toxic Substances and Disease Registry (ATSDR) have all issued expert panel reports and public health notifications that urge health care providers to use DEHP and PVC alternatives, particularly in procedures for especially vulnerable patients. The actions of these regulatory agencies helps to encourage and motivate hospitals and medical centers to recognize the significance of the threat posed by DEHP and PVC and to work to transition away from their use in medical procedures.

In July 2002, the FDA issued a public health notification about PVC devices that contain DEHP. This notice reflected the results of a safety assessment that took place in 2001 and advised readers of steps to reduce exposure to DEHP. It specifically focused on DEHP exposure in hospitals and reported that DEHP is most often found in intravenous bags and tubing, umbilical artery catheters, blood bags and infusion tubing, enteral

feeding bags, nasogastric tubes, peritoneal dialysis bags and tubing, as well as tubing used in cardiopulmonary bypass procedures, extracorporeal membrane oxygenation (ECMO), and hemodialysis. While the FDA acknowledged that everyone is exposed to small amounts of DEHP in everyday life, they reported that DEHP is inclined to leach out of medical devices and that individuals that are seriously ill and require multiple procedures are often exposed to elevated levels of DEHP. Based on the prevalence of studies that reported adverse reproductive effects in young, male, laboratory animals and the absence of any studies that ruled out adverse events in humans, the FDA concluded that DEHP poses a risk in medical settings.

Upon this conclusion, the FDA evaluated significant risk determinants and high risk procedures. The sensitivity of the patient and the received dose were found to be the largest risk determining factors and male fetuses, male neonates, and peripubertal males were found to be the highest risk group, as these patients are small, still developing, and frequently hospitalized for a significant and continuous length of time. Furthermore, the FDA found that exchange transfusion, total parenteral nutrition, and ECMO in neonates, hemodialysis in peripubertal males and pregnant or lactating women, enteral nutrition in neonates and adults, heart transplantation, and massive blood transfusions pose the highest risk of exposure to DEHP. While they don't recommend avoiding these procedures in order to prevent DEHP exposure, they do suggest that alternative devices be substituted if available.

A few months following this report in September 2002, ATSDR released their own report paralleling the findings of the FDA. While this report focused on environmental ramifications of phthalate production and exposures from the natural

environment, they do suggest that phthalate exposure from medical procedures are likely to be significantly higher than any environmental exposure. They call particular attention to the risks from blood products that are stored in plastics and used for transfusions and tubing used to deliver nutrition and medications. They also indicate that young males are likely to be more susceptible to toxicity from DEHP exposure than older ones.

In 2006, the National Toxicology Program released a 300-page monograph on the potential human reproductive and developmental effects of DEHP. This assessment evaluated over 70 peer reviewed papers and concluded that most people are exposed to DEHP and that this exposure has probably adverse effect for reproduction. Importantly, they concluded that high DEHP exposures of fetuses and infants can occur when breast-feeding women undergo medical procedures involving PVC medical devices. They also find that medical treatments of male infants with PVC-devices warrant a serious concern for the adverse development of the male reproductive tract. They find that there is also concern for male infants up to one year of age and some concern for older male children as well.

The widespread acknowledgement of the health risks posed by PVC and DEHP-containing medical devices combined with the necessity of medical procedures that require flexible plastics forces the question: if PVC is bad, what should we use instead? Fortunately, research on the feasibility of a wide variety of alternative materials occurred in tandem with research on the health effects of phthalate plasticizers. As a result, recommendations to remove PVC and DEHP can be accompanied with recommendations for replacements.

As noted previously, transfusions, total parenteral nutrition, ECMO, hemodialysis, and the delivery of medications through PVC tubing present the highest risk situations for patients. Discussion and concern surrounding research on the health effects from phthalates has generated a demand for alternatives and medical manufacturers have been quick to respond. Companies including Baxter Healthcare, B. Braun, Kendall/Tyco Healthcare, Smiths-Medical, and Cook Critical Care among many others provide a long list of alternative equipment that is functional and non-toxic, if slightly more expensive. Alternatives for blood transfusions are often made of polyurethane and silicone, total parenteral nutrition devices are now being made of glass and ethylene vinyl acetate, while tubing used for drug delivery can be made of silicone, polyurethane, or Non-DEHP PVC. Hemodialysis presents a unique challenge due to the ability of DEHP to stabilize and increase the shelf life of blood products, but alternatives exist for this procedure as well. Alternative hemodialysis equipment is now being made from Non-DEHP PVC and polyurethane. Currently, ECMO presents the greatest challenge for transitioning to a completely PVC/DEHP free hospital and alternatives are not readily available.

The effort to remove phthalates from medical settings is challenging, but not impossible. While labeling of medical equipment is not always clear or complete, several organizations are committed to helping hospitals tackle the transition. Many alternative devices are more expensive and require adaptation on the part of the hospital staff. However, many hospitals are beginning to realize that the threat to human health posed by phthalate containing materials ultimately is of a greater cost than the switch to alternative medical devices. Furthermore, researchers in environmental and reproductive toxicology, green chemistry, and chemical engineering are constantly working to identify

the molecular qualities that make phthalates problematic and identify ways of making better options more readily available.

Conclusion

Green health care encompasses lofty goals and significant challenges. The ideals that make up this movement gained strength in the late 20th and early 21st century and are based on the belief that healthcare professionals are inherently obligated to prevent harm through the reduction of risks in medical settings. This philosophy faces many challenges and obstacles. Perhaps the most significant barrier to success is the fact that the nature of scientific research is naturally uncertain. Toxicologists explore health effects of chemicals but are almost always limited to animal models which do not always correlate to human health consequences. The green healthcare movement embraces the intrinsic uncertainty that accompanies scientific endeavor and employs the precautionary principle in instances where experimental evidence is not entirely sufficient. In addition to this philosophical foundation, certain regulatory agencies have supported the notions of green healthcare through the release of reports and recommendations that consolidate the current scientific reports. In the case of phthalate plasticizers used in medical equipment, the consensus of scientists, advocates, and regulatory agencies is that these compounds pose a significant risk in a significant number of medical procedures. Manufacturers have responded accordingly, making a large number of alternative, non-DEHP materials available. PVC has been used in medical settings for over seventy years. As a result, transitioning away from this material is a daunting task. As scientists, advocates, industry, and public health officials continue to work together, the green healthcare philosophy gains strength and an ever increasing number of hospitals decide to take on the challenge.

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CHAPTER 4

Understanding the Transition Away from PVC and DEHP-Containing Medical Devices

Introduction

For many decades, PVC has been used widely in hospitals (1). The DEHP that makes these PVC hospital devices flexible has been shown repeatedly to leach out of the materials that contain them. A vast collection of animal research suggests that these chemicals interact with the body in a way that results in significant detrimental reproductive consequences (2, 3). Emerging epidemiological data suggests that this animal data can be corroborated in human studies and demonstrates that certain vulnerable patients are receiving physiologically relevant doses as a result of procedures that require PVC and DEHP-containing equipment (4). In recent years, this data has been discussed widely in a variety of peer-reviewed journals, popular magazines, print media, and television news stories (2-4). As doctors, patients, and advocates have increased their awareness of the risks from DEHP, several campaigns have developed to help inform hospitals and aid in their transition away from PVC and DEHP (5). These efforts have met some success (6), but the majority of hospitals are currently unaware or unmotivated to inventory their use of these materials. Thus, the purpose of this study is to identify the qualities of hospitals that have committed to reducing their use of PVC and DEHP-containing medical devices, to assess the influence of a variety of factors, and to determine what motivates hospitals to take such an important step, and what challenges they face along the way.

In order to accomplish this goal, a survey was conducted to get hospitals talking about their practices and their initiatives. We asked hospitals if they were aware of the FDA's public health advisory about DEHP in hospitals, and if so, how they responded to it (8). We asked hospitals that have made efforts to remove these risky materials about

the things that motivated these changes (8). We evaluated the devices that are the easiest to replace and the one's that are more challenging (8). Finally, we asked hospitals to talk about the challenges they faced, the reasons they've struggled to make changes, and the kind of help they think they need (8).

In answering these questions and in determining what qualities make a hospital more likely to remove PVC and DEHP-containing medical devices, we hope to improve awareness about DEHP and the availability of safer alternatives, and ultimately reduce this risk to vulnerable patients. The simple process of talking to hospitals forces even the most unaware practitioners to think about these issues and how they are being addressed. Currently, efforts to aid hospitals are broad and generally focused on only the highest ranked hospitals. Hopefully, this assessment of the qualities that define hospitals that are already taking the right steps will inform future efforts and help advocates to target the audiences in a more effective way. Additionally, allowing hospitals to talk about the approaches that motivate them and the stumbling blocks they deal with lets advocates learn from the parties they are trying to help and thereby adjust their strategies and create an atmosphere of collaboration and mutual respect.

Materials and Methods

Study Population Hospitals for one segment of this study were recruited for the study based on the 2008 US News and World Report rankings of the top 25 pediatric hospitals in the United States (10), and hospitals that have completed Health Care Without Harm's online 'NICU No Harm' survey (8). Hospital officials were contacted via phone and e-mail and asked to answer a uniform set of questions about their hospitals use of PVC and DEHP-containing medical devices. The total study population totaled 18 hospitals. Only hospitals within the United States were considered, and private practices were excluded from analysis. Ultimately, 16 hospitals were included in this study. Four hospitals included were ranked in the top 20 pediatric hospitals by the 2008 US News World Report. For a list of surveyed hospitals, see Appendix 1.

In addition, hospitals that signed Health Care Without Harm's NICU Pledge, a list of hospitals committed to reducing the use of PVC and DEHP-containing medical devices, were also evaluated for this study. These hospitals were assessed for hospital characteristics, but were not interviewed. This study population included 107 hospitals that were also limited to non-private practice, United States hospitals. For a complete lists of NICU Pledge hospitals, see Appendix 2.

Study Design and Survey

The baseline data collection was divided into two separate studies – one that evaluated the qualities of hospitals that have signed the NICU pledge, the other that evaluated the quantitative influence of hospital age, the population of the city in which the hospital is located, university affiliation on hospital awareness of the FDA advisory on DEHP-medical devices, the extent of knowledge sharing within and amongst hospital

departments, and the presence of a non-DEHP purchasing policy, as well as the motivations of hospitals that have transitioned away from DEHP-containing medical devices. This second study also qualitatively assessed the challenges these hospitals face.

For evaluation of NICU pledge hospitals, a basic data set was collected. Hospitals on this list were identified using Health Care Without Harm's publicly accessible lists, which can be found on their website (1). Location, age, population, and university affiliation were determined for each hospital. LEED certification was also considered but ultimately not included in analysis due to the fact that no hospitals on the list had achieved certification and only a very select few are currently pursuing LEED status.

Data on hospitals that participated in the survey (Appendix 2) was collected in a variety of ways. Most hospitals filled out an online survey through Health Care Without Harm's website (2). This survey asked 18 questions which included detailed information about hospital awareness of DEHP concerns, intra-hospital communication, DEHP-purchasing policies, and hospital actions including whether or not the hospital has been inventoried for PVC and DEHP-containing devices, whether any devices have been replaced, and whether or not the hospital asks manufacturers about the presence of DEHP before purchasing. 15 of the included hospitals were surveyed in this manner and data was used with permission from Health Care Without Harm. In addition, one survey was conducted via e-mail. This survey was nearly identical to the online version. For qualitative analysis, six hospitals participated in follow-up interviews via e-mail. The hospitals that participated were those that were actively implementing non-DEHP policies. Interviews asked for more information about the motivations and challenges of the transition.

Assessment of Age Hospital age was determined based on the reported foundation date. This information was acquired from hospital websites. Hospitals that are affiliated with larger parent institutions were dated based on the year of their individual founding, not on the date of foundation of the parent hospital. For analytical purposes, hospitals were grouped in to three categories: > 100 years, 50 – 99 years, and <50 years.

Assessment of Population The population of the city in which each hospital is located was determined based on data from the 2006 US Census (11). Population for hospitals located in metropolitan areas was determined based on the population of the entire metropolitan area, not just the city itself. Seven hospitals that signed the NICU pledge were located in cities that were not evaluated in the 2006 Census. 2000 US Census data was used to determine population in these cases. For analytical purposes, hospitals were grouped into three categories: > 600,000, 250,000 – 599,999, and < 250,000.

Regional Identification Hospitals were assigned to be located in one of the following regions: North East, South East, Mid West, and West. See Figure 1 for regional borders.

Definition of University Affiliation University affiliation was determined based on information gathered from individual hospitals. Hospitals that serve as teaching hospitals for medical schools or were founded as a result of a university charter were considered to have a major affiliation. Those that house biomedical researchers from neighboring universities were considered to have a limited affiliation. Hospitals that are not teaching hospitals, which do not support university researchers, and were not founded as a part of a university are considered to be unaffiliated.

Determination and Definition of Membership in Major Health Care System For the purposes of this study, ‘major health care system’ was defined as health maintenance

organization or hospital management system that oversees the operations of hospitals in more than one state. Health care systems that oversee the operations of multiple hospitals within one state were not considered major health care systems.

Statistical Analysis

Standard error was calculated using Microsoft Excel and reported in figures.

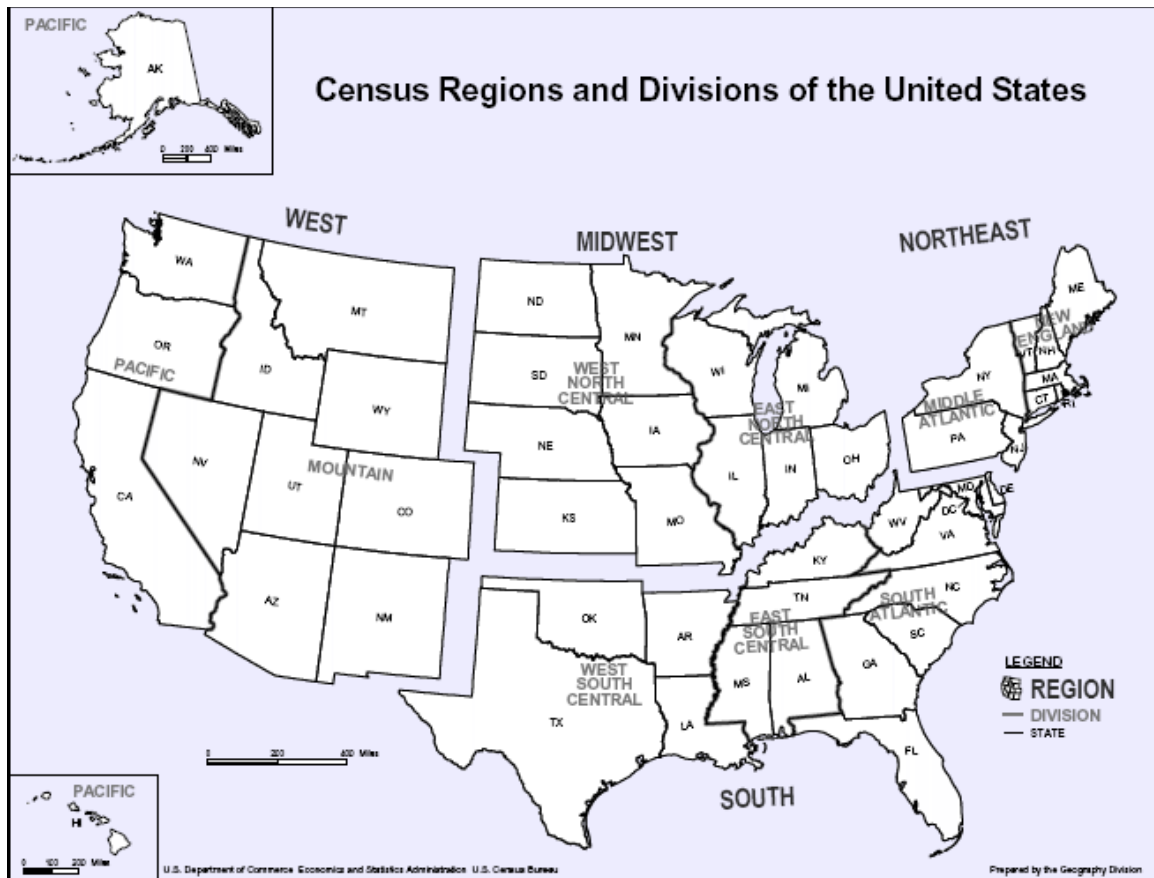


Figure 1. Regional categorization. US Census Regional demarcation was used to identify regional trends in hospital awareness and action regarding the the risks posed by PVC and DEHP-containing medical devices.

Results

Age of Hospital has a limited influence on hospital awareness of the FDA DEHP advisory, intra-hospital communication, or existence of non-DEHP purchasing policy.

To determine the influence of hospital age on a hospital's actions and initiatives surrounding the use of PVC and DEHP-containing medical devices, the percentage of hospitals in three age divisions that were aware of the FDA DEHP advisory, that had taken measures to inform other hospital departments about the concern about DEHP, that have a non-DEHP purchasing policy, and that have discussed DEHP-free initiatives with the hospital engineering department were compared. This data can be seen in Figure 2.

Of the seven hospitals that participated in the NICU No Harm survey and were 100 years old or older, 57.1% were aware of the FDA advisory. Of five survey participants that were between 50 and 99 years old, 60% were aware of the FDA advisory. Of the four survey participants that were less than 50 years old, 75% were aware of the FDA advisory. This indicates a moderate trend that indicates that younger hospitals may be more likely to be aware of the FDA advisory. However, the increase is small, as is the survey population.

Of the surveyed hospitals greater than 100 years old, 85.7% showed evidence of interdepartmental communication about DEHP-related issues. Of those between 50 and 99 years old, 40% indicated that their hospital had participated in discussion about DEHP between departments. Of the hospitals less than 50 years old, 50% demonstrated interdepartmental communication about DEHP. Again, this data does not suggest a very strong relationship between age and communication about DEHP, although it does indicate that communication in older hospitals may be more effective.

Of the surveyed hospitals greater than 100 years old, 57.1% had a non-DEHP purchasing policy. Of those between 50 and 99 years old, 60% indicated such a non-DEHP purchasing policy. Of the hospitals less than 50 years old, 25% had a non-DEHP purchasing policy. Thus, it seems that the differences between old, and moderately old hospitals is negligible, that younger hospitals are less likely to have implemented a non-DEHP purchasing policy.

Of the hospitals that have signed the NICU pledge, 18.6% are greater than 100 years old, 56.7% are between 50 and 99 years, and 24.7% are less than 50 years old (Figure 3). Overall, this data indicates the potential for some trends, but generally suggests that age has negligible impact on hospital awareness, policy, or communication.

Influence of age on awareness of FDA advisory on DEHP, interhospital knowledge sharing, and presence of non-DEHP purchasing policy

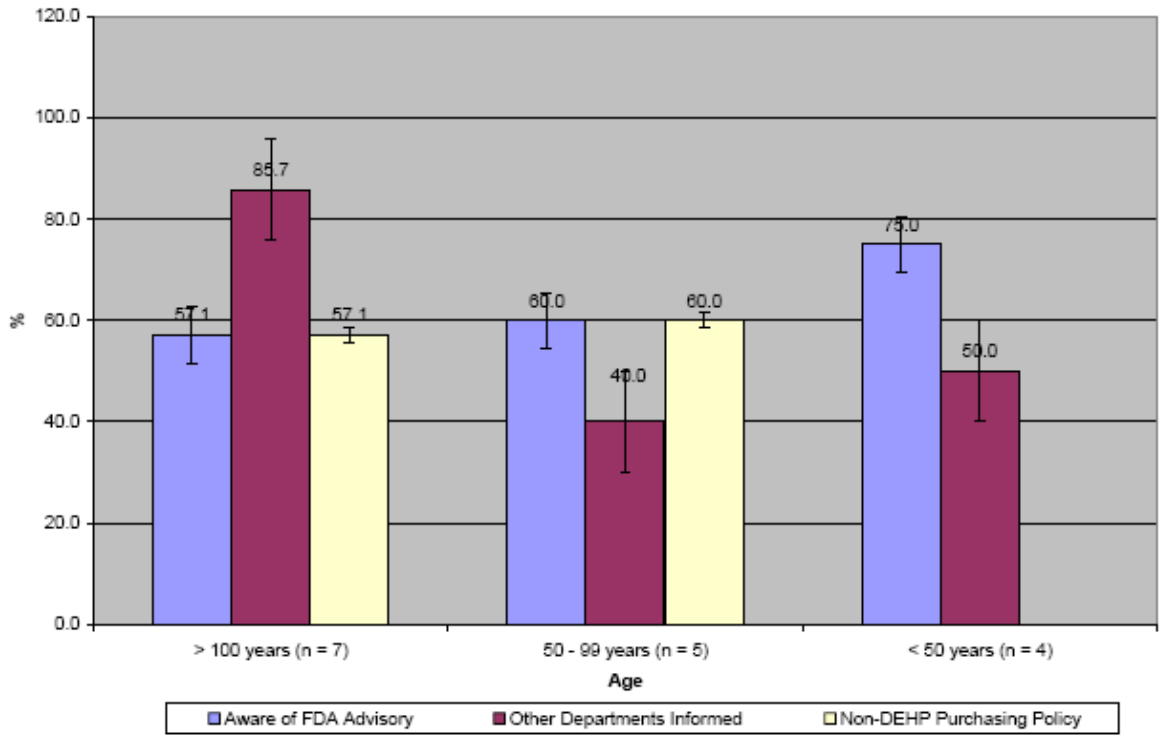


Figure 2. Age has minimal impact on awareness of FDA advisory on DEHP, inter-hospital knowledge sharing, and the presence of a non-DEHP purchasing policy. Shown is a bar graph representing the percent of hospitals in each of three age ranges that report being aware of the FDA advisory on DEHP in hospitals (blue), that report sharing information about DEHP with more than one hospital department (purple), and that have implemented a non-DEHP purchasing policy (yellow). Error bars represent standard error.

Age Distribution of NICU Pledge Hospitals
(n = 97)

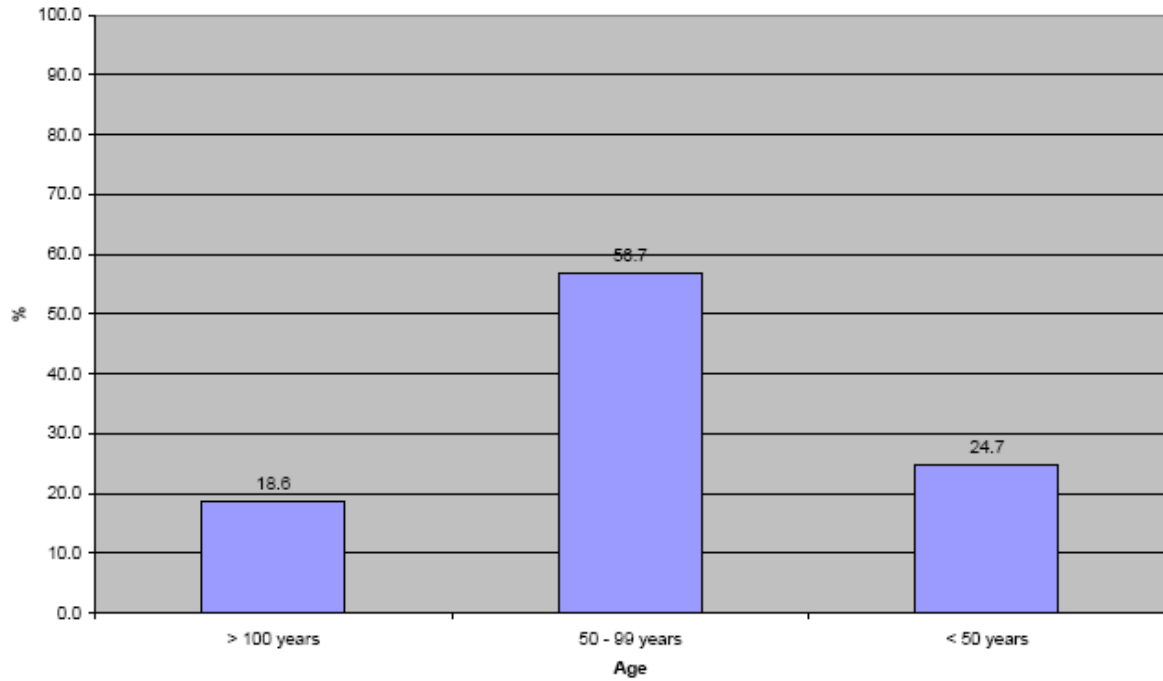


Figure 3. Hospitals that have signed the NICU pledge are predominantly between 50 and 99 years old, although no trend in age is apparent. Shown are bars representing the percentage of hospitals that have signed the NICU pledge (n = 97) that fall into each of the tree age categories.

The population of the city in which a hospital is located has some influence on its awareness of the FDA DEHP advisory, inter-hospital knowledge sharing, and the presence of a non-DEHP purchasing policy. In order to determine the influence of city population on a hospital's actions and awareness of the risks from DEHP-containing medical devices, we compared the awareness of the FDA DEHP advisory, the extent to which the hospital has discussed the risks surrounding DEHP with a variety of different hospital departments, and the presence of a non-DEHP purchasing policy in hospitals in three different population ranges. Hospitals in cities with more than 600,000 people were considered 'large,' hospitals in cities with population between 250,000 and 599,999 people were considered 'medium,' and hospitals with fewer than 250,000 people were considered 'small.'

Of the four hospitals that responded to the NICU No Harm survey and are located in large cities, 75% were aware of the FDA DEHP advisory, 75% had taken action to inform a variety of hospital departments, and 50% had non-DEHP purchasing policies. Of the seven hospitals in medium sized cities, 57.1% were aware of the FDA DEHP advisory, 85.7% had informed multiple hospital departments, and 14.3% had non-DEHP purchasing policies. Of the remaining five hospitals that we are located in cities considered small, 60% were aware of the FDA DEHP advisory, 20% had informed multiple hospital departments of the concerns surrounding DEHP, and 20% had non-DEHP purchasing policies. This data indicates that hospitals located in larger cities may be more likely to implement DEHP removal and replacement initiatives. This data can be found in Figure 4.

Amongst hospitals that signed the NICU pledge, 18.9% were located in large cities, 24.5% were located in medium cities, 56.6% were located in small cities. This data indicates an opposite trend, but may be a result of bias in the types of hospitals that were asked to sign the pledge. This data can be seen in Figure 5.

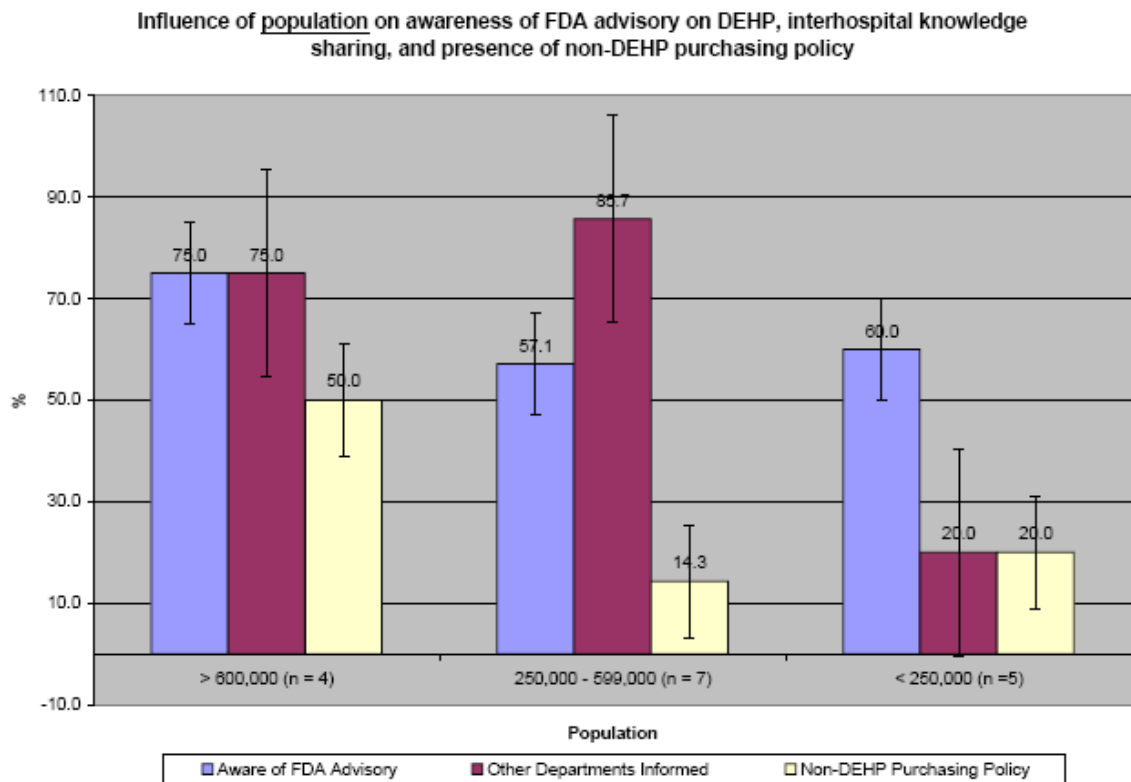


Figure 4. Population has some impact on awareness of FDA advisory on DEHP, inter-hospital knowledge sharing, and the presence of a non-DEHP purchasing policy. Shown is a bar graph representing the percent of hospitals located in cities in each of three population ranges that report being aware of the FDA advisory on DEHP in hospitals (blue), that report sharing information about DEHP with more than one hospital department (purple), and that have implemented a non-DEHP purchasing policy (yellow). Error bars represent standard error.

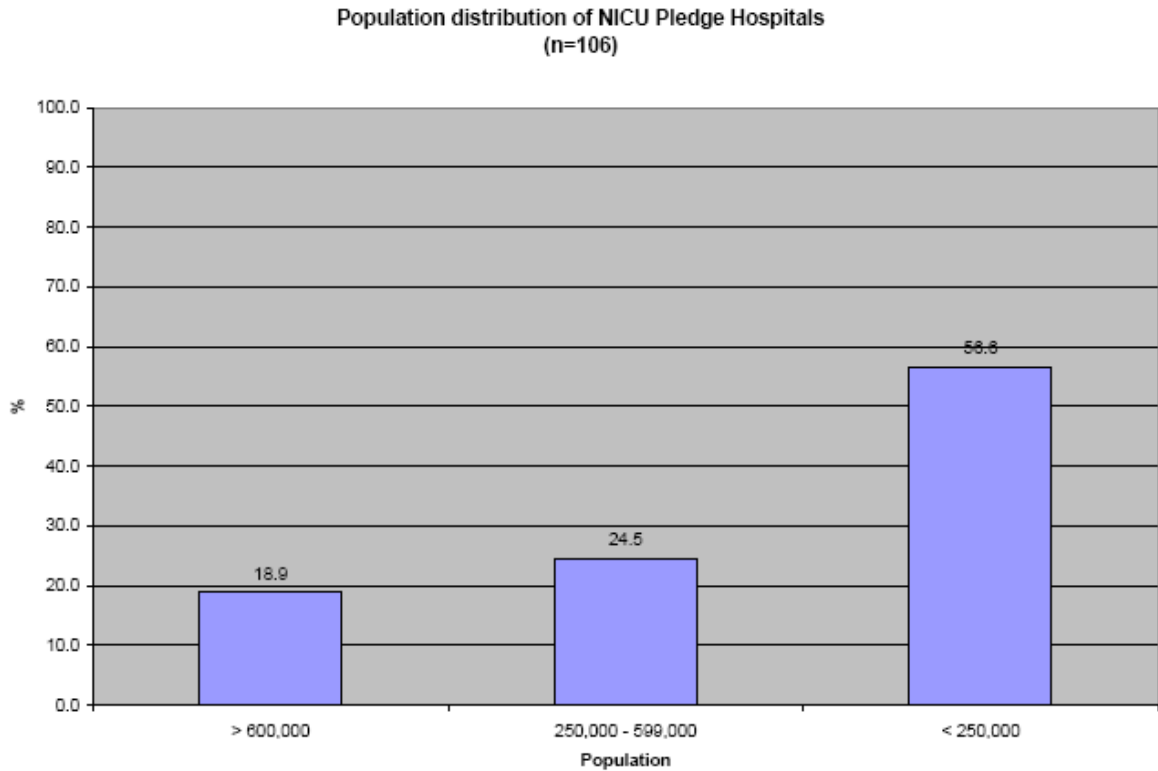


Figure 5. The majority of hospitals that have signed the NICU pledge are located in cities that have less than 250,000 people. Shown are bars representing the percentage of hospitals that have signed the NICU pledge that are located in cities of three different population ranges (n = 106). An upward trend is evident suggesting that hospitals in larger cities are less likely and hospitals in smaller cities are more likely to sign the NICU pledge.

Extent of university affiliation has minimal impact on a hospitals awareness of the FDA DEHP advisory, inter-hospital knowledge sharing, and the presence of a non-DEHP purchasing policy. Universities are frequently hot beds of cutting edge research and scientific discovery. It seems reasonable to predict that hospitals affiliated with such research institutions would be more aware of emerging research in toxicology and more likely to have engaged in initiatives to remove and replace PVC and DEHP-containing medical devices. In order to evaluate this hypothesis, hospitals were grouped in to three categories – those that have a major university affiliation, those that have a limited university affiliation, and those that have no university affiliation. Awareness of the FDA advisory on DEHP in hospitals, the extent to which DEHP awareness is hospital-wide, and the presence of a non-DEHP purchasing policy were then compared across the three groups. This data can be found in Figure 6.

Of the ten hospitals that responded to the NICU No Harm survey and have a major university affiliation, 70% were aware of the FDA advisory, 50% had communicated with multiple hospital departments about the risks of DEHP, and 20% had implemented a non-DEHP purchasing policy. Of the three hospitals with limited university affiliation, 66.7% percent were aware of the FDA advisory, 66.7% had shared knowledge about DEHP throughout the hospital, and 33.3% had a non-DEHP purchasing policy. Of the four hospitals with no university affiliation 50% were aware of the FDA advisory, 75% had discussed DEHP and its health risks with a variety of hospital departments, and 25% had implemented a non-DEHP purchasing policy. Based on this data, it seems that university affiliation plays a realitvely insignificant role in the

likelihood of a hospital to be engaged in the removal and replacement of PVC and DEHP-containing medical devices.

Amongst hospitals that signed the NICU pledge, the vast majority had no university affiliation. 14.4% had major university affiliation, 7.2% had limited affiliation, and 78.4% had no university affiliation. This data can be seen in Figure 7. Again, it appears that university affiliation does not make a hospital more likely to take action against DEHP.

Influence of university affiliation on awareness of FDA advisory on DEHP, interhospital knowledge sharing, and presence of non-DEHP purchasing policy

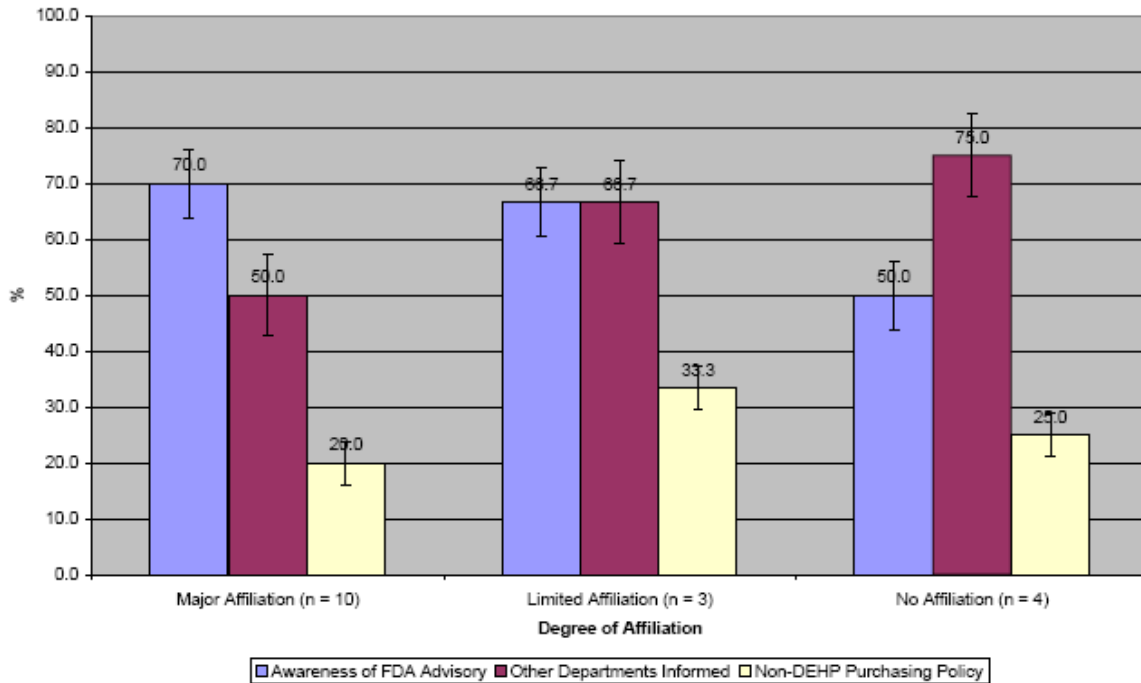


Figure 6. University affiliation has minimal impact on awareness of FDA advisory on DEHP, inter-hospital knowledge sharing, and the presence of a non-DEHP purchasing policy. Shown is a bar graph representing the percent of hospitals with major university affiliation, limited university affiliation, and no university affiliation that report being aware of the FDA advisory on DEHP in hospitals (blue), that report sharing information about DEHP with more than one hospital department (purple), and that have implemented a non-DEHP purchasing policy (yellow). Error bars represent standard error.

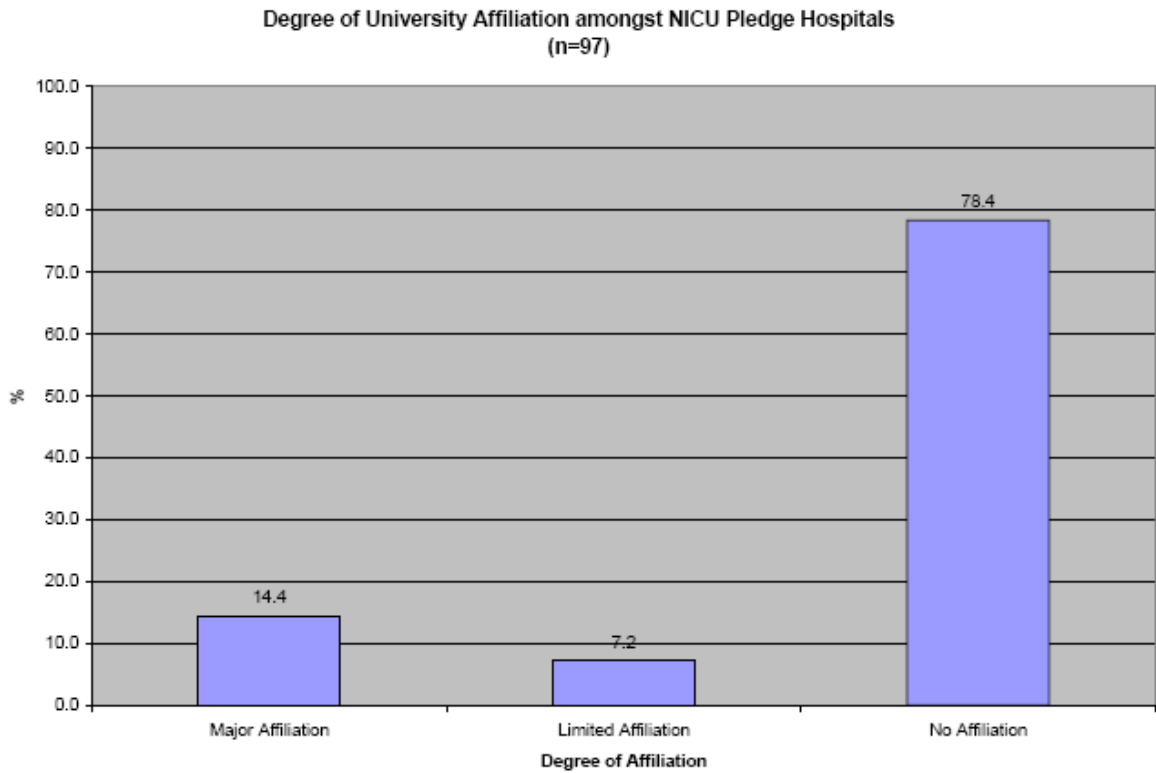


Figure 7. The vast majority of hospitals that signed the NICU pledge have no university affiliation. Shown are bars representing the percentage of hospitals that have signed the NICU pledge that have major university affiliation, limited university affiliation, or no university affiliation (n = 97).

Most hospitals that have signed the NICU Pledge are located in the western part of the United States. In order to gain an understanding of the regions of the United States that are more likely to have initiated efforts to remove and reduce the use of PVC and DEHP-containing medical devices, we evaluated the regional distribution of the hospitals that signed the NICU pledge. This information has the potential to inform future advocacy efforts and give advocacy organization an idea of regions that require more attention than others. Our data shows that 12.1% of NICU pledge hospitals are located in the Northeast, 3.7% are located in the Southeast, 3.7% are located in the Midwest, and an overwhelming 80.4% are located in the West (Figure 8).

Regional Distribution of NICU Pledge Hospitals
(n = 107)

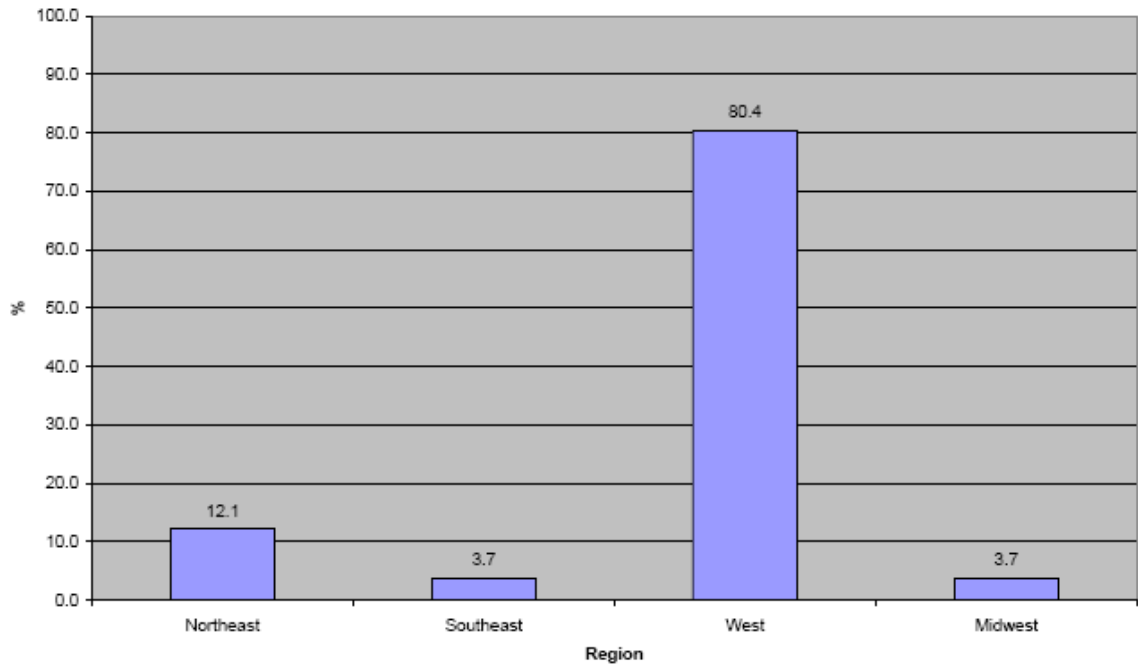


Figure 8. The majority of hospitals that have signed the NICU pledge are located in the Western United States. Shown are bars representing the regional break down of hospitals that have signed the NICU pledge (n = 107).

Membership in a major healthcare system has a strong influence on a hospital's awareness of the FDA DEHP advisory, inter-hospital knowledge sharing, and the presence of a non-DEHP purchasing policy. Previous data indicates that neither age, population, nor university affiliations have a very profound impact on a hospital's use of PVC and DEHP-containing medical devices. In the quest to determine what hospital qualities do have an effect on these hospital decisions, we evaluated the role of membership in a major, multi-state healthcare system on hospitals' awareness of the FDA advisory on DEHP in hospitals, inter-hospital knowledge sharing, and the presence of non-DEHP purchasing policy. Of the hospitals that responded to the NICU No Harm survey, four were members of major, nationwide healthcare systems. Of these four 75% were aware of the FDA advisory on DEHP in hospitals, 75% had taken measures to inform a variety of hospital departments about the issues that surround DEHP, and 25% had implemented non-DEHP purchasing policies. In contrast, of the twelve surveyed hospitals that were not members of major healthcare systems, 58.3% were aware of the FDA advisory, 58.3% had communicated the risks of DEHP throughout the hospital, and 16.7% had implemented non-DEHP purchasing policies. In all three categories, hospitals that are members of major healthcare systems had higher positive rates. This data can be seen in Figure 9. Of the hospitals that signed the NICU pledge, 59.8% were members of major multi-state health care systems. Thus, membership in a major healthcare system appears to play a major role in the likelihood of a hospital to be aware of the FDA's statement on DEHP in hospitals, to share DEHP knowledge throughout the hospital, and to implement a non-DEHP purchasing policy.

Influence of membership in a major healthcare system on awareness of FDA advisory on DEHP, interhospital knowledge sharing, and presence of non-DEHP purchasing policy

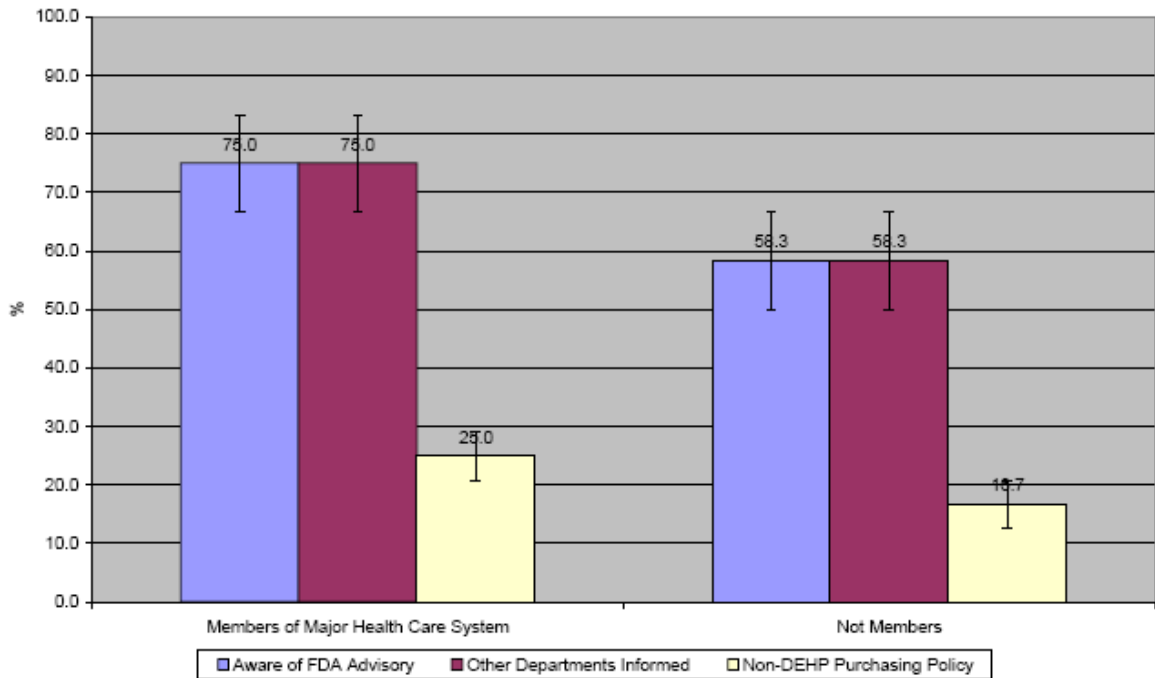


Figure 9. Membership in a major healthcare system has an important impact on awareness of the FDA advisory on DEHP in hospitals, inter-hospital knowledge sharing, and the presence of a non-DEHP purchasing policy. Shown are bars representing the percent of hospitals that are and are not members of major, multi-state health care systems that report being aware of the FDA advisory on DEHP in hospitals (blue), that report sharing information about DEHP with more than one hospital department (purple), and that have implemented a non-DEHP purchasing policy (yellow). Error bars represent standard error.

Hospitals that have transitioned away from PVC and DEHP-containing medical devices are motivated by the FDA advisory and internal awareness. In order to assess the factors that motivate hospitals to initiate efforts to remove and replace PVC and DEHP-containing medical devices we asked hospitals to select as many of four options that best describe their motivations. Hospitals were asked to choose from 1) The FDA advisory on DEHP in hospitals 2) Internal Awareness 3) External Education, and 4) Other. Of the thirteen surveyed hospitals that had made any effort to remove or reduce the use of PVC and DEHP-containing medical devices, 30.8% identified the FDA advisory as their motivation, 38.5% selected internal awareness, 15.4% selected External Education, and 15.4% identified some other source of motivation (Figure 10). Hospitals that selected ‘other’ were asked to identify these other motivators. Responses to this question included patient advocacy and efforts from advocacy organizations like Health Care Without Harm and Hospitals for Healthy Environments (H2E), a non-profit organization that gives hospitals the tools they need to create healthy medical environments (12).

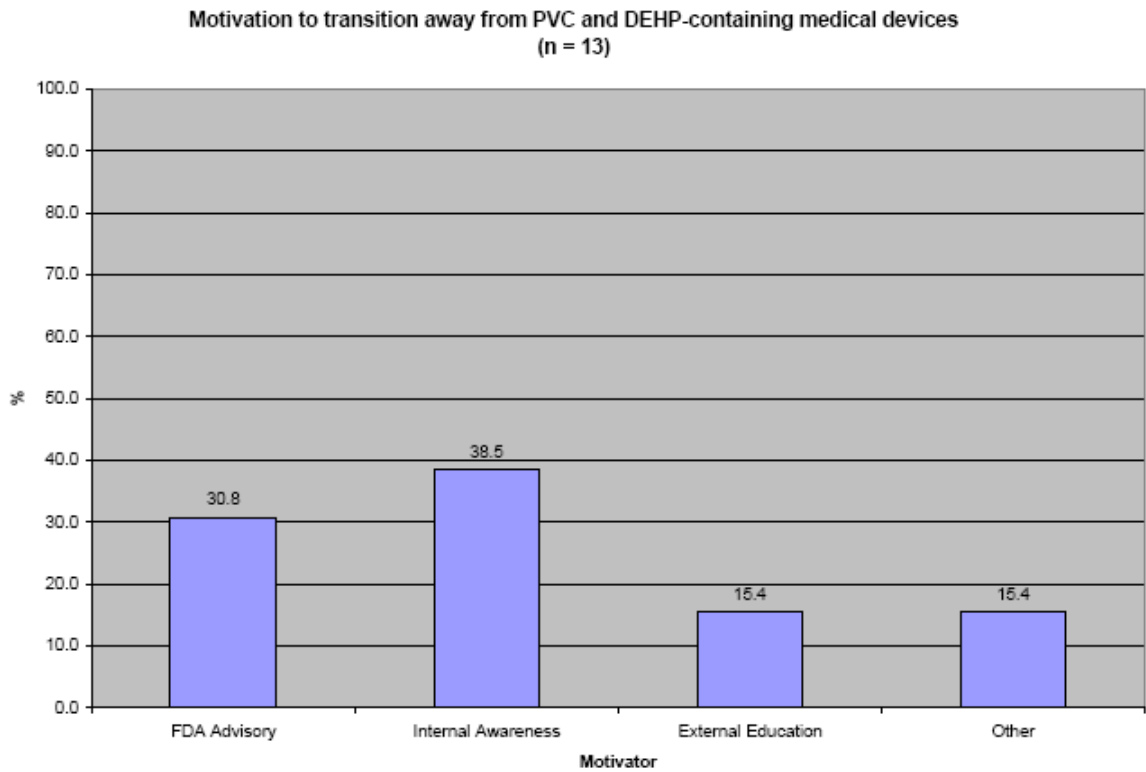


Figure 10. Hospitals that have signed the NICU pledge appear most likely to be motivated by the FDA advisory on DEHP in hospitals and internal awareness. Shown are bars representing the percentage of hospitals that report having made tangible efforts to remove and reduce the use of PVC and DEHP-containing medical devices that report being motivated to do so by the FDA advisory, internal awareness, external education, or other factors (n = 13).

Hospitals that have initiated efforts to remove and replace PVC and DEHP-containing medical devices are most challenged by cost and availability. Qualitative assessment of interview and survey data indicate that hospitals that want to replace their DEHP-containing equipment are most often prevented from doing so because of alternative materials are almost universally more expensive. Additionally, alternative materials were occasionally considered difficult to find for certain procedures including ECMO, hemodialysis, and other procedures involving blood storage. Some survey responders also reported being challenged by the complicated and occasionally incomplete material information on certain devices.

Case Study: Rhode Island Women and Infants Hospital

This study was initiated and motivated by conversations with Rhode Island Women and Infants Hospital, one of the nations leading specialty hospitals for women and newborns. While the data presented in this study comes from conversation and analysis of many different hospitals, information from Rhode Island Women and Infants hospital laid the ground work for the study and this hospital is an excellent example of a successful transition away from PVC and DEHP-containing medical devices. Rhode Island Women and Infants Hospital was founded in 1885 and thus falls into the oldest hospital age bracket in this study. It is located in Providence, RI, a relatively small city of 175,255 people. It has major University affiliation with Brown University's Warren Alpert School of Medicine and is also a member of the Care New England Health Care System. This hospital provides an excellent case study of a hospital that has successfully implemented PVC and DEHP removal initiatives.

According to the Neonatal Intensive Care Unit Nurse Manager, this transition began in February 2004 as a result of an article about phthalate exposure and health consequences for boys. After further research to become more aware of the issues surrounding PVC and DEHP, the purchasing department was contacted and manufacturers were identified that sell DEHP-free medical equipment. While these devices were significantly more expensive, the hospital faced very little opposition in implementing a transition and had no trouble acquiring the appropriate approvals. The entire transition took just two months as manufacturers and suppliers were very cooperative in the effort. The hospital did face challenges in the transition. Staff expressed some difficulty using the new, stiffer, equipment but eventually adapted to the

change. With the exception of blood products, the Rhode Island Women and Infants NICU is DEHP-free. This study of an instance in which a toxicant was successfully identified, assessed, and removed from the NICU can serve as an example for other hospitals trying to transition away from PVC and DEHP. Furthermore, as new toxicants are identified, this case study can provide a general model for management of environmental exposures in medical settings.

Discussion

Despite several widely circulated and strongly worded statements and advisories on the risks posed by exposure to DEHP in medical settings, an alarmingly high number of hospitals have not taken action to remove and reduce their use of PVC and DEHP-containing medical devices. Health care advocacy organization like Hospitals for Healthy Environments and Health Care Without Harm have made concerted efforts to increase awareness of the potential health consequences of DEHP exposure and motivate hospitals and physicians to take precautionary action against the use of PVC and DEHP-containing devices. The purpose of this study is to identify what qualities make a hospital more or less likely to be aware of these issues and to respond to them with removal and reduction initiatives. Furthermore, this study sought to identify the tactics that are effective motivators of change in hospital settings and the challenges that hospitals face when trying to implement these changes. By engaging nurses, physicians, and hospital administrators in a dialogue with researchers and advocates, this study also seeks to cultivate open lines of communication, increased knowledge sharing, and an atmosphere of collaboration and respect. Ideally, this study will raise awareness surrounding the risks of exposure to DEHP and will inform future advocacy efforts.

The collected data indicates that the age of a hospital and its university affiliation have little influence on its likelihood to be aware of the FDA public health statement on DEHP in medical devices, hospital knowledge sharing, and the presence of a non-DEHP purchasing policy. While the lack of correlation between age and DEHP-initiative is not surprising, the lack of correlation between university affiliation and DEHP awareness is. Many universities conduct research on phthalate toxicity and are doubtlessly aware of the

reproductive health concerns surrounding these compounds. It seems reasonable to assume that university-affiliated hospitals, as participators in this environment of discovery, would be more aware of emerging data and more likely to apply it within their hospital walls. Our data indicates that this is not the case, suggesting that knowledge sharing between scientific researchers and hospital employees is underdeveloped. This also suggests that there may be some disconnect in the way that scientists and medical doctors view emerging research and the ways that they motivate and implement hospital policy decisions.

While age and university affiliation were found to have a minimal impact on hospital actions regarding DEHP, the population of the city in which a hospital is located appeared to be somewhat linked to the likelihood of a hospital to be aware of the FDA public health notification, to engage in knowledge sharing, and to implement a non-DEHP purchasing policy. Hospitals located in larger cities were found to be more likely to be aware, more likely to share information, and more likely to have a non-DEHP purchasing policy than hospitals located in smaller cities. This may be due to the fact that hospitals in larger cities are usually larger hospitals that serve a larger patient base, have a larger budget, and are more likely to receive assistance from health care advocacy groups. By virtue of the city size, any hospital located in a large city will likely be visited by a larger number of people. Phthalate plasticizers have recently received significant media attention. The likelihood that a patient will read or hear about the health issues associated with phthalate exposure and advocate for change within their hospital increases with an increased patient base. Furthermore, hospitals in larger cities may also have larger budgets and therefore be more financially able to phase out PVC and DEHP-

containing medical devices. Finally, health care advocacy organizations may be more likely to reach out to hospitals in larger cities due to the perception of a greater impact. This data is compelling and suggests that hospitals in smaller cities may warrant greater advocacy attention in order to increase their awareness.

Perhaps the most striking data suggests that the factor that plays the largest role in a hospital's likelihood to be aware of the FDA's notification on DEHP in hospitals, to engage in knowledge sharing, and to implement a non-DEHP purchasing policy is hospital membership in a major health care system. Hospitals that are members of such a system were 25% more likely to be aware of the FDA notification and to inform multiple hospital departments about it, and nearly 10% more likely to have implemented a non-DEHP purchasing policy. These results reflect the fact that advocacy organizations like Hospitals for a Healthy Environment and Health Care Without Harm have effectively targeted large health care systems like Kaiser Permanente and Catholic Health Care West. This data suggests that increased advocacy outreach to smaller health care systems might also be an effective way of influencing positive change in a greater number of hospitals.

Individual interviews with hospitals willing to participate in follow up conversations highlight the importance of support from large health care systems. In many cases, departmental efforts to remove or reduce the use of PVC and DEHP-containing medical devices are initiated by nurses who are generally well versed on emerging issues and have significant interactions with patients. Unfortunately, efforts from these individuals are only limitedly successful due to a lack of institutional support. For instance, one NICU nurse reported that she considered the issues surrounding

DEHP plasticizers “interesting and important,” but reported that she “got limited buy-in from my boss.” Despite being aware of DEHP-removal efforts that took place two years prior, this nurse concluded that “this is just not something I can push any farther here until there is more institutional buy-in.” In situations such as these, support from a major health care system has the potential to help nurses and doctors achieve the goals of conducting DEHP inventories and ultimately removing and replacing PVC and DEHP-containing materials.

Future efforts can also be improved through the analysis of the challenges that hospitals face in their efforts to remove and replace PVC and DEHP-containing medical devices. Survey and interview data indicates that hospitals are most challenged by the cost of alternative equipment, the availability of products used in blood storage and delivery, and general institutional support. This suggests that future advocacy efforts should focus on identifying available alternatives for blood storage and delivery and making this information readily available. Additionally, effort needs to be focused on identifying why alternatives are more expensive and how manufacturers can make their production more cost efficient. Furthermore, hospitals identify the FDA advisory and internal awareness as the most effective means of motivating the transition away from PVC and DEHP-containing medical devices. Advocacy organizations can use this information in their outreach efforts to offer more compelling reasons why transitioning is necessary.

While this data is interesting and exciting, it is important to remember the limitations of a study so small. Potential confounders must be acknowledged and addressed. Our data indicates that hospitals that are located in larger cities may be more

likely to have implemented initiatives surrounding the removal of DEHP. This conclusion may be confounded by the locations of the hospitals that are located in larger cities, or by any of the other factors. Analysis of regional location of large hospitals reveals that only two are located in the same region, suggesting that geographic location is not a significant confounder in this case. Age may be a possible confounder, as 50% of large hospitals are older than 100 years. However, age was found to have little influence on hospital behavior towards DEHP, so this finding is not particularly concerning. Similarly, 75% of large hospitals have major university affiliations. Like age, university affiliation was found to be inconsequential. Thus, this finding does not warrant a loss of confidence in the observed correlation between population size and hospital action. Finally, an equal number of large hospitals are affiliated with major health care systems as are not. Thus, this potential confounder seems minor as well.

Our data also suggested that membership in a major health care system is a significant predictor of DEHP awareness and action. This finding could be confounded by any of the hospital characteristics we evaluated. Of the interviewed hospitals that were also members of major health care systems, all were located in the Midwest or Northeast. This indicates that regional location may be a confounder of these results. However, the population of the cities these hospitals were located ranged fairly evenly between large and small. Thus, population size is likely not a significant confounder. These hospitals were predominantly older hospitals. As previously stated, hospital age was not found to have a significant influence on hospital behavior. While age may be a confounder of these results, it is doubtful that there is a significant effect. Similarly, most of the hospitals that were members of major healthcare systems also had major university

affiliation. While university affiliation was found to have negligible consequence, it may have confounded these results.

While this study was small, the results do suggest that hospitals that are members of major health care systems are more likely to take the initiative to remove and reduce their use of PVC and DEHP-containing medical devices, and that hospitals in small cities may need more help and attention from advocacy organizations. This study is intended only to inform future outreach efforts, to improve communication between hospitals, scientists, and advocates, and ultimately to help in the mission to get PVC and DEHP out of hospitals and away from vulnerable populations. While it is by no means comprehensive it does provide a starting place for future analysis. From this study it is apparent that there is still much to be done in many hospitals, that certain advocacy strategies are working, and that others can be improved.

Appendix 1:

Surveyed Hospitals:

Shriners Hospital for Children – Philadelphia, Philadelphia, PA
Children's Healthcare of Atlanta, Atlanta, GA
Presbyterian St. Luke's Medical Center, Denver, CO
Cleveland Clinic Main Campus, Cleveland, OH
Massachusetts General Hospital, Boston, MA
McKay-Dee Hospital, Ogden, UT
John's Hopkins Hospital, Baltimore, MD
The Children's Hospital Denver, Denver CO
University of New Mexico Children's Hospital, Albuquerque, NM
Seattle Children's Hospital, Seattle, WA
University of Alabama Birmingham School of Nursing, Birmingham, AL
Christ Hospital, Oaklawn, IL
Children's Hospital, Columbus, OH
Ochsner Hospital, New Orleans, LA
University of Florida and Shands Jacksonville Hospital, Jacksonville, FL
Hackensack University Medical Center, Hackensack, NJ

Appendix 2:

NICU Pledge Hospitals

Arroyo Grand Community Hospital, CHW	Arroyo Grande, CA
Bakersfield Memorial Hospital	Bakersfield, CA
Baldwin Park Medical Center, Kaiser Permanente	Baldwin Park, CA
Barrow Neurological Institute	Phoenix, AZ
Bellflower Medical Center Kaiser Permanente	Bellflower, CA
California Hospital Medical Center	Los Angeles, CA
Catholic Healthcare West	San Francisco, CA
Chandler Regional Hospital	Chandler, AZ
Community Hospital of San Bernardino	San Bernardino, CA
Dana-Farber Cancer Institute	Boston, MA
Fontana Medical Center, Kaiser Permanente	Fontana, CA
Fremont medical Center, Kaiser Permanente	Fremont, CA
French Hospital Medical Center,	San Luis Obispo, CA
Fresno Medical Center, Kaiser Permanente	Fresno, CA
Glendale Memorial Hospital	Glendale, CA
Hayward medical Center, Kaiser Permanente	Hayward, CA
Kaiser Permanente	Oakland, CA
Los Angeles Medical Center, Kaiser Permanente	Los Angeles, CA
Manteca Medical Center, Kaiser Permanente	Manteca, CA
Marian Medical Center	Santa Maria, CA
Mark Twain St. Joseph's Hospital	San Andreas, CA
Mercy General Hospital	Sacramento, CA
Mercy Gilbert Medical Center	Gilbert, AZ
Mercy Hospital of Folsom	Folsom, CA
Mercy Medical Center Redding	Redding, CA
Mercy Medical Center, Merced Community Campus	Merced, CA
Mercy Medical Center, Mt. Shasta	Mt. Shasta CA
Mercy San Juan Medical Center	Carmichael, CA
Methodist Hospital of Sacramento	Sacramento, CA
Moanalua Medical Center, Kaiser Permanente	Honolulu, HI
Northridge Hospital Medical Center	Northridge, CA
Oakland Medical Center, Kaiser Permanente	Oakland, CA
Orange Coast Medical Center, Kaiser Permanente	Fountain Valley, CA
Providence St. Vincent Medical Center, Kaiser Permanente	Portland, OR
Redwood City Medical Center, Kaiser Permanente	Redwood City, CA
Richmond Medical Center, Kaiser Permanente	Richmond, CA
Riverside Medical Center, Kaiser Permanente	Riverside, CA
Roseville Medical Center, Kaiser Permanente	Roseville, CA
Sacramento Medical Center, Kaiser Permanente	Sacramento, CA
Saint Bernadine Medical Center	San Bernardino, CA
Saint Elizabeth Community Hospital	Red Bluff, CA

Saint Francis Memorial Hospital	San Francisco, CA
Saint John's Pleasant Valley Hospital	Camarillo, CA
saint John's Regional Medical Center	Oxnard, CA
Saint Joseph Medical Center	Tacoma, WA
Saint Joseph's Behavioral Health Center	Stockton, CA
Saint Joseph's Hospital and Medical Center	Phoenix, AZ
Saint Josphe's Medical Center	Stockton, CA
Saint Mary Medical Center	Long Beach, CA
Saint Rose Dominican Hospitals	Henderson, NV
San Diego Medical Center, Kaiser Permanente	San Diego, CA
San Gabriel Valley Medical Center	San Gabriel, CA
San Raphael Medical Center, Kaiser Permanente	San Rafael, CA
Santa Rosa Medical Center, Kaiser Permanente	Santa Rosa, CA
Santa Teresa Medical Center, Kaiser Permanente	San Jose, CA
Sequoia Hospital	Redwood, CA
Sierra Nevada Memorial Hospital	Grass Valley, CA
South Bay Medical Center, Kaiser Permanente	Sacramento, CA
	South San Francisco,
	CA
South San Francisco Medical Center, Kaiser Permanente	Clackamas, OR
Sunnyside Medical Center, Kaiser Permanente	Vallejo, CA
Vallejo Medical Center, Kaiser Permanente	Walnut Creek, CA
Walnut Creek Medical Center, Kaiser Permanente	Los Angeles, CA
West Los Angeles Medical Center, Kaiser Permanente	Woodland, CA
Woodland Healthcare	Woodland Hills, CA
Woodland Hills Medical Center, Kaiser Permanente	Abington, PA
Abington Memorial Hospital	Philadelphia, PA
Albert Einstein Medical Center	Berkeley, CA
Alta Bates Summit Medical Center	<i>St. Louis, MO</i>
<i>Amerinet, Inc.</i>	Boston, MA
Brigham and Women's Hospital	Irving, TX
Broadlane	Bryn Mawr , PA
Bryn Mawr Hospital	Seattle, WA
Children's Hospital and Regional Medical Center	Oakland, CA
Children's Hospital and Research Center at Oakland	<i>Chicago, IL</i>
<i>Consorta</i>	Santa Cruz, CA
Dominican Hospital	Doylestown, PA
Doylestown Hospital	Kirkland, WA
Evergreen Hospital Medical Center/Children's Services	Philadelphia, PA
Frankford Hospitals	Seattle, WA
Group Health Cooperative, Special Care Nursery	Philadelphia, PA
Hahnemann University Hospital	Philadelphia, PA
Hospital of the University of Pennsylvania	Walnut Creek, CA
John Muir Medical Center	Portland, OR
Legacy Emanuel Children's Hospital	Palo Alto, CA
Lucile Packard Children's Hospital	Pittsburgh, PA
Magee-Women's Hospital, University of Pittsburgh	

Medical Center	Alpharetta, GA
MedAssets	Bakersfield, CA
Mercy Hospitals of Bakersfield	Grand Rapids, MI
Metro Health	Long Beach, CA
Miller Children's Hospital	Dallas, TX
Novation	Oakdale, CA
Oak Valley District Hospital	Portland, OR
Oregon Health and Science University	Panorama City, CA
Panorama City Medical Center	Paoli, PA
Paoli Memorial Hospital	Philadelphia, PA
Pennsylvania Hospital	Charlotte, NC
Premier Inc	Eugene, OR
Sacred Heart Medical Center, NICU	Lakewood, WA
Saint Clare Hospital	Federal Way, WA
Saint Francis Hospital	Ann Arbor, MI
Saint Joseph Mercy Health System	San Francisco, CA
Saint Mary's Medical Center	San Francisco, CA
San Francisco Medical Center	Santa Clara, CA
Santa Clara Medical Center	Portland, OR
Shriners Hospital for Children	Philadelphia, PA
The Children's Hospital of Philadelphia	Voorhees, NJ
Virtua Health	

Appendix 3:

NICU No Harm Survey –

1. Contact Information

2. Are you aware of the Food and Drug Administration (FDA) DEHP Public Health Notification? Or are you aware of any ongoing research that addresses concerns with chemicals and plastics in hospital or medical activities?

* Are you aware of any advocacy organizations that have asked hospitals to phase out certain chemicals and plastics?

3. Have you taken measures to inform hospital departments?

4. If yes, please explain:

5. Do you ask vendors if their products contain: DEHP? PVC?

6. Do you have a purchasing policy that prefers non-DEHP and/or non-PVC products?

7. Have you inventoried use of DEHP and PVC products in:

- NICU
- PICU
- Ob/Gyn
- Maternal and Child Health
- Pediatrics
- Labor and Delivery
- Full Term Nursery
- Hospital Wide
- Other (please specify)

8. Have you eliminated the purchase of DEHP containing:

- IV Administration Sets?
- ECMO Tubing?
- Blood Bags?

9. Have you eliminated the purchase of PVC containing:

- IV Bags
- IV tubing
- IV administration sets
- TPN delivery
- enteral feeding sets?
- umbilical vessel catheters?
- examination gloves?

feeding tubes, including nasogastric tubes?
ECMO tubing?
Peritoneal dialysis bags and tubing?
hemodialysis tubing?
irrigation & drainage products?
urinary catheters?
vascular catheters?

10. If possible, list the manufacturers of your PVC/DEHP free products:

11. Some hospitals are switching to non-PVC products for other uses beyond medical devices. Are you aware of such efforts within your hospital to purchase PVC-free:
carpets?
flooring?
wall coverings?
wall guards?
shower curtains?
furniture/upholstery?
window treatments?

12. Have you made Engineering/Facilities or your Construction/Renovation/Design team aware of concerns around PVC and DEHP-containing products?

* If you have transitioned away from PVC and DEHP:
a. Did any outside organizations facilitate this effort?
b. How long did the transition take?

13. Why did your facility move away from DEHP?
FDA advisory
Internal Awareness
External Education
Other (please specify)

14. For which products have you had difficulty purchasing DEHP or PVC-free alternatives? (please list):

15. What were the challenges you encountered?

16. Are you interested in receiving assistance in DEHP/PVC elimination? To learn more about these issues, visit www.noharm.org.

17. If yes, who should HCWH contact? Please provide name, title, telephone number and email address.

18. Is your hospital engaged in any other environmental/sustainability initiatives that you would like us to know about?

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CHAPTER 5

Conclusions, Future Directions, and Recommendations

Part I: Biological Responses to Phthalate Exposure

Introduction

The work presented in this thesis contributes to the understanding of the role of gene-environment interactions in germ cell survival and apoptosis in the rodent testis. Specifically, the described results indicate a role for the PI3K/Akt and NF- κ B signaling pathways in a germ cell survival response following MEHP-induced postnatal testicular injury. We have found that Akt1 kinase activity increases in the testes of Akt1-wild type mice following exposure to MEHP, and that loss of Akt1 results in the premature onset of germ cell apoptosis. We measured markers of NF- κ B activation and demonstrated an Akt1-dependent increase in I- κ B α phosphorylation at 1 hour and 3 hours after MEHP exposure. Interestingly expression of both p50 and p65 NF- κ B subunits is elevated basally in Akt1-deficient mice suggesting that Akt1 may play a role in the regulation of p50 and p65 transcriptional regulation. Collectively, this data indicates an Akt1-dependent promotion of NF- κ B transactivation potential in Akt1-wild type mice that ultimately results in a transient protective response following MEHP-induced postnatal testicular injury.

In our studies, we have observed a base line trend for increased germ cell apoptosis in the postnatal testes of Akt1-deficient mice. We hypothesize that this higher basal level of germ cell apoptosis may lead to the activation of pathways associated with oxidative damage and/or inflammation. Therefore we have conducted preliminary gene array analyses to examine differentially expressed genes in the postnatal testis. We compared Akt1 wild type mice with Akt1-deficient mouse testes at 3 hours post MEHP exposure (500mg/Kg). Table 1 illustrates the differences found to date. Although there

are approximately 70 genes listed in Table 1, relatively few of the genes are significantly changed by over 2-fold. Therefore we examined one gene identified in our analyses which was been previously implicated is apoptotic signaling in the testis, SMAC/DIABLO.

In the gene expression analyses, SMAC/DIABLO was found to be decreased in Akt1-deficient mouse testes relative to wild type mouse testes exposed to MEHP. Diablo is implicated in apoptotic signaling pathways, and this result was initially counterintuitive. One would expect elevated levels in Akt1-deficient mice. However, examination at various time points by RT-PCR demonstrated that DIABLO is significantly elevated by 5-fold basally in the testes of Akt1-deficient mice (Figure 1). The data suggests that there is increased mitochondrial stress in the testes of mice deficient for Akt1. At 6 hours, a 1.5 –fold increase is observed in wild type mice exposed to the MEHP which may suggest a role for mitochondrial injury following MEHP exposure. Experiments to address this possibility are ongoing.

Table 1. Differential gene expression in Akt1 wild type versus Akt1-deficient mouse testes 3 hours post MEHP exposure.

Differentially Expressed Genes (r^2)

locusID	symbol	WT.tr	KO.tr	Diff	P1	P2
243302	LOC243302	1.62	-1.41	-3.03	0.000151	0.000529
15430	Hoxd10	-0.349	0.0373	0.386	0.000183	9.68e-05
73473	Iwsl	0.188	-0.0394	-0.227	0.00129	0.00133
18430	Oxtr	-0.896	-0.00996	0.886	0.00135	0.000381
66593	Diablo	0.342	-0.144	-0.486	0.00141	0.00288
52150	Kcnk6	0.84	0.238	-0.602	0.00151	2.57e-05
270166	Clpx	0.532	-0.185	-0.717	0.00166	0.00283
71891	Cdadc1	-0.523	0.076	0.599	0.00221	0.00175
28109	D10Wsu102e	0.528	0.0603	-0.468	0.00227	0.000292
22695	Zfp36	0.748	0.115	-0.632	0.00238	0.000208
116940	Ncoa6ip	0.573	-0.0235	-0.596	0.00272	0.00122
28001	D16Wsu65e	0.389	-0.13	-0.519	0.00282	0.00468
73625	1810008I18Rik	-0.208	-0.0138	0.195	0.00351	0.000755
319584	9330107J05Rik	0.434	-0.0927	-0.526	0.00403	0.00454
216850	Jmjd3	0.494	0.0407	-0.453	0.00403	0.000778
235293	Sc5d	-0.175	0.0514	0.227	0.00427	0.00637
68067	3010026O09Rik	0.251	-0.0623	-0.314	0.00431	0.00554
27359	Sytl4	0.364	-0.115	-0.479	0.00434	0.00691
67420	Mlstd2	-0.222	0.0429	0.265	0.00459	0.00482
12153	Bmp1	-0.4	0.00799	0.408	0.00463	0.00197
227325	Dner	-0.362	0.00865	0.37	0.00466	0.00203
434778	6330534C20Rik	0.288	-0.042	-0.33	0.00514	0.00445
229681	St7l	-0.245	0.0739	0.319	0.00521	0.00804
16796	Lasp1	-0.208	0.0137	0.221	0.00535	0.00308
67298	Gprasp1	0.389	-0.0108	-0.4	0.0056	0.00259
69274	Ctdspl	0.485	-0.0807	-0.566	0.00591	0.00568
20874	Slk	0.156	-0.00868	-0.165	0.00593	0.00327
	NA	-0.478	0.0173	0.496	0.00663	0.00332
328099	AU021838	-0.135	0.0236	0.159	0.00669	0.00674
192136	AF397014	0.435	0.0403	-0.395	0.00669	0.00134
70568	Cpne3	-0.549	0.113	0.662	0.00674	0.00771
74386	Rmi1	0.126	-0.0258	-0.152	0.007	0.00795

230810	Slc30a2	0.368	0.09	-0.278	0.00742	0.000341
106877	AI173486	-0.118	0.0202	0.139	0.00817	0.00824
18718	Pip5k2a	0.293	-0.00017	-0.293	0.00824	0.00345
30046	Zfp292	0.625	-0.0852	-0.71	0.00852	0.00747
242687	Wasf2	0.563	0.0245	-0.538	0.00905	0.00286
19933	Rpl21	0.51	-1.44	-1.95	4.51e-05	7.34e-05
27993	Imp4	0.0443	0.492	0.447	9.05e-05	9.31e-06
574530	LOC574530	0.612	-1.12	-1.73	0.000167	0.00044
224024	Scarf2	-0.589	0.719	1.31	0.00051	0.00171
66952	2310030G06Rik	-0.762	1.05	1.81	0.000564	0.00179
77087	Ankrd11	-0.529	1.23	1.76	0.000634	0.00132
319211	Nol4	-0.359	0.767	1.13	0.00069	0.00156
59069	Tpm3	-0.117	0.656	0.773	0.000925	0.000807
72762	2810436B12Rik	-0.134	0.377	0.511	0.000956	0.00164
	NA	2.42	-2.86	-5.28	0.00119	0.00391
191578	Hel308	0.00951	0.512	0.502	0.00124	0.000327
73512	1700085D22Rik	-0.131	0.18	0.311	0.0013	0.00403
66830	Btbd14b	-0.0159	-0.339	-0.323	0.00152	0.000329
13609	Edg1	0.0311	-0.209	-0.24	0.00164	0.00129
108097	Prkab2	0.0394	0.435	0.396	0.00239	0.000387
14961	H2-Ab1	-0.115	0.198	0.313	0.00249	0.00666
17311	Kitl	-0.0341	0.716	0.75	0.00279	0.00131
212514	Ccdc52	-0.0197	0.185	0.205	0.00306	0.00205
	NA	-0.0175	0.427	0.445	0.00315	0.00144
93686	Rbm9	-0.00441	0.23	0.234	0.00356	0.00144
72147	Btbd4	-0.0771	0.24	0.317	0.00376	0.00607
244329	McpH1	-0.0958	0.326	0.422	0.00415	0.0062
22017	Tpmt	-0.0377	-0.363	-0.325	0.00441	0.000725
328977	Zfp532	0.229	-0.449	-0.678	0.00481	0.0115
320398	Lrig3	0.321	-0.81	-1.13	0.00525	0.0103
16797	Lat	-0.0487	0.304	0.353	0.00542	0.00502
70974	Pgm211	0.163	-0.523	-0.687	0.0063	0.0101
230098	E130306D19Rik	-0.0907	0.262	0.352	0.00639	0.0112
236930	Erc61	-0.0126	0.323	0.336	0.00695	0.00357
12464	Cct4	-0.0951	0.433	0.528	0.00759	0.00918
407817	BC059050	-0.0543	0.364	0.418	0.00774	0.0071
78807	4930544L04Rik	-0.248	0.73	0.978	0.00786	0.0136
546201	LOC546201	-0.0114	0.364	0.375	0.0087	0.00444

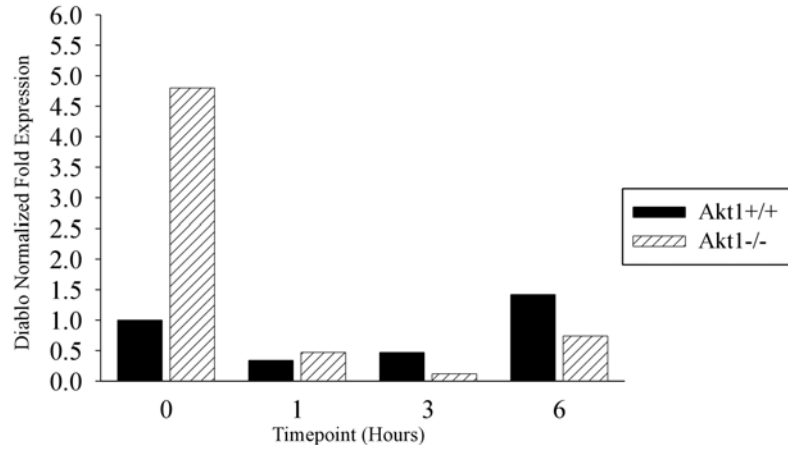


Figure 1. Basal Expression of Diablo is Elevated in Akt1-Deficient Mouse Testes.

Does Akt1 suppress Oxidative Damage and/or Inflammation following postnatal testicular injury?

Based on our gene expression analysis, we can now ask questions about the role of Akt1 in oxidative damage and inflammation following postnatal testicular injury. Our data indicates that Akt1-deficient control mice have elevated levels of germ cell apoptosis compared to Akt1-wild type mice. While our evidence does suggest an important role for Akt and NF-kB in maintaining germ cell homeostasis following MEHP insult, it is also possible that oxidative damage and inflammation contribute to the increased sensitivity to MEHP in Akt1-deficient mice. Biological systems maintain a delicate balance between the production of reactive oxygen species and the ability to detoxify the reactive intermediates and repair the resulting cellular damage (11). The maintenance of this balance is critical and is thought by some to be the most important method of protection from cell death in biological systems (13). Thus, the exploration of oxidative stress and the role that it plays in germ cell death is a relevant future direction for this project.

While reactive oxygen species play an important role in cell signaling pathways, disruption of the balance between reactive oxygen species and detoxification can have significant negative consequences and is termed oxidative stress (12). One consequence of oxidative stress is chronic inflammation and occurs when free radicals are synthesized in quantities that overwhelm the biological systems ability to neutralize and eliminate them (13). Reactive oxygen species and their intermediates have been shown to activate nuclear factors like NF-kB and cause the production of proinflammatory cytokines (13). These cytokines enhance inflammation and generate more reactive oxygen species (13). This response can be overcome by antioxidant defenses, however if these mechanisms

are overwhelmed inflamed cells activate a series of caspases that cause apoptosis (13). The testis is particularly vulnerable to oxidative damage and as a result has relatively high concentrations of antioxidants (14). The ability of the testis to use antioxidants to defend itself against oxidative damage is evidenced by studies that show a correlation between ascorbic acid and Vitamin E deficiencies and disruption of spermatogenesis (15, 16). The vulnerability of the testis to oxidative damage, the relationship between NF- κ B and reactive oxygen species, the germ cell death that results from Akt1 deficiency, and the necessity for antioxidant defenses systems makes oxidative damage and inflammation interesting areas to pursue in an attempt to more adequately delineate the interplay between Akt1 and NF- κ B, and their functional roles in MEHP-induced germ cell apoptosis.

These further studies can be accomplished in a variety of ways. Measurement of cytokine levels, reactive oxygen species, and other inflammatory cells may help determine the degree of inflammation in Akt1-deficient mice, while analysis of antioxidants and caspases would give an indication of the cellular response to oxidative stress and inflammation. Interleukin-6 (IL-6) is an important cytokine associated with the inflammation due to the activation of T cells, differentiation of B Cells (17), and induction of the acute phase response (18). The binding of IL-6 to its receptor (IL-6R or gp80) leads to the dimerization of gp130 and IL6-R and activates signal transduction pathways that lead to a variety of responses including cell growth arrest and apoptosis (19). Cytokines like IL-6 have been found to stimulate the production of reactive oxygen species and elevated NF- κ B activation. Thus, elevated expression of IL-6 at the transcriptional and translational level in Akt1-deficient mice might suggest that elevated

levels of apoptosis in these mice are causing an inflammatory response that may be responsible for increased NF-kB expression prior to MEHP exposure.

Researchers from Japan have measured antioxidant concentrations in rat testes as a means of elucidating apoptotic mechanisms in rat germ cells exposed to DEHP. The techniques they employ may be useful in furthering the understanding the role of Akt in mouse germ cell apoptosis. This study measured concentrations of total glutathione, low molecular mass thiols, and ascorbic acid in the testis and found that rats exposed to DEHP had reduced levels of all three (14). They hypothesize that this decrease in antioxidants may contribute to apoptosis because biological systems rely on antioxidants to defend themselves from reactive oxygen species (14). Similarly, decreased levels of critical antioxidants in Akt1-deficient mice might suggest that Akt deficiency deprives these mice of the necessary antioxidants to counteract damage from reactive oxygen species.

It is well established that oxidative stress is a significant inducer of germ cell apoptosis. It is also commonly known that oxidative stress can be a direct result of cytokine production and inflammation, and that this process has the potential to upregulate the NF-kB signaling pathway. Finally, antioxidants are a known defense against these insults. Thus, it is important and necessary to further the previously discussed work by evaluating the extent of inflammation and oxidative damage as well as antioxidant production in Akt1-deficient mice. This area of study has the potential to explain the observed elevation of NF-kB in Akt1-deficient control mice, despite elevated levels of germ cell apoptosis.

Is there a Preemptive Stress Response in the postnatal testis following injury ?

RT-PCR and Western Blot analysis indicates that Akt1-deficient control mice have elevated levels of NF-kB subunits p50 and p65 compared to Akt1-wild type controls. NF-kB is known to be a protective signaling pathway (1) and thus, it is counter-intuitive that Akt1-deficient control mice have increased NF-kB activation and increased germ cell apoptosis compared to Akt1-wild type control mice. One possible explanation for this observation involves a preemptive stress response to the elevated levels of germ cell apoptosis. . In short, it is possible that Akt-deficient control mice are recruiting protective pathways prior to injury from MEHP because they are trying to compensate for their Akt1-deficient state. The work presented in this thesis supports this hypothesis, but there are still many unanswered questions. Evaluation of basal levels of traditional stress responses in both Akt1-deficient and Akt1-wild type mice would help elucidate this idea.

Transcription of heat shock genes is a common stress response that helps facilitate cellular recovery by limiting the damage caused by stress-induced injury and maintaining cellular homeostasis (2). Evaluation of the expression of genes in this family in Akt1-wild type mice compared to Akt1-deficient mice might help determine the extent to which preemptive stress response is playing a role in MEHP-induced apoptosis in Akt1-deficient mice. Hsp90 is particularly interesting because it interacts with and maintains the activation of Akt by preventing its dephosphorylation (10). Hsp90 promotes cell survival primarily through the stabilization of Receptor Interacting Protein (RIP), a protein required for sustained NF-kB activity (2). Hsp90 also acts with Cdc37 to aid in the formation of IKK and Akt complexes and has the ability to cause NF-kB subunits to

disassociate from their inhibitor (3). Hsp90 works with Akt to indirectly promote cell survival by the phosphorylation and inactivation of ASK-1 which inhibits JNK-mediated cell death (2). Additionally, Hsp90 has the ability to prevent the formation of active apoptosome complexes by preventing the oligomerization of Apaf-1(4) and the subsequent activation of initiator caspases 9 and executioner caspases 3,6, and 7 (9). Thus, Hsp90 makes a compelling target for the future study of stress responses and elevated NF-kB activity in Akt1-deficient control mice. If mRNA and protein levels are elevated in Akt1-deficient mice compared to Akt1-wild type mice, it might be concluded that Akt1-deficient mice activate the NF-kB pathway prior to exogenous injury as a part of a larger stress response to their genetic instability.

While base line NF-kB transcription is elevated in Akt1-deficient mice compared to Akt1-wild type mice, our data shows that these levels do not increase following exposure to MEHP despite an increase in apoptotic germ cells. This suggests that toxicant exposure overwhelms the Akt1-deficient mouse's defense mechanisms leaving it vulnerable to massive cell death. This theory could be evaluated by measuring levels of stress-inducible proteins like Hsp90 and others following MEHP exposure. Heat shock protein 70 (Hsp70) would be an interesting protein to evaluate as it is primarily anti-apoptotic and has been found to mediate susceptibility to apoptosis following injury from exogenous stimuli (5). Researchers have previously shown that cell lines that over-express Hsp70 are less susceptible to apoptosis following exposure to heat treatments, while those that have x-ray induced Hsp70 impairment had greater induction of apoptosis following the same heat treatment (5). Significant work from several different research teams has shown that Hsp70 is an inducible stress responder (6-7) and that this protein

facilitates cellular survival (6). Exploration of the expression of this protein may shed light on the behavior of traditional stress responders in genetically unstable organisms following toxicant insult. Furthermore, it may help increase the understanding of the role of Akt in genetic susceptibility to toxicant exposure.

Activation of NF- κ B is widely considered a protective response and has been correlated to decreases in apoptosis in rat germ cells insulted with MEHP (8). However, in our study we observed increased levels of NF- κ B activation in the testes of Akt1-deficient mice relative to Akt1-wild type testes, despite a significant increase in apoptotic germ cells. We also observed no change in the levels of NF- κ B constituents following MEHP exposure despite steadily increasing levels of germ cell apoptosis. These observations are unexpected and worthy of further investigation in order to completely characterize the role of Akt in MEHP induced testicular injury. Hsp90 is linked to both the PI3K/Akt and NF- κ B signaling pathways and is known to facilitate cellular survival during times of stress. Hsp70 is similarly considered a classic stress responder. These two proteins, as well as others from the heat shock gene family, have the potential to clarify the role of genetic susceptibility in testicular injury as well as elucidate the role of Akt in MEHP-induced toxicity.

In conclusion, the cell signaling pathways and stress responses that maintain the critical balance between apoptosis and cell survival in the testis are intricate, complex, and overlapping. Our data shows that Akt1-deficient mice are more susceptible to germ cell apoptosis in both the presence and absence of postnatal toxicant-induced injury. We hypothesize that there is crosstalk between Akt1 and NF- κ B signaling pathways in the testis and that this crosstalk facilitates germ cell survival in the MEHP-exposed testis.

Part II: Medical Responses to Phthalate Exposure

Introduction

The evaluation of the ways that hospitals have and have not responded to emerging information about the risks posed by the use of PVC and DEHP-containing medical devices reveals important information about the level of awareness surrounding these issues and the efficacy of existing advocacy and outreach efforts. Our study demonstrated that many hospitals are not aware of the concerns about PVC and DEHP and that most are still buying and using these materials. Furthermore, we observed a trend in the likelihood of a hospital to be aware of the FDA public health statement about DEHP in medical devices, to engage in knowledge sharing about this information, and to have implemented a non-DEHP purchasing policy that suggests that hospitals that are members of large, multi-state health care systems are more likely to have done these things. These results can inform future efforts to increase awareness and action on these issues. Based on the conducted surveys, it is possible to identify the steps that are most effective in the effort to remove and reduce the use of PVC and DEHP-containing medical devices. The information hospitals have shared about the challenges they face in these efforts allows advocates to more effectively tailor their aid and to provide information that is useful. However, it is important that this study be vastly expanded in the future, and that the data is applied to education and advocacy efforts. Finally, this study and the conclusions that can be made based on it warrant several recommendations for action.

Recommendations

The effort to transition away from PVC and DEHP-containing medical devices is an understandably intimidating task. Through discussion with hospitals that have taken on this challenge, it is possible to identify an effective strategy that can be implemented by hospitals interested in making this transition. Once a hospital is aware of the risks surrounding PVC and DEHP-containing medical equipment and has made a commitment to remove and reduce their use of these products, most begin by conducting a PVC/DEHP audit in order to identify the products and materials that need to be replaced (28). This process typically requires cooperation with the hospital purchasing department, which can provide a complete list of all devices and equipment purchased for hospital use. This list is then reviewed with an emphasis on identifying items that are made of PVC or contain DEHP.

Next, the purchasing department or nurse manager typically contacts their preferred manufacturers to identify available alternative materials. Because these alternative materials are often more costly, it may be necessary to form a committee within the hospital to conduct regular reviews of hospital use of PVC and DEHP-containing devices and to assert the importance of investing in alternative materials. These committees are typically made up of nurse managers, environmental health and safety professionals, purchasing department employees, as well as doctors and nurses. This committee must both prioritize the materials that are used in procedures with the most susceptible patient populations. Eventually, this committee can also develop and implement a non-PVC/DEHP-free purchasing policy as well as a PVC/DEHP disclosure policy.

Hospitals that participate in a group purchasing organization (GPO) can take further action. These hospitals should contact their GPO and first request information about non-PVC/DEHP-free materials that are available. They should also include language in their GPO contracts that specifies a preference for products and materials that are DEHP-free. In order to facilitate full disclosure of the presence of PVC and DEHP in materials, hospitals should also ask their GPO to identify products that contain (or don't contain) these materials. Survey data indicates that the challenges that hospitals face in their efforts to transition away from PVC and DEHP are significant but not unique. The two primary challenges are the identification of alternative materials and the ability to garner the institutional support necessary to commit to such an undertaking. The recommended steps identified above address the challenge of availability as they provide a proven mechanism to identify available replacement options. Furthermore, the development of a hospital committee and the involvement of the GPO has the potential to help advocates within the hospital find larger institutional support.

In addition to these recommendations for hospitals, the described study generates recommendations for advocacy groups as well. Our survey data indicated that hospitals that are members of large health care systems are more likely to make initiatives to remove and reduce their use of PVC and DEHP-containing materials. Furthermore, evaluation of NICU pledge hospitals indicated that the vast majority of hospitals that have signed the pledge are members of such organizations, namely Kaiser Permanente and Catholic Health Care West. This suggests that previous advocacy work has targeted these systems and has met success. Based on this success, efforts to increase awareness in other health care systems seem warranted. Furthermore, this success indicates that these

efforts might be effectively expanded to include health care systems that operate multiple hospitals within single states. It is thusly recommended that advocacy organizations move away from tedious and often ineffectual efforts to target individual hospitals in favor of a strategy that focuses on health care systems that oversee more than one hospital.

Finally, this study indicates that there is limited knowledge sharing between medical professionals and researchers and within hospital departments. Thus, it is recommended that educational efforts be increased in order to improve awareness. This might be achieved by the implementation of joint seminar series with medical professionals and researchers in the same city. Additionally, increased efforts in improving environmental literacy amongst researchers, doctors, nurses, and medical school staff has the potential to build a skill set that allows these people to think about the overlaps between scientific research and public health (29). As medical professionals are busy people and do not always have time to follow the progression of emerging scientific research, it is also recommended that a brochure be printed and distributed to inform hospital boards of directors, doctors, nurses, and hospital administrators of the most striking research and to equip them with the information, strategies, and tools to initiate a transition away from PVC and DEHP-containing medical devices. A design for a brochure that would achieve these goals can be found in Appendix 1.

Future Directions

This study has the potential to grow in many directions. In order to improve the quantitative power of the study, it must be expanded. Ideally, all hospitals in the US News and World Report's Top 25 Pediatric Hospitals would be contacted and interviewed on their practices regarding PVC and DEHP. In addition, it would be helpful to interview additional hospitals in the same cities as those on the Top 25 list in order to facilitate a comparison. The conclusions reached regarding membership in a major health care system are interesting and useful, but need to be further explored. In order to fully understand this trend, an analysis of the ways health care systems influence policies within individual hospitals is necessary. The described study identifies some of the things that motivate individual hospitals to transition away from PVC and DEHP, but says nothing of the motivations of major health care systems. To achieve the maximum potential impact of advocacy efforts, these things must be identified. Furthermore, the challenges that face individual hospitals are likely different from the challenges that face major health care systems. If the challenges that face major health care systems could be identified public health advocacy organizations might be able to find ways of overcoming stumbling blocks and helping a greater number of health care systems to implement initiatives to remove and reduce the use of PVC and DEHP-containing medical devices.

Conclusion

This evaluation of hospital practices concerning PVC and DEHP is exciting and informative, but barely scratches the surface of many larger issues. From the numerous conversations with nurses, managers, doctors, and environmental health and safety officers it becomes abundantly clear that much has been done and that there is much left to do. Many of these conversations were encouraging. The interviewees at hospitals that were engaging in efforts to find alternatives to these toxic materials were informed, aware, and passionate about removing this risk to hospital patients. Some of the interviewees had never heard of DEHP or the concerns surrounding PVC. However, these people were often happy to learn about a new public health concern and excited at the prospect of being able to make a positive impact. Overall, this study demonstrated that our medical community is working hard to remove risks. That being said, this study also highlighted the failing of medical and research communities to collaborate on these issues. Many hospitals directly affiliated with Universities that produce peer reviewed publications on phthalate toxicity reported that they were not even aware of the concerns about phthalates. This indicates a tangible disconnect between those doing research and those applying it in medical settings. Ultimately, the study needs to be expanded, the results applied to future advocacy efforts, and perhaps most importantly, education about environmental exposures must be emphasized.

Appendix 1:

Stories of Success

Rhode Island Women and Infants Hospital:

Rhode Island Women and Infants Hospital, one of the nation's leading specialty hospitals for women and newborns, was founded in 1888, is the primary teaching hospital for Brown University's Warren Alpert School of Medicine and is a member of the Care New England Health Care System. This hospital provides an excellent case study of a hospital that has successfully implemented PVC and DEHP removal initiatives.

According to the Neonatal Intensive Care Unit Nurse Manager, this transition began in February 2004 as a result of an article about phthalate exposure and health consequences for boys. After further research to become more aware of the issues surrounding PVC and DEHP, the purchasing department was contacted and manufacturers that sell DEHP-free medical equipment were identified.

While these devices were more expensive, the hospital faced very little opposition in instigating a transition and had no trouble acquiring the appropriate approvals.

The entire transition took just two months as manufacturers and suppliers were very cooperative in the effort. The hospital did face challenges in the transition. Staff expressed some difficulty using the new, stiffer, equipment but quickly adapted to the change. With the exception of blood products, the Rhode Island Women and Infants NICU is now DEHP-free.

Removing Phthalates: A Health Care Imperative

Understanding the transition away from PVC medical devices containing DEHP




What We Know About Phthalate Toxicity

Di-(2-ethylhexyl) Phthalate (DEHP) is a plasticizer used to make polyvinyl chloride (PVC) soft and flexible. A primary metabolite of this compound, Mono-(2-ethylhexyl) Phthalate (MEHP) is a known toxicant of the male reproductive system.

Animal Studies

- Studies in rats show that repeated oral exposure to high doses of DEHP causes lethality in 21-day old animals, but not in adults (Richburg and Boelkeheide, 1996). This indicates that age influences susceptibility to DEHP.
- In utero administration of phthalates to male rats during critical window of development resulted in malformations of the reproductive tract (Foster, FM 2006).

Epidemiological Studies

- Researchers at Harvard University's School of Public Health report that increasing concentrations of MEHP were correlated to an increasing degree of sperm DNA damage (Hauser R, et al. 2007)
- Male infants of mothers with higher phthalate metabolite concentrations were found to be more likely to have a lower than average anogenital distance which is considered a marker of feminization (Marsee K, et al. 2006).



What the FDA says about PVC and DEHP

For certain high risk procedures including exchange transfusion, total parenteral nutrition, ECMO, hemodialysis, enteral nutrition, heart transplantation, and massive blood transfusions, PVC devices that do not contain DEHP or devices made of other materials (such as ethylene vinyl acetate (EVA), silicone, polyethylene or polyurethane) should be used, if available.

The FDA recommends considering the use of alternatives especially with male neonates, pregnant women who are carrying male fetuses, and peripubertal males.

What the American Medical Association says about PVC and DEHP

In 2001, the AMA passed a resolution strongly urging all hospitals to phase out their use of PVC products containing DEHP in Neonatal Intensive Care Units.

Furthermore, this resolution calls upon health professionals to encourage the institutions with which they are associated to adopt purchasing policies that will lead to the exclusive use of non-DEHP medical devices in Neonatal Intensive Care Units.

What the National Toxicology Program says about PVC and DEHP

In 2006, the NTP released a monograph on the potential human reproductive and developmental effects of DEHP. This assessment concluded that most people are exposed to DEHP and that this exposure has probable adverse effect for reproduction. Importantly, they find that medical treatments of male infants with PVC devices warrant a serious concern for the adverse development of the male reproductive tract. They also concluded that high DEHP exposures of fetuses and infants can occur when breast-feeding women undergo medical procedures involving PVC medical devices.

Steps for Removal

1. Perform a system-wide audit to determine the presence of PVC and DEHP-containing medical devices.
2. Form a committee to regularly review the use of PVC and DEHP-containing medical devices and advocate for a transition away from these materials.
3. Replace any products that contain PVC or DEHP. Prioritize those materials used with high risk patient populations.
4. Implement a non-DEHP purchasing policy.
5. Require PVC/DEHP disclosure on all products.
6. Talk to your group purchasing organization (GPO) about products that don't contain PVC or DEHP and include language in your contracts that specifies a preference for DEHP-free products.

Alternative Materials

- Ethylene vinyl Acetate	- Non-DEHP PVC
- Polyolefin	- Nylon
- Polypropylene	- Teflon
- Polyethylene	- Nitrile
- Glass	- Ethylene propylene
- Polyurethane	- Non-latex rubber
- Silicone	- Fluoropolymer

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