Gene Expression Changes from Exposure to Phthalates in Testicular Cells

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ABSTRACT

Phthalates are industrial plasticizers with a wide range of applications. Di-(2-ethylhexyl) phthalate (DEHP) is one of the most highly produced and frequently studied phthalates. Its metabolite, mono-(2-ethylhexyl) phthalate (MEHP) is known as a testicular toxicant. The objective of this study was to examine expression of the genes of interest in testicular germ cells exposed to MEHP in a dose- and time-dependent manner at concentrations of 1μM, 10μM, and 100μM at 24, 48, 72 and 96hr time points**.** The genes consisted of Testisin, GSPT1, and MGMT genes which are a tumor suppressors, phase II xenobiotic metabolizing enzyme and DNA repair gene respectively. These genes were analyzed by Quantitative Real Time PCR (RT-PCR). The results revealed an overall down-regulation for each gene as the concentration and/or time increased. Testisin was the focus of the gene expression analysis. Testisin is epigenetically silenced in testicular germ cell tumors (TGCT) by DNA methylation at the 5'CpG island of the gene. To investigate if MEHP is capable of DNA hypermethylation, a co-exposure with 5-azacytidine (demethylating agent) was conducted. Compared with the 5-azacytidine treatment alone, there was a significant down-regulation of the Testisin gene in the co-exposure. This suggests that MEHP may down-regulate Testisin gene expression by DNA methylation. These findings provide evidence that MEHP can alter the expression of Testisin, GSTP1 and MGMT, genes that are associated in the risk of developing testicular germ cell tumors. In addition, results indicated that MEHP may cause DNA methylation leading to the down-regulation/silencing of genes such as Testisin.

Introduction 1
Male Reproductive System 1
Phthalates as Testicular Toxicants 4
Metabolism of Phthalates in Humans 5
Toxicity of Phthalates in Animals and Humans 7
Phthalates Toxicity in Adult Human Males 9
Testicular Cancer 11
Genes Associated with Testicular Cancer 13
GSTP1 13
MGMT 14
Testisin 15
Objective and Rationale 16
Materials and Methods 17
Chemicals and Solutions 17
Cell Culture 18
Exposure 18
Cell Viability Assessment (MTT Assay) 19
Gene Expression Analysis (Quantitative RT-PCR) 19
Statistical Analysis 21
Results 21
MTT Cell ViabilityAssay 21
Gene Expression in Ntera-2 cells Exposed to MEHP 23
Restoration of Testisin Expressionwith 5-azacytidine and MEHP. 23
Discussion 30
Cytotoxicity 30
Gene Expression 30
Testisin 30
GSTP1 33
MGMT 37
Conclusion 40
References 41
Appendix 58

List of Tables

Table 1: Quantitative Real-Time PCR Primers……..…….....…20

List of Figures

Figure 1: The cytoxicity of MEHP assessed by MTT cell viability assay…….....22

Figure 2A: Testisin mRNA expression post MEHP exposure (Dose dependent).....24

Figure 2B: GSTP1 mRNA expression post MEHP exposure (Dose dependent).....24

Figure 2C: MGMT mRNA expression post MEHP exposure (Dose dependent).....24

Figure 2D: Testisin mRNA expression post MEHP exposure (Time dependent).....25

Figure 2E: GSTP1 mRNA expression post MEHP exposure (Time dependent).....25

Figure 2F: MGMT mRNA expression post MEHP exposure (Time dependent).....25

Figure 3A: Overall representation of Testisin gene expression post MEHP exposure.....26

Figure 3B: Overall representation of GSTP1 gene expression post MEHP exposure.....27

Figure 3C: Overall representation of MGMT gene expression post MEHP exposure.....28

Figure 4: Testisin mRNA expression in NT2 cells exposed to MEHP and/or 5-azacytidine…….……...……...……...29

List of Abbreviations

5-aza	- 5-azacytidine
ARE	-Antioxidant response element
AP1	- Activating protein-1
AR	- Androgen receptor
BTB	- Blood testis barrier
CIS	- Carcinoma in situ
CREB-1	- cAMP response element binding proteins-1
DBP	- Dibutyl phthalate
DEHP	- Di-(2-ethylhexyl) phthalate
DEP	- Diethyl phthalate
DHT	- 5- α-dihydrotestosterone
DiDP	- Di-isodecyl phthalate
DiNP	- Di-isononyl phthalate
DMP	- Dimethyl phthalate
FSH	- Follicle stimulating hormone
GCLC	- Glutathione cysteine ligase catalytic subunit
GCLM	- Glutathione cysteine ligase modifier subunit
GnRH	- Gonadotrophin releasing hormone
GPX2	- GSH-dependent antioxidant enzymes, glutathioneperoxidase 2
GSH	- Glutathione
GST	- Glutathione S-transferase
GSTP1	- Glutathione S-transferase pi
HPRT1	- Hypoxanthine phosphoribosyltransferase 1
INSL3	- Insulin like 3
ITGCN	- Intra-tubular germ cell neoplasia

Keap1 - Kelch-like ECH-associated protein 1

LH - Luteinizing hormone

MBP - Mono-*n*-butyl phthalate

MCMHP /2cx-MMHP - Mono(2-carboxymethylhexyl) phthalate

MECPP/ 5cx-MEPP - Mono(2-ethyl-5-carboxypentyl) phthalate

MEHHP/5OH-MEHP - Mono(2-ethyl-5-hydroxyhexyl) phthalate

MEHP - Mono(2-ethylhexyl) phthalate

MEOHP/5oxo-MEHP - Mono(2-ethyl-5- oxohexyl) phthalate

MEP - Mono-ethyl phthalate

MGMT - O^6-methylguanine-DNA methyltransferase

MMP - Mono-methyl phthalate

MTT - (3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide)

NF-kB - Nuclear factor kB

Nrf2-ARE - Nrf2-Antioxidant response element

NT2 - Ntera-2/cl.D1, a human embryonic cell line (embryonal carcinoma)

PGC - Primordial germ cells

PKA - Protein kinase A

ROS - Reactive oxygen species

S.E.M - Standard Error of Mean

SHBG - Sex hormone binding globulin

TBP - Tata box binding protein

TDS - Testicular genesis syndrome

TGCT - Testicular germ cell tumors

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INTRODUCTION

Male Reproductive System

The male reproductive system comprises of a number of reproductive organs including the penis, scrotum, testis, prostate, seminal vesicles, vas deferens, and ejaculatory ducts (Fütterer, Heijmink, & Spermon, 2008). The main function of the reproductive system is to produce sperm cells (Spermatozoa) with the coordination and contribution by a number of reproductive organs. The testis is made of anouter fibrous membrane called the tunica albuginea and consists of seminifeous tubules lined with germ cells and somatic cells (Sertoli and Leydig cells) (Fütterer et al., 2008; Seli, Mahutte, & Arici, 2002). Spermatozoa are produced in the seminiferous tubules by a process known as spermatogenesis which then migrate to the epididymis where they mature (Sikka, 2001; F. C. W. Wu, 1996). From ejaculation and into the female reproductive tract, the spermatozoa travels from the epididymis to the vas deferens and is released through the urethra. The ejaculate consist of fluids from the vas deferens, the prostate gland,the seminal vesicles, and the Cowper's gland, which forms semen (Bronson, 2011). The production of healthy spermatozoa maybe the most important exocrine function of the male reproductive system; however, this process relies on the endocrine function in the testis (F. C. W. Wu, 1996)

The function of the testis is twofold, the endocrine and exocrine function. Both these functions are linked and work in conjunction to regulate the production of male hormones and to influence the development of the male secondary sexual characteristics and expression of male sexuality through the production of sperm through the actions of the Hypthalamic-Pituitary-Gonadal Axis. This process consists of a complex negative feedback system that involves

hormones secreted from the hypothalamus, the anterior pituitary gland, and the testis (Mostafa et al., 2007; F. C. W. Wu, 1996). From the hypothalamus, gonadotrophin releasing hormone (GnRH) is released into the portal blood circulation and triggers the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from gonadotrophs in the anterior pituitary gland (F. C. W. Wu, 1996). Within the testis, LH acts on the Leydig cells stimulating the production of the principle androgenic hormone, testosterone (Seli et al., 2002). Testosterone, as well as its metabolites, 5- α-dihydrotestosterone (DHT) among others, have significant influences on the male developmental and physiological processes. They act on a wide range of biological processes and mediate their effects by binding to specific androgen receptors (AR) inducing transcriptional gene expression of androgen responsive genes in target cells (F. C. W. Wu, 1996). This results in sexual maturation during puberty, development of sexual phenotype and function, and maintenance of spermatogenesis (Luccio-Camelo & Prins, 2011). Testosterone also engages in a negative feedback response to the pituitary gland and hypothalamus to regulate secretion of GnRH and LH (Heemers & Tindall, 2007). In addition, inhibin produced by Sertoli cells signals a feedback to the anterior pituitary gland to regulate the secretion of FSH (Bronson, 2011). Like the Leydig cells, Sertoli cells have a role in endocrine regulation and they also support spermatogenesis.

Germ cell development relies greatly on the structure and function of the Sertoli cells. The tight morphological association between the Sertoli cells and germ cells provide comprehensive interactions and communications between these cells to influence cell function. This action occurs at various stages of spermatogenesis by coordinating many biochemical and molecular events (Cheng & Mruk, 2002). Sertoli cells have several functions such as nourishing germ cells, creating the blood testis barrier, and aiding in germ cell movement. Each of these

functions is essential for germ cell development and spermatogenesis (Mruk & Cheng, 2004). For instance, Sertoli cells provide nutrients, and secrete a variety of proteins that are involved in repair, maintenance, growth, differentiation and development. These proteins include proteases and protease inhibitors, hormones and growth factors, and basement membrane proteins (McGuinness & Griswold, 1994). In addition, the blood testis barrier (BTB) is a physical barrier that separates the blood vessels from the seminiferous epithilium and protects the germ cells from harmful substances by restricting the pathway of small molecules (Mruk, Silvestrini, & Cheng, 2008). The BTB consists of three main functions: establishing a suitable environment to support normal germ cell development, regulating nutrients, waste, and other molecules both into and out of the seminiferous epithelium, and lastly, it provides an immunological barrier by preventing the germ cells from being recognized and attacked by the host's own immune system (Mruk & Cheng, 2004). Sertoli cells also take part in translocating the germ cells across the seminiferous epithelium releasing the mature spermatids during spermatogenesis (Cheng & Mruk, 2002; Mruk & Cheng, 2004). Therefore, the structure and function of the Sertoli cells is vital for spermatogenesis and normal male reproductive development.

It is evident that the male reproductive system is a sophisticated network of cellular events that coordinate and regulate the interaction and signaling of a wide range of molecular functions. By understanding the endogenous dynamic processes that occur during male reproductive development and functioning, the effects of exogenous chemicals on the system can be studied and characterized.

Phthalates as Testicular Toxicants

Phthalates have a wide range of industrial and commercial applications and are primarily used as industrial plasticizers (Se & Byung, 2005). Phthalates are used as plasticizers in the production of polyvinyl chloride (PVC) products. The addition of phthalates to plastic provides flexibility to products such as medical devices, consumer products, and vinyl flooring (Schettler et al., 2006). Di-(2-ethylhexyl) phthalate (DEHP), di-isodecyl phthalate (DiDP), and di-isononyl phthalate (DiNP) are some of the most highly produced and widely used phthalates and are commonly used in commerce. Low molecular weight phthalates such as diethyl phthalate (DEP), dimethyl phthalate (DMP), and dibutyl phthalate (DBP) are used to manufacture personal care products, such as cosmetics, lotions, perfumes, and shampoos (Frederiksen et al., 2007). Phthalates are not covalently bound to the plastic matrix and thus can leach out from the products overtime, migrating out into the environment (Lyche et al., 2009). Ecological and human contamination by phthalates occurs from their ubiquitous presence in the environment.

In recent years the use of phthalates in trade, commerce and personal care products is on the increase leading to a concomitant increase in environmental and human contamination. The primary routes of exposure to phthalates include pathways such as ingestion, inhalation, and skin absorption with major human exposure occurring through inhalation and ingestion (Hauser & Calafat, 2005). There are multiple sources of phthalates for environmental contamination and there are multiple routes of exposure, therefore, it is difficult to make an estimate of the direct exposure to humans (Frederiksen et al., 2007)

Metabolism of Phthalates in Humans

The metabolism of phthalates involves hydrolysis through phase I metabolizing enzymes followed by conjugation through phase II metabolizing enzymes. During the hydrolysis phase the diester phthalates (eg. DEHP) are hydrolyzed into their primary hydrolytic monoesters (eg. MEHP), which occurs in the gut, liver and blood (Frederiksen et al., 2007; Rusyn et al., 2006). Low molecular weight phthalates are rapidly metabolized and excreted in urine and feces as monoesters. However, high molecular weight phthalates are complex and are initially metabolized into their monoester forms before undergoing further biotransformation which includes oxidation (ω,ω-n, α or β oxidation) and hydroxylation (Frederiksen et al., 2007; Silva et al., 2006; Silva et al., 2007; Silva et al., 2006; Silva et al., 2007). Monoesters and oxidative products can either be excreted changed or unchanged in the urine or feces or they can proceed to phase II conjugation. This phase produces glucuronide conjugates which are more water soluble and can be easily eliminated from the body via urinary excretion (Hauser et al., 2007)

The toxicology of phthalates including DEHP has been most studied and it has been reported that DEHP is metabolized rapidly in biological systems. Phthalates are primarily oxidized into metabolites and the oxidative metabolism of phthalates in humans has received the most attention (Hauser et al., 2007). The metabolism of such a high molecular weight compound produces many metabolites which have been identified and characterized. Monoester mono-(2-ethylhexyl) phthalate (MEHP) is the primary metabolite of DEHP and can be detected in urine, serum, breast milk, saliva, ovarian follicular fluid, seminal plasma, and amniotic fluid (Hauser et al., 2006; Silva et al., 2006). The metabolism of DEHP also yields four major secondary oxidative metabolites which includes mono-(2-ethyl-5-hydroxyhexyl) phthalate (5OH-MEHP or

MEHHP), mono-(2-ethyl-5-oxohexyl) phthalate (5oxo-MEHPor MEOHP), mono-(2-ethyl-5-carboxypentyl) phthalate (MECPP or 5cx-MEPP) and mono-(2-carboxymethylhexyl) phthalate (2cx-MMHP or MCMHP) (Frederiksen et al., 2007; Koch et al., 2004; Silva et al., 2006). Measuring urinary concentration of phthalate metabolites is the most common biomonitoring method in assessing human exposure to phthalates. Biomonitoring studies on DEHP exposure assessment have been mostly dependent on the urinary concentrations of MEHP. However, it has been reported that three oxidative metabolites of DEHP (MEHHP, MEOHP and MECPP) have higher urinary levels than MEHP indicating that these metabolites are more potent biomarkers than MEHP for determining the exposure to DEHP. Furthermore, the sum of all four secondary metabolite of DEHP represents about 70% of the DEHP excreted, whereas only 6% was excreted as MEHP (Hauser & Calafat, 2005; Lyche et al., 2009; Wittassek et al., 2011).The difference in urinary concentration between MEHP and the hydrolytic monoesters of other diesters of phthalates may indicate an alternative metabolic pathway that form the oxidative metabolites are more susceptible to urinary excretion than MEHP (Hauser & Calafat, 2005)

Monoester, mono-(2-ethylhexyl) phthalate has been associated with reproductive adverse health effects in humans, more specifically it is recognized as a testicular toxicant (Gray & Gangolli, 1986). A correlation between urinary levels of MEHP and secondary metabolites has been linked to DNA sperm damage. It was found that the higher the ratio of MEHP/MEHHP or MEHP/MEOHP the more DNA sperm damage occurred. This indicated that metabolism of MEHP into secondary metabolites protects against DNA sperm damage (Frederiksen et al., 2007). Nonetheless, MEHP has been implicated in male reproductive abnormalities and is known as a testicular toxicant (Lambrot et al., 2009; Sharpe, 2001)

Toxicity of Phthalates in Animals and Humans

Scientific literature describes a number of adverse health effects from exposure to phthalates and the scope and range of the type of adverse effects from exposure to different metabolites of phthalates differing widely from one another (Kamrin, 2009). Chronic phthalate toxicity is believed to induce hepatocarcinogenesis and also cancer of thyroid and the testis. However, these adverse effects show significant differences among species as well as between genders (Heudorf et al., 2007). With regards to hepatocarcinogenesis, certain phthalates such as di-(2-ethylhexyl) phthalate (DEHP) and di-n-butyl phthalate (DBP) along with their primary metabolites exert their toxic effects in rodents through the activation of peroxisome proliferator-activated receptor (PPAR) alpha-receptor (Lapinskas et al., 2005). However, the adverse effect associated PPAR pathway does not occur in humans and thus, hepatocarcinogenesis caused by DEHP is irrelevant to humans. Overall, DEHP is not classifiable as carcinogenic in humans (IARC, 2000).

Among the other observed adverse health effects of phthalates, reproductive and developmental toxicity have received the most attention. It has been well reported that exposure to elevated levels of phthalates and its metabolites in male laboratory animals have the potential to interfere with normal reproduction and development (Heudorf et al., 2007). Pubertal rats and rats exposed *in utero* are more sensitive to phthalates compared to adult rats (Hauser & Calafat, 2005). Recent findings have provided evidence that the developing testis is more vulnerable to phthalates than adult testis (Li, Jester, & Ortho, 2000). A number of studies have reported that *in utero* exposure of rat fetus to phthalates can disrupt Leydig cell function and reduce testosterone production (Habert et al., 2009). The reduction of testosterone levels is believed to be caused by down-regulation of the genes involved in cholesterol metabolism and steroidogenesis

(Howdeshell et al., 2007; Lyche et al., 2009). In addition, phthalates are also found to decrease INSL3 mRNA expression which produces the proteins necessary for testis descent (Wilson et al., 2004). The adverse health effects of phthalates have been shown to produce symptoms that are comparable to the human testicular genesis syndrome (TDS) which include cryptorchidism, hypospadias, retention of nipples, epididymal agenesis, and decreased anogenital distance, and impaired spermatogenesis (Habert et al., 2009; Mylchreest et al., 1998; Mylchreest et al., 1999).

Diester phthalates and their monoester metabolites do not interact with or bind to AR receptors suggesting that they are not direct AR antagonists (Parks et al., 2000). However, these compounds act as endocrine disruptors and possess antiandrogenic properties that can interfere with androgen signaling causing reproductive and developmental abnormalities similar to the effects of AR antagonists, flutamide and vinclozolin (Mylchreest et al., 1999). It has been shown that *in utero* exposure to phthalates can disrupt the differentiation of androgen dependent tissues in male rats leading to symptoms that have TDS like phenotypes. The disruption of the androgen dependent tissue development is mainly due to the inhibition of fetal testicular testosterone biosynthesis (Dalsenter et al., 2006; Martino-Andrade & Chahoud, 2010). Numerous studies have focused on the relationship between reduced testosterone production and its effect on fetal testicular development. For instance, DBP and DEHP have been shown to reduce or inhibit testosterone levels in fetal rats resulting demasculinization (Fisher et al., 2003; Latini et al., 2004). However, an in vitro study on human fetal testis revealed that MEHP reduced the number of germ cells, but did not influence steroidogenesis leaving testosterone production unchanged (Lambrot et al., 2009). Then again, MEHP was found to induce testosterone production in mouse fetal testis and depending on the stage of development MEHP can stimulate, inhibit or have no impact on testosterone production (Lehraik et al., 2009). This may suggest that the effects of

phthalates on steroidogenesis can vary among species and that the mechanism of phthalates is still not fully understood (Lehraiki et al., 2009)

Phthalates have been also reported to alter germ cell morphology and affect Sertoli cell function. It has been reported that *in utero* exposure to certain phthalates such as DBP and DEHP can impair Sertoli cell development and function (Fisher et al., 2003). There is also evidence that phthalates can interrupt Sertoli and germ cell interaction resulting in germ cell detachment from the underlying Sertoli monolayer. This suggests a disturbance in cell adhesion leading to fundamental changes at the molecular level (Fisher et al., 2003; Li et al., 1998). These effects have been observed in rats exposed during neonatal and postnatal development and during adult life (Creasy et al., 1987; Fisher et al., 2003; Li et al., 1998; Li et al., 2000). In addition, similar results were also observed in organ culture and co-culture (Sertoli and germ cells) experiments when exposed to MEHP (Gray & Gangolli, 1986; Li & Kim, 2003).

Phthalate Toxicity in Adult Human Males

Studies on human exposure to phthalates and their association with male reproduction and development are scarce compared to the vast amount of evidence in animal studies (Habert et al., 2009). Nonetheless, a few studies have generated some data on human exposure to phthalates. For instance, a study on phthalate exposure through breast milk revealed that monoester phthalates can have an effect on hormones related to Leydig cell function. A significant inverse relationship with mono-*n*-butyl phthalate (MBP) and free testosterone levels was observed in male offspring. In the same study, there was a positive correlation between mono-ethyl phthalate (MEP) and MBP with SHBG (sex hormone binding globulin). This trend

was also noted between mono-methyl phthalate (MMP), MEP, and MBP with LH: free testosterone level ratio. However, no association between phthalates and cryptorchidism was observed (Main et al., 2006). Habert *et al.* (2009) investigated the effects of MEHP on the development of human somatic and germ cells using organ culture system. The organ culture was developed from human fetal testis acquired during the first trimester of pregnancy, a period known to be a critical stage for reproductive development. It was observed that at any dose, MEHP did not affect basal or LH-stimulated testosterone produced by the testis. Also, MEHP did not affect mRNA expressions of genes involved in steroidogenesis and testosterone biosynthesis (P450c17, P450scc, StAR) neither did it effect Insl3 which is involved in testicular descent. It was therefore observed that MEHP had no influence on the proliferation and apoptosis in Sertoli cells; however, it decreased mRNA expression of anti-Müllerian hormone, a hormone which acts on Müllerian ducts inducing their regression (Seli et al., 2002). However, MEHP(10^{-4}M) was observed to increase germ cell apoptosis which lead to a reduction in germ cell numbers, but their proliferation was unaffected (Habert et al., 2009).

Numerous studies in adult males have suggested a potential association between phthalates with sperm function and quality. The observed effects of phthalates in humans include low sperm count, increased DNA damage, decreased sperm motility and morphological abnormalities (Hauser et al., 2006; Hauser et al., 2007; Pant et al., 2011). For example, a study from Hauser *et al*. (2006) reported that sperm DNA damage is associated with exposure to MEP and MEHP. The results for MEP showed an increase in DNA damage which was also observed in MEHP treatment after adjusting to oxidative DEHP metabolites (Hauser et al., 2007). In addition, a dose-response relationship with DBP and DEHP in both *in-vivo* and *in-vitro* experiments showed a significant decrease in sperm motility. Data from *in-vitro* experiments

reflected results from epidemiological findings (Pant et al., 2011). The same study also reported an increase in sperm morphological abnormalities and a decrease in sperm count. These results coincided with animal studies that also displayed these characteristics following exposure to DEHP and DBP (Pant et al., 2011).

Testicular Cancer

Testicular cancer is the most common malignancy in young men (ages 15 -35) and accounts for 1% of all cancer in men. The incidence of this disease has been steadily growing in Western countries and is most common in northern European populations (Garner et al., 2008; Khan & Protheroe, 2007). Testicular cancer is comprised of 95% germ cell tumors, and the rest of the tumors are tumors of Leydig and Sertoli cells. Testicular germ cell tumors (TGCTs) are subcategorized into seminoma (50%), non-seminoma (40%) or combined (10%) tumors. Seminoma and non-seminoma subtypes are prevalent among adolescents and adults, and are the most common form of testicular cancer (Garner et al., 2008).

Seminomas are similar to primordial germ cells (PGC) and usually occur later in life at a mean age of 35 whereas non-seminomas occur at 25 years of age (Gilbert, Rapley, & Shipley, 2011). Non-seminomas consist of various differentiating embryonic lineages such as embryonal carcinoma, teratocarcinoma, choriocarcinoma, yolk sac carcinoma, and teratoma (Winter & Albers, 2011). These TGCTs originate from premalignant precursor lesion in the testis known as carcinoma in situ testis (CIS), also referred to as intra-tubular germ cell neoplasia (ITGCN) (Skakkebaek et al., 1987). It has been proposed that CIS developed from the malignant transformation of PGC or gonocytes during early fetal development (Skakkebaek et al., 1987).

Evidence from immunohistochemical studies have revealed that proteins identified in CIS were also present in PGCs and it was shown that CIS originated before onset of puberty (Kristensen et al., 2008; Muller et al., 1984). In addition, comparative studies on gene expression profiles and imprinting patterns confirmed that TGCTs derived from PGC or gonocytes (Gilbert et al., 2011). Further evidence from epidemiology studies has shown that an increase in testicular cancer was associated with birth cohort (Kristensen et al., 2008; Moller, 1989). Thus, it has been implicated that CIS have fetal origin. Nonetheless, the etiology of testicular cancer has yet to be fully understood (Garner et al., 2008). However, many risk factors have been found to be associated with the development of testicular germ cell tumors which includes CIS, cryptochordism, environmental and occupational exposures, maternal risk factors, and hereditary factors. Among the reported risk factors, cryptorchidism, CIS, and *in utero* exposure are the most established risk factors that occur in early life (Garner, Turner, Ghadirian, & Krewski, 2005).

Testicular cancer is the most severe condition that may be related to the symptoms of testicular dysgenesis syndrome (TDS) (Olesen et al., 2007). As mentioned previously, cryptorchidism poses a risk for developing testicular germ cell tumors (Garner et al., 2005). Other symptoms that also increase the risk of testicular cancer include poor semen quality and reduced fertility (Bay et al., 2006). In more extreme cases, infertility combined with a history of genital malformation (uni- or bilateral cryptorchidism and/or hypospadias) can lead to testicular cancer in patients (Skakkebaek et al., 2003). Environmental factors have been suggested to play a role in the origin of TDS. As described earlier, environmental factors such as endocrine disrupting chemicals can alter the production of androgens effecting the normal development of the male reproductive tract and increasing the risk of developing TDS (Mylchreest et al., 1999). For example, anti-androgen compounds such as flutamide can lead to the development of

symptoms of TDS in male rats (Mylchreest et al., 1999). Moreover, DBP exposure can lead to cryptorchidism, hypospadias, infertility and abnormalities of the testis, which resembles human TDS (Fisher et al., 2003). Therefore, data from animal models for exposure to phthalates can provide a possible indication for the cause of TDS in humans (Sonne et al., 2008).

Human testicular embryonal carcinoma (EC) cells are derived from testicular germ cell tumors and the most widely used embryonal carcinoma cell line is the Ntera-2/cl.D1 (NT2).This cell line is the clone of the parental NTera-2 cell line which was established from a nude mouse xenograft of the Tera-2 cell line. NT2 cells differ from other embryonal carcinoma cell lines by their sensitivity to differentiation induced by agents such as retinoic acid and hexamethylene bis-acetamide (HMBA). Retinoic acid induced differentiation has been best studied which results in the development of neurons and unlike NT2, other human EC cell lines don't respond to retinoic acid.

Ntera-2/cl.D1 cell line has been primarily used in research on testicular germ cell tumors and thus, these cells would make an appropriate in-vitro model to study the etiology of testicular cancer.

Genes Associated with Testicular Cancer

GSTP1

Glutathione S-transferase pi (GSTP1) is an important multifunctional detoxifying enzyme of the glutathione S-transferase family that acts as phase II xenobiotic metabolizing enzyme (Anuradha et al., 2000). Its action depends on the supply of GSH which is synthesized from glutamate cysteine ligase and glutathione synthase. GSTP1plays a major role in protecting cells by inactivating electrophilic substrates of GSTs such as carcinogens, toxins, hydrogen peroxides,

mutagens, and anti-cancer drugs by conjugation with glutathione (H.W. Lo & Ali-Osman, 2002; Mannervik et al., 1985). The glutathione conjugates are then removed from the cell by transporters. GSPT1 is also involved in oncogenesis, tumorigenesis, and drug resistance as well as the regulation of cell signaling, DNA repair and apoptosis pathways (Kraggerud et al., 2009; Lo et al., 2008; McLellan & Wolf, 1999). Expression of GSTP1 is present in both normal and tumor tissues. Increased expression in normal cells is often associated with the response to physiological stress and there is significant increase in GSTP1 expression in tumors compared to normal tissue (H.W. Lo & Ali-Osman, 2002). GSTP1 gene expression is regulated at many levels including transcriptional, post-transcriptional and post-translational level (Slonchak et al., 2009). Furthermore, genetic polymorphisms in GSTP1 have also been associated with the susceptibility to numerous cancers such as testicular, prostate and bladder cancer (Harries et al., 1997). Nonetheless, the mechanism involved in elevated GSTP1 expression in normal cells and many tumors has still yet to be clarified (Lo et al., 2008).

MGMT

O^6-methylguanine-DNA methyltransferase (MGMT) plays an important role in the defense against mutagenic, carcinogenic, and cytotoxic effects ofO^6-alkylguanine adducts (Bhakat & Mitra, 2000). Consequently, it also provides resistance to anticancer drug treatments (Passagne et al., 2006). As a DNA repair enzyme, MGMT repairs O^6-alkylguanine by transferring the alkyl group to a specific cysteine residue in the catalytic pocket of MGMT, thus removing the alkyl group from O^6 guanine resulting in repaired DNA and the inactivation of MGMT. However, the extent of repair is determined by the quantity of pre-existing MGMT molecules and the rate in which MGMT can be resynthesized (Nagel et al., 2003). MGMT plays significant part in the etiology of cancer. For instance, other than protecting cells from alkylating

agents, it has been shown to be down-regulated in many cancers, suggesting that its function is important in malignant transformation. In addition, it has many applications in preventing cancer as it can assist in assessing the sensitivity of drugs in patients (Gerson, 2004).

The MGMT gene in testicular cancer has been shown to be regulated epigenetically by DNA methylation. For instance, a study by Smith-Sorenson *et al.* (2002) revealed that frequent promoter hypermethylation of MGMT in testicular germ cell tumors was associated with down-regulation of the gene (Smith-Sorensen et al., 2002). In non-seminoma TGCTs, MGMT was found to be 44% methylated (Honorio et al., 2003). Methylation frequency was significantly higher in non-seminomas compared to seminomas. Also, immunohistochemistry analysis of MGMT protein expression supported the notion of gene silencing being associated with promoter hypermethylation. Thus, it was concluded that silencing of MGMT gene is a key event in the development of testicular cancer, particularly non-seminomatous testicular cancer (Smith-Sorensen et al., 2002)

Testisin

Serine proteases are involved in many proteolytic events and are essential in a multiple range of biological processes which includes blood coagulation, wound healing, digestion, and immune responses (Antalis et al., 2010). These enzymes are also associated with many diseases and are implicated in tumorigenesis (Netzel-Arnett et al., 2003). Testisin has recently been recognized as a serine protease that has a hydrophobic C-terminal domain which is linked to the membrane through a glycosyl-phosphatidylinositol anchor (Scarman et al., 2001). It is abundantly expressed in the testis by premeiotic testicular germ cells during spermatogenesis and it has been suggested that Testisin regulates proteolytic reactions involved in testicular germ cell

maturation (Hooper et al., 2000). Furthermore, abnormal Testisin expression may be linked to defective germ cell development, leading to infertility and sterility (Hooper et al., 1999). Although, Testisin is present in normal testicular germ cells, its expression is lost in testicular germ cell tumors suggesting that it may be a novel tumor suppressor gene (Kempkensteffen et al., 2006). Several potential regulatory elements at the 5'flanking region of the gene have been identified and are likely to participate in the basal transcriptional machinery of the gene. For example, two potential binding sites for the male sex determining gene SRY and the CCCCACCC element have been identified. These elements at the 5'flanking region may be involved in the regulation of testis specific expression of Testisin (Hooper et al., 2000). In addition, DNA hypermethylation at the promoter region has been found to silence Testisin expression in testicular germ cell tumors (Manton et al., 2005).

Objective and Rationale

Mono-(2-ethylhexyl) phthalate (MEHP) is known as a testicular toxicant, however its potential role in the development of testicular germ cell tumors is still unknown (Lambrot et al., 2009).In this study, we aimed to investigate the effects of MEHP on expression of Testisin, MGMT, and GSTP1 genes which are a tumor suppressor, DNA repair, and phase II metabolizing enzyme genes respectively. These genes are known to be associated with the risk of the development of testicular germ cell tumors. Examining the effect of time and dose dependant exposures to MEHP on these genes of interest can provide greater insight on the role MEHP plays in the development of testicular germ cell tumors.

Ntera-2/cl.D1 (NT2) is a human embryonic cell line (embryonal carcinoma) that has been used in a wide range of research, particularly in the research of testicular germ cell tumors

(TGCT) (Cheung et al., 2010). The objective of this study is to examine the molecular effects of MEHP on testicular NT2 cells in an *in vitro* cell model, to assess cell viability and examine the relative gene expression changes associated with TGCT upon treatment with MEHP. Additionally, to investigate DNA hypermethylation by MEHP, a co-exposure with demethylating agent (5-azacytidine) and MEHP will be examined and Testisin will be the gene of interest for this experiment as DNA methylation is known to regulate its expression (Manton et al., 2005).

MATERIALS AND METHODS

Chemicals and Solutions

The culture medium was Dulbecco's modified Eagle's medium (DMEM) purchased from American Type Culture Collection (Manasass, VA) and completed with 10% FBS (Sigma, Oakville, ON) and 1% Penicillin/Streptomycin (Invitrogen, Burlington, ON). Phenol Red Free RPMI1640 (Wisent, St-Bruno, QC) was supplemented with 5% Charcoal stripped FBS (Wisent), and 1% L-Glutamine (Wisent). Mono-(2-ethyhexyl) phthalate (MEHP) in neat form (100mg) with 99.9% purity was purchased from Accustandard (New Haven, CT) and 200mM stock solution was prepared in 100% ethanol. 5-azacytidine (A2385) was purchased from Sigma (Sigma, Oakville, ON). The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was obtained from Invitrogen (Eugene, OR). The Aurum Total RNA Mini Kit, iScript cDNA Synthesis Kit and SsoFast EvaGreen Supermix were all purchased from Biorad (Mississauga, ON). All primers used were ordered from Eurofins MWG Operon **(**Huntsville, AL)**.**

Cell Culture

NTera 2/cl.D1 (NT2) pluripotent human testicular embryonal carcinoma cell line was purchased from American Type Culture Collection (Manasass, VA). The cells were grown in 75 cm^2 culture flasks and maintained at 37˚C in a 5% CO2 atmosphere with complete Dulbecco's modified Eagle's medium until 80-90% confluency and were trypsinized with 0.05% Trypsin, 0.53mM EDTA (Wisent).

Exposure

The NTera 2/cl.D1 (NT2) cells were cultured in 100x20mm plates (80% confluency) with DMEM. The control plate contained the media with 0.1% EtOH as the solvent, while the exposure samples contained the test compound with a range of concentrations (10nM, 1uM, 10uM, 100uM, and 1mM of MEHP - diluted with EtOH with final concentration of 0.1%). Exposures were conducted for different time points (24hr, 48hr, 72hr and 96hr). For exposures beyond 48 hours each of the sample was re-exposed every 48hr. Following the time- and dose-based exposures the cells were harvested, collected and stored at -80^oC until further analysis.

NT2 cells were also exposed to 5-azacytidine (5-aza) as positive control at 2uM, 4uM, and 10uM for 96hrs. The 5-aza was prepared fresh for each experiment by dissolving it in autoclaved ddH20. The stock solution was sterilized by filtration in 0.2 μm filter. In addition, a co-exposure with a combination of 5-aza at 2uM, 4uM, and 10uM each with 100uM MEHP was exposed to NT2 cells for 96hrs. The control plate consisted of autoclaved ddH20 for 5-aza treatment and combination of ddH20 and ethanol.

Cell Viability Assessment (MTT Assay)

MTT assay is a colorimetric assay which determines cell viability through a colorimetric reaction. MTT ((3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide) is converted to insoluble formazan crystals by the mitochondrial dehydrogenase of viable cells. These crystals are then solubilized in a 10% SDS-HCl solution and absorbance is measured at 570nm and 690nm.

For each time point, NTera 2/cl.D1 (NT2/D1) cell lines were seeded in 24 well plates (Greiner) at a density of ~4 x 10^5 cells/well and grown in DMEM until 70-80% confluency. Cells were then exposed to each MEHP concentration with a control and each were performed in three technical replicates. A final MTT solution was prepared by adding 1.2 ml of 12 mM MTT stock solution (5mg/ml with PBS) in 12ml RPMI1640 phenol red free media At the end of each experimental time point, DMEM was removed and 500ul of the final MTT solution was added to each well with a blank included. The plate was incubated at 37^{o}C for 4hrs followed by the addition of 500ul of SDS-HCl solution (1.2g of sodium dodecyl sulfate dissolved in 12ml of 0.01M of HCl). The plate was incubated at 37^{o}C in a shaker overnight for 18hrs and the absorbance was read at 570nm and 690nm by a microplate reader (PolarStar Omega).

Gene Expression Analysis (Quantitative RT-PCR)

Total RNA was extracted from all experimental samples using the Aurum Total RNA Mini Kit according to the manufacturer's instructions (Biorad). RNA concentration was determined using the Nanodrop reader (Thermofisher). The quality and integrity of the RNA samples were determined by running a formaldehyde denaturing gel. The quality of RNA was

determined by observing sharp bands for 28S and 18SrRNA as well as the intensity with 28S band being twice as intense as the 18S bands.

cDNA was synthesized from 1ug of total RNA using iScript cDNA Synthesis Kit according to the manufacturers protocol (Biorad). With CFX96 Real-Time PCR Detection System (Biorad) quantitative real-time PCR was performed using cDNA in SsoFast EvaGreen Supermix containing primers for Testisin, GSTP1, and MGMT genes (see table 1 for primer details). Testisin and MGMT primer sequences were adopted from Manton et al. (2005) and Passagne et al. (2006) respectively. Housekeeping genes, HPRT1 and TBP, were used to normalize the expressions for each gene of interest. These housekeeping genes were the most stable compared to three other housekeeping genes when analyzed using the GeNorm software. Primer blast was used to design GSTP1 (NM_000852.3) and TBP (NM_003194.4) primer sequences. HPRT1 was obtained from RTPrimerDB (ID:8095). The relative gene expression was quantified using the $2^{-\Delta\Delta CT}$method.

Table 1. Quantitative Real-Time PCR primers.

Gene	Primer Sequence	Expected product size (bp)
Testisin	5’-CTGACTTCCATGCCATCCTT-3’	460
	5’-GCTCACGACTCCAATCTGAT-3’	
GSTP1	5’-AGGTCCTAGCCCCTGGCTGC-3’	103
	5’-TCAGGGGAGGCCAGGAAGGC-3’	
MGMT	5’-TTCACCATCCCGTTTTCCAG-3’	161
	5’-ATTGCCTCTCATTGCTCCTC-3’	
HPRT1	5’-TGACACTGGCAAAACAATGCA-3’	94
	5’-GGTCCTTTTCACCAGCAAGCT-3’	
TBP	5’-TCGCTTCCGCTGGCCCATAGT-3’	165
	5’-ACCAGAAACCCTTGCGCTGGAAC’-3	

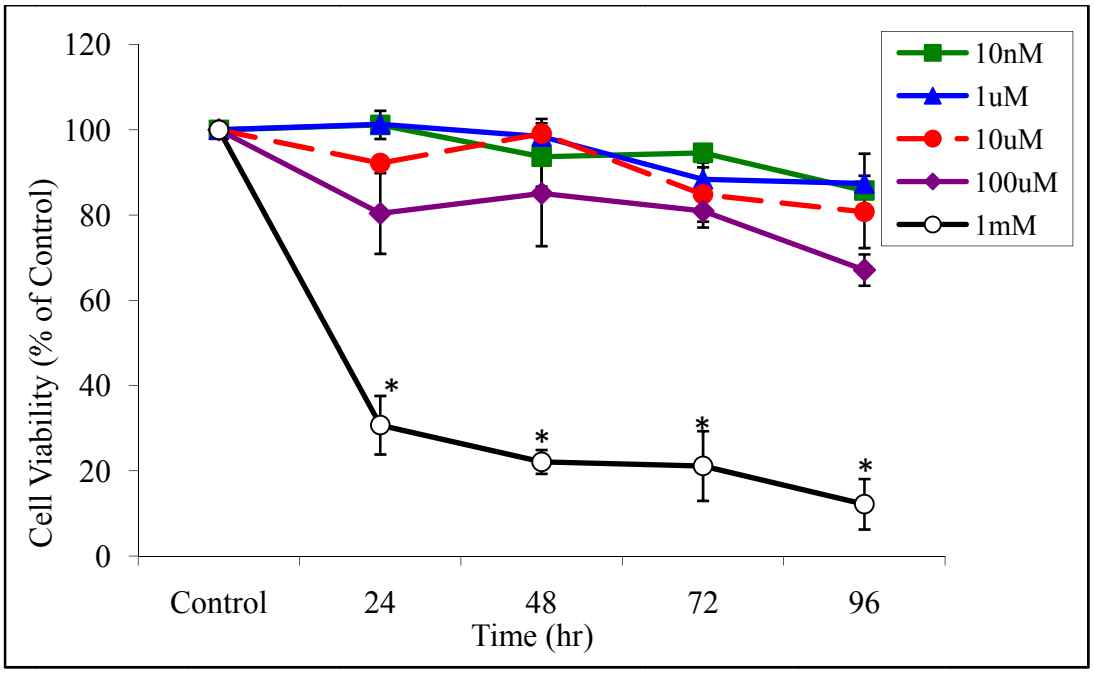
Statistical Analysis

Data are represented as means ± S.E.M of three independent experiments. SPSS software was used to conduct an independent samples t-test, One-Way and Two-Way Anova on the raw data to determine the statistical significance ($P < 0.05$) of the quantitative real-time PCR and MTT assay data (Refer to Appendix for the raw data and statistical data). Statistical analysis was performed to compare the expression of the Control and each of the doses of MEHP as well as the expression in between the time points.

RESULTS

MTT Cell Viability Assay

To study the cytotoxicity of MEHP, a MTT cell proliferation assay was performed for all time points (24, 48, 72, and 96hr) at each concentration ranging from 10nM to 1mM. The induction of cytotoxicity of MEHP and the decrease in cell number was most significant at 1mM for each time point (Fig. 1). These results are in agreement with the expectations and indicate that germ cells are highly sensitive to MEHP (Kasahara et al., 2002). In the earlier time points (24hr and 48hr) for 10nM, 1uM, 10uM, and 100uM, it seems as though cell viability was unaffected significantly unlike at 1mM.



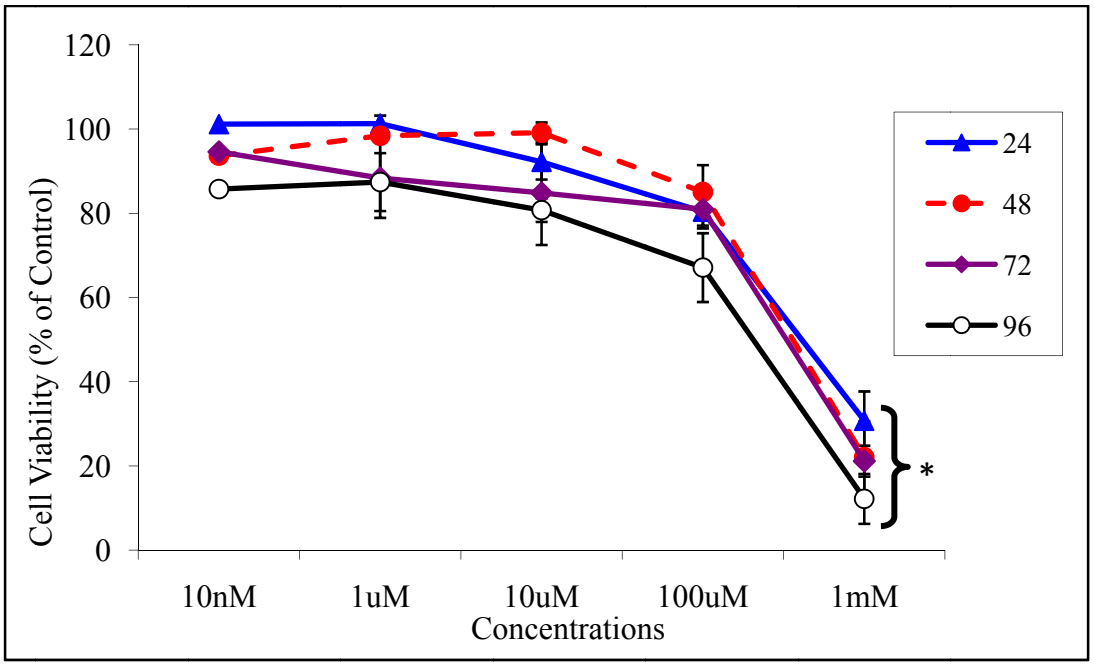


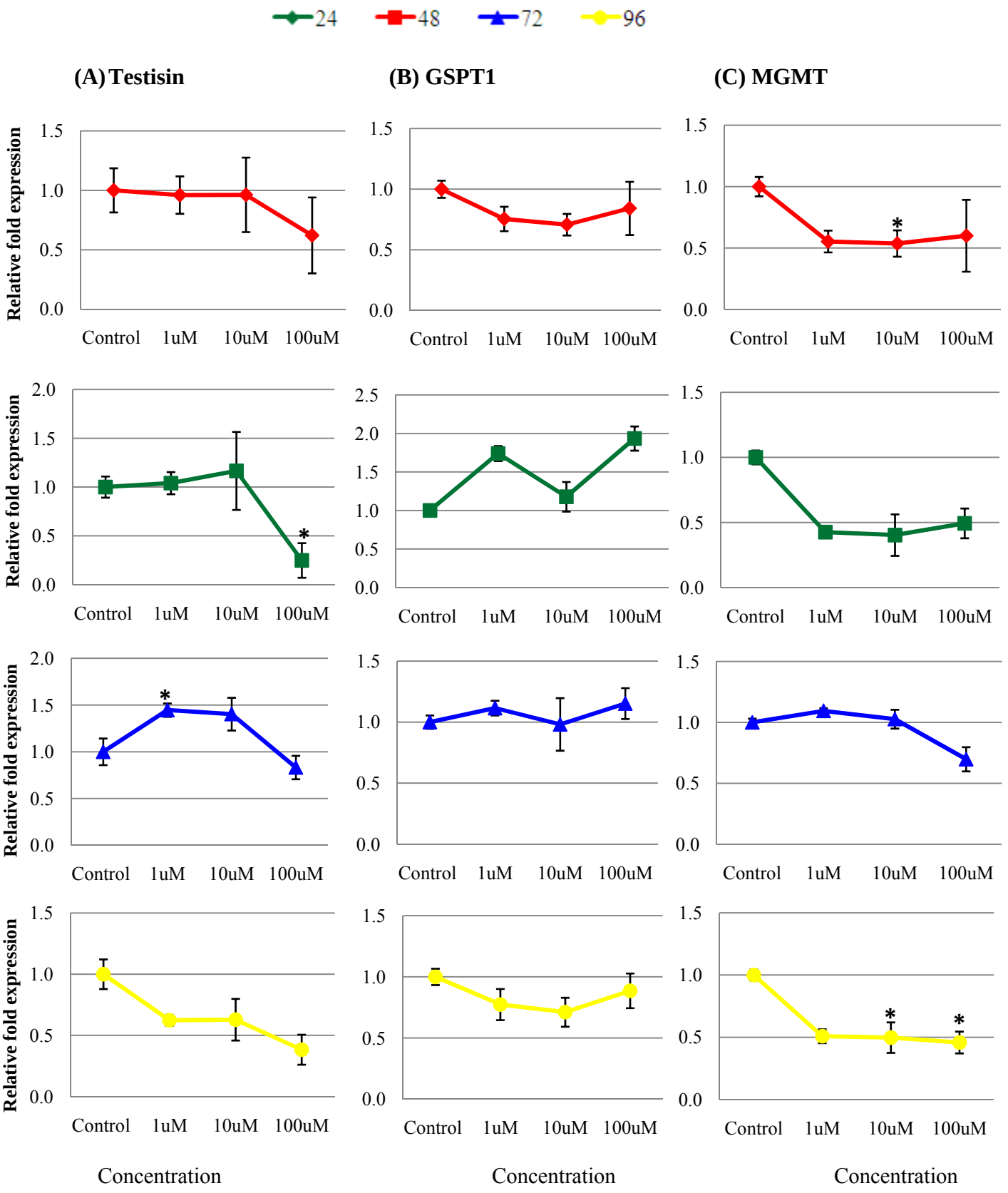
*Figure 1.Time and dose dependent effects of MEHP in NT2 cells. The cytotoxicity of MEHP was assessed by MTT cell proliferation assay for each time concentration and time period. Bars represent means ± S.E.M of three independent experiments. *Significant ($p<0.05$) compared to control as determined by Independent t-test. Refer to Appendix for additional details on statistical data.*

Gene Expression in Ntera-2 Cells Exposed to MEHP

At the highest concentration of 100uM, the expression levels of Testisin decreased for all time points compared to control. The expression level is generally lowest at 96hrs with the highest at 72hrs for all concentrations. However, there is no significant change between 1uM and 10uM. Overall, the expression levels show a decreasing trend (Fig. 2A, 2D, and 3A). A pattern is noticeable for GSTP1 with an increase in expression from 24hr to 48hr time points followed by a gradual decrease in expression for all concentrations. The highest expression is seen at the 48hr time point for 100uM with an approximately two fold increase (Fig. 2B, 2E, and 3B). MGMT also shows a pattern for all concentrations in which there is a decrease in expression at 24hr to 48hr time point compared to control followed by an increase in expression at 72hrs then a gradual decrease in expression at the last time point. The highest expression levels for each concentration peaks at 72hr time point with 1uM and 10uM returning to control levels. However, at 100uM, the expression levels are below control for each time points (Fig. 2C, 2F, and 3C).

Restoration of Testisin Expression with 5-azacytidine and MEHP

The concentration at 100uM MEHP was chosen for the co-exposure experiment because it had the most significant effect in down-regulating Testisin (Fig. 2A, 2D and 3A). Treatment of 5-aza to NT2 resulted in an increase in expression levels (Fig.4). It seems that the optimal concentration is at 4uM showing the highest expression level. The co-exposure revealed a down-regulation of Testisin gene expression. These data show that MEHP may be involved in DNA methylation.


24
48
72
96
(A) Testisin
(B) GSPT1
(C) MGMT
Relative fold expression
Control
1uM
10uM
100uM
Concentration
*

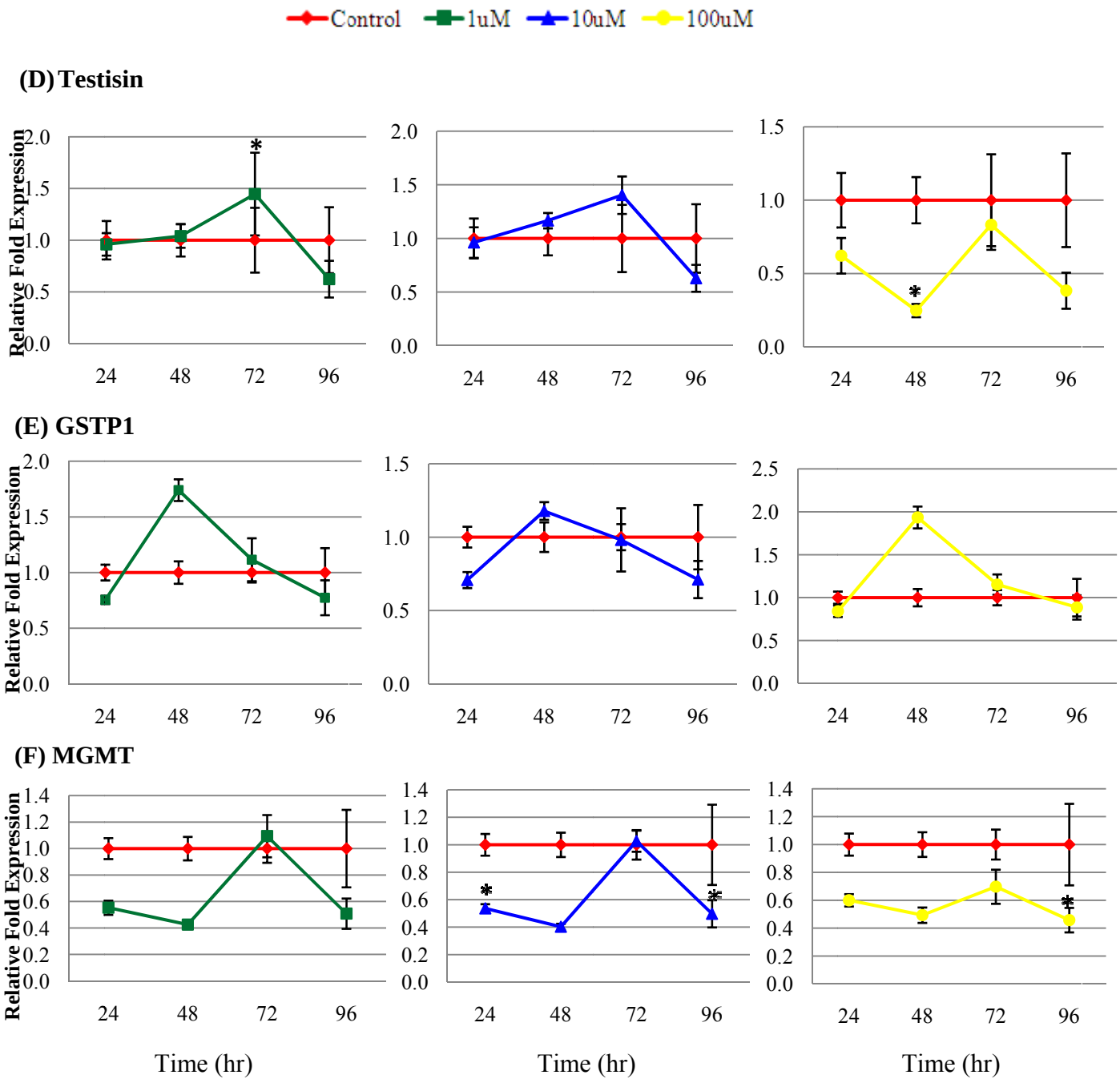
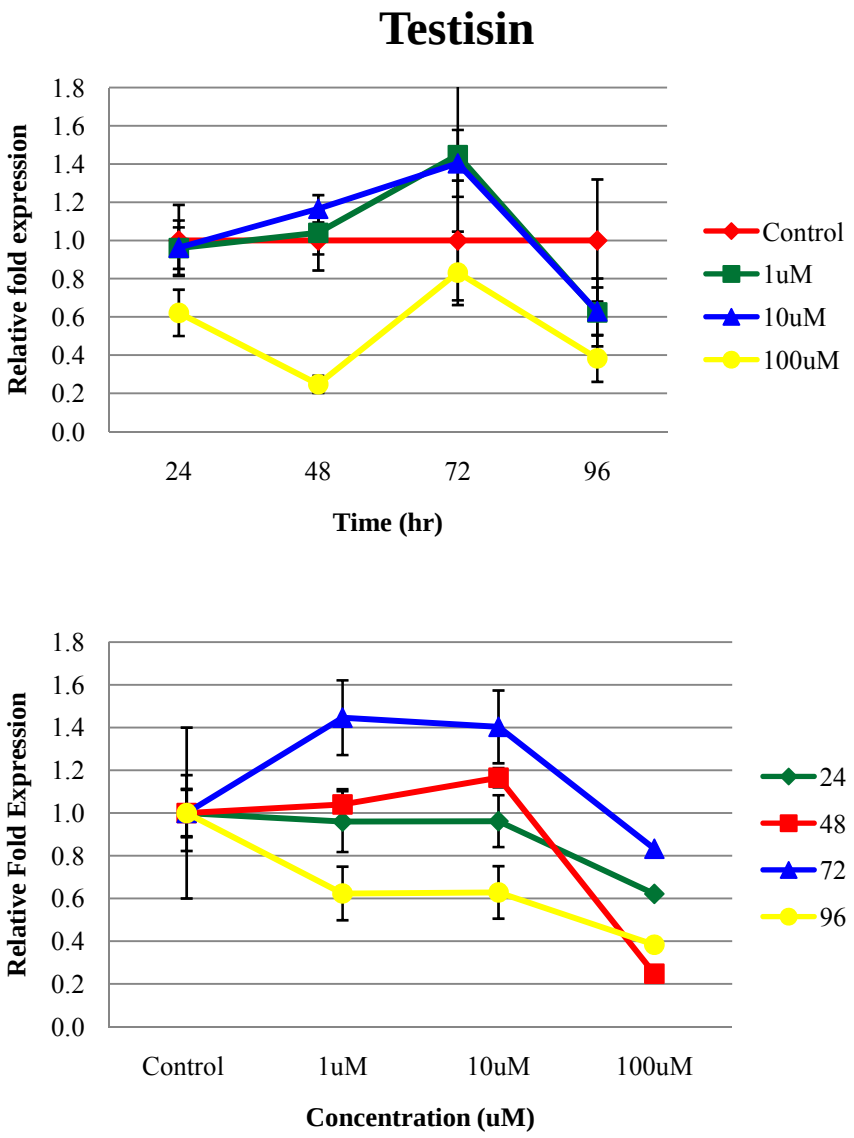
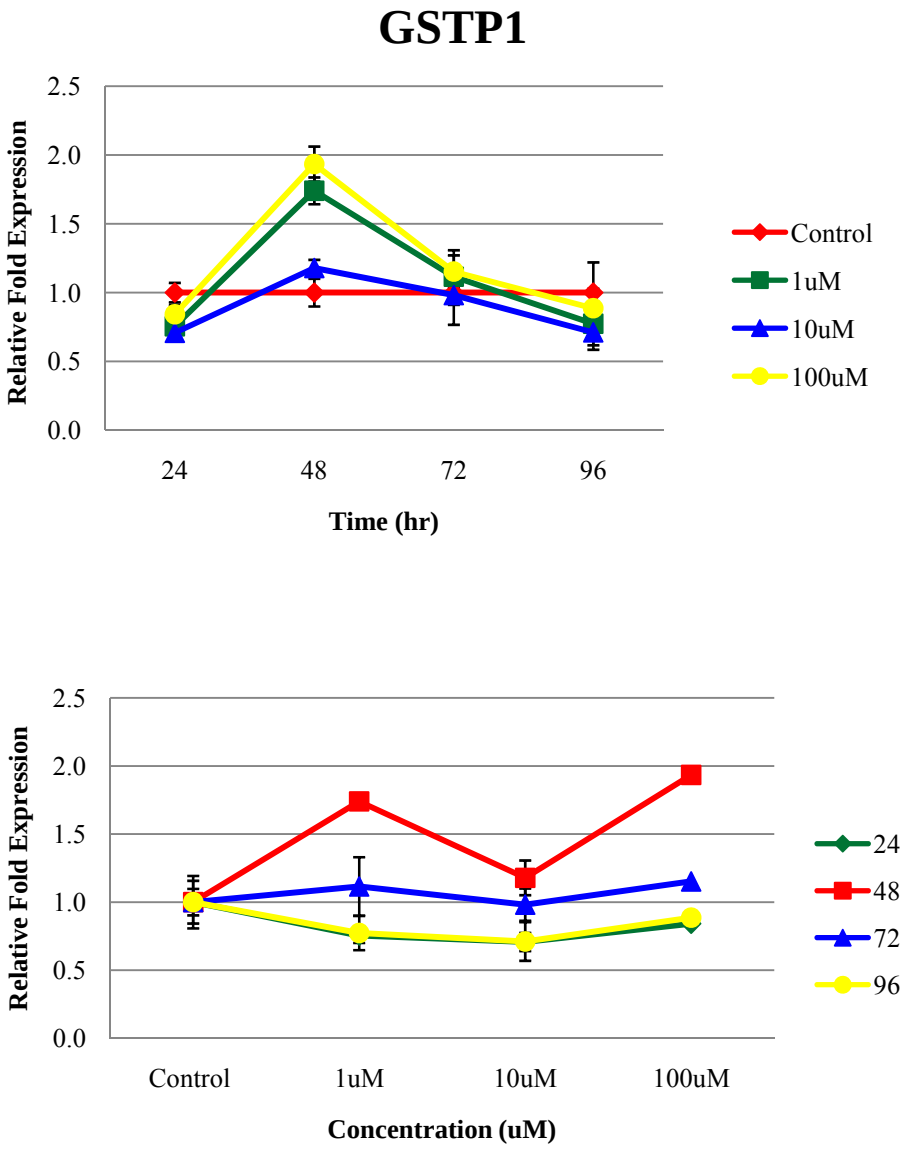


*Figure 2.Dose dependent (A, B, C) and time dependent (D,E,F) effects of MEHP on mRNA expression at 1uM, 10uM, and 100uM for 24, 48, 72, and 96hr time points for Testisin, GSTP1, and MGMT. Bars represent means ±S.E.M of three independent experiments. *Significant ($P < 0.05$) compared to control as determined by Independent t-test. Refer to Appendix for additional details on the raw data and statistical data.*

(A)


Testisin
Relative fold expression
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
24
48
72
96
Time (hr)
Control
1uM
10uM
100uM
Relative Fold Expression
Control
1uM
10uM
100uM
Concentration (uM)
24
48
72
96

(B)


GSTP1
Relative Fold Expression
2.5
2.0
1.5
1.0
0.5
0.0
24
48
72
96
Time (hr)
Control
1uM
10uM
100uM
Relative Fold Expression
2.5
2.0
1.5
1.0
0.5
0.0
Control
1uM
10uM
100uM
Concentration (uM)
24
48
72
96

(C)

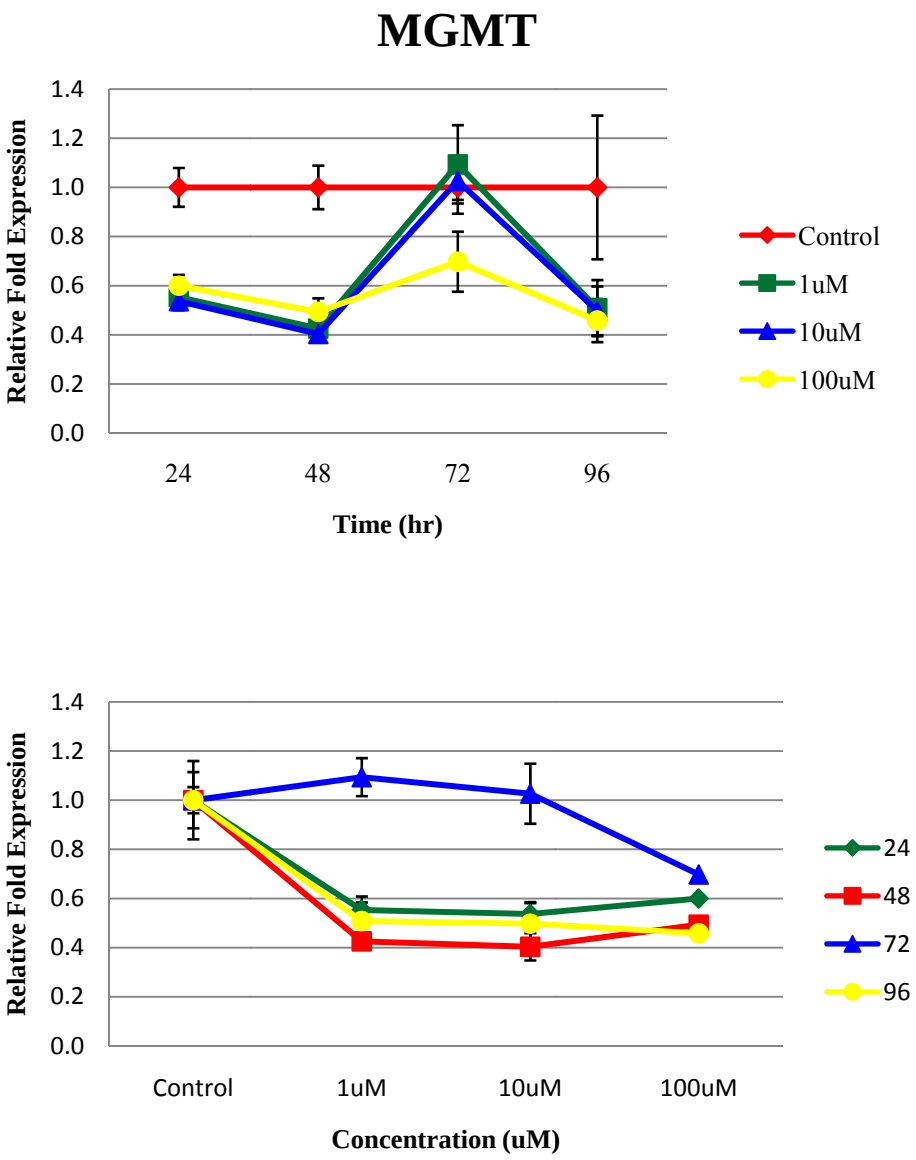


Figure 3. Time dependent and dose dependent effects of MEHP. Overall representation of mRNA expression post MEHP exposure at 1uM, 10uM, and 100uM for 24, 48, 72, and 96hr time points. (A) Testisin (B) GSTP1 (C) MGMT. Bars represent means ± S.E.M of three independent experiments.

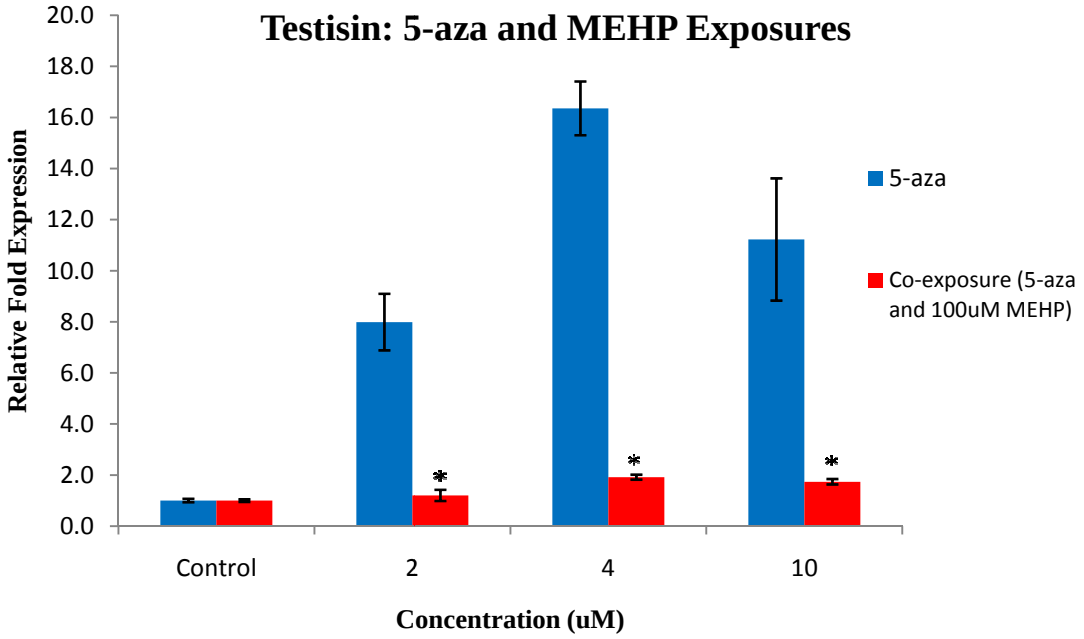


*Figure 4. Testisin mRNA expression in NT2 cells determined by quantitative real-time PCR. Restoration of Testisin gene in NT2 cells after treatment with5-azacytidine at 2uM, 4uM, and 10uM for 96hrs. Co-exposure consists of a combination of 5-azacytidine at 2uM, 4uM, and 10uM each with 100uM MEHP for 96hrs. The graph represents the raw data normalized to housekeeping genes and relative to control. *Significant ($p<0.05$) compared to control as determined by Independent t-test. Refer to Appendix for additional details on the raw data and statistical data.*

DISCUSSION

Cytotoxicity

The results indicate cytotoxicity in NT2 cells from exposure to MEHP which may be from oxidative stress or apoptosis. In organ cultures of human fetal testis, MEHP at a concentration of 10^{-4}M reduced germ cell numbers by increasing apoptosis (Habert et al., 2009). One of the factors involved in inducing apoptosis is the release of cytochrome *c* from the mitochondria into the cytosol. It has been shown that oxidative stress elicited from MEHP at low concentrations can impair mitochondrial function and is responsible for apoptosis via release of cytochrome *c* (Kasahara et al., 2002). Thus, a reduction in NT2 cells by apoptosis may reflect the cytotoxic effects of MEHP. A dose and time-dependent cytotoxicity has been observed in these exposures of NT2 cells.

Gene Expression

Testisin

Testisin is specifically and abundantly expressed in premeiotic testicular germ cells in the testis, but is absent in testicular germ cell tumors, indicating that it may act as a tumor suppressor gene (Hooper et al., 1999). Ntera-2/cl.D1 (NT2) is a human testicular embyronal carcinoma cell line derived from cloning the parental NTera-2 cell line which was established from a nude mouse xenograft of the Tera-2 cell line. NT2 showed low Testisin mRNA expression similar to Tera-2 cell line, which also displayed low expression. The low expression in Tera-2 cell line was correlated with the extensive methylation at the transcription initiation site and has been implicated in the down-regulation of Testisin mRNA in TGCT (Kempkensteffen et al., 2006).

The aim of this study is to investigate the changes in relative gene expression upon MEHP treatment as well as examine the effects of 5-azacytidine.

Epigenetics has been recognized as an important mechanism associated with the development of many cancers. One epigenetic mechanism is DNA methylation, a process involved in the covalent addition of methyl groups by DNA methyltransferase (Dnmt) enzymes to the C5 position of the cytosine residue, forming the 5-methylcytosine (S. Li et al., 2003). The main target for methylation is at the CpG site, a region in which a cytosine is immediately followed by a guanine (Lind et al., 2007). This epigenetic mechanism has been known to silence gene expression of tumor suppressor genes. For example, Manton *et al.* (2005) have proposed that the mechanism most likely to inactivate Testisin expression in testicular tumors is the hypermethylation of the 5' CpG island. This was demonstrated by correlating the methylation status with the gene expression in both primary and tumor cell lines of testicular tumors and re-activating Testisin mRNA expression by treatment with demethylating agent and histone deacetylase inhibitor. These findings suggested that Testisin gene is regulated by DNA methylation and other indirect epigenetic processes that may work synergistically such as histone modification enzymes and chromatin remodeling complexes (Manton et al., 2005).

In this study, NTera 2/cl.D1 (NT2) cells were exposed to mono-(2-ethyhexyl) phthalate (MEHP) at 1uM, 10uM and 100uM. A decrease in Testisin gene expression was noticeable as the concentration of MEHP increased. These results indicate a dose-dependence for the gene expression changes. It appears that exposure to MEHP decreases the expression of Testisin which may be due to epigenetic modifications induced by MEHP. For instance, Wu *et al.* (2009) investigated the relationship between DEHP induced testicular toxicity and DNA methylation in

mice by examining the changes in global methylation and DNA methytransferase (Dmnt) expression levels. The results revealed a significant increase in global methylation levels in the testis. In addition, mRNA expression for DNA methyltransferase maintenance Dmnt1a and *de novo* DNA methyltransferases, Dmnt3a and Dmnt3b all increased significantly relative to control group in DEHP exposed mice. Results from protein expression were consistent with the mRNA expression with DEHP exposed mice showing increased levels of Dmnts. These Dmnts are essential for establishing genomic methylation patterns which is responsible for transcriptional silencing (Burgers et al., 2002). Dmnt1 is necessary for the maintenance of global methylation by restoring methylation patterns on hemimethylated DNA after DNA replication (Burgers et al., 2002; S. Li et al., 2003). Dnmt3a and dnmt3b are *de novo* DNA methyltransferase enzymes that can methylate hemimethylated and unmethylated DNA (S. Li et al., 2003). Thus, these enzymes are important in the regulation of DNA methylation and are associated with gene silencing.

Although DEHP is the parent compound of MEHP and was found to increase expression for Dnmts in mice testis, it can still provide a possible explanation for MEHP and its role in down-regulating Testisin expression by DNA methylation. Transcriptional gene silencing by DNA methylation at the promoter CpG island can occur either by interfering with the binding of transcription factors or by recruiting methylated CpG binding proteins to the transcription site thereby inhibiting transcription (Curradi et al., 2002; Jones &Takai, 2001). It has also been suggested that gene silencing is strongly correlated with methylation of CpG islands near the transcription start site by inhibiting the basal transcription machinery of genes (De Smet et al., 1999)

.

DNA hypermethylation is involved in the initiation and progression of cancer by silencing tumor suppressor genes as well as other essential genes required for normal development (Bird, 1996; Müller & Florek, 2010). Nonetheless, reactivation of methylated silenced genes can be achieved through treatment with a demethylating agent (Müller & Florek, 2010). Regulation of Testisin gene by DNA methylation has been confirmed by the reactivation of Testisin gene by 5-azacytidine (5-aza), a DNA methyltransferase inhibitor (Manton et al., 2005). 5-azacytidine is able to demethylate newly synthesized DNA by incorporating itself onto DNA and covalently binding to the DNA methyltransferase enzyme. This traps the enzyme and inhibits its progression on the DNA duplex leading to the degradation and depletion of it from the cells (Müller & Florek, 2010). To investigate the role of MEHP on Testisin gene and its relationship with DNA methylation and gene expression, an independent exposure with 5-aza and a co-exposure with the combination of 5-aza and MEHP were performed on NT2 cells. Treatment with 5-aza resulted in the up-regulation of Testisin mRNA with the highest expression at 4uM. In comparison with the exposure to 5-aza only, the co-exposure with MEHP revealed a down-regulation of Testisin gene. The effects of MEHP may resemble the effects of DEHP in which levels of DNA methyltransferase enzymes (DNMT1, 3a and 3b) in mice testis increased (S. Wu et al., 2010). If so, it is possible that the down-regulation of Testisin mRNA maybe explained by the increased levels of DNA methyltransferase activity overwhelming the demethylating effects of 5-aza. Thus, these results suggest that MEHP may play a role in down-regulating or silencing genes by DNA methylation.

GSTP1

Glutathione S-transferase pi (GSTP1) is a phase II xenobiotic metabolizing enzyme involved in the detoxification of toxins (Anuradha et al., 2000). In this study, the affects of

MEHP on gene expression was investigated. GSTP1 showed the same trend for all concentrations (1uM, 10uM, and 100uM). The results showed an increase in expression from 24hr to 48hr time point which then gradually declined at subsequent time points. It seems that the metabolism of MEHP takes place between 24hr and 72hr time point with a maximum peak at 48hr for all concentrations (Fig. 2B, 2F and 3B). In addition, these results indicate that the change in expression might reflect the survival of the cells. For instance, at the highest expression of GSTP1 at 48hrs for all concentrations there is an increase in cell viability compared to the other stages of exposure (Fig. 1). After 48hrs, the expression levels seem to gradually decrease and return to control level. However, the cell viability declined after 48hrs for each concentration. This may suggest a delayed response which can possibly be explained by the rate of re-synthesis of the GSTP1 enzymes and/or the rate of detoxification. Nonetheless, the detoxification system was activated and GSTP1 may have catalyzed the removal of MEHP from the cell. This also suggests that GSTP1 may have protective roles against MEHP in testicular cells. Furthermore, MEHP can also produce oxidative stress in the testis which is a major component inducing germ cell apoptosis (Kasahara et al., 2002).

Reactive oxygen species (ROS) are derivative forms of oxygen-derived oxidants which are associated with a condition known as oxidative stress (Maneesh & Jayalekshmi, 2006). ROS has been shown to effect the regulation of cell growth and induce apoptosis by influencing many cellular signaling pathways (Rahman & MacNee, 2000; Özgen et al., 2009a). The antioxidant mechanism is one of many processes involved in defending against ROS; however, increase ROS formation and impairment of antioxidant defense can lead to cellular apoptosis (Syng-Ai et al., 2004). MEHP has been known to generate ROS in testicular cells of rats (Kasahara et al.,

2002). Thus, ROS may indirectly alter GSTP1 expression in the same manner as previously described.

The molecular mechanism responsible for the regulation of GSTP1 has yet to be fully understood. However, recent studies have shown that the Nrf2-Antioxidant response element (Nrf2-ARE) signaling pathway is an important mechanism involved in the cellular defense against oxidative and electrophilic stress by regulating phase II detoxification and antioxidant genes (Lee & Johnson, 2004). Nrf2 is a basic leucine zipper transcription factor that is bound to Kelch-like ECH-associated protein 1 (Keap1) which tethers Nrf2 in the cytoplasm (Nguyen et al., 2009). Nrf2 is activated in response to oxidative stress by dissociating from Keap1 protein. Nrf2 then translocates into the nucleus where it binds to the ARE in the promoter of target genes and induce transcription of detoxifying genes such as glutathione transferases (GSTs) and other genes involved in the antioxidant glutathione pathway (Harvey et al., 2009). Many studies have provided evidence that Nrf2 plays a critical role in regulating the genes associated with the antioxidant glutathione pathway. These genes include glutathione (GSH) biosynthetic enzymes such as glutathione cysteine ligase modifier subunit (GCLM) and glutathione cysteine ligase catalytic subunit (GCLC) as well as GSH-dependent antioxidant enzymes, glutathione peroxidase 2 (GPX2) and glutathione S-transferases (GST). For instance, it was found that $Nrf2^{-/-}$cells and tissues attained low levels GSH or loss of function to produce GSH. Nrf2 is also involved in maintaining intracellular levels of GSH by regulating cysteine/glutamate exchange transporter (Kensler et al., 2007).

As mentioned previously, Nrf2 is bound to the Keap1 protein and is activated by the dissociation of the Nrf2-Keap1 complex when under stress leading to transcriptional induction of

target genes (Harvey et al., 2009). Dissociated Nrf2 is an unstable protein that is susceptible to proteolytic degradation by the ubiquitin-dependent pathway and in non-stress cells it is continually degraded (Nguyen et al., 2009). Keap1 has been found to not only repress the activity of Nrf2, but it also seems to contribute to the ubiquitylation of Nrf2 (Kobayashi et al., 2006). Keap1 has been suggested to be capable of shuttling into the nucleus and ubiquitylating Nrf2. In addition, it has also been suggested that Keap1 escorts Nrf2 out of the nucleus into the cytoplasm for degradation (Itoh et al., 2003; Nguyen et al., 2009). Nevertheless, this implies that Nrf2 is likely to rely on a mechanism that increases its stability and reduces its degradation allowing for its accumulation in the nucleus in response to stress signals. The regulation of the stability of Nrf2 by Keap1 is modulated by a mechanism that limits their interaction in a coordinate fashion. It is believed that Nrf2 stabilization in response to oxidative/electrophilic stress is controlled by mechanisms that either restrict or decrease the access of Keap1 protein to Nrf2 (Kobayashi et al., 2006; Nguyen et al., 2009). However, the exact mechanism of how Keap1 targets Nrf2 for degradation and the process of the stabilizing Nrf2 still remains to be clarified (Nguyen et al., 2009). Thus, Nrf2-ARE pathway may be involved in regulating GSPT1 expression in response to MEHP directly or indirectly.

Regulation of GSTP1 has also been reported to be mediated by cAMP signaling pathway. The GSTP1 transcription is regulated by cAMP response element binding proteins-1 (CREB-1) where it is activated by protein kinases (PKA) which is released by cAMP signaling (Andrisani, 1999; Taylor et al., 1990). CREB-1 activates transcription by binding to the cAMP response element (CRE) present in the 5'regulatory region near the transcription start site of the GSTP1 gene (Andrisani, 1999; H. W. Lo & Ali-Osman, 2002; Özgen et al., 2009b). Since GSTP1 gene has a high affinity for CREB-1, the activation of CREB-1 and induction GSTP1 transcription

may include other signaling pathways such as cellular stress, growth factors, and Ca^{2+}influx (H. W. Lo & Ali-Osman, 2002).

Another pathway that regulates GSTP1 expression is the nuclear factor kB (NF-kB) pathway. The activation of NF-kB and activating protein-1 (AP1) transcription factors are known to induce transcription of pro-inflammatory cytokine genes and cytoprotective antioxidant genes (Morceau et al., 2004). NF-kB and AP1 are regulated by tumor necrosis factor alpha TNFα which is involved in controlling GSTP1 expression (Duvoix et al., 2003).Furthermore, oxidative stress has been known to activate various signaling pathways which include the NF-kB pathway(Bowie & O'Neill, 2000; Morceau et al., 2004).

MGMT

O^6-methylguanine-DNA methyltransferase (MGMT) encodes a DNA repair enzyme that removes alkyl groups from the *O*^{6}-guanine by transferring the alkyl group to a specific cysteine residue in the catalytic pocket of MGMT resulting in repaired DNA and the inactivation of MGMT (Lind et al., 2007; Nagel et al., 2003). The extent of repair is determined by the quantity of MGMT molecules pre-existing and the rate MGMT can be resynthesized (Nagel et al., 2003). To understand the effects of MEHP on the activity of MGMT in NT2 cells, gene expression analysis was examined.

According to the results in Fig. 2C, 2F, and 3C, the gene expression analysis for MGMT may reflect the cells capacity to repair DNA adducts which in turn may possibly, in combination with GSTP1, contribute to the sensitivity of NT2 cells to MEHP. The mRNA levels for 1uM, 10um, and 100uM MEHP exposure are slightly lower than control and seem to be in a steady

state in the first 48hr cycle. MGMT levels seem to have undergone a phase of decline followed by a restoration phase of the lost activity. The initial decline in expression can be explained by the pre-existing MGMT molecules currently in the process of DNA repair in which one molecule is capable of repairing one DNA adduct. The amount of MGMT in a cell can suddenly decrease and be momentarily depleted due to excess DNA adducts and if DNA adducts continue to form MGMT molecules can be permanently depleted (Gouws & Pretorius, 2011). At the 72hr time point an increase in expression was observed for 1uM, 10uM and 100uM. This increase in expression may have resulted in response to the depletion of MGMT molecules followed by a period of MGMT resynthesis; however, from 72hr onwards the expression levels decreased. This may suggest that enough MGMT molecules have been generated to continue the DNA repair which explains the decline in MGMT expression. However, at 100uM concentration, the expression level is relatively lower compared to the other concentrations at 72hr time point. It seems that the expression level is dose dependent at this particular time point. This may suggest that MEHP is capable of suppressing MGMT gene.

MGMT expression can be regulated by several transcription-factor-recognition sequences located at the promoter region which includes SP1,GRE and AP-1 sites allowing MGMT expression to be induced by glucocorticoids, cyclic AMP and protein kinase C activators (Gerson, 2004). In addition, nuclear factor kB (NF-kB) has been suggested to play a significant role in MGMT regulation and is most likely the major component involved in NF-kB mediated drug resistance to alkylating agents (Lavon et al., 2007). Furthermore, DNA methylation is an important mechanism that has an influence on MGMT expression and plays a role in carcinogenesis (Danam et al., 2005).

As previously mentioned, DNA hypermethylation has been described to be one of the major mechanisms that is associated with MGMT gene expression (Smith-Sorensen et al., 2002). MGMT is the most frequently altered gene in testicular germ cell tumors (TGCT) (Smith-Sorensen et al., 2002). Methylation at the CpG island of the promoter region is known to down-regulate or silence MGMT gene expression which can lead to the development of many cancers including TGCTs (Lind et al., 2007; Smith-Sorensen et al., 2002). Carcinogenesis can result from the loss of MGMT function by permitting mutagenesis in the DNA adducts particularly in oncogenes and tumor suppressor genes (Gerson, 2004).

As described earlier, DEHP has been known to increase DNA methyltransferases (Dmnts) in rats at the mRNA and protein level. As a metabolite of DEHP, MEHP may possess the same effect and may contribute to the down-regulation of MGMT via promoter methylation (S. Wu et al., 2010). In Ntera 2 cell line, MGMT was found to be partially methylated (Honorio et al., 2003). It may be possible that enhanced methylation can lead to further down-regulation of MGMT. Nonetheless, the exact mechanism by which MEHP affects MGMT via methylation-mediated transcriptional inactivation has still yet to be elucidated.

In addition to this study, future exploration to further investigate the effects of MEHP association with these genes could involve examining protein expression studies and/or methylation status analyses to observe consistency with mRNA expression and to confirm that MEHP is associated with DNA methylation.

CONCLUSION

The results from this study seem to present plausible evidence that Mono-2-ethyhexyl phthalate (MEHP) can alter the expression levels of Testisin, GSTP1, and MGMT in NT2 cells. For instance, Testisin was down-regulated at the highest dose. GSTP1 was induced at 48hr time point and MGMT displayed a general decline in expression. Therefore, it may be possible that MEHP may be associated with the increased risk of developing this disease. However, further research is necessary to confirm the relationship between MEHP and the development of TGCT. In addition, MEHP, at the highest dose, down-regulated Testisin gene expression in a co-exposure experiment. This suggests that MEHP can epigenetically down-regulate genes by DNA methylation of genes such as Testisin. Thus, it would be ideal to confirm the methylation potential of MEHP on the genes that are known to be inactivated by promoter hypermethylation. Nonetheless, the role of MEHP on the mechanistic pathway of Testisin, GSTP1 and MGMT still needs to be further defined.

REFERENCES

Andrisani, O. M. (1999). CREB-mediated transcriptional control. *Critical Reviews in Eukaryotic Gene Expression, 9*(1), 19-32.

Antalis, T. M., Buzza, M. S., Hodge, K. M., Hooper, J. D., & Netzel-Arnett, S. (2010). The cutting edge: Membrane-anchored serine protease activities in the pericellular microenvironment. *Biochemical Journal, 428*(3), 325-346.

Anuradha, D., Reddy, K. V., Kumar, T. C., Neeraja, S., Reddy, P. R. K., & Reddanna, P. (2000). Purification and characterization of rat testicular glutathione S-transferases: Role in the synthesis of eicosanoids. *Asian Journal of Andrology, 2*(4), 277-282.

Bay, K., Asklund, C., Skakkebaek, N. E., & Andersson, A. -. (2006). Testicular dysgenesis syndrome: Possible role of endocrine disrupters. *Best Practice and Research: Clinical Endocrinology and Metabolism, 20*(1), 77-90.

Bhakat, K. K., & Mitra, S. (2000). Regulation of the human O6-methylguanine-DNA methyltransferase gene by transcriptional coactivators cAMP response element-binding protein-binding protein and p300. *Journal of Biological Chemistry, 275*(44), 34197-34204.

Bird, A. P. (1996). The relationship of DNA methylation to cancer. *Cancer Surveys, 28*, 87-101.

Bowie, A., & O'Neill, L. A. (2000). Oxidative stress and nuclear factor-kappaB activation: A reassessment of the evidence in the light of recent discoveries. *Biochemical Pharmacology, 59*(1), 13-23.

Bronson, R. (2011). Biology of the male reproductive tract: Its cellular and morphological considerations. *American Journal of Reproductive Immunology, 65*(3), 212-219.

Burgers, W. A., Fuks, F., & Kouzarides, T. (2002). DNA methyltransferases get connected to chromatin. *Trends in Genetics, 18*(6), 275-277.

Cheng, C. Y., & Mruk, D. D. (2002). Cell junction dynamics in the testis: Sertoli-germ cell interactions and male contraceptive development. *Physiological Reviews, 82*(4), 825-874.

Cheung, H. H., Lee, T. L., Davis, A. J., Taft, D. H., Rennert, O. M., & Chan, W. Y. (2010). Genome-wide DNA methylation profiling reveals novel epigenetically regulated genes and non-coding RNAs in human testicular cancer. *British Journal of Cancer, 102*(2), 419-427.

Creasy, D. M., Beech, L. M., Gray, T. J. B., & Butler, W. H. (1987). The ultrastructural effects of di-n-pentyl phthalate on the testis of the matura rat. *Experimental and Molecular Pathology, 46*(3), 357-371.

Curradi, M., Izzo, A., Badaracco, G., & Landsberger, N. (2002). Molecular mechanisms of gene silencing mediated by DNA methylation. *Molecular and Cellular Biology, 22*(9), 3157-3173.

Dalsenter, P. R., Santana, G. M., Grande, S. W., Andrade, A. J. M., & Araújo, S. L. (2006). Phthalate affect the reproductive function and sexual behavior of male wistar rats. *Human and Experimental Toxicology, 25*(6), 297-303.

Danam, R. P., Howell, S. R., Brent, T. P., & Harris, L. C. (2005). Epigenetic regulation of O6-methylguanine-DNA methyltransferase gene expression by histone acetylation and methyl-CpG binding proteins. *Molecular Cancer Therapeutics, 4*(1), 61-69.

De Smet, C., Lurquin, C., Lethé, B., Martelange, V., & Boon, T. (1999). DNA methylation is the primary silencing mechanism for a set of germ line- and tumor-specific genes with a CpG-rich promoter. *Molecular and Cellular Biology, 19*(11), 7327-7335.

Duvoix, A., Morceau, F., Delhalle, S., Schmitz, M., Schnekenburger, M., Galteau, M., Diederich, M. (2003). Induction of apoptosis by curcumin: Mediation by glutathione S-transferase P1-1 inhibition. *Biochemical Pharmacology, 66*(8), 1475-1483.

Fisher, J. S., Macpherson, S., Marchetti, N., & Sharpe, R. M. (2003). Human 'testicular dysgenesis syndrome': A possible model using in-utero exposure of the rat to dibutyl phthalate. *Human Reproduction, 18*(7), 1383-1394.

Frederiksen, H., Skakkebæk, N. E., & Andersson, A. (2007). Metabolism of phthalates in humans. *Molecular Nutrition and Food Research, 51*(7), 899-911.

Fütterer, J. J., Heijmink, S. W. T. P. J., & Spermon, J. R. (2008). Imaging the male reproductive tract: Current trends and future directions. *Radiologic Clinics of North America, 46*(1), 133-147.

Garner, M., Turner, M. C., Ghadirian, P., Krewski, D., & Wade, M. (2008). Testicular cancer and hormonally active agents. *Journal of Toxicology and Environmental Health - Part B: Critical Reviews, 11*(3-4), 260-275.

Garner, M. J., Turner, M. C., Ghadirian, P., & Krewski, D. (2005). Epidemiology of testicular cancer: An overview. *International Journal of Cancer, 116*(3), 331-339.

Gerson, S. L. (2004). MGMT: Its role in cancer aetiology and cancer therapeutics. *Nature Reviews Cancer, 4*(4), 296-307.

Gilbert, D., Rapley, E., & Shipley, J. (2011). Testicular germ cell tumours: Predisposition genes and the male germ cell niche. *Nature Reviews Cancer, 11*(4), 278-288.

Gouws, C., & Pretorius, P. J. (2011). O6-methylguanine-DNA methyltransferase (MGMT): Can function explain a suicidal mechanism? *Medical Hypotheses, 77*(5), 857-860.

Gray, T. J. B., & Gangolli, S. D. (1986). Aspects of the testicular toxicity of phthalate esters. *Environmental Health Perspectives, VOL. 65*, 229-235.

Habert, R., Muczynski, V., Lehraiki, A., Lambrot, R., Lécureuil, C., Levacher, C., Rouiller-Fabre, V. (2009). Adverse effects of endocrine disruptors on the foetal testis development: Focus on the phthalates. *Folia Histochemica Et Cytobiologica, 47*(5), S67-S74.

Harries, L. W., Stubbins, M. J., Forman, D., Howard, G. C., & Wolf, C. R. (1997). Identification of genetic polymorphisms at the glutathione S-transferase pi locus and association with susceptibility to bladder, testicular and prostate cancer. *Carcinogenesis, 18*(4), 641-644.

Harvey, C. J., Thimmulappa, R. K., Singh, A., Blake, D. J., Ling, G., Wakabayashi, N., Biswal, S. (2009). Nrf2-regulated glutathione recycling independent of biosynthesis is critical for cell survival during oxidative stress. *Free Radical Biology and Medicine, 46*(4), 443-453.

Hauser, R., & Calafat, A. M. (2005). Phthalates and human health. *Occupational and Environmental Medicine, 62*(11), 806-818.

Hauser, R., Meeker, J. D., Duty, S., Silva, M. J., & Calafat, A. M. (2006). Altered semen quality in relation to urinary concentrations of phthalate monoester and oxidative metabolites. *Epidemiology, 17*(6), 682-691.

Hauser, R., Meeker, J. D., Singh, N. P., Silva, M. J., Ryan, L., Duty, S., & Calafat, A. M. (2007). DNA damage in human sperm is related to urinary levels of phthalate monoester and oxidative metabolites. *Human Reproduction (Oxford, England), 22*(3), 688-695.

Heemers, H. V., & Tindall, D. J. (2007). Androgen receptor (AR) coregulators: A diversity of functions converging on and regulating the AR transcriptional complex. *Endocrine Reviews, 28*(7), 778-808.

Heudorf, U., Mersch-Sundermann, V., & Angerer, J. (2007). Phthalates: Toxicology and exposure. *International Journal of Hygiene and Environmental Health, 210*(5), 623-634.

Honorio, S., Agathanggelou, A., Wernert, N., Rothe, M., Maher, E. R., & Latif, F. (2003). Frequent epigenetic inactivation of the RASSF1A tumour suppressor gene in testicular tumours and distinct methylation profiles of seminoma and nonseminoma testicular germ cell tumours. *Oncogene, 22*(3), 461-466.

Hooper, J. D., Bowen, N., Marshall, H., Cullen, L. M., Sood, R., Daniels, R., Antalis, T. M. (2000). Localization, expression and genomic structure of the gene encoding the human serine protease testisin. *Biochimica Et Biophysica Acta, 1492*(1), 63-71.

Hooper, J. D., Nicol, D. L., Dickinson, J. L., Eyre, H. J., Scarman, A. L., Normyle, J. F., Antalis, T. M. (1999). Testisin, a new human serine proteinase expressed by premeiotic testicular germ cells and lost in testicular germ cell tumors. *Cancer Research, 59*(13), 3199-3205.

Howdeshell, K. L., Furr, J., Lambright, C. R., Rider, C. V., Wilson, V. S., & Gray Jr., L. E. (2007). Cumulative effects of dibutyl phthalate and diethylhexyl phthalate on male rat reproductive tract development: Altered fetal steroid hormones and genes. *Toxicological Sciences, 99*(1), 190-202.

IARC Monographs on the Evaluation of Carcinogenic Risks to Humans / World Health Organization, International Agency for Research on Cancer (2000). *Some Industrial Chemicals, 77,* 41-148.

Itoh, K., Wakabayashi, N., Katoh, Y., Ishii, T., O'Connor, T., & Yamamoto, M. (2003). Keap1 regulates both cytoplasmic-nuclear shuttling and degradation of Nrf2 in response to electrophiles. *Genes to Cells, 8*(4), 379-391.

Jones, P. A., & Takai, D. (2001). The role of DNA methylation in mammalian epigenetics. *Science, 293*(5532), 1068-1070.

Kamrin, M. A. (2009). Phthalate risks, phthalate regulation, and public health: A review. *Journal of Toxicology and Environmental Health - Part B: Critical Reviews, 12*(2), 157-174.

Kasahara, E., Sato, E. F., Miyoshi, M., Konaka, R., Hiramoto, K., Sasaki, J., Inoue, M. (2002). Role of oxidative stress in germ cell apoptosis induced by di(2-ethylhexyl)phthalate. *Biochemical Journal, 365*(3), 849-856.

Kempkensteffen, C., Christoph, F., Weikert, S., Krause, H., Kollermann, J., Schostak, M., Schrader, M. (2006). Epigenetic silencing of the putative tumor suppressor gene testisin in testicular germ cell tumors. *Journal of Cancer Research and Clinical Oncology, 132*(12), 765-770. doi:10.1007/s00432-006-0124-6

Kensler, T. W., Wakabayashi, N., & Biswal, S. (2007). *Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway*

Khan, O., & Protheroe, A. (2007). Testis cancer. *Postgraduate Medical Journal, 83*(984), 624-632.

Kobayashi, A., Kang, M., Watai, Y., Tong, K. I., Shibata, T., Uchida, K., & Yamamoto, M. (2006). Oxidative and electrophilic stresses activate Nrf2 through inhibition of ubiquitination activity of Keap1. *Molecular and Cellular Biology, 26*(1), 221-229.

Koch, H. M., Bolt, H. M., & Angerer, J. (2004). Di(2-ethylhexyl)phthalate (DEHP) metabolites in human urine and serum after a single oral dose of deuterium-labelled DEHP. *Archives of Toxicology, 78*(3), 123-130.

Kraggerud, S. M., Oldenburg, J., Alnaes, G. I., Berg, M., Kristensen, V. N., Fossa, S. D., & Lothe, R. A. (2009). Functional glutathione S-transferase genotypes among testicular germ cell tumor survivors: Associations with primary and post-chemotherapy tumor histology. *Pharmacogenetics and Genomics, 19*(10), 751-759. doi:10.1097/FPC.0b013e3283304253

Kristensen, D. M., Sonne, S. B., Ottesen, A. M., Perrett, R. M., Nielsen, J. E., Almstrup, K., Meyts, E. R. -. (2008). Origin of pluripotent germ cell tumours: The role of

microenvironment during embryonic development. *Molecular and Cellular Endocrinology, 288*(1-2), 111-118.

Lambrot, R., Muczynski, V., Lécureuil, C., Angenard, G., Coffigny, H., Pairault, C., Rouiller-Fabre, V. (2009). Phthalates impair germ cell development in the human fetal testis in vitro without change in testosterone production. *Environmental Health Perspectives, 117*(1), 32-37.

Lapinskas, P. J., Brown, S., Leesnitzer, L. M., Blanchard, S., Swanson, C., Cattley, R. C., & Corton, J. C. (2005). Role of PPARalpha in mediating the effects of phthalates and metabolites in the liver. *Toxicology, 207*(1), 149-163. doi:10.1016/j.tox.2004.09.008

Latini, G., Verrotti, A., & De Felice, C. (2004). DI-2-ethylhexyl phthalate and endocrine disruption: A review. *Current Drug Targets: Immune, Endocrine and Metabolic Disorders, 4*(1), 37-40.

Lavon, I., Fuchs, D., Zrihan, D., Efroni, G., Zelikovitch, B., Fellig, Y., & Siegal, T. (2007). Novel mechanism whereby nuclear factor κB mediates DNA damage repair through regulation of O6-methylguanine-DNA-methyltransferase. *Cancer Research, 67*(18), 8952-8959.

Lee, J., & Johnson, J. A. (2004). An important role of Nrf2-ARE pathway in the cellular defense mechanism. *Journal of Biochemistry and Molecular Biology, 37*(2), 139-143.

Lehraiki, A., Racine, C., Krust, A., Habert, R., & Levacher, C. (2009). Phthalates impair germ cell number in the mouse fetal testis by an androgen- and estrogen-independent

mechanism.*Toxicological Sciences : An Official Journal of the Society of Toxicology, 111*(2), 372-382. doi:10.1093/toxsci/kfp153

Li, H., & Kim, K. H. (2003). Effects of mono-(2-ethylhexyl) phthalate on fetal and neonatal rat testis organ cultures. *Biology of Reproduction, 69*(6), 1964-1972.

Li, L., Jester Jr., W. F., & Orth, J. M. (1998). Effects of relatively low levels of mono-(2-ethylhexyl) phthalate on cocultured sertoli cells and gonocytes from neonatal rats. *Toxicology and Applied Pharmacology, 153*(2), 258-265.

Li, L., Jester W.F., J., Laslett, A. L., & Orth, J. M. (2000). A single dose of di-(2-ethylhexyl) phthalate in neonatal rats alters gonocytes, reduces sertoli cell proliferation, and decreases cyclin D2 expression. *Toxicology and Applied Pharmacology, 166*(3), 222-229.

Li, S., Hursting, S. D., Davis, B. J., McLachlan, J. A., & Barrett, J. C. (2003). *Environmental exposure, DNA methylation, and gene regulation: Lessons from diethylstilbesterol-induced cancers*

Lind, G. E., Skotheim, R. I., & Lothe, R. A. (2007). The epigenome of testicular germ cell tumors. *APMIS : Acta Pathologica, Microbiologica, Et Immunologica Scandinavica, 115*(10), 1147-1160. doi:10.1111/j.1600-0463.2007.apm_660.xml.x

Lo, H. W., & Ali-Osman, F. (2002). Cyclic AMP mediated GSTP1 gene activation in tumor cells involves the interaction of activated CREB-1 with the GSTP1 CRE: A novel mechanism of cellular GSTP1 gene regulation. *Journal of Cellular Biochemistry, 87*(1), 103-116. doi:10.1002/jcb.10275

Lo, H., Stephenson, L., Cao, X., Milas, M., Pollock, R., & Ali-Osman, F. (2008). Identification and functional characterization of the human glutathione S-transferase P1 gene as a novel transcriptional target of the p53 tumor suppressor gene. *Molecular Cancer Research, 6*(5), 843-850.

Luccio-Camelo, D. C., & Prins, G. S. (2011). Disruption of androgen receptor signaling in males by environmental chemicals. *Journal of Steroid Biochemistry and Molecular Biology,*

Lyche, J. L., Gutleb, A. C., Bergman, A., Eriksen, G. S., Murk, A. J., Ropstad, E., Skaare, J. U. (2009). Reproductive and developmental toxicity of phthalates. *Journal of Toxicology and Environmental Health.Part B, Critical Reviews, 12*(4), 225-249. doi:10.1080/10937400903094091

Main, K. M., Mortensen, G. K., Kaleva, M. M., Boisen, K. A., Damgaard, I. N., Chellakooty, M., Skakkebæk, N. E. (2006). Human breast milk contamination with phthalates and alterations of endogenous reproductive hormones in infants three months of age. *Environmental Health Perspectives, 114*(2), 270-276.

Maneesh, M., & Jayalekshmi, H. (2006). Role of reactive oxygen species and antioxidants on pathophysiology of male reproduction. *Indian Journal of Clinical Biochemistry, 21*(2), 80-89.

Mannervik, B., Alin, P., & Guthenberg, C. (1985). Identification of three classes of cytosolic glutathione transferase common to several mammalian species: Correlation between structural data and enzymatic properties. *Proceedings of the National Academy of Sciences of the United States of America, 82*(21), 7202-7206.

Manton, K. J., Douglas, M. L., Netzel-Arnett, S., Fitzpatrick, D. R., Nicol, D. L., Boyd, A. W., Antalis, T. M. (2005). Hypermethylation of the 5' CpG island of the gene encoding the serine protease testisin promotes its loss in testicular tumorigenesis. *British Journal of Cancer, 92*(4), 760-769. doi:10.1038/sj.bjc.6602373

Martino-Andrade, A. J., & Chahoud, I. (2010). Reproductive toxicity of phthalate esters. *Molecular Nutrition & Food Research, 54*(1), 148-157. doi:10.1002/mnfr.200800312

McGuinness, M. P., & Griswold, M. D. (1994). Interactions between sertoli cells and germ cells in the testis. *Seminars in Developmental Biology, 5*(1), 61-66.

McLellan, L. I., & Wolf, C. R. (1999). Glutathione and glutathione-dependent enzymes in cancer drug resistance. *Drug Resistance Updates, 2*(3), 153-164.

Moller, H. (1989). Decreased testicular cancer risk in men born in wartime. *Journal of the National Cancer Institute, 81*(21), 1668-1669.

Morceau, F., Duvoix, A., Delhalle, S., Schnekenburger, M., Dicato, M., & Diederich, M. (2004). Regulation of glutathione S-transferase P1-1 gene expression by NF-kappaB in tumor necrosis factor alpha-treated K562 leukemia cells. *Biochemical Pharmacology, 67*(7), 1227-1238.

Mostafa,RM., Mirghani, Z.; Moustafa ,K.M. , Moustafa ,Y.M., and El Hefnawi, M.H. (2007) New Chapter in old story : endocrine disruptors and male reproductive system Journal of Medical Sciences Research, 1 (2): 33-42

Mruk, D. D., & Cheng, C. Y. (2004). Sertoli-sertoli and sertoli-germ cell interactions and their significance in germ cell movement in the seminiferous epithelium during spermatogenesis. *Endocrine Reviews, 25*(5), 747-806.

Mruk, D. D., Silvestrini, B., & Cheng, C. Y. (2008). Anchoring junctions as drug targets: Role in contraceptive development. *Pharmacological Reviews, 60*(2), 146-180.

Müller, A., & Florek, M. (2010). 5-Azacytidine/Azacitidine. *Small Molecules in Oncology: Recent Results in Cancer Research, 184*,159-170.

Muller, J., Skakkebeak, N. E., Nielsen, O. H., & Graem, N. (1984). Cryptorchidism and testis cancer. atypical infantile germ cells followed by carcinoma in situ and invasive carcinoma in adulthood. *Cancer, 54*(4), 629-634.

Mylchreest, E., Cattley, R. C., & Foster, P. M. D. (1998). Male reproductive tract malformations in rats following gestational and lactational exposure to di(n-butyl) phthalate: An antiandrogenic mechanism? *Toxicological Sciences, 43*(1), 47-60.

Mylchreest, E., Sar, M., Cattley, R. C., & Foster, P. M. (1999). Disruption of androgen-regulated male reproductive development by di(n-butyl) phthalate during late gestation in rats is different from flutamide. *Toxicology and Applied Pharmacology, 156*(2), 81-95. doi:10.1006/taap.1999.8643

Nagel, G., Brenner, W., Johnsson, K., & Kaina, B. (2003). DNA repair protein O^6-methylguanine-DNA methyltransferase in testis and testicular tumors as determined by a novel nonradioactive assay. *Analytical Biochemistry, 321*(1), 38-43. doi:10.1016/S0003-2697(03)00432-9

Netzel-Arnett, S., Hooper, J. D., Szabo, R., Madison, E. L., Quigley, J. P., Bugge, T. H., & Antalis, T. M. (2003). Membrane anchored serine proteases: A rapidly expanding group of cell surface proteolytic enzymes with potential roles in cancer. *Cancer and Metastasis Reviews, 22*(2-3), 237-258.

Nguyen, T., Nioi, P., & Pickett, C. B. (2009). The Nrf2-antioxidant response element signaling pathway and its activation by oxidative stress. *Journal of Biological Chemistry, 284*(20), 13291-13295.

Olesen, I. A., Sonne, S. B., Hoei-Hansen, C. E., Rajpert-DeMeyts, E., & Skakkebaek, N. E. (2007). Environment, testicular dysgenesis and carcinoma in situ testis. *Best Practice and Research: Clinical Endocrinology and Metabolism, 21*(3), 462-478.

Özgen, N., Guo, J., Gertsberg, Z., Danilo Jr., P., Rosen, M. R., & Steinberg, S. F. (2009a). Reactive oxygen species decrease cAMP response element binding protein expression in cardiomyocytes via a protein kinase D1-dependent mechanism that does not require Ser133 phosphorylation. *Molecular Pharmacology, 76*(4), 896-902.

Özgen, N., Guo, J., Gertsberg, Z., Danilo Jr., P., Rosen, M. R., & Steinberg, S. F. (2009b). Reactive oxygen species decrease cAMP response element binding protein expression in cardiomyocytes via a protein kinase D1-dependent mechanism that does not require Ser133 phosphorylation. *Molecular Pharmacology, 76*(4), 896-902.

Pant, N., Pant, A. B., Shukla, M., Mathur, N., Gupta, Y. K., & Saxena, D. K. (2011). Environmental and experimental exposure of phthalate esters: The toxicological consequence on human sperm. *Human and Experimental Toxicology, 30*(6), 507-514.

Parks, L. G., Ostby, J. S., Lambright, C. R., Abbott, B. D., Klinefelter, G. R., Barlow, N. J., & Gray Jr., L. E. (2000). The plasticizer diethylhexyl phthalate induces malformations by decreasing fetal testosterone synthesis during sexual differentiation in the male rat. *Toxicological Sciences, 58*(2), 339-349.

Passagne, I., Evrard, A., Depeille, P., Cuq, P., Cupissol, D., & Vian, L. (2006). O6-methylguanine DNA-methyltransferase (MGMT) overexpression in melanoma cells induces resistance to nitrosoureas and temozolomide but sensitizes to mitomycin C. *Toxicology and Applied Pharmacology, 211*(2), 97-105.

Rahman, I., & MacNee, W. (2000). Oxidative stress and regulation of glutathione in lung inflammation. *European Respiratory Journal, 16*(3), 534-554.

Rajpert-De Meyts, E. (2006). Developmental model for the pathogenesis of testicular carcinoma in situ: Genetic and environmental aspects. *Human Reproduction Update, 12*(3), 303-323.

Rusyn, I., Peters, J., & Cunningham, M. (2006). Modes of action and species-specific effects of di-(2-ethylhexyl)phthalate in the liver. *Critical Reviews in Toxicology, 36*(5), 459-479.

Scarman, A. L., Hooper, J. D., Boucaut, K. J., Sit, M. L., Webb, G. C., Normyle, J. F., & Antalis, T. M. (2001). Organization and chromosomal localization of the murine testisin gene encoding a serine protease temporally expressed during spermatogenesis. *European Journal of Biochemistry / FEBS, 268*(5), 1250-1258.

Schettler, T., Skakkebæk, N. E., De Kretser, D., & Leffers, H. (2006). Human exposure to phthalates via consumer products. *International Journal of Andrology, 29*(1), 134-139.

Se, C. K., & Byung, M. L. (2005). DNA methylation of estrogen receptor α gene by phthalates. *Journal of Toxicology and Environmental Health - Part A, 68*(23-24), 1995-2003.

Seli, E., Mahutte, N. G., & Arici, A. (2002). The reproductive system from an immunologic perspective. *Immunology and Allergy Clinics of North America, 22*(3), 383-405.

Sharpe, R. M. (2001). Hormones and testis development and the possible adverse effects of environmental chemicals. *Toxicology Letters, 120*(1-3), 221-232.

Sikka, S. C. (2001). Relative impact of oxidative stress on male reproductive function. *Current Medicinal Chemistry, 8*(7), 851-862.

Silva, M. J., Reidy, J. A., Preau Jr., J. L., Samandar, E., Needham, L. L., & Calafat, A. M. (2006). Measurement of eight urinary metabolites of di(2-ethylhexyl) phthalate as biomarkers for human exposure assessment. *Biomarkers, 11*(1), 1-13.

Silva, M. J., Samandar, E., Reidy, J. A., Hauser, R., Needham, L. L., & Calafat, A. M. (2007). Metabolite profiles of di-n-butyl phthalate in humans and rats. *Environmental Science and Technology, 41*(21), 7576-7580.

Skakkebaek, N. E., Berthelsen, J. G., Giwercman, A., & Muller, J. (1987). Carcinoma-in-situ of the testis: Possible origin from gonocytes and precursor of all types of germ cell tumours except spermatocytoma. *International Journal of Andrology, 10*(1 SPEC.), 19-28.

Skakkebaek, N. E., Holm, M., Hoei-Hansen, C., Jorgensen, N., & Rajpert-De Meyts, E. (2003). Association between testicular dysgenesis syndrome (TDS) and testicular neoplasia:

Evidence from 20 adult patients with signs of maldevelopment of the testis. *APMIS : Acta Pathologica, Microbiologica, Et Immunologica Scandinavica, 111*(1), 1-9; discussion 9-11.

Slonchak, A. M., Chwieduk, A., Rzeszowska-Wolny, J., & Yu Obolenska, M. (2009). Transcription regulation in differential expression of the human GSTP1 gene in breast and choriocarcinoma cells. *Ukrainskii Biokhimicheskii Zhurnal, 81*(4), 48-58.

Smith-Sorensen, B., Lind, G. E., Skotheim, R. I., Fossa, S. D., Fodstad, O., Stenwig, A. E., Lothe, R. A. (2002). Frequent promoter hypermethylation of the O6-methylguanine-DNA methyltransferase (MGMT) gene in testicular cancer. *Oncogene, 21*(57), 8878-8884. doi:10.1038/sj.onc.1205978

Sonne, S. B., Kristensen, D. M., Novotny, G. W., Olesen, I. A., Nielsen, J. E., Skakkebæk, N. E., Leffers, H. (2008). Testicular dysgenesis syndrome and the origin of carcinoma in situ testis. *International Journal of Andrology, 31*(2), 275-287.

Syng-Ai, C., Kumari, A. L., & Khar, A. (2004). Effect of curcumin on normal and tumor cells: Role of glutathione and bcl-2. *Molecular Cancer Therapeutics, 3*(9), 1101-1108.

Taylor, S. S., Buechler, J. A., & Yonemoto, W. (1990). cAMP-dependent protein kinase: Framework for a diverse family of regulatory enzymes. *Annual Review of Biochemistry, 59*, 971-1105.

Wilson, V. S., Lambright, C., Furr, J., Ostby, J., Wood, C., Held, G., & Gray Jr., L. E. (2004). Phthalate ester-induced gubernacular lesions are associated with reduced insl3 gene expression in the fetal rat testis. *Toxicology Letters, 146*(3), 207-215.

Winter, C., & Albers, P. (2011). Testicular germ cell tumors: Pathogenesis, diagnosis and treatment. *Nature Reviews Endocrinology, 7*(1), 43-53.

Wittassek, M., Koch, H. M., Angerer, J., & Bruning, T. (2011). Assessing exposure to phthalates - the human biomonitoring approach. *Molecular Nutrition & Food Research, 55*(1), 7-31. doi:10.1002/mnfr.201000121; 10.1002/mnfr.201000121

Wu, F. C. W. (1996). Male contraception. *Bailliere's Clinical Obstetrics and Gynaecology, 10*(1), 1-23.

Wu, S., Zhu, J., Li, Y., Lin, T., Gan, L., Yuan, X., Wei, G. (2010). Dynamic epigenetic changes involved in testicular toxicity induced by di-2-(ethylhexyl) phthalate in mice. *Basic & Clinical Pharmacology & Toxicology, 106*(2), 118-123. doi:10.1111/j.1742-7843.2009.00483.x

APPENDIX

The effect of MEHP on cell viability and gene expression of Testisin, GSTP1, and MGMT in NT2 cells following qRT-PCR. Independent t-test and One-Way Anova was performed to determine statistical significance between the control and each of the doses at each time point and between the time points at each dose. Two-Way Anova was performed to determine statistical significance of all time points and doses. Significant p-values (<0.05) are listed.

Cell Viability: MTT Assay

Raw data

	24hr		48hr		72hr		96hr	
	Mean	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**
Control	1.485	0.123	1.587	0.140	1.901	0.288	1.950	0.402
10nM	1.495	0.084	1.478	0.133	1.805	0.297	1.663	0.321
1uM	1.503	0.121	1.532	0.083	1.660	0.178	1.650	0.194
10uM	1.369	0.113	1.530	0.082	1.582	0.141	1.506	0.130
100uM	1.171	0.070	1.276	0.152	1.561	0.315	1.328	0.323
1mM	0.439	0.066	0.333	0.059	0.359	0.122	0.189	0.089

Independent samples t- test

P-values:

	24hr	48hr	72hr	96hr
10nM	0.948	0.600	0.828	0.609
1uM	0.920	0.754	0.522	0.551
10uM	0.527	0.748	0.396	0.388
100uM	0.109	0.207	0.469	0.298
1mM	0.005	0.006	0.021	0.013

Significance between control and each of the doses of MEHP.

	Time (hr)					
	24 vs 48	**24 vs 72**	**24 vs 96**	**48 vs 72**	**48 vs 96**	**72 vs 96**
Control	0.612	0.284	0.368	0.401	0.469	0.927
10nM	0.919	0.408	0.656	0.393	0.634	0.762
1uM	0.855	0.511	0.562	0.563	0.620	0.971
10uM	0.317	0.306	0.470	0.769	0.884	0.712
100uM	0.581	0.341	0.679	0.476	0.893	0.633
1mM	0.300	0.603	0.094	0.862	0.260	0.330

Significance between time points for each dose of MEHP.

One-Way Anova

	24hr	**48hr**	**72hr**	**96hr**
P-values	P<0.0001	P<0.0001	0.0065	0.0084
F-values	17.44	17.91	5.673	5.315

Significance across all doses of MEHP for each time point.

	Control	**10nM**	**1uM**	**10uM**	**100uM**	**1mM**
P-values	0.5497	0.7283	0.8355	0.6384	0.7125	0.3086
F-values	0.7553	0.4437	0.2843	0.5904	0.4684	1.413

Significance across all time points for each dose of MEHP.

Two-Way Anova

P-values	0.987
F-values	0.343

Significance across all time points and doses of MEHP.

Quantitative Real-time PCR

Testisin

Raw data

	24hr		**48hr**		**72hr**		**96hr**	
	Mean	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**
Control	1.099	0.182	1.095	0.128	0.941	0.077	1.440	0.510
1uM	0.921	0.124	1.103	0.051	0.761	0.091	0.876	0.031
10uM	0.937	0.162	0.946	0.180	1.073	0.318	0.812	0.206
100uM	0.656	0.219	0.365	0.095	0.642	0.158	0.501	0.148

Independent samples t-test

P-values:

	24hr	48hr	72hr	96hr
1uM	0.47	0.962	0.206	0.33
10uM	0.543	0.541	0.722	0.345
100uM	0.198	0.012	0.19	0.199

Significance between control and each of the doses of MEHP.

	Time (hr)					
	24 vs 48	**24 vs 72**	**24 vs 96**	**48 vs 72**	**48 vs 96**	**72 vs 96**
Control	0.987	0.487	0.579	0.371	0.570	0.385
1uM	0.281	0.361	0.755	0.043	0.028	0.332
10uM	0.973	0.730	0.657	0.751	0.649	0.534
100uM	0.318	0.961	0.601	0.221	0.512	0.563

Significance between time points for each dose of MEHP.

One-Way Anova

	24hr	48hr	72hr	96hr
P-values	0.4061	0.0084	0.4262	0.2124
F-values	1.093	8.049	1.039	1.874

Significance across all doses of MEHP for each time point.

	Control	1uM	10uM	100uM
P-values	0.6497	0.0978	0.8761	0.5795
F-values	0.5711	2.958	0.2254	0.697

Significance across all time points for each dose of MEHP.

Two-Way Anova

P-values	0.654
F-values	0.759

Significance across all time points and doses of MEHP.

Raw data

5-azacytidine and co-exposure with 100uM MEHP

	96hr			
	5-aza		Co-exposure	
	Mean	SEM	Mean	SEM
Control	0.001005	0.00065	0.727	0.0041
2uM	0.00159	7.3E-05	0.875	0.00404
4uM	0.003375	6.1E-05	1.402	0.00058
10uM	0.002285	6.9E-05	1.232	0.03256

Independent samples t-test

P-values:

	5-aza	Co-exposure
2uM	0.541	0.002
4uM	0.097	0
10uM	0.251	0.006

Significance between control and each of the doses of 5-azacytidine

One-Way Anova

	5-aza	Co- Exposure
P-values	0.094	0.050
F-values	3.947	6.017

Significance across all doses of 5-aza and co-exposure at 96hr time point.

MGMT

Raw data

	24hr		48hr		72hr		96hr	
	Mean	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**
Control	1.498	0.206	1.065	0.158	1.208	0.215	1.209	0.152
1uM	0.837	0.151	0.989	0.175	1.047	0.089	0.751	0.191
10uM	0.788	0.024	0.709	0.099	1.045	0.136	0.570	0.088
100uM	0.898	0.134	0.930	0.277	0.676	0.023	0.519	0.103

Independent samples t-test

P-values:

	24hr	48hr	72hr	96hr
1uM	0.066	0.764	0.544	0.137
10uM	0.027	0.143	0.562	0.032
100uM	0.081	0.7	0.13	0.025

Significance between control and each of the doses of MEHP.

	Time (hr)					
	24 vs 48	**24 vs 72**	**24 vs 96**	**48 vs 72**	**48 vs 96**	**72 vs 96**
Control	0.176	0.386	0.328	0.623	0.548	0.997
1uM	0.548	0.311	0.741	0.786	0.409	0.258
10uM	0.513	0.135	0.124	0.122	0.355	0.051
100uM	0.922	0.238	0.093	0.412	0.274	0.213

Significance between time points for each dose of MEHP.

One-Way Anova

	24hr	48hr	72hr	96hr
P-values	0.0271	0.6002	0.1097	0.0297
F-values	5.248	0.6584	2.786	5.061

Significance across all doses of MEHP for each time point.

	Control	1uM	10uM	100uM
P-values	0.4556	0.5469	0.0427	0.3065
F-values	0.9638	0.7609	4.352	1.421

Significance across all time points for each dose of MEHP.

Two-Way Anova

P-values	0.381
F-values	1.115

Significance across all time points and doses of MEHP.

GSTP1

Raw data

	24hr		48hr		72hr		96hr	
	Mean	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**
Control	1.350	0.171	1.274	0.372	0.996	0.129	1.288	0.296
1uM	1.005	0.015	0.958	0.067	1.027	0.080	1.021	0.038
10uM	0.948	0.079	0.954	0.073	0.899	0.157	0.905	0.129
100uM	1.151	0.044	1.042	0.045	1.104	0.076	1.024	0.060

Independent samples t-test

P-values

	24hr	48hr	72hr	96hr
1uM	0.115	0.450	0.848	0.421
10uM	0.128	0.447	0.658	0.363
100uM	0.322	0.570	0.518	0.431

Significance between control and each of the doses of MEHP.

	Time (hr)					
	24 vs 48	**24 vs 72**	**24 vs 96**	**48 vs 72**	**48 vs 96**	**72 vs 96**
Control	0.864	0.179	0.866	0.541	0.977	0.438
1uM	0.557	0.808	0.725	0.543	0.469	0.946
10uM	0.954	0.798	0.870	0.770	0.850	0.983
100uM	0.156	0.625	0.168	0.527	0.824	0.456

Significance between time points for each dose of MEHP.

One-Way Anova

	24hr	48hr	72hr	96hr
P-values	0.0724	0.633	0.6667	0.5571
F-values	3.432	0.5998	0.5425	0.7404

Significance across all doses of MEHP for each time point.

	Control	1uM	10uM	100uM
P-values	0.7796	0.815	0.9892	0.4297
F-values	0.366	0.314	0.03833	1.029

Significance across all time points for each dose of MEHP.

Two-Way Anova

P-values	0.965
F-values	0.312

Significance across all time points and doses of MEHP.