In Vitro Effects of Bisphenol A on Prostate Cells: Searching for Clues of Environmental Carcinogenesis

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ABSTRACT

Estrogens maintain the appropriate androgen-estrogen balance for normal regulation of the structure and function of the male reproductive tract, including the prostate gland. This research investigated viability of cells and expression of selected genes in prostate carcinoma cells (PC-3) exposed to bisphenol A (BPA), an estrogen-like substance present in a number of plastic materials. PC-3 cells are able to metabolize BPA at concentrations below 100 μM. BPA exposure at concentrations between 1nM and 100 μM does not increase or significantly reduce cell viability of these cells. Although the genes investigated in this study (GSTP1 and MGMT) did not show a significant change in expression following *in vitro* exposure to BPA, the positive control ethinyl estradiol (EE2) caused an increase in GSTP1 expression at mRNA level. These results indicate that BPA does not affect the viability of prostate cells, and motivate a need for further research to identify other genes that could be affected by BPA.

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Acronym	Definition	Acronym	Definition
ADT	androgen deprivation therapy	UGE	urogenital sinus epithelium
AR	androgen receptor	UGM	urogenital sinus mesenchyme
BPA	bisphenol A	UGS	urogenital sinus
BPH	benign prostatic hyperplasia		
DHT	dihydrotestosterone		
EE2	17-α-ethinyl estradiol		
ER-alpha	estrogen eeceptor-alpha		
ER-beta	estrogen receptor-beta		
EtOH	ethanol		
DES	diethylstilbestrol		
GST	glutathione –S-transferase		
GSTP1	glutathione S-transferase		
MGMT	O^6-methylguanine DNA methyltransferase		
OCs	oral contraceptives		
PCa	prostate cancer		
PIA	proliferative inflammatory atrophy		
PIN	prostatic intraepithelial neoplasia		
PSA	prostate specific antigen		
PBS	phosphate buffered saline		

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INTRODUCTION

1. PROSTATE CANCER

Prostate cancer is a leading cause of death in men in the Western World. The past two decades have witnessed an increase in the reported incidence of prostate cancer, due to widespread use of prostate specific antigen (PSA) as a screening tool for the early detection of prostate cancer. Although race, age and family history of prostate cancer (PCa) are recognized as risk factors for the disease, the potential role of genetic and environmental risk factors in the etiology of prostate cancer remain to be explored (Ruth and Leibovich, 2005; Wigle et al., 2008).

The natural history of PCa has not been fully elucidated, however it is believed to arise from mutations in prostate epithelium that result in development of malignancy over a period of years (Rosenberg et al, 2010). Prostate cancer is known to be a hormone-dependent cancer. Huggins and Hodges (1941) reported that the growth of prostate cancer is influenced by androgens. The observation that castration is an effective treatment for prostate cancer led to development of androgen deprivation therapy (ADT) which has remained a standard treatment for advanced prostate cancer for over 60 years. Unfortunately, in many cases ADT is very often ineffective, and the initial response to treatment is often followed by relapse of the disease to hormone refractory PCa (Lucas and Petrylak, 2006). The 1990s brought more insight into the importance of genetic alterations in the induction of human cancer including PCa. Initiation of human cancers was explained in terms of accumulation of multiple mutations that inactivate tumor suppressor genes and activate proto-oncogenes. Research in hormonal carcinogenesis and genetics provided considerable insight into the etiology of PCa but failed to describe it as a distinct disease. Schulz et al. (2003) concluded that the biological mechanism underlying the

development of prostate cancer cannot be understood simply by applying knowledge of the mechanisms of other types of cancer. Therefore, there is a need for the development of a biological model of carcinogenesis specific to prostate carcinoma.

1.3 Prostate gland and hormones

Development of prostate gland in humans is largely complete by the 4th month of gestation and its maturation reinitiates and continuous at puberty. The prostate develops from the urogenital sinus (UGS), part of the caudal terminus located in the hindgut named the cloaca. The male and female UGS have the same morphology until about 10-12 weeks of fetal growth, at which time prostate morphogenesis begins. The first step in its development is the outgrowth of five solid buds of urogenital sinus epithelium (UGE) within the urogenital sinus mesenchyme (UGM) that encloses it. The UGE buds undergo active branching to form a lobular arrangement of tubuloalveolar glands surrounded by urethra and ejaculatory ducts. The lobular subdivision of the prostate is then established, with inner and outer zones of the mature prostate.

Testosterone is essential to the development of the prostate from the urogenital sinus (UGS). Dihydrotestosterone (5α-DHT) is the biologically active androgen in the prostate, with a higher affinity for the androgen receptor (AR) relative to testosterone. Androgens bind to the AR located in the UGM to control the process of branching morphogenesis, proliferation, and cell differentiation (Cowin et al, 2007). Humans and rodents without functioning ARs do not develop a prostate gland, a fact which further underlines the importance of androgens and receptor ligand binding in prostate growth and function. At puberty, the prostate undergoes rapid growth, which correlates with levels of serum testosterone increasing to adult levels (Cowin et al, 2007).

With the stabilization of androgen levels in mature adults, prostate size remains constant until the fourth decade of life when growth is reinitiated (Cowin et al, 2007). Androgens maintain proliferation and survival of the secretory epithelial cells by counterbalancing the cell death rates of 1-2% per day. The secretory epithelial cells show high AR expression, whereas basal epithelial cells do not express AR. Appropriate androgen levels are needed to maintain normal epithelial function and prevent epithelial apoptosis (Heinlein and Chang, 2004).

Prostate development is also highly sensitive to estrogens. An appropriate androgen-estrogen balance is required for normal regulation of the structure and function of male reproductive tract, including the prostate (Cowin et al, 2007). Estrogens interfere with androgen production by suppressing the hypothalamic-pituitary-gonadal axis. Neonatal treatment of rodents with estrogen causes adverse effects in prostate development and function later in life (Harkonen and Makala, 2004).

1.4 Natural history of prostate cancer

Inflammation, infection, and exposure to carcinogens are suspected to lead to the formation of proliferative inflammatory atrophy (PIA), thought to be a precursor to PCa. Prostatic intraepithelial neoplasia (PIN) characterized by accumulation of epithelial cells and thickening of the epithelial layer is considered to be a more defined precursor of prostate cancer. High grade prostatic intraepithelial neoplasia is recognized as an intermediate step to PCa as compared to low-grade PIN. The majority of PCa develop in the peripheral zone of the prostate gland where PIA and PIN are often found. (Rosenberg et al, 2010; Miller and Torkko, 2001) There has been a long debate as to whether or not benign prostatic hyperplasia (BPH), a nodular disease

composed of epithelial and stromal components, is related to PCa. Considering the fact that the majority of PCa develop in the peripheral zone, the association of benign prostatic hyperplasia-prone transition zone with PCa seems unlikely (Miller and Torkko, 2001).

As mentioned above, DHT is the main prostatic androgen capable of stimulating prostatic growth at higher rate than testosterone. Expression of 5α-reductase, a key component of DHT production, is highly elevated in PCa (PIN, localized, recurrent, and metastatic) when compared with BPH. It is believed that elevated levels of DHT play a role in PCa initiation. The role of DHT in PCa development suggests that inhibition of 5α-reductase activity may reduce the risk of PCa. 5α-reductase inhibitors are currently being investigated as potential therapeutic agents for inhibition of DHT production in prostate disease. These agents show promising results, reducing circulating and intraprostatic DHT levels by 70 % and 68-86% respectively. Although, the therapy bypasses the adverse effects of reduced testosterone levels common in patients undergoing standard androgen deprivation therapy, its use in PCa treatment is not yet accepted as safe for patients (Rosenberg et al, 2010).

It is important to emphasize that androgens alone do not promote prostate carcinogenesis; rather, androgens and the functional status of AR act together in prostate cancer progression. Men with newly diagnosed prostate cancer have low serum testosterone levels, but higher AR expression, increased capillary vessel density, and higher Gleason score. AR activity is often blocked as a therapy of choice by administration of antiandrogens. Although this therapy eventually fails in a manner similar to ADT, its initial positive effects suggest that the AR is an important modulator of prostate cancer growth and survival. To further complicate matters studies in human suggest that increased AR expression is not associated with prostate cancer initiation (Heinlein and Chang, 2004).

Male hormone levels change throughout adulthood. Most men over 50 years old experience a decline in androgen levels, a phenomenom referred to as “androgen decline in ageing males” (ADAM) or “andropause”. On the other hand, estrogen levels remain constant resulting in a decrease in the testosterone-estrogen ratio (Cowin et al, 2007). The change in the ratio of serum steroid concentrations has been suggested to stimulate progression of prostate cancer. Although there is no consensus on this topic, there is evidence of correlation between an increased estrogen to androgen ratio and increased incidence of prostate cancer. In addition, studies show that exposure to estrogens can cause changes in prostate development, functional impairment, and malignancies (Harkonen and Makala, 2004).

2. XENOESTROGENS

Xenoestrogens are environmental agents such as pesticides or synthetic industrial chemicals that have a structure similar to estradiol and have the ability to act as estrogen agonists or antagonists of estrogens. The mechanism of action of xenoestrogens and the manifestation of toxicity in animals and humans remains to be confirmed and is being debated. Studies show that xenoestrogens could be linked to altered hormonal regulation and cancer initiation in humans and wildlife. In this study, we have considered one specific xenoestrogen, Bisphenol A, an industrial product used in production of polycarbonate and epoxy resins, in more detail.

2.1 BISPHENOL A

2.1.1 BISPHENOL A- CHEMISTRY, USE AND HUMAN EXPOSURE

As illustrated in Figure 1, bisphenol A (BPA), 2,2-bis(4-hydroxyphenyl)propane is an organic compound composed of two phenol rings connected by a methyl bridge with two methyl functional groups attached to the bridge (Kang, Kondo, & Katayama, 2006). BPA is used in the manufacture of phenol resins, polyacrylates, and polyesters, but most often in the production of polycarbonate plastics and epoxy resins. Polycarbonate plastics have variety of applications in products such as food packaging, plastic bottles, automobile parts, household appliances, medical tubing, and toys due to their high impact strength, hardness, toughness, transparency, resistance to temperatures between about −40 °C and about 145 °C, and resistance to many acids and oils. Epoxy resins are used as coatings for cans, jar lids, protective coatings and finishes, automobile parts, adhesives, and aerospace products. BPA is also used in resin-based dental sealants and bonding agents (Kang et al, 2006; Welshons et al, 2006).

BPA can be found in wastewater from factories that produce it because of its incomplete removal during wastewater treatment. Wastewater containing BPA from water treatment plants finds its way into rivers, leading to contamination of the aquatic environment. The aquatic environment is also contaminated by BPA leaching from landfills. BPA in fresh water can be degraded in aerobic conditions by bacteria such as *Pseudomonas* sp and *Streptomyces* sp., although this biodegradation is insufficient to prevent adverse effects of BPA on aquatic species. Seafood has been found to be highly contaminated with BPA due to the longer persistence of BPA in seawater than in fresh water. Although inhalation of high levels of BPA is difficult, workers involved in production of BPA were found to have higher BPA concentrations in urine

than other workers. BPA released to ground or surface waters is readily absorbed by soil and sediments, representing another medium where BPA levels are much higher than those in surface waters (Kang et al, 2006).

Routes and levels of human exposure to BPA are not well characterized. It is assumed that the main sources of human exposure to BPA involve hydrolysis of BPA molecules. This process is accelerated by increased temperatures and contact with acidic or basic substances (see Fig 1) during washing, sterilization or general wear and use of cans and polycarbonate containers (Welshons et al, 2006). Used polycarbonate containers have higher migration of BPA than new ones due to gradual degradation of the polymer. Migration of BPA from the surface of cans takes place during heating used in manufacturing processes (Kang et al, 2006).

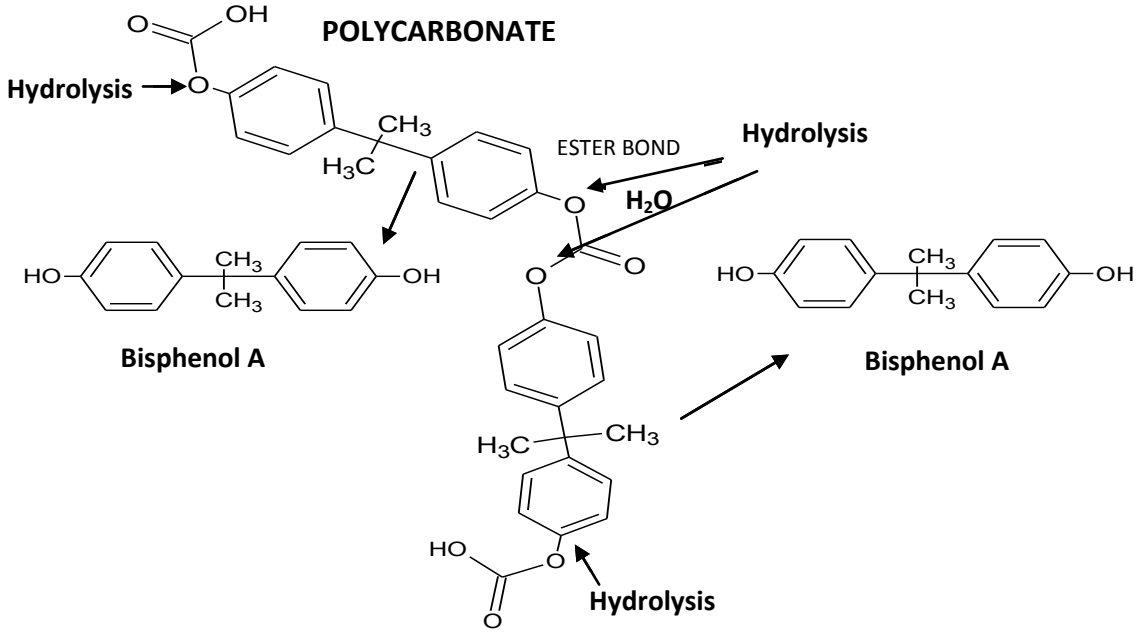


Fig. 1. Hydrolysis of the ester bond linking BPA molecules to form polycarbonate plastics (Welshons et al, 2006).

Analysis of human urine samples is used to evaluate the magnitude of human exposure to bisphenol A. Many studies have shown adult BPA levels in urine samples in the nanomolar range, with virtually all subjects examined demonstrating detectable levels of BPA or its metabolites (Welshons et al, 2006). Higher levels of BPA in urine tend to correlate with the consumption of canned foods. These results suggest that although the environment is one source of BPA exposure the main source may be foods (Kang et al, 2006).

2.1.2 GENERAL TOXICOLOGY AND BIOLOGICAL EFFECTS OF BISPHENOL A

2.1.2.1 Biological effects of bisphenol A

The estrogenic activity of BPA was first observed by Krishnan et al (1993) who demonstrated that leaching of BPA from autoclaved polycarbonate flasks increased the rate of proliferation of MCF-7 breast cancer cells. It has been reported that BPA has weak estrogenic activity of approximately 1/1000-1/10000 fold less than 17-β estradiol, and interacts with estrogen receptor alpha (ERα) and beta (ERβ) (Miyakoshi et al, 2008). Exposure to BPA at nanomolar concentrations can also initiate proliferation in androgen dependent human prostatic adenocarcinoma (LNCaP) cells; this stimulatory effect is mediated through interaction of BPA with a mutant tumour derived androgen receptor called AR-TA77A (Wetherill, 2002).

Although BPA-gluc, the major metabolite of BPA, does not show estrogenic activity, there is evidence that some metabolites of BPA can be 250-fold more potent than the parent BPA itself. Yoshihara et al (2004) identified the structure of a BPA metabolite, 4-methyl-2,4-bis(*p*-

hydroxyphenyl)pent-1-ene, formed in rat liver S9 fractions. The estrogenic activity of this metabolite was several fold to several thousand fold greater than that of BPA itself. Although the investigators were not certain what could cause this high potency, they suggest that the structural similarity of this metabolite to estradiol could be important in this regard (Yoshihara et al, 2004).

Developmental exposure to low doses of BPA has been reported to alter prostate growth as reflected by increasing prostate size or weight. The effects of BPA are somewhat comparable to diethylstilbestrol (DES), increasing the size and number of dorsolateral ducts with enhanced proliferation in the basal epithelia. These findings suggest that BPA can influence growth of the prostate gland on its own without the requirement for androgen stimulation (Hess-Wilson and Knudsen, 2006). Dolinoy et al. (2007) showed that maternal exposure to BPA modified methylation of the metastable loci A^{vy} and $Capb^{IAP}$ in rats, changing the offspring coat color. This result suggests that the epigenome is sensitive to BPA exposure, and can lead to permanent phenotypic effects on offspring (Dolinoy et al, 2007).

Studies show that BPA can disrupt the function of hepatic cytochrome P450s in rat, pig and humans by inhibition of activities at high micro-molar concentrations. For example, exposure of rats to BPA at 100 µM caused inhibition of testosterone 16ß-hydroxylase and testosterone 2α-hydroxylase activities (69% and 74% respectively). Treatment of rats with BPA was also found to suppress UDP-glucuronosyltransferase activities disrupting the metabolism of BPA and testosterone as well as estradiol. These findings suggest that BPA can affect the metabolism of hormones and consequently disrupt endocrine balance (Welshons, 2006).

BPA can interfere with steroid synthesis. Huang and Leung (2009) demonstrated that BPA suppresses CYP19 mRNA expression in the placental JEG-3 cells acting at the protein and

gene expression levels. This provides evidence of endocrine disruption where aromatase, responsible for the synthesis of estradiol, estriol, and estrone from androgenic steroidal compounds, is inhibited. Enzyme inhibition at the protein level can be temporary and reversible, but alterations in gene expression could have long term effects on estrogen levels and androgen secretion (Huang and Leung et al, 2009).

Atkinson and Roy (1995a, b) found that BPA metabolites can have a negative effect on DNA integrity through the induction of DNA adducts. The BPA metabolite 3-hydroxybisphenol A (3-OH-BPA) is oxidized to bisphenol-*o*-quinone in rat hepatic microsomal suspension (Figure 2). Their study showed that bisphenol-*o*-quinone (BPAQ) could react with DNA and form DNA adducts *in vitro* and *in vivo*. They suggested that covalent modifications in DNA by in vivo exposure to BPA may be a factor in the induction of hepatotoxicity. Edmonds et al (2004) also have shown the formation of stable adducts between BPA -3,4-quinone and deoxyguanosine (dG) using the method of Atkinson and Roy and crystallography (see Figure 3). The adduct of BPAQ and guanine was described as BPAQ-N7Gua based on their ^{1}H and ^{13}C NMR spectra. This is the first study in which the chemical nature of the adduct was determined. These results provide evidence that BPA, through metabolic activation, could be as effective as estrogens in the generation of ortho-quinones that can covalently bind to DNA (Edmonds et al, 2004).

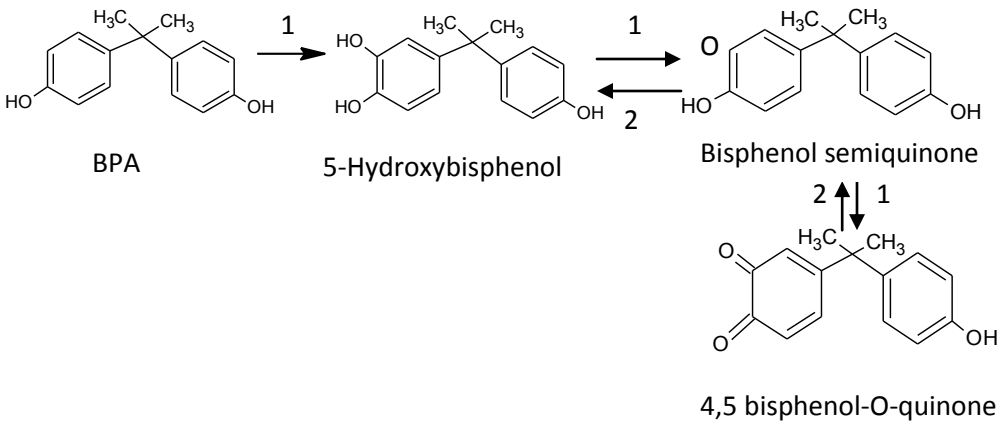


Figure 2: Conversion of BPA to hydroxylated BPA and then to bisphenol o-quinone. 1. Cytochromes P450 or peroxidases; 2. Cytochromes P450 reductase (Atkinson and Roy, 1995)

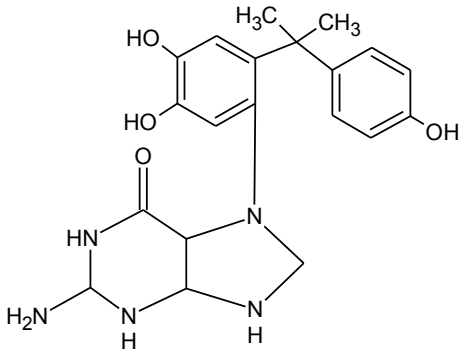


Figure 3: BPAQ-N7Gua (Edmonds et al, 2004)

BPA has the ability to adversely influence the prostate gland, the organ of interest in this thesis. Previous studies have shown that prenatal exposure to BPA as well as other estrogenic compounds leads to altered development of hormonally targeted organs. Fetal exposure to BPA leads to increased prostate weight in adulthood, decreased sperm production, and early puberty based on in vivo studies in mice showing effects identical to those of estradiol, ethinyl estradiol, and DES (Gupta, 2000; vom Saal, 1997). It is still unclear whether prostate size change is associated with increased cancer susceptibility. Some studies in which histopathology of

enlarged prostates have been evaluated have failed to demonstrate a link with development of prostate cancer in rodents (Keri et al, 2007). However, some rat models do show association between neonatal exposure to BPA and increased tumour susceptibility. Ho and colleagues (2006) have shown that post-natal BPA injections at days 1, 3 and 5 and subsequent chronic exposure to BPA and testosterone for 16 weeks starting at 90 days of age, causes a significant increase in the incidence of PIN. Their data provide evidence that early exposure to BPA has the ability to alter genes, which increases sensitivity of the prostate gland to BPA and other estrogenic compounds later in life.

The pathway involved in this process appears to involve phosphodiesterase type 4 variant 4 (PDE4D4), an enzyme involved in regulating the levels of cellular cAMP. The gene is normally silenced through methylation with age but early exposure to BPA could alter this process causing upregulation of this gene in adult animals (Ho et al., 2006). The alteration in the activity of this gene could play a role in the early stages of carcinogenesis. The conflicting results from different studies may be explained by the different age of BPA exposure (fetal versus neonatal), strain of rats, and dosage. These significant differences underscore the need for more studies to evaluate the ability of BPA to cause drastic changes in prostate tissue leading to carcinogenicity.

As noted previously, BPA stimulation of prostate cancer cells with the AR-T877A mutation can impact therapeutic response in patients undergoing androgen deprivation therapy (ADT). BPA exposure can inappropriately activate AR in tumours in patients treated with ADT and induce AR activity in untreated patients (Hess-Wilson and Knudsen, 2006). Therefore, BPA

decreases the therapeutic potential of ADT and enhances the morbidity associated with prostate cancer.

The biological effects of BPA documented above span a broad spectrum, ranging from estrogenic activity in MCF-7 breast cancer cells to DNA adduct formation in rats. There is increasing evidence that BPA has the potential to not only disrupt the endocrine balance by affecting hormone synthesis and metabolism, but also to play a role in the induction of cancer. Although BPA is found to be a weak estrogen having little effect on ER-mediated cell proliferation, it is possible that through metabolic activation and oxidation, BPA can be effective as an *ortho*-quinone that can bind covalently to DNA and disrupt its integrity. In the prostate, early life exposure to BPA can predispose to pre-neoplastic lesions in adult life. In patients with advanced prostate cancer and androgen receptor mutation, BPA can cause early recurrence of this disease and stimulate tumour progression.

2.1.2.2 Biotransformation of BPA

BPA undergoes first pass metabolism and is rapidly converted to BPA-gluc in humans and rats (Figure 4). In humans, BPA conjugation with glucuronic acid in the presence of glucoronyltransferase takes place in the liver to form glucuronide, which is rapidly cleared from blood by the kidneys and eliminated in urine. In rats the elimination is delayed because glucoronide is transported through the bile into the feces and might undergo enterohepatic circulation (Volkel et al., 2002). Other metabolic differences between species could include glucoronidation of both BPA and hydroxylated BPA (Zalko et al., 2003). Taylor et al (2010)

concluded, based on their study, that the rodent is a valid model for investigating conjugated and unconjugated levels of BPA in humans.

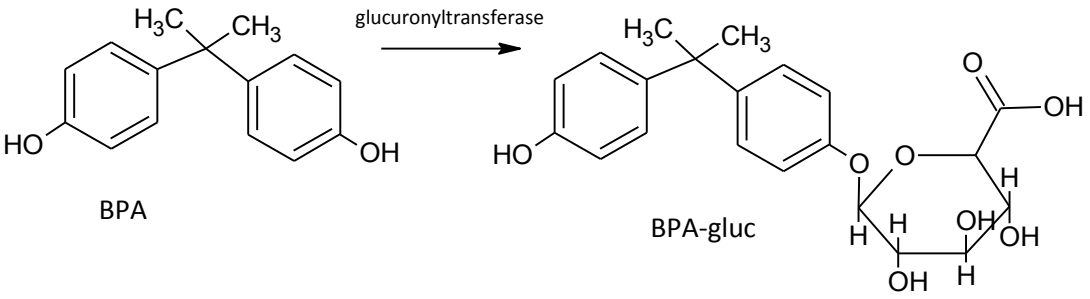


Fig. 4. Biotransformation of BPA in humans (Volkel et al., 2002).

The comforting results from Volkel's group of complete elimination of BPA from humans are questionable, because the assay used in their study was not sensitive enough to detect unconjugated metabolites; only the conjugated levels of BPA have been reported. Nevertheless, their findings have been widely cited in the literature and used as a guideline for BPA clearance from blood in humans (Taylor et al, 2010). A number of other studies have detected free BPA by a variety of methods not only in blood but also in placenta and fetal samples (Ginsberd and Rice, 2009). Volkel et al (2008) argued that these findings represent background noise. Ye et al. (2005) have investigated the composition of 30 urine samples collected from a diversified adult population in the U.S using HPLC. The levels of BPA in samples were found to be 9.5 % BPA, 69.5% bisphenol A glucuronide, and 21 % bisphenol A sulfate conjugate (Ye et al, 2005). A similar study conducted in Korea by Kim et al. (2003) reported urinary compound composition at 29.1 % BPA, 66.2 % bisphenol A glucuronide and 4.78% bisphenol sulfate conjugate. These

findings suggest that not all bisphenol A becomes conjugated to undergo safe detoxification pathways and that free BPA remains circulating in the blood.

The presence of unconjugated BPA metabolites is yet to be explored. Jaeg et al. (2004) identified nine metabolites from the metabolism of BPA by CD1 mice liver microsomal and S9 fractions. These metabolites were isopropyl-hydroxyphenol, BPA glutathione conjugate, glutathionyl-phenol, glutathionyl 4-isopropylphenol and BPA dimers. The formation of glutathione conjugates drew our attention as a novel metabolism pathway in BPA biotransformation. Jeag et al (2004) hypothesized two mechanisms that can explain the formation of BPA glutathione conjugates. First, BPA could first be oxidized into an arene epoxide and subsequently conjugated to glutathione, this reaction being catalyzed by a glutathione S transferase (GST). The second mechanism can be a first-step CYP mediated oxidation of BPA into 5-hydroxybisphenol A, this intermediate possibly being converted into bisphenol-*o*-quinone. *o*-Quinones are electrophile-reactive species that can covalently bind to nucleophilic sites and glutathione, a nucleophile compound, may react directly with bisphenol-o-quinone to produce glutathione BPA conjugates.

2.1.2.3 Mode of action of BPA through estrogen receptor and other pathways

BPA is known to act through the pathway of binding to estrogen receptor-alpha (ER-α) and estrogen receptor-beta (ER-β), a finding that has been confirmed in a number of *in vitro* and *in vivo* assays. The ER is a member of the nuclear receptor superfamily and functions as a transcription factor to modulate gene expression in a ligand-dependent manner. BPA has been shown to induce number of biological effects through binding to ER-α and ER-β, including ER-α

and ER-β mediated reporter gene activity. Proliferation of the MCF-7 human breast cancer cell, progesterone receptor expression, and prolactin release in pituitary tumour cell lines all occur through ER binding (Matthews et al, 2001).

ER-α and ER-β come from two distinct genes and differ in their tissue distribution, and in their ligand preferences. In the rat, ER-α is expressed at higher levels in the uterus, kidney, and epididymis, whereas ER-β levels are higher in the prostate, ovaries, and lung (Matthews et al, 2001). Therefore, the activity of BPA through estrogen receptor binding may vary from tissue to tissue and although the estrogenic/antiestrogenic activity of BPA may occur in tissues which express the ER, the magnitude of such activity is unknown because the distribution and concentration of BPA in specific tissues is unclear (Kurosawa et al, 2002).

A number of studies have shown that BPA induces a unique ER conformation, which then can cause downstream effects on interaction of ER with coregulatory proteins that act as coactivators or corepressors of ER-mediated transcription (Welshons et al, 2006). BPA can also act through activation of cell signaling systems that have receptors located in the cell membrane. In rat pituitary tumour cells, BPA was reported to stimulate a rapid influx of calcium at doses of 1pM and prolactin release triggered by calcium influx similar to the response to estradiol. Influx of calcium was also observed in human MCF-7 breast cancer cells (Welshons, 2006).

BPA also increases expression of the nuclear transcription factor Nur77 in mouse Leydig cells, which is involved in testosterone synthesis controlled by luteinizing hormone. BPA-induced expression of Nur77 in Leydig cells is mediated by the activation of protein kinase A and MAPK. In this pathway, phosphorylation of MAPK becomes detected within 5 min after

administration of BPA and reaches a maximum at 10 min resulting in altered steroidogenesis (Welshons, 2006).

Sui et al (2011) reported that BPA is a potent agonist for pregnane X receptor (PXR), a nuclear receptor that functions as a master regulator of xenobiotic mechanism. In addition, their group identified the structural requirements of BPA analogs that activate the human receptor. Although the details of the mechanisms by which BPA and its analogs cause this activation remain unknown, their findings may explain some of the endocrine disrupting effects of BPA. As the effects caused by BPA cannot be explained in terms of estrogenic activity alone (Sui et al, 2011).

2.2 ETHINYL ESTRADIOL

17-α-Ethinyl Estradiol (EE2) is a widely used synthetic estrogen used in the production of oral contraceptives (OCs), which are a combination of estrogen and progestin. There is increasing concern that EE2 from OCs is a major estrogenic disrupting compound contributing to estrogenicity of surface water and downstream contamination of drinking water. EE2 enters the water system through domestic sewage excreted by women prescribed OCs. It is reported that women metabolize only 20-40 % of the ingested dose of EE2 (Wise et al, 2011). The major pathway of EE2 metabolism is a phase–I aromatic 2-hydroxylation to 2-OH-EE (Bolt, 2004). The remaining components of OCs are excreted as free EE2, EE2 sulfate, or glucuronide conjugate. However, studies show that treatment of water with chlorine and ozone removes up to 99% of EE2 (Wise et al, 2011). Ethinyl estradiol was chosen as a positive control in our study

because of its wide use in studies evaluating disruption of male reproductive system by bisphenol A exposure. EE2 is referred to as xenoestrogen and endocrine disruptor exerting adverse effects on reproductive tract and hormonal imbalance (Timms et al. 2005).

3.0 MOLECULAR PROCESSES AND PATHWAYS OF PROSTATE CARCINOGENESIS

The molecular pathology of prostate cancer is thought to involve interconnections among many factors and molecular processes. Hereditary factors play a role in development of this disease in 10% of the cases. The role of environmental factors, including diet, and chronic inflammation still needs to be better understood. Reported molecular abnormalities involved in development of prostate cancer include chromosomal loss or gain, gene amplification, mutations causing an increase or decrease in gene expression, and functional mutations. Reduced expression of genes with important roles in apoptosis and cell cycle regulation (p53), cell proliferation (PTEN), intracellular detoxification (glutathione S-transferase pi, GSTP1) (Murphy et al, 2008) and DNA damage repair (O-6-methylguanine DNA methyltransferase, MGMT) have been identified throughout all stages of prostate cancer (Li et al, 2004).

4. GENES OF INTEREST IN BPA EXPOSURE

The π-class glutathione-S-transferase (GSTP1) is one of the superfamily of glutathione-S-transferase (GST) genes that play an important role in protection against DNA damage from electrophilic compounds and oxidants (Xiang et al, 2008). Hypermethylation and inactivation of

GSTP1 occurs in 90 % of cancerous samples and about 70% of prostatic intraepithelial neoplasia (PIN), but this inactivation is rarely detected in normal prostate or BPH tissue where GSTP1 expression is normally high. This suggests that silencing of this gene is associated with both the development and progression of prostate carcinogenesis. Loss of GSTP1 function may increase the susceptibility of prostate cells to damage by environmental carcinogens that might be GSTP1 substrates (Nelson et al., 2005). It is possible that hypermethylation of GSTP1 and other genes in the prostate is an age related phenomenon. However, analyses of genes from non-neoplastic prostate samples show no promoter methylation. This suggests that the methylation of these genes might be tumour related (Kang et al 2004).

Glutathione transferases (GSTs), including GSTP1, catalyze the conjugation of the tripeptide glutathione (GSH) with xenobiotics. This process takes place in cytosolic, microsomal and mitochondrial cell fractions. GSH is composed of glycine, cysteine and glutamic acid. Glutathione conjugates are thioethers, which form by nucleophilic attack of glutathione thiolate anion (GS^-) with an electrophilic carbon atom in the xenobiotic (Figure 5). Glutathione can also conjugate xenobiotics containing electrohilic heteroatoms (O, N, and S). Substrates for glutathione transferases are hydrophobic, contain an electrophilic atom and react nonenzymatically with glutathione at some measurable levels (Parkinson and Ogilvie, 2008).

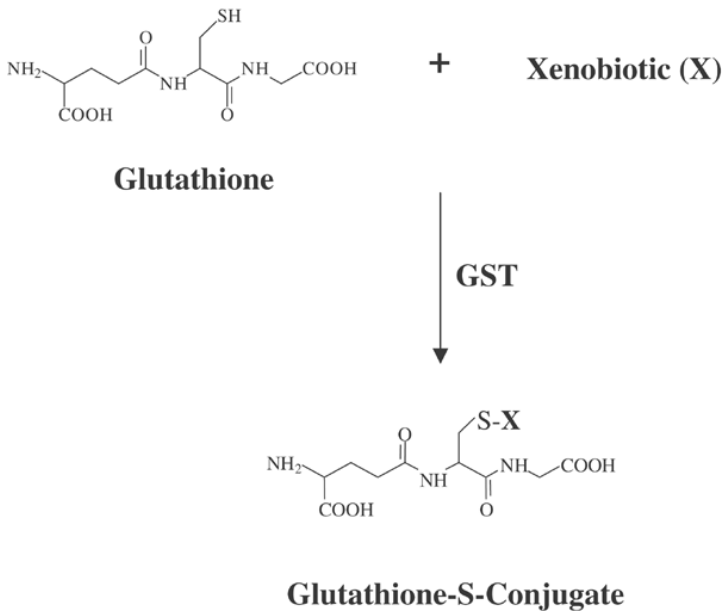


Figure 5: Glutathione conjugation to a generic xenobiotic (X) via GST results in the formation of a glutathione-*S* conjugate.

Conjugation with glutathione is important in the detoxification of electrophiles, potentially toxic species that can bind to nucleophiles, such as proteins and nucleic acids, causing cellular damage and genetic mutations. All enzymes involved in xenobiotic biotransformation have the potential to produce reactive intermediates, most of which are detoxified to some extent by conjugation with glutathione. Loss of glutathione transferase function in prostate tissue makes the cells more vulnerable to the effects of electrophilic compounds, eventually causing the progression of PIN to prostate cancer (Nelson et al., 2005).

O^6-methylguanine DNA methyltransferase, or MGMT (also referred to as ATase, AGT, AGAT) is an example of another gene with known abbreviated expression in prostate cancer. MGMT repairs O^6-alkylation adducts in a one step alkyl transfer reaction. It transfers the alkyl group from the oxygen in the DNA to a cystein residue in the catalytic pocket of MGMT (see

Figure 6). This reaction restores DNA integrity and inactivates (sacrifices) MGMT. As MGMT can remove only one alkyl group at a time the removing capacity of DNA adducts strictly depends on the abundance of the MGMT molecule per cell (Kaina et al., 2007).

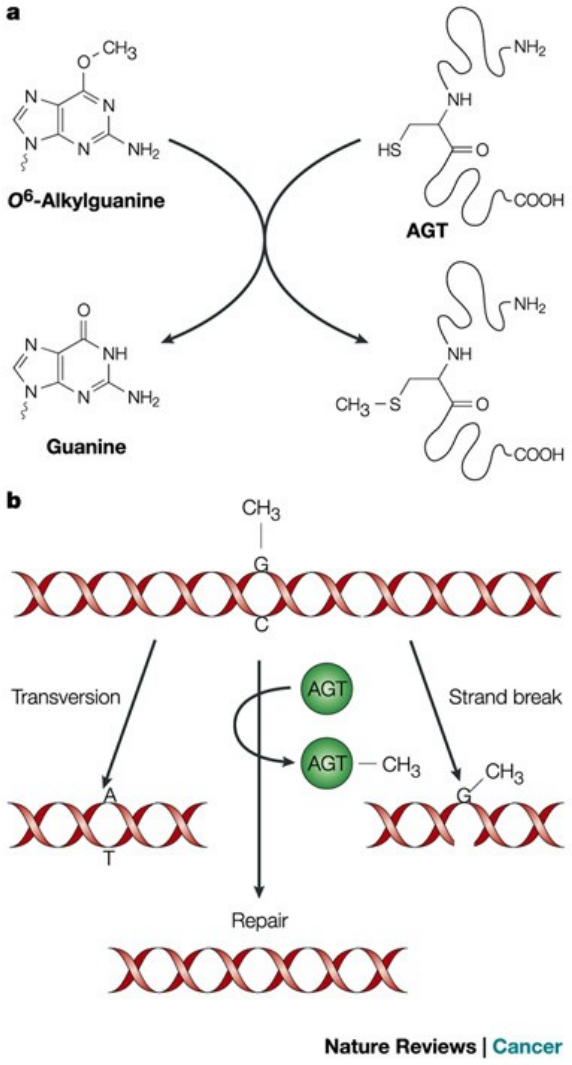


Figure 6: O^6-methylguanine DNA methyltransferase, MGMT, DNA repair process (Gerson, 2004).

Studies show that cells without MGMT expression are more sensitive to the effects of alkylating agents than cells with normal MGMT expression. MGMT plays a role in both cancer development and cancer prevention. The endogenous metabolites and environmental genotoxic compounds are known to produce reactive electrophilic species that alkylate DNA. These alkylating agents can generate O^6-MeG which is able to mispair with thymine causing point mutations, sister chromatid exchanges and chromosomal aberrations by double strand breaks in DNA replication. These abnormal events eventually lead to cell death, mutations and cancer (Kaina et al., 2007). Proper MGMT function is important in prevention of cancerous lesions.

Rhee et al (2011) presented results from their study showing certain endocrine disrupting compounds (EDCs), including BPA, causing decrease in expression of MGMT in liver of *Kryptolebias marmoratus*, a species of fish that is often used in toxicological studies and cancer research. This is one of the early studies suggesting that estrogenic compounds can cause cancer by altering DNA repair processes.

Clearly the role of both GSTP1 and MGMT genes in prostate cancer prevention is crucial relative to other genes involved in detoxification of xenobiotics and DNA repair. Based on current evidence it is assumed that inactivation of these genes through methylation is a consequence of aging, and that the role of xenoestrogens in this process is not known. GSTP1 function is highly impaired in prostate cancer due to methylation. This makes the prostate tissue vulnerable to genotoxic compounds such as BPA, which is believed to serve as substrate in the GSTP1 pathway. Exposure to xenoestrogens such as BPA could cause progression of PIN to malignant state as suggested by Nelson et al. (2005). Expression of MGMT was reported to be decreased in fish following exposure to BPA. Although BPA is not a substrate in the MGMT

pathway, BPA could potentially affect MGMT by altering its capacity for DNA repair, which could lead to cancer initiation.

HYPOTHESIS AND RESEARCH OBJECTIVES

The objectives of this thesis are to determine the effects of bisphenol A on the viability of prostate cells and to evaluate its ability to cause alterations in expression of specifically selected genes. These genes are known to be important in prostate carcinogenesis and play an important role in the detoxification of electrophilic and oxidative compounds as well as in DNA repair.

We hypothesize that BPA metabolite can be converted into glutathione conjugates. Therefore, the expression of the GSTP1 gene responsible for conjugation of BPA metabolite with glutathione, should increase with BPA exposure. Studies show that certain endocrine disrupting compounds such as BPA can affect expression of MGMT through unknown mechanisms. Our specific goal was to determine whether BPA can influence the expression of GSTP1 and MGMT in prostate cells. This information could improve our understanding of the mode of action of environmentally produced estrogen-like substances such as Bisphenol A. This information could help us understand the role of endocrine disruptors such as BPA in the initiation and development of prostate cancer.

MATERIALS AND MATHODS

Refer to Appendix for further details.

Chemicals

Bisphenol A (BPA) and Ethinyl Estradiol (EE2) were purchased from Sigma-Aldrich (Sigma, Mississauga, ON, Canada). The chemicals were dissolved in ethanol (Ethanol, Fisher) and diluted within a cell culture medium to yield the desired final concentration. The concentration of ethanol in the culture medium was kept at 0.1 %

Ethinyl estradiol (EE2) is a well known synthetic estrogen used as a positive control when conducting studies with agents of unknown estrogenic activity. The synthetic structure of EE2 is similar to that of 17-β estradiol (E2), although EE2 is estimated to be twice as potent as E2 (Wise et al, 2011). EE2 was chosen as a positive control in our study, with the intention that it will produce the expected effect in the same manner as the test compound, but with greater efficiency.

Cell Culture

The study was based on PC-3 cells, a human cell line purchased from American Type Cell Culture Collection (ATTC). PC-3 cells are prostate cancer cells derived from bone metastases expressing both estrogen receptors (ERα and ERβ) (Kaighn, et al., 1979). PC-3 cells were grown in F-12 Ham's medium (Sigma, Oakville, Canada) containing 10% fetal bovine serum (Sigma, Saint Louis, USA) and 5 % antimycotic (Sigma, Oakville, Canada). PC-3 cells

were seeded at a density of $5x10^4$ cells/well in 24 well plates for MTT assay and $5x10^5$/60mm plate for cell harvest. The cells were left undisturbed for 48 hours to allow attachment to the surface. Twenty-four hours before treatment, the cell culture medium was replaced with phenol-red free F-12 medium (Wisent, Canada) with 5% charcoal treated FBS for PC-3 cells in order to remove any factors that could modify the activity of the cells. Cells were cultured further with or without 10^{-9} -10^{-4} M of BPA and 10^{-9} -10^{-4} EE2 for 8, 24, 48, 72 and 120 hrs (Miyakoshi et al, 2009; Kurosawa et al, 2002).

Cell Harvest

At the end of incubation, cells were washed with phosphate-buffered saline (PBS) (Sigma, Oakville, Canada), and dead cells were removed from the culture flask. Cells were harvested using Trypsin (Sigma, Oakville, Canada). Cells were counted using a Countess instrument (Invitrogen) by mixing 10 µl of cell suspension and 10 µl of trypan blue and adding it into the chamber of the slide. For gene and protein analysis, two vials of approximately 2 $x10^6$ cells from each treatment were stored at -80 C. For long term cell preservation, cells were stored in cryovials with 5% DMSO in liquid nitrogen.

MTT assay

Cell viability was measured by the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Following exposure to the test compounds (BPA or EE2), cells were incubated for 4 hours in phenol-red free media with MTT. The purple formazan crystals formed were dissolved in SDS-HCL solution and incubated for 4-18 hours in a

humdidified chamber at 37° C. Absorbance was measured at 570 nm and background at 690nm. Cell viability was expressed as a percentage of the number of control cells treated with EtOH.

Real-Time PCR

Total RNA was extracted from cells using Aurum Total RNA Mini kits (BioRad) according to the manufacturer's instructions. Quantification of extracted RNA was performed using NanoDrop (Thermo Scientific) by adding 2µl of RNA onto the pedestal of the instrument. The quality of the RNA preparations was assessed by electrophoresis using a denaturating agarose-formaldehyde gel. The formaldehyde gel and running buffer were prepared using DEPC treated water and a MOPS buffer (pH 7.0). The RNA samples were prepared by adding RNA to a loading buffer, sterile water, and ethidium bromide. Samples were heat denatured for 5 min. prior to loading. The RNA marker was loaded alongside the samples. The gel was run for 60 min at 80 V and examined visually for presence of RNA quality markers (18S and 28S RNA molecules).

Total RNA (1 µg) from each sample was reverse-transcribed using the iScript™ cDNA Synthesis kit (Bio-Rad). Real-time analysis was carried out using a CFX96 Real-Time PCR Detection System (Bio-Rad). Reactions were performed in a 15 µL volume containing cDNA generated from 1 µg of original RNA template RT-PCR, 300 nM each of specific forward (F) and reverse (R) primers and 10.5 µl of SsoFast EvaGreen Supermix (Bio-Rad). The amplification protocol used was as follows: initial 3 min denaturation and enzyme activation at 95°C, 40 cycles at 95°C for 30 s, 60 ^{o}C for 30 s and 72°C for 30 s; 72 °C for 5 min and melt curve 65°C to 95°C, increment 0.5°C for 5 s. Primers for PCR amplification of GSTP1 and MGMT shown in

Table 1, were designed using Primer BLAST software and selecting appropriate parameters. GAPDH and HPRT1 primer sequences were found in article by Vandesamele et al, (2002). The melting curve for each gene was determined to verify the quality of the primers. Gene expression was normalized relative to HPRT-1 and GAPDH, following which the expression in each treatment sample was calculated by setting the control expression level as 100%.

Table1: Primers used in the study

Gene	**Sequence (5'--->3')**
HPRT1-forward HPRT1- reverse	TGACACTGGCAAAACAATGCA GGTCCTTTTCACCAGCAAGCT
GAPDH-forward GAPDH-reverse	TGCACCACCAACTGCTTAGC GGCATGGACTGTGGTCATGAG
GSTP1-forward GSTP1-reverse	AGGTCCTAGCCCCTGGCTGC TCAGGGGAGGCCAGGAAGGC
MGMT-forward MGMT-reverse	TTCACCATCCCGTTTTCCAG ATTGCCTCTCATTGCTCCTC

Western Blot Analysis

The protein concentration was determined using bicinchoninic acid (BCA) assay. In this assay, BSA was used as a standard with a lysis solution used as a blank. The BCA solution was mixed with the protein extract in 96 well plates and incubated for 15 min at 37 ^{o}C. Optical density was read at 562 nm.

Protein samples were electrophoresed and transferred to polyvinylidene difluoride (PVDF) membranes. Immunoblotting was performed using antibody dilutions for GSTP1 (BD Bioscience) at 1:5000 and GAPDH (Sigma) at 0.05µg/ml. Horseradish peroxidease HRP-conjugated anti-mouse secondary antibody (Bio-Rad) was used a dilution of 1:5000. Detection of protein bands was accomplished using Immun-Star WesternC Kit (Bio-Rad). Signals were visualized using Versa Doc instrument (Bio-Rad). Densitometric analysis was performed using Quantity One software (Bio-Rad).

Statistical Analysis

Data are presented as means ± standard error. Levene's test was used to assess homogeneity of variances. A one-way analysis of variance (ANOVA) was used to assess statistical significance of different durations and doses of exposure. Dunnet's test was used to compare duration and exposure groups to vehicle treated controls. Differences with p-value <0.05 versus vehicle treated control were considered statistically significant. For real-time PCR results, a 2-fold or more change in mRNA levels was considered to be significant.

RESULTS

Cell viability by MTT assay

A cell viability assay was used to assess the effects of estrogenic compounds of interest (ethinyl estradiol, EE2 and bisphenol A, BPA) on the viability of PC-3 cells. Cell viability was measured by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay.

Dunnet's test was used to compare duration and exposure groups to vehicle treated controls in cases where ANOVA rejected the equality of means. Levene's test of homogeneity of variances was not significant for all cell viability results.

Ethinyl estradiol reduced the viability of PC-3 cells at the highest concentration of 100 μM after 72 hours exposure (see **Figure 7**) but not at other concentrations. Although EE2 is known to stimulate cell proliferation through ER, PC-3 cells in this experiment did not exhibit significant growth stimulation in these experiments. Although the decrease in cell viability caused by BPA at the highest concentration tested is apparent in microscope imaging (**Figure 8B**), BPA did not show statistically significant decrease in viability in MTT assay (see **Figure 8A**). At a concentration of 100 μM of BPA (**Figure 8B**), the microscope image shows that cells seem to form fewer connections, their network is disrupted, and the cells appear deformed. At lower concentrations cells appear similar to controls and form denser networks. BPA appeared to act in the same way as EE2, the positive control in this study, having no effect on cell viability at lower concentrations. However, a statistically significant decrease in cell viability at 100 μM was not observed.

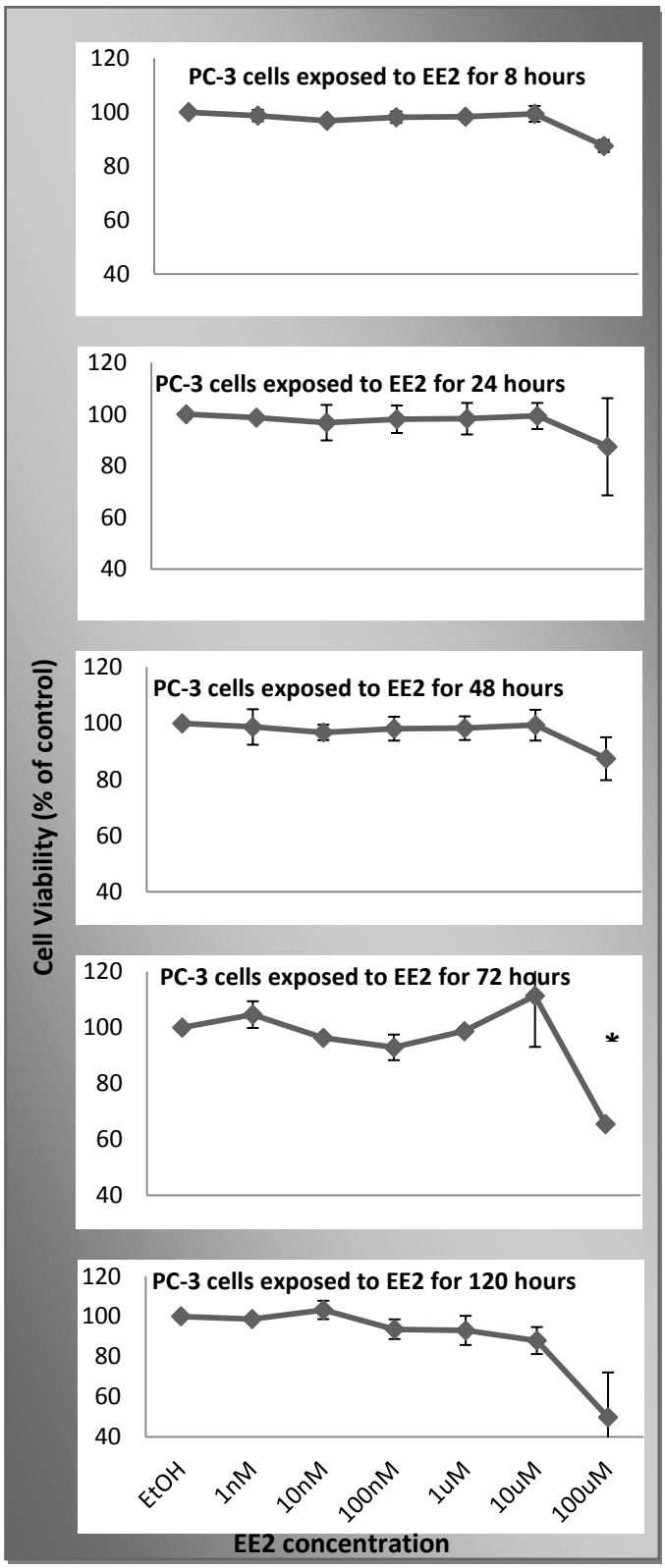


Figure 7: Viability of PC-3 cells exposed to ethinyl estradiol (EE2), as assesed by the MTT assay. Viability was assesed with the MTT assay after cell treatment with ethinyl estradiol (1nM-100 μM), for 8, 24, 48, 72 and 120 h. Values are the mean values ±s.e. from two separate experiments.*$p<0.05$.

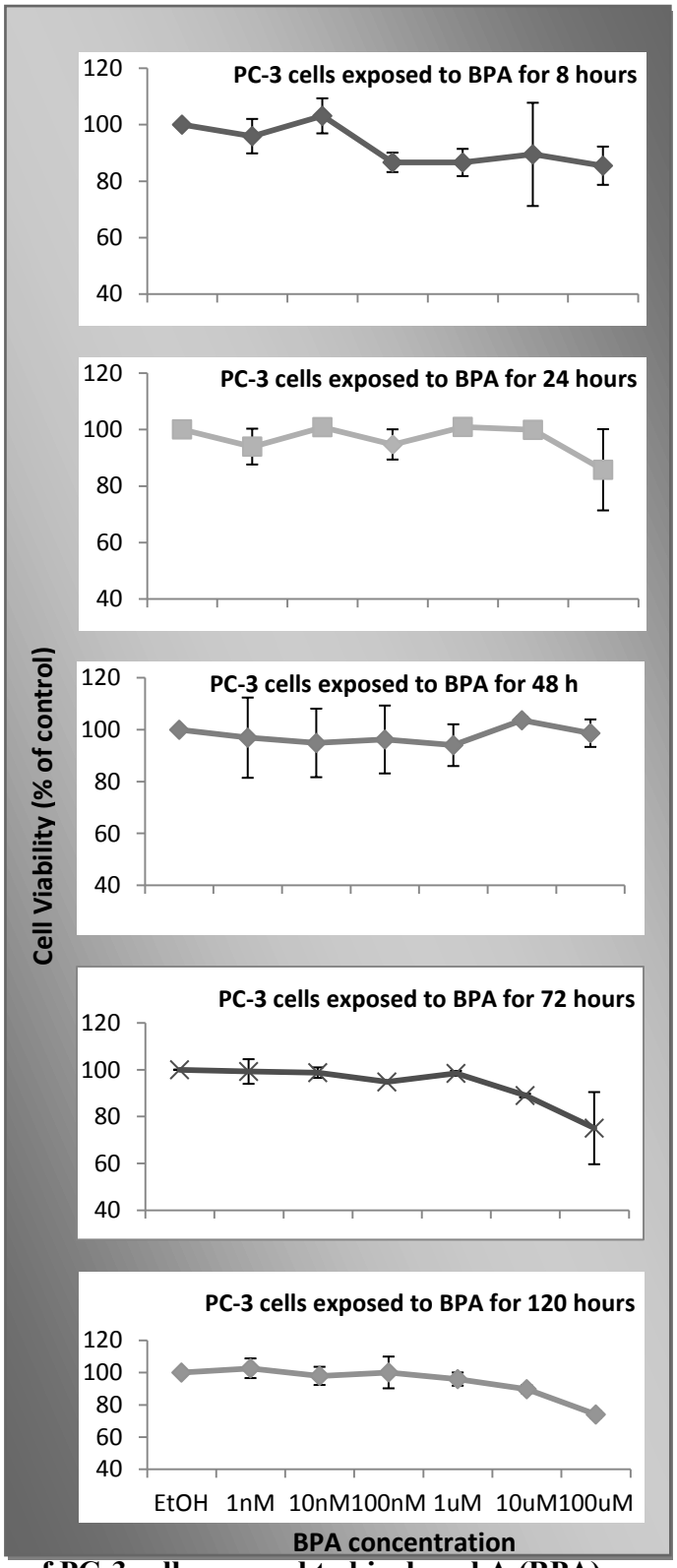


Figure 8A: Viability of PC-3 cells exposed to bisphenol A (BPA), as assesed by the MTT assay. Viability was assesed with the MTT assay after cell treatment with bisphenol A (1 nM - 100 μM), for 8, 24, 48, 72 and 120 h. Values are the mean values ±s.e. from two separate experiments.*$p<0.05$.

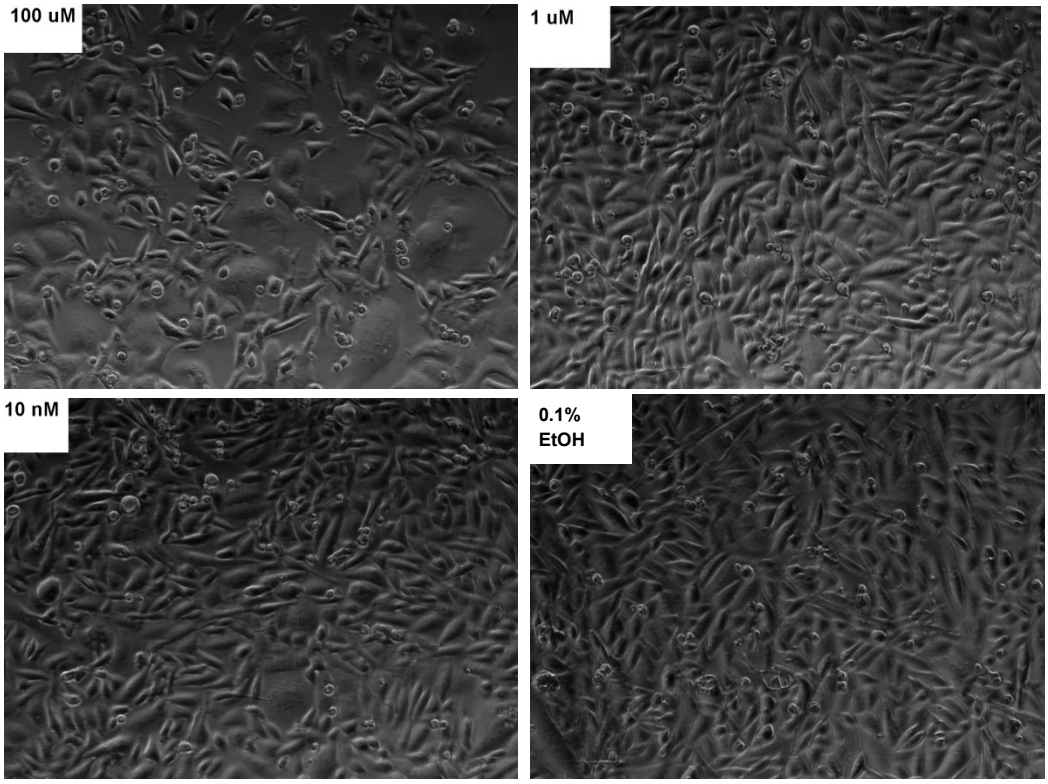


Figure 8B. Images of PC-3 cells exposed to bisphenol A (100 µM, 1µM, and 10 nM) and ethanol for 72 hours.

Real-time PCR

To evaluate the possible involvement of GSTP1 and MGMT in detoxification pathways of bisphenol A, the expression of these genes was analyzed using real-time PCR. The quality of RNA was assessed before each reverse transcription by running a denaturating agarose gel capable of detecting possible RNA degradation and confirming integrity of the 18S and the 28S rRNA signals (see **Figure 9**). The consistency in expression of the housekeeping genes hypoxanthine-guanine phosphoribosyl transferase (HPRT) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was assessed by exposing the cells to bisphenol A (1nM, 100nM and 10 μM) for 48 hours and 120 hours respectively. The expression of HPRT1 and GAPDH genes was found to be comparable to ethanol treated cells (**Figure 10A**). Since the chemicals used in all experiments were solubilized in ethanol, cells exposed to ethanol only were used as negative controls. The negative control employed a culture medium containing 0.1 % ethanol. To confirm and validate the negative controls, real-time PCR was performed for cells exposed to 0.1 % ethanol for 0, 24, 48 and 72 hours. The negative control was found to be valid since no change in gene expression was observed between EtOH treated and non-treated cell cultures (see **Figure 10B**).

Gene expression in cells exposed to BPA and EE2 are shown in **Figures 11-14**. It is presented relative to the vehicle treated control (negative control) and normalized to two housekeeping genes HPRT1 and GAPDH. Dunnet's test was used to compare duration and exposure groups to vehicle treated controls in cases where ANOVA rejected the equality of means. Levene's test of homogeneity of variances was not significant for all real time PCR results.

GSTP1 expression increased significantly by 2 fold in cells exposed to 10 µM (or 10000 nM) of EE2 (**Figure 11**). The expression of MGMT was not changed (**Figure 12**) in cells exposed to EE2. GSTP1 and MGMT was not altered at the 72 hour exposure time across the three different concentrations of BPA tested (**Figures 13, 14**).

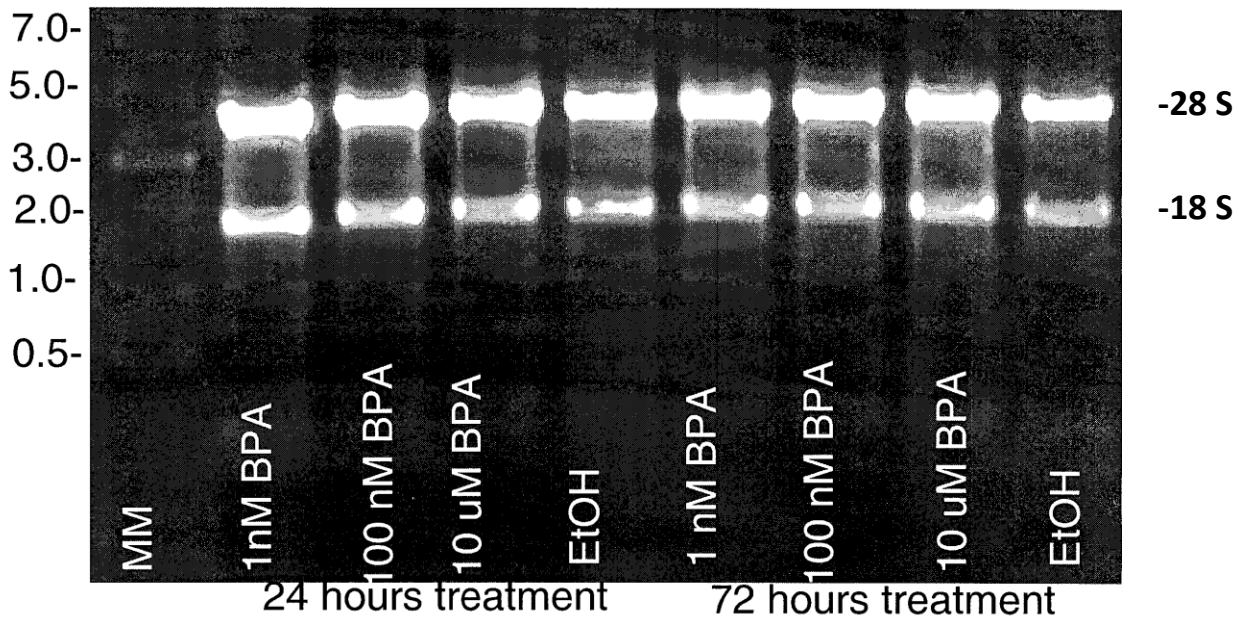


Figure 9: Quality and purity of extracted RNA. RNA quality was assessed by denaturating agarose gel electrophoresis and visual assessment of the 28S: 18S rRNA ratio. Above results represent RNA quality of cells exposed to BPA (1 nM, 100 nM, 10 µM) and ethanol (EtOH) for 24 or 72 hours.

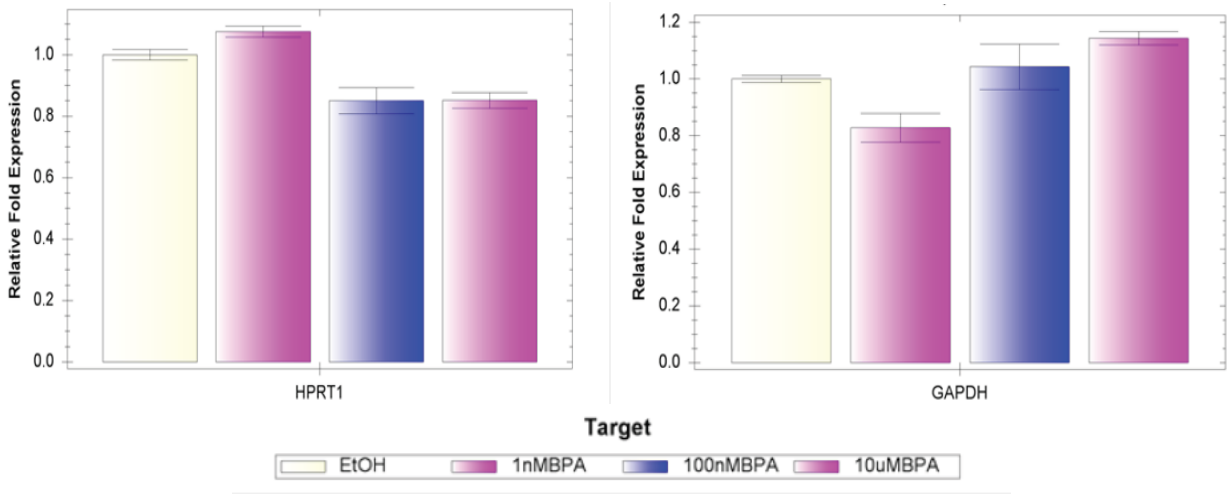


Figure 10A: Validation of housekeeping genes. Expression of HPRT1 and GAPDH housekeeping genes in PC-3 cells exposed to bisphenol A (1nM, 100nM and 10 μM) for 48 hours and 120 hours respectively. Gene expression is shown relative to the vehicle treated control (EtOH). Values are from one experiment.

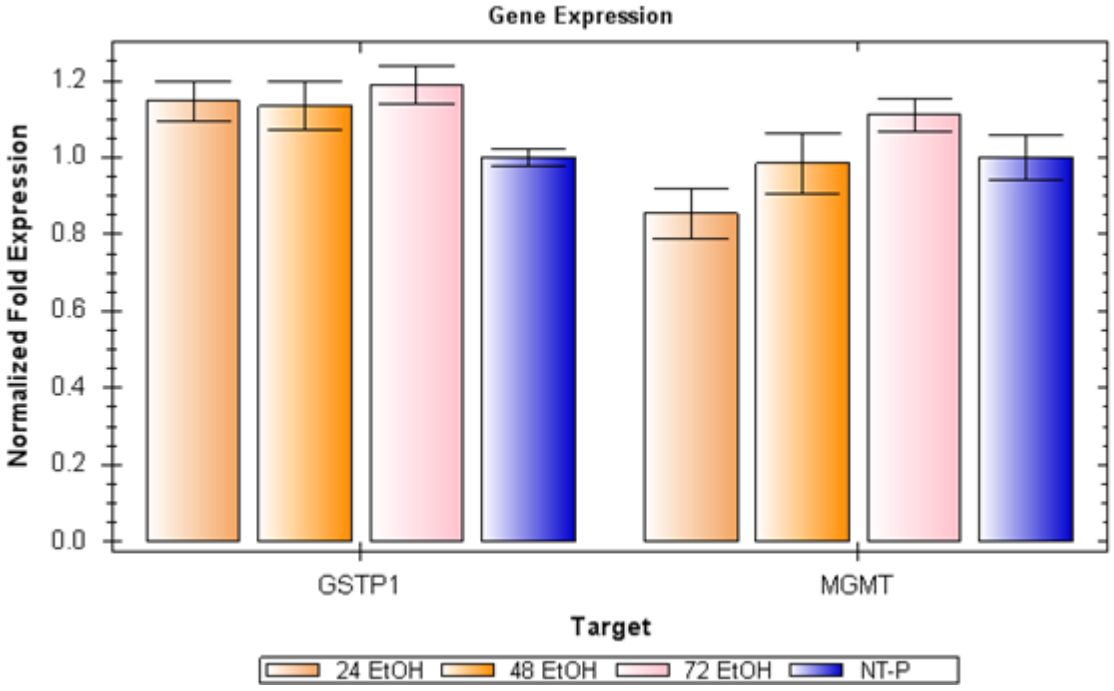


Figure 10B: Validation of negative control (ethanol). Cells were treated with ethanol (EtOH) for 24, 48 and 72 hours to determine its effect on expression of GSTP1 and MGMT. Gene expressions is shown relative to non-treated control (NT-P) and normalized to two housekeeping genes HPRT1 and GAPDH. Values are from one experiment.

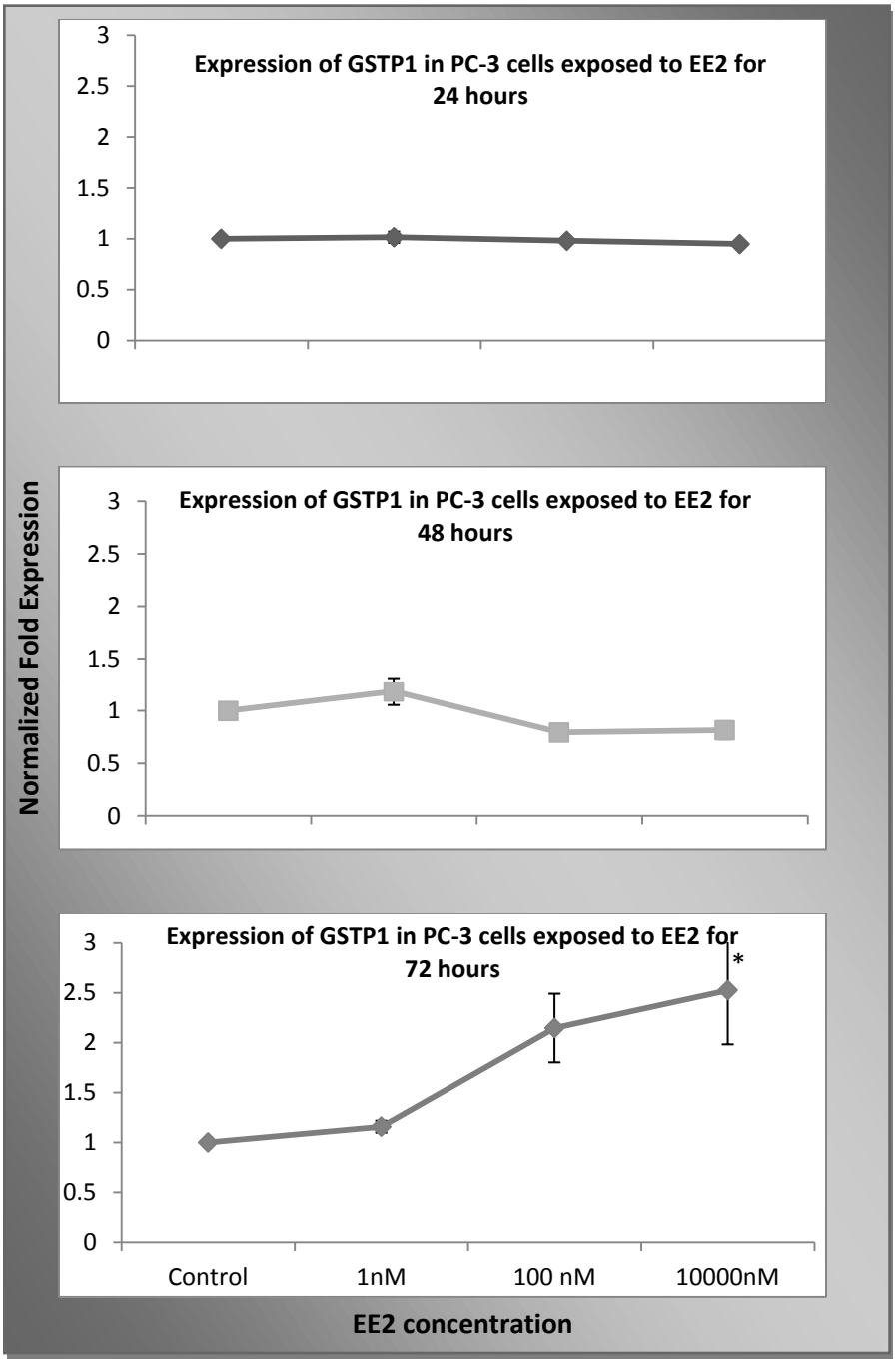


Figure 11: Expression of GSTP1 in PC-3 cells exposed to ethinyl estradiol for 24, 48 and 72 hours. Gene expression is shown relative to the vehicle treated control and normalized to two housekeeping genes HPRT1 and GAPDH. Values are the mean values ±s.e. from two separate experiments.*$p<0.05$ and 2-fold or greater changes compared with the control.

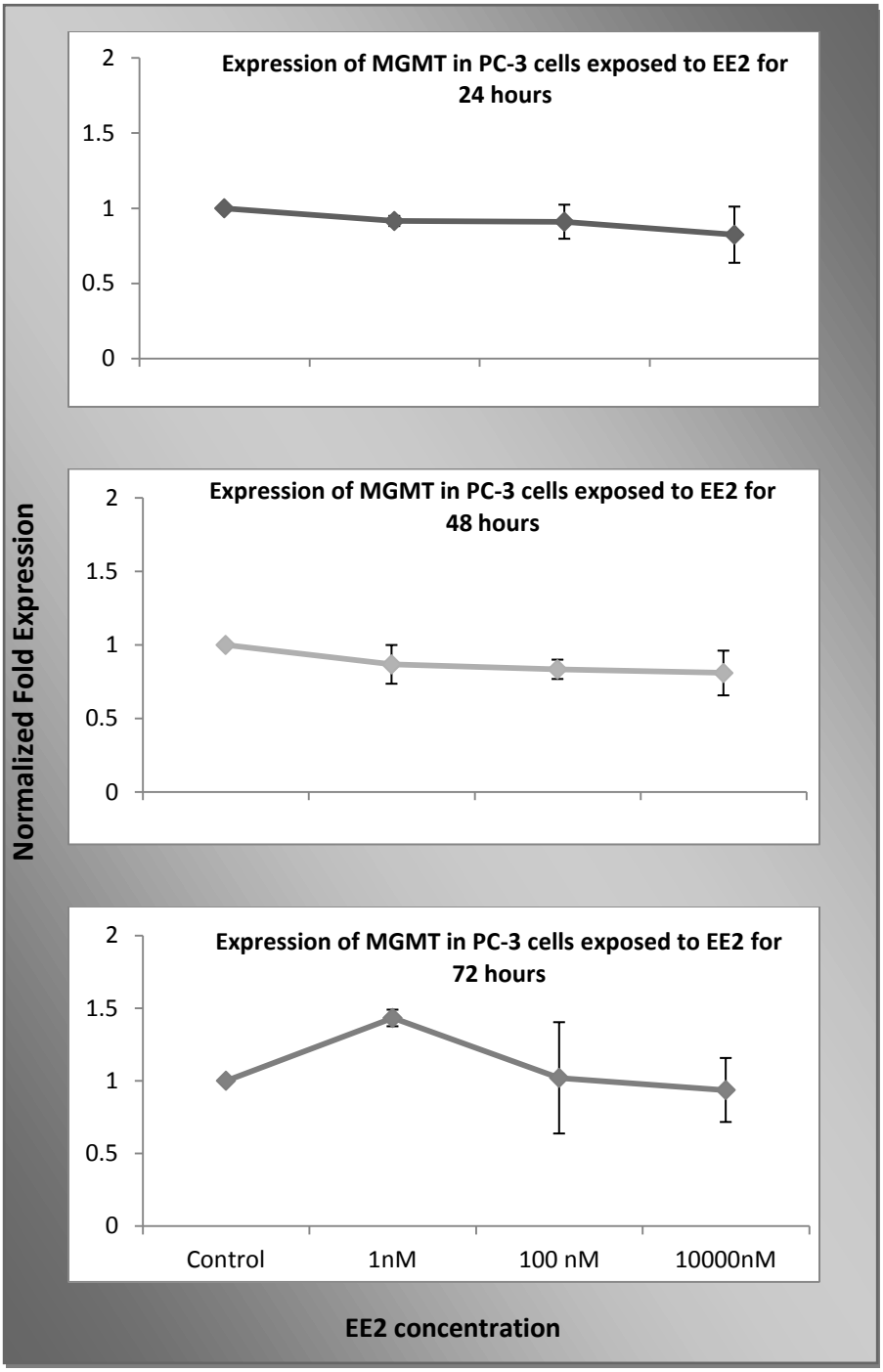


Figure 12: Expression of MGMT in PC-3 cells exposed to ethinyl estradiol for 24, 48 and 72 hours. Gene expression is shown relative to the vehicle treated control and normalized to two housekeeping genes HPRT1 and GAPDH. Values are the mean values ±s.e. from two separate experiments.*$p<0.05$ and 2-fold or greater changes compared with the control.

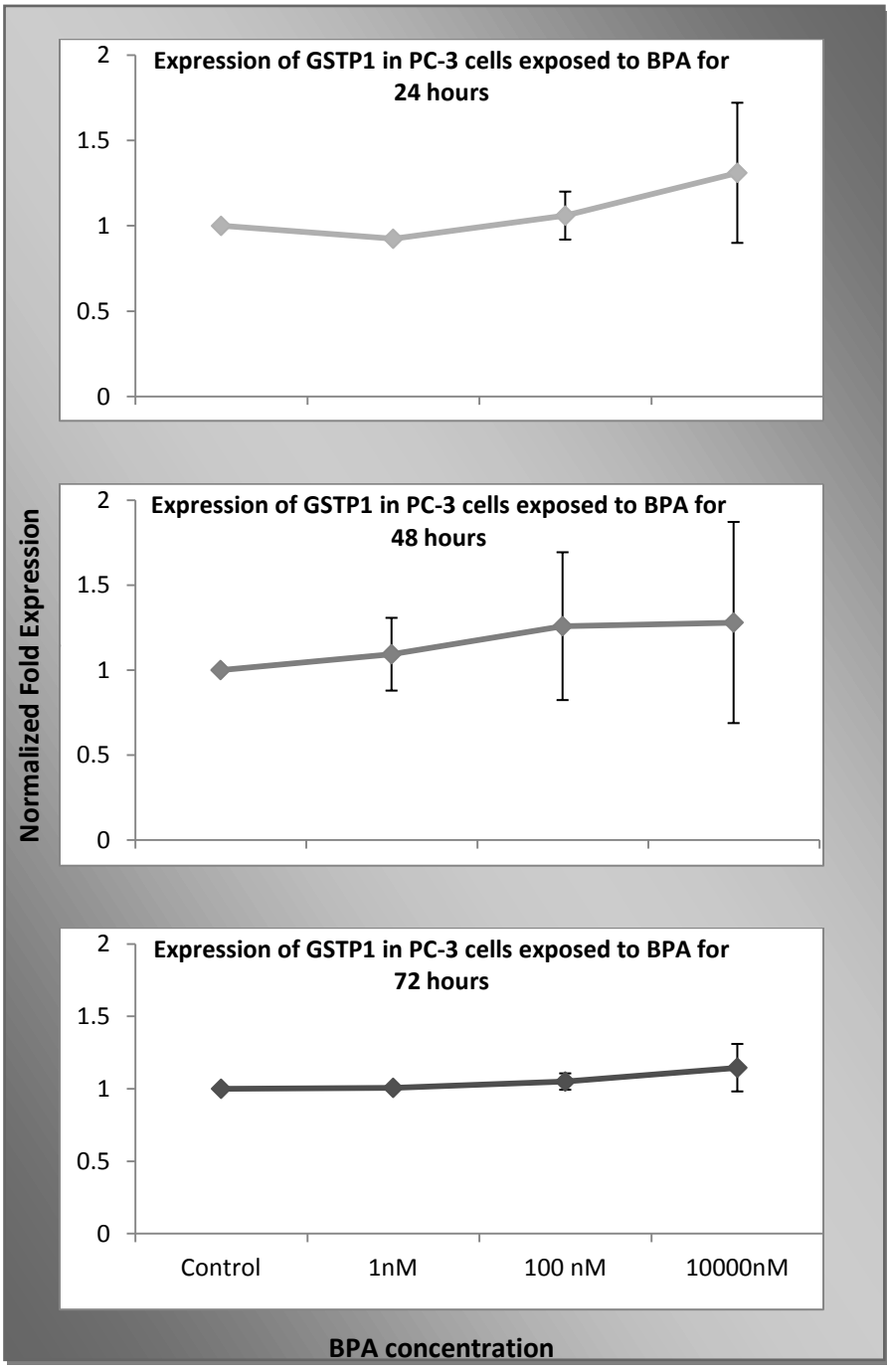


Figure 13: Expression of GSTP1 in PC-3 cells exposed to bisphenol A for 24, 48 and 72 hours. Gene expression is shown relative to the vehicle treated control and normalized to two housekeeping genes HPRT1 and GAPDH. Values are the mean values ±s.e. from two separate experiments.*$p<0.05$ and 2-fold or greater changes compared with the control.

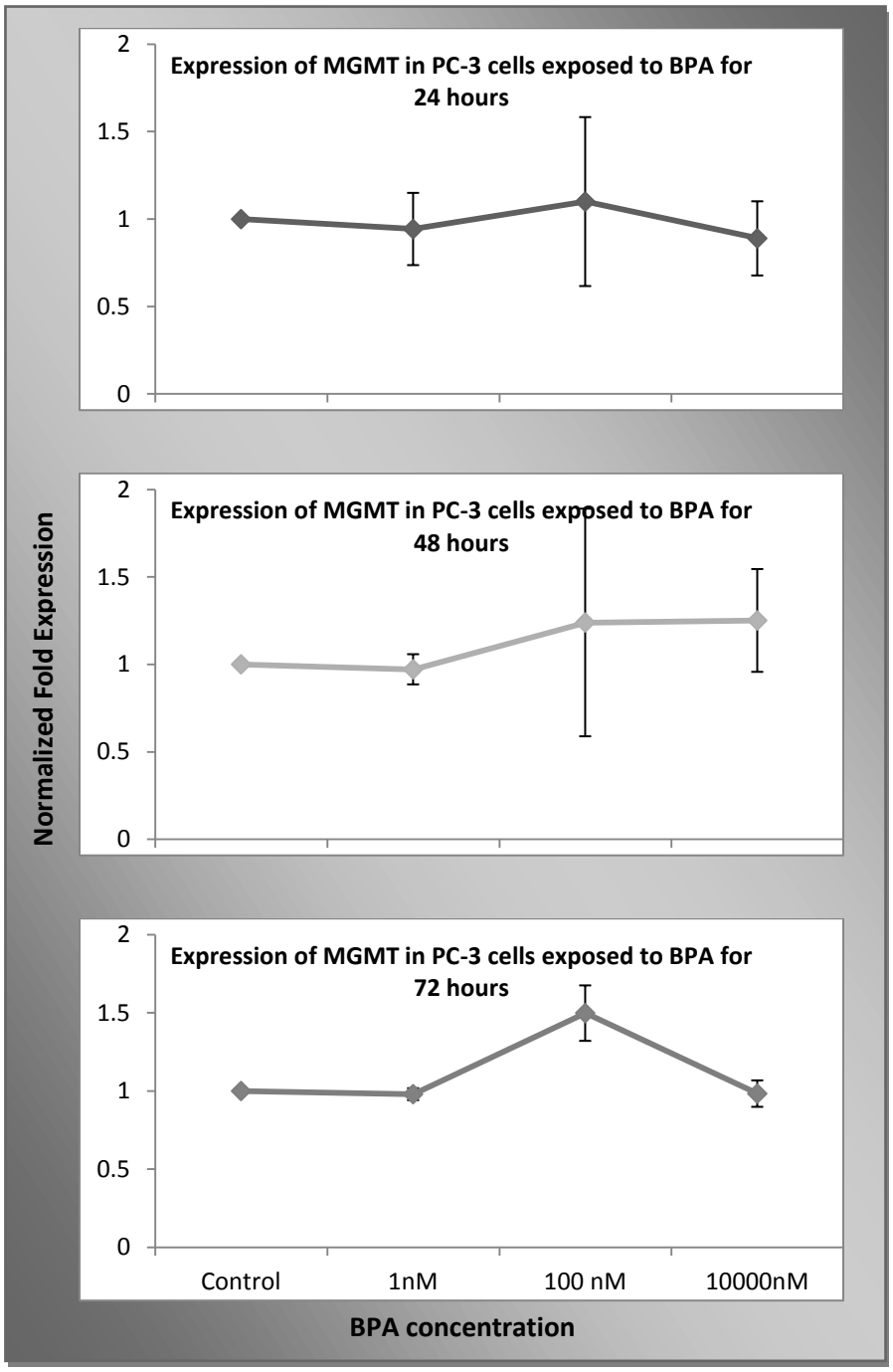
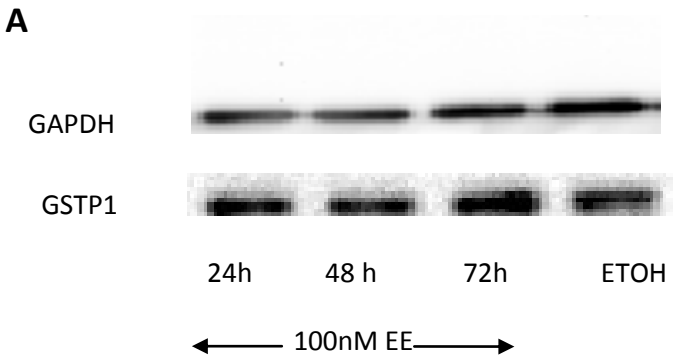


Figure 14: Expression of MGMT in PC-3 cells exposed to bisphenol A for 24, 48 and 72 hours. Gene expression is shown relative to the vehicle treated control and normalized to two housekeeping genes HPRT1 and GAPDH. Values are the mean values ±s.e. from two separate experiments.*$p<0.05$ and 2-fold or greater changes compared with the control.

Western Blot Analysis

Western blot analysis was performed to determine if the expression levels of GSTP1 protein change in PC-3 cells as result of exposure to ethinyl estradiol and bispheol A. As seen in **Figures 15** and **16**, GSTP1 protein levels were comparable in both control and treated cells, indicating no effect of exposure to EE2 and BPA on protein expression. Densitometric data were normalized to GAPDH and presented as percentage of EtOH treated control cells (**Figures 15B, 16B**).



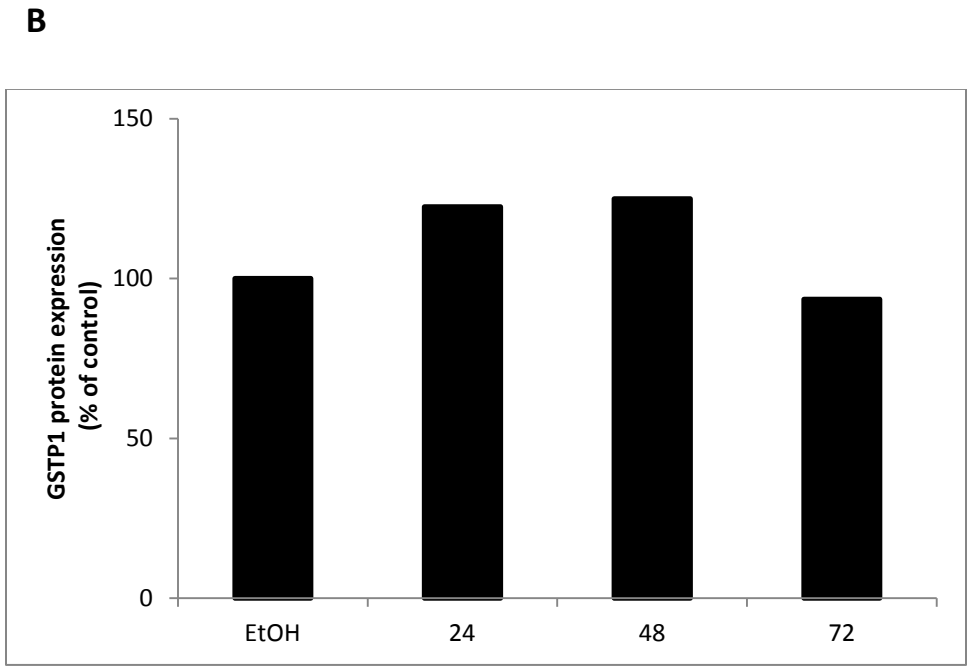
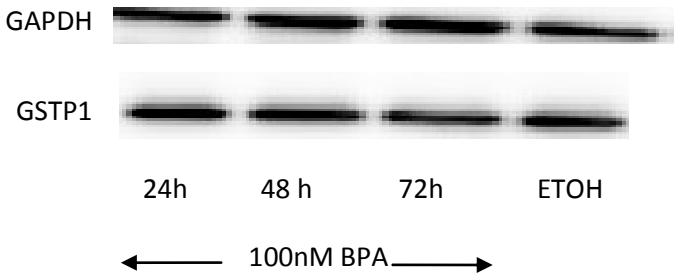


Figure 15 A, B: Western blot analysis of GSTP1 protein expression in PC-3 cells after administration of ethinyl estradiol (EE2) at 100 nM for 24, 48 for 72 hours. **B:** Densitometric data normalized to GAPDH are presented as percentage of control EtOH treated control cells. Values are from one experiment.

A



B

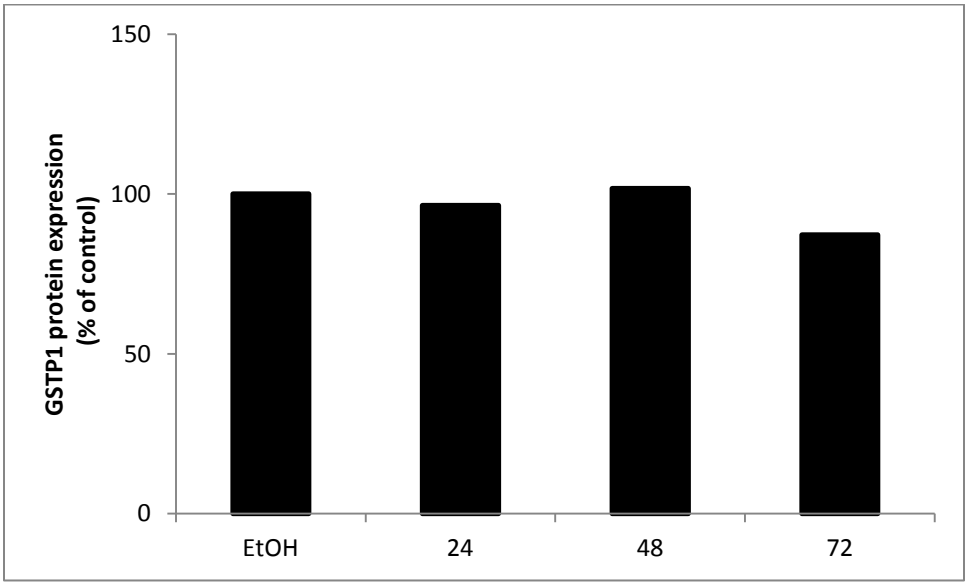


Figure 16 A, B: Western blot analysis of GSTP1 protein expression in PC-3 cells after administration of bisphenol A at 100 nM for 24, 48 for 72 hours. **B:** Densitometric data normalized to GAPDH are presented as a percentage of EtOH treated control cells. Values are from one experiment.

DISCUSSION

Bisphenol A has previously been shown to demonstrate estrogenic activity (Krishnan et al, 1993).It is possible that through metabolic activation and oxidation BPA can be effective as an *ortho*-quinone that can bind covalently to DNA and disrupt its integrity (Atkinson and Roy, 1995). The molecular biology and toxicology of BPA in prostate cells is not well understood. To better understand the role of this xenobiotic in prostate carcinogenesis, it is important to determine how chronic exposure to BPA affects cell viability and expression of genes involved in prostate cancer development. In this thesis, the viability of PC-3 prostate carcinoma cells derived from a bone metastasis was examined. In addition, the expression of two key genes known to play a role in prostate carcinogenesis was assessed.

Glucuronidation is the major phase II detoxification pathway for metabolism and excretion of bisphenol A (BPA) in humans (Volkel et al., 2002). Studies in humans and experimental animals show that glucuronidation of bisphenol A occurs in the liver and that most of its metabolites are excreted in urine as bisphenol A glucuronide. Although most of the metabolites of BPA found in urine are produced in the liver, peripheral tissues such as the prostate also have some capacity for metabolizing estrogenic compounds. Prostate cells express phase I and phase II metabolizing enzymes. The phase I enzyme CYP1B1 is known to metabolize endogenous hormones (estrogens), converting them into 2-OH and 4-OH estrogen metabolites, the latter of which is known to have genotoxic potential. It is of interest that CYP1B1 has been reported to be highly expressed in the PCa prone peripheral zone of the prostate. CYP1A1 and CYP1A2 metabolise exogenous compounds such as polycyclic aromatic

hydrocarbons (PAH). In general, CYP enzymes have overlapping substrates that can give rise to genotoxic compounds. Phase II enzymes include glutathione-S-transferases (GST), UDP-glucuronyltransferase (UGT), sulfotransferase (SULT), N-acetyltransferases (NATs) and catechol-O-methyltransferase (COMT); their predominant role is to safely deactivate/conjugate phase I metabolites in preparation for their excretion *in vivo* (John et al. 2009).

Studies in rats show that BPA can be converted to a hydroxylated metabolite following oral administration. Atkinson and Roy (1995) suspected that some BPA can be converted by phase I enzymes to 3-OH-BPA (see Figure 2 in the introduction) which could bind to DNA through the formation of bisphenol-o-semiquinone or bisphenol-o-quinone. Since this process can be inhibited by α-naphthoflavone, an inhibitor of the family of CYP1 enzymes it is possible that one of CYP1 enzymes is responsible for BPA hydroxylation. Nakagawa and Suzuki (2001) reported that 3-OH-BPA is in fact quite toxic in the MCF-7 breast cancer cell line, presumably acting via a reactive semi-quinone or quinone intermediate. The process of DNA adduct formation causing mutations in critical genes is believed to be one of the major pathways in the initiation of cancer by estrogenic compounds.

Our results (Figure 8A) show that PC-3 cells are able to metabolize BPA administered at concentrations at or below 100 μM. Although our MTT assay results failed to show significant reduction in cell viability at higher concentration of BPA, qualitatively cell growth was visibly reduced (Figure 8B): cells formed fewer connections, their networks were disrupted, and some cells appeared deformed. At lower concentrations, treated cells appeared to be similar to control

cells, and formed denser networks. This reduction in cell growth could be due to accumulation of DNA adducts causing apoptosis, resulting in a visible reduction in cell viability.

We hypothesized that expression of glutathione S-transferase pi (GSTP1) might be increased when PC-3 cells are exposed to higher concentrations of BPA. It is expected that GSTP1 should be activated to prevent DNA damage from bisphenol-*o*-quinone, an electrophilic species that can covalently bind to DNA (Jaeg et al, 2004). GSTP1 is a phase II enzyme which conjugates the products of phase I metabolism, generating metabolites that are less toxic and readily excreted *in vivo*. In our experiments, GSTP1 expression was unchanged at the 72 hour exposure time across the three different concentrations of BPA tested (1 nM, 100 nM and 10 μM) based on real time PCR and western blot results (Figure 13 and Figure 15). BPA was expected to increase expression of GSTP1 at concentrations of 10 μM, just below the threshold where reductions in cell viability were observed (Figure 8). Although the expression of GSTP1 was unchanged when PC-3 cells were exposed to BPA, it is possible that phase I metabolites of BPA were produced to undergo a different pathway of conjugation. Future studies should include assays which could detect the presence of GSH conjugates to confirm these results. Another possible explanation is that insufficient metabolites were produced to activate GSTP1 expression, resulting in a failure to observe an increase in RNA and protein expression.

Interestingly, PC-3 cells exposed to EE2, the positive control used in this study, showed increased expression of the GSTP1 gene. Real time PCR analysis showed that the expression of GSTP1 significantly increased by 2 fold in cells exposed to 10 μM of EE2 (Figure 11). This

suggests that GSTP1 expression can be increased in response to a more potent estrogenic compound such as EE2. EE2 is known to undergo first pass phase I aromatic 2-hydroxylation to 2-OH-EE (Bolt, 2004), affording an opportunity for the formation of electrophilic DNA damaging quinone intermediates. It is possible that EE2 metabolites are the substrates for GSTP1, which resulted in the observed increase of GSTP1 activity observed in our experiments. However, western blot results did not correlate with the real time PCR results in cells exposed to EE2 (Figures 15AB). The protein levels of GSTP1 were comparable across all exposed samples. This discrepancy between mRNA and protein expression is in fact very common in molecular biology for the following reasons. First, since cells have the ability to control the levels of protein at the transcriptional level and the translational level, mRNA and protein levels do not always correlate with each other. Second, this lack of correlation can be caused by different rates of protein synthesis (Greenbaum et al, 2003).

Estrogens are believed to participate in the process of carcinogenesis by over-stimulation of cells through receptor-mediated processes. The covalent binding of quinones and semi-quinones to DNA bases mentioned above can lead to formation of DNA adducts and loss of bases from DNA strands, suggesting an alternate way in which estrogens might induce cancer. In our experiment, BPA did not increase GSTP1 expression that could indicate presence of quinones, but this does not mean that they were not produced. It is suggested that future experiments should include assays to assess DNA adduct production following BPA exposure.

O^6-methylguanine DNA methyltransferase (MGMT) is responsible for removing certain type of DNA adducts caused by alkylating agents. The ability of MGMT to remove DNA adducts formed in prostate tissue is hindered by its methylation. This decrease in the activity of MGMT is known to be present in tissue samples from patients with PCa, suggesting a role for MGMT in PCa initiation and progression. There is emerging toxicological evidence showing that certain EDCs, including BPA, could cause decrease in MGMT expression. Our goal was to determine if exposure to BPA could further decrease expression of already suppressed MGMT in PCa. The expression of this gene in PC-3 cells was unaltered as a result of exposure to BPA (Figure 14). Similar results were obtained when PC-3 cells were exposed to the positive control ethinyl estradiol (figure 12). These results suggest that exposures to the estrogenic compounds BPA and EE2 have no effect on MGMT expression in prostate cells, as previously suggested by experimental studies in different biological systems.

Conclusion and Future Consideration

This thesis derived a methodology to study the *in vitro* effects of exposure to estrogenic compounds in prostate carcinoma cells. The MTT cell viability assay was introduced to study viability and cytotoxicity of cells exposed to BPA. Analysis of gene expression at the mRNA and protein level was conducted using real time PCR and western blotting techniques.

One of the major limitations in this study was the use of single cell line PC-3, which failed to show an increase in cell proliferation due to BPA exposure. Other *in vitro* studies have shown that BPA can stimulate breast and prostate cell proliferation (Krishnan et al, 1993; Wetherill, 2002). Future experiments should include analysis of different prostate cell lines expressing ER to investigate potential increases in cell proliferation and to determine whether such increases are time and dose dependent. Future studies should include in depth analyses of expression of genes involved in phase I and phase II metabolism of BPA and the mechanisms underlying DNA adduct formation by BPA both *in vitro* and *in vivo*. Indirect evidence suggests that BPA can be converted by phase I enzymes to 3-OH-BPA, which could disrupt DNA integrity through the formation of bisphenol-o-semiquinone or bisphenol-o-quinone. Studies should be designed to test which candidate CYP enzymes are responsible for conversion of BPA to phase I metabolites.

In conclusion, the present study was designed to investigate the effects of an estrogenic compound, bisphenol A, used in production of polycarbonate plastics and other household products. Prostate carcinoma PC-3 cells are able to metabolize BPA administered at concentrations at or below 100 μM. Based on microscopy images, higher concentrations of BPA appear to cause cytotoxicity due to the formation of the metabolite 3-OH-BPA. Gene expression analyses showed that BPA does not produce a toxic response that can be measured in terms of altered expression of GSTP1 and MGMT. It is possible that BPA metabolites in prostate tissue are not a substrate for GSTP1. It also appears that BPA has no effect on MGMT expression, as previously suggested in other studies.

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APPENDIX A

I. CELL CULTURE

Initial growth:

1. Warm desired amount of growth medium at 37 ^{O}C

2. Add 9-10 ml of growth medium into pre-labeled culture flasks (10 cm^2)

3. Remove cells from frozen storage and quickly thaw in a 37 ^{O}C waterbath by gently agitating vial.

4. As soon as the ice crystals melt, take out gently using micropipette into the culture flask containing growth medium.

5. Incubate the dishes at 37 ^{O}C in humidified atmosphere with 5 % CO_2.

6. Observe the cells for growth under microscope.

7. Once the cells reach 80 % confluency, adhering to the surface and growing in the monolayer, can be subcultured.

Suculturing:

1. Warm required volume of culture medium (with 10 % FBS), Trypsin-EDTA and 1X PBS at 37 ^{O}C in water bath for 15 minutes.

2. Thoroughly rinse media/reagent bottles, culture flasks with ethanol, wipe them off an place in BSC.

3. Aspirate the media from culture dishes using aspirating apparatus.

4. Gently rinse the cells with 4-5ml of 1XPBS; then aspirate the PBS.

5. Add 1ml of Trypsin-EDTA to the cells and allow them to sit for 5-7min to facilitate detachment.

6. Observe the cells under the microscope to ensure complete detachment of cells from surface.

7. Add 3-4 ml of culture medium and pipette out the cells into a culture tube.

8. Spin down the cells by centrifuging at ~1000rpm/2 min

9. Carefully remove the media without disturbing the pellet.

10. Add 3-4ml of culture medium, gently vortex the tube to resuspend pellet.

11. Use 10 µl of cells for cell counting using cell countess.

12. Record all observations.

13. Use the following formula for calculating the total cells:

Total cells=cells/ml x the original volume of media from which the cell sample is removed

14. Split the cells into 1:3 ratio using total cell count and add desired growth media into each pre-labeled flask.

15. Incubate the culture flasks at 37 ^{O}C in humidified atmosphere with 5 % CO_2.

Cell counting method using Countess (Invitogen)

1. Take a countess slide.

2. Gently vortex the cell suspension

3. Mix 10 µl cell sample with 10 µl of trypan blue in a 200 µl tube.

4. Using a micropipette carefully add 10 µl of cell suspension into the chamber on the countess slide; make sure the sample is spread evenly in the slot.

5. Insert slide with the sample side into the instrument.

6. Focus the cells using "ZOOM IN" function on touchpad and fix the focus where there is clear contrast between live and dead cells

7. Press the "Count Cells" button and wait for processing, record all the observations.

Changing the media:

1. Check the culture flask every day.

2. Whenever the color of the media is changed, take out the flasks.

3. Aspirate the growth media and add same volume of fresh growth medium.

4. Incubate the dishes at 37 ^{O}C in humidified atmosphere with 5 % CO_2.

Freezing cells:

1. Harvest cells at the desired passage using usual method (trypsinization)

2. Spin down and resuspend cells in growth medium and determine cell count and viability.

3. Prepare freezing medium by adding 5% DMSO (v/v) to growth medium

4. Transfer 1 ml aliquots to cryo-vials on ice

5. Place vials at -20 for 3 hours, then transfer to -80 C freezer overnight.

6. Next day, transfer to liquid nitrogen tank.

Exposure methods:

1. Design the experiment with desired concentrations and durations of exposure. Include vehicle control group (ethanol).

2. Subculture 3 x 10^4 cells /cm^2 in culture dishes (60 mm or 100 mm)

3. 48 hours after seeding (or when cells reach 60 % confluency) change the medium to phenol red free medium and incubate for 24 hours.

4. Dissolve the estrogenic compound in ethanol.

5. Expose the cells to the estrogenic compound. Add ethanol (vehicle control) to cell cultures not treated with estrogenic compound. Concentration of ethanol in each culture should never exceed 0.1%.

5. Change the media every 48 hours adding the chemical of interest.

6. At the end of treatment collect the cells for future analysis. For gene and protein analysis store 2 x10^6 cells/vial at -80 ^oC.

II. MTT ASSAY

Reagent preparation

1. Prepare a 12 mM MTT stock solution by adding 1.3ml of sterile PBS to 6.5 mg of MTT.Mix by vortexing until dissolved. Remove any particles by centrifugation. Store MTT solution at 4^oC for up to four weeks.

2. Add 10 ml of 0.01 M HCl to one tube containing 1 g of SDS. Mix the solution by vortexing. Once prepared, the solution should be used promptly.

Culturing Cells

1. Seed cells in 24 well plates. Culture cells in phenol red free medium.

Labeling cells

1. Remove the medium and replace it with 500 µl of medium and MTT mixture (1:10).

2. Incubate at 37 C for 4 hours.

3. Add 100 µl of SDS-HCl solution to each well and mix thoroughly using the pipette.

4. Incubate the plate at 37 ^{o}C for 4018 hours in a humidified chamber.

5. Mix each sample again using a pipette and read absorbance at 570 nm and background at 690nm.

III. REAL TIME PCR

RNA Isolation

Aurum Total RNA Mini Kit:

Note: All centrifugation steps are performed at maximum speed (12000xg) at room temperatures

A1

-Rinse the growth vessel once with PBS and aspirate. Proceed with lysis if the expected number of cells in the vessel does not exceed $2x10^6$ cells; otherwise, release the cells from the plate and transfer up to $2x10^6$ cells into a 2 ml capped microcentrifuge tube.

-Centrifuge the tube for 2 min.

-Decant the supernatant and blot the tube with the paper towels.

A2

-Add 350µl of lysis solution (already supplemented with 1 % β-mercaptoetahnol) to each tube or growth vessel.

-Pipette up and down several times to lyse cells thoroughly.

A3

-Add 350µl of 70 % ethanol to each tube. Pipet up and down to mix thoroughly.

-Make sure that no bilayer is visible and that the viscosity is substantially reduced.

B1. Insert an RNA binding column into a 2ml capless wash tube.

B2.

-Dicant or pipet the homogenized lysate into RNA binding column. Centrifuge for 30 sec.

-Remove the RNA binding column from the wash tube, discard the filtrate from the wash tube and replace the column into the same wash tube.

B3. The low stringency wash solution is provided as a 5x concentrate. Add 4 volumes (80 ml) of 95-100% ethanol to the low stringency wash solution concentrate before initial use.

B4. Add 700 µl of low stringency wash solution to the RNA binding column. Centrifuge for 30 sec. Discard the low stringency wash solution from the wash tube, and replace the column into the same wash tube.

B5. The RNase-free DNase I is provided as a lyophilized powder. Reconstitute the DNase I by adding 250 µl 10 mM Tris, pH 7.5 (not provided) to the vial and pipetting up and down briefly to mix.

B6. For each column processed, mix 5 µl of reconstituted DNase I with 75 µl of DNase dilution solution in a 1.5 ml microcentrifuge tube (not provided).

Scale up proportionally if processing more than one column. Add 80 µl of diluted DNase I to the membrane stack at the bottom of each column. Allow the digest to incubate at room temperature for 15 min.

B7. Add 700 µl of high stringency wash solution to the RNA binding column. Centrifuge for 30 sec. Discard the high stringency wash solution from the wash tube, and replace the column into the same wash tube.

B8. Add 700 µl of low stringency wash solution to the RNA binding column. Centrifuge for 1 min. Discard the low stringency wash solution from the wash tube, and replace the column into the same wash tube.

B9. Centrifuge for an additional 2 min to remove residual wash solution.

B10. Transfer the RNA binding column to a 1.5 ml capped microcentrifuge tube (provided). Pipette 40 µl of the elution solution onto the membrane stack at the bottom of the RNA binding column, and allow 1 min for the solution to saturate the membranes. Centrifuge for 2 min to elute the total RNA.

The eluted total RNA samples can be used immediately in RT-PCR reactions or in any other application. Alternatively, the total RNA can be stored at 4°C for later use.

RNA concentration determination

Thermo Scientific NanoDrop

1. Start Nanodrop Software.

2. Rinse pedestal with distilled water (clean off top and bottom with chem wipe afterward each sample)

3. Blank with 2 µL of elution solution (whatever RNA was eluded with)

4. Run 2 µL of each sample. After each sample rinse with distilled water and wipe with chem wipe.

5. Record the concentration values.

RNA quality determination

1. Prepare the gel.

-heat 0.5 g agarose in 44.1ml DEPC treated water, 5 ml 10X MOPS running buffer, and 0.9 ml 37% formaldehyde until dissolved

WARNING: Formaldehyde is toxic through skin contact and inhalation of vapors. Manipulations involving formaldehyde should be done in a chemical fume hood.

10X MOPS buffer:

0.4 M MOPS, pH 7.0
0.1 M sodium acetate
0.01 M EDTA

-assemble the gel in the tank, and add enough 1X MOPS running buffer to cover the gel by a few millimeters. Then remove the comb.

2. Prepare the RNA sample.

- to 1-3 µg RNA, add 0.5-3X volumes Formaldehyde Load Dye.

-add ethidium bromide at a final concentration of 10 µg/ml

3. Heat denature samples at 65-70°C for 5-15 min.

4. Run gel for 2-3 hours at 80V or, for finer resolution, run at 5V/cm for 4-5 hours.

cDNA synthesis

iScript cDNA Synthesis Kit:

1. For each reaction use 4µl 5x iScript Reaction Mix and 1 µl iScript Reverse Transcriptase. Scale up proportionally if preparing more than one reaction and pipette up and down before alliquoting 5 µl into separate PCR tubes.

2. Calculate the volume of eluted RNA needed to obtain 1 µg of RNA. Add appropriate volume to each tube.

3. Add appropriate volume of sterile water for the total volume of 20 µl.

4. Incubate complete reaction mix:

5 minutes at 25ºC

30 minutes at 42ºC

5 minutes at 85ºC

Hold at 4ºC (optional)

Real-Time PCR

1. Thaw all components at room temperature. Mix thoroughly by inverting the tube multiple times to ensure homogeneity. Centrifuge to collect the content at the bottom of the tube.

2. Assemble all required components of the reaction cocktail except the sample template and dispense equal aliquots into each reaction tube.

SooFast mix - 10uL

Forward primer - 500 nM

Reverse primer – 500 nM

DNA - 1 uL

Water - 5 uL

3. Mix thoroughly and centrifuge briefly to ensure all components are at the bottom of the reaction tube.

4. Place the plate in the real time PCR instrument and run the program.

IV. WESTEN BLOT ANALYSIS

Ingredients

10% Separating Gel:

3ml of 4x Separating Buffer

4ml of 30% Acryl

5.1ml of H_20

7ul of TEMED

100ul of 10% APS (Ammomium persulfate)

8% Stacking gel:

1ml of 4x Separating Buffer

0.6ml of 30% Acryl

2.4ml of H_20

7ul of TEMED

50ul of 10% APS (Ammomium persulfate)

Procedure

1. Make 10% separating gel and stacking gel each at the same time in 15ml tubes (Put TEMED and APS last as they initiate the crosslinking reaction). Complete the separating gel first by adding TEMED and APS.
2. Using a pipette slowly add the separating gel solution into the gel apparatus and let it sit for 15-20min or until the remaining solution in the tube has solidified.

3. Once the separating gel has solidified complete the stacking gel by adding TEMED and APS. Slowly add the stacking gel into the gel apparatus, but leave enough space for the comb: ~1cm. Insert the comb and let it sit for 15-20min.
4. Prepare samples to be loaded.

Reagents

Lysis buffer

- Get RIPA 2x from fridge and add PMSF and Protease inhibitor cocktail.

1x Runing Buffer

- Make 1L of of 1x running buffer from 10x

1x Transfer Buffer

- For 1L: 700ml of H_20 + 100ml of 10x Transfer buffer + 200ml of MeOH (last).

Procedure

1. Add lysis buffer to samples depending on amount of cells.
2. Vortex/sonicate samples then let them sit for 15min on ice.
3. Spin at top speed for 5min.
4. Perform BCA assay for determining protein concentration.
5. Calculate volume of protein to be loaded.
6. Make sample in 1:1 with loading dye. Add remaining volume with Millipore water to each sample to get final volume of 20ul.
7. Vortex before and after heating samples for better resolution.
8. Heat each sample at ~100^{o}C for 5min.
9. Then centrifuge rapidly for 10-15 seconds at highest speed.
10. Return to prepared gel and remove the comb. Rinse it with water to remove residue. Try to remove as much water as possible.

11. Place gel in holder and put it in the electrophoresis apparatus containing running buffer (Dilute to 1x with H_20)
12. Load samples into gel along with ladder (10ul of Precision plus). Load dye in last lane.
13. Put on the lid and make sure the electrodes are correctly placed.
14. Run gel at 30millamps per gel for 35-45min (Keep an eye on the gel!!)

Membrane Transfer

Preparation of membrane

1. Cut a piece of PVDF membrane and label it on corner with a pencil.
2. Soak it in methanol for 5min.
3. Remove methanol and add 1x transfer buffer until ready to use.
4. Assemble "sandwich" for Bio-Rad's Transblot.
5. Prewet the sponges, 4x filter papers in 1x Transfer buffer.

Sandwich: Sponge – 2x filter paper - gel - membrane – 2x filter paper - sponge

(Make sure there are no air bubbles in the sandwich)

6. In the sandwich, the gel should be on the black side and the membrane should be on the clear side. Place the sandwich in the electrophoresis apparatus containing 1x Transfer buffer and run at 100v for 60-90min.
7. When finished, immerse membrane in Blocking buffer (5% BSA or milk powder in TBST; 10ml is sufficient for 3 membranes) and block for 1hr at room temp.

Antibodies and Detection

1. Once transfer is complete incubate the membrane with diluted primary antibody in Blocking buffer overnight and store in 4^oC.
2. Quickly rinse membranes in TBST then wash 3 x 5 min with TBST.
3. Incubate with secondary antibody diluted in Blocking buffer for 1hr min at room temp. (Same as in step 1)
4. Quickly rinse membranes in TBST then wash 3 x 5 min with TBST.
5. Detection:
   a. Place membrane in the middle of the clear overhead projector sheet.
   b. Get the Immun-Star WesternC Kit
   c. Add 1:1 of the peroxide solution and the luminol/enhancer solution. Take 350 µ l of peroxide solution and mix it with 350ul of luminol solution in a microcentrifuge tube. Pipette all and squirt across the membrane. Place another clear sheet over the membrane to disperse the solution.
   d. Detect with the Versa Doc.

Stripping blot

1. Dilute 10x Stripping solution into 1x with H_2O and incubate membrane(s)
2. Rock for 20min and wash it 2x with TBST for 5min each.
3. Repeat step 1 of "Antibodies and Detection"
4. If you want to preserve it for another day, put it in TBST and wrap it up with parafilm at 4 ^oC.

BCA measurement of proteins

1. Make fresh solution:

- 1 ml BCA (Bicinchoninic Acid solution, Sigma #B9643-1L)

- 1 ml ddH_2O

- 40 µl of 4% $CuSO_4$ (solution kept at 4ºC, and made with water)

2. Add 4 µl of standard, sample or blank (water) in each 96-well plate wells:

* For the sample only, keep in mind that you must add a total of 4 µl, but it does not have to be 4 µl of sample only. You can use 1, 2, 3 µl of sample and complete with water up to 4 µl.

- Blank (ddH_2O) (1 blank of 4 µl)

- BSA (standards): 0.20, 0.5, 0.75, 1.0, 1.5 µg/µl (4 µl each) – or different, depends on your sample concentration

- *Sample (in triplicate for each sample to measure, 1, 2, 3 or 4 µl of sample and complete with water up to 4 µl if necessary) – might need to dilute them prior to taking 1-4 ul.

3. Add 100 µl of BCA mix solution prepared fresh in #1 to each 96-well plate well.

Keep in mind that if you are missing solution to complete all your conditions, you cannot prepare another batch and use it. All the samples have to be prepared with the same BCA solution if compared together.

4. Homogenize with pipette each well.

5. Incubate 15 minutes, 37 ºC, in incubator (or more if samples are not very concentrated, usually stop it when samples are reaching a light purple color). The color complex is stable.

6. Read optical density (O.D.) at 562 nm

7. Calculate the mean O.D. for each sample and use that for protein concentration.