Is gene expression altered by exposure to DBP in a cell culture model?

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Abstract

Phthalates are widely used in industrial processes and household products and as a result are amongst the most commonly encountered environmental pollutants worldwide. Humans are exposed to them directly via their use in cosmetics, medicines, as plasticisers in various products, medical devices and in children’s toys, and indirectly via the environment where they are ubiquitous contaminants due to their industrial, domestic, and agricultural uses. Dibutylphthalate (DBP) was widely used as an insecticide by New Zealand troops stationed in Malaya during the 1950s, and there are reports of increased rates of reproductive abnormalities in children of these veterans. This thesis set out to explore the possible effects of DBP exposure on genes that control testosterone metabolism. To do this, an appropriate cell line was selected, and DBP exposure experiments were conducted. Quantitative reverse transcriptase polymerase chain reaction assays for several key steroidal pathway genes were established, and used to explore possible effects of DBP exposure on expression of these genes. The \textit{HSD17}, \textit{CYP17}, \textit{CYP11 HSD3} and \textit{StAR} genes were examined, and only changes in \textit{StAR} gene expression due to DBP in high (10\,\mu{g}/mL), medium (1\,\mu{g}/mL) and low (0.1 \mu{g}/mL) doses were studied. The results indicated that \textit{StAR} mRNA was upregulated following exposure to a high dose of DBP in the THP1 cell line. On the other hand, exposure to medium and low doses of DBP showed no effect on \textit{StAR} mRNA expression. Despite the limited results obtained in this study, analysis of gene expression changes in the THP1 cell line after exposure to DBP represents a potential model system that may be useful for future work in this area.
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III. Abbreviations

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<th>Abbreviations</th>
</tr>
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<tbody>
<tr>
<td>Dibutylphthalate</td>
<td>DBP</td>
</tr>
<tr>
<td>Endocrine disrupting compounds</td>
<td>EDCs</td>
</tr>
<tr>
<td>Glucocorticoid</td>
<td>GC</td>
</tr>
<tr>
<td>bis (2-ethylhexyl)phthalate</td>
<td>DEHP</td>
</tr>
<tr>
<td>Fetal bovine serum</td>
<td>FBS</td>
</tr>
<tr>
<td>Steroidogenic acute regulatory protein</td>
<td>STAR</td>
</tr>
<tr>
<td>Cytochrome P450 17α-hydroxylase/17-20 lyase</td>
<td>CYP17</td>
</tr>
<tr>
<td>3 beta- hydroxy-delta-5-steroid dehydrogenase</td>
<td>HSD3</td>
</tr>
<tr>
<td>17-hydroxysteroid dehydrogenase</td>
<td>HSD17</td>
</tr>
<tr>
<td>Cytochrome P450 side chain cleavage enzyme</td>
<td>CYP11</td>
</tr>
<tr>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>GAPDH</td>
</tr>
<tr>
<td>Glutaminyl-tRNAsynthetase,</td>
<td>QARS</td>
</tr>
<tr>
<td>beta-2-microglobin</td>
<td>B2M</td>
</tr>
<tr>
<td>c-abl oncogene 1, non-receptor tyrosine kinase</td>
<td>ABL1</td>
</tr>
<tr>
<td>Universal Probe Library</td>
<td>UPL</td>
</tr>
</tbody>
</table>
1 Introduction

1.1 Historical background

The environment has many chemicals that have the potential to interact with essential biological functions and cause adverse effects (Wittassek et al., 2011). Accordingly, there is growing concern about the effects of environmental toxins on human reproductive health (Lam et al., 2014; National Research Council (NRC) 2009; Rupnik, 2011; Sengupta, 2014). Most of the studies in this area have been on animals like rats, and as rats are physiologically closely related to humans, there is reason for concern that there will be similar negative effects on human reproduction caused by environmental toxins such as endocrine disruption compounds (EDCs) (Carlsen et al., 1992; Schettler et al., 2009).

A study by Carlsen et al. (1992) was the first to suggest the potential effects of endocrine disruption compounds (EDCs) on humans, reporting a decline in semen quality in the past 50 years. Studies carried out in the United Kingdom and New Zealand have supported Carlsen et al.’s (1992) results on the decline of sperm number, concentration and motility (Irvine et al., 1996; Shine et al., 2008). It is significant that there is an increase in the number of couples being treated for fertility related problems in today’s world (Kondaveeti et al., 2011), indicating that infertility is on the rise. A study of 150 individuals from a reproductive health clinic in China was carried out to measure the correlation between an environment toxin (phthalates) and semen quality, and found a borderline-significant dose–response relationship between the toxin and sperm concentration (Liu et al., 2012).
According to the Environmental Working Group (2003) and Physicians for Social Responsibility (2003), synthetic chemicals amounting to 85,000 in number have been registered and deemed fit for use in the United States (US) alone; additionally, close to 2,000 more are being added on a daily basis world-wide. Research conducted by the Environmental Working Group (2003) and Physicians for Social Responsibility (2003) has suggested that manmade chemicals are found in tissue, amniotic fluid and umbilical cord blood in levels high enough to cause concern, undermining reproductive health by bypassing the placenta and reaching the developing foetus. These toxins endure in the next generation, potentially affecting following generations’ reproductive health (Huang et al., 2009). Duty et al. (2003a) critique the research conducted about manmade chemicals as unreliable because, in the real world, humans are exposed to multiple combinations of chemicals in the environment, not the one or two at a time tested in experiments. In addition, how an adult reacts to environmental toxins is not necessarily the same as the way a foetus or child will react to the same toxins (Landrigan & Goldman, 2011).

At high doses, environmental toxins cause effects that are expected (Schettler et al., 2009). However, at lower, but continuous levels, these toxins cause diverse effects that are generally not expected, especially on the offspring of those exposed (Shaw et al., 2009). Such effects have been further argued to be as a result of low levels of toxins affecting hormone levels, therefore turning genes off or on and potentially causing significant deformities (Vandenberg et al., 2012). It is common biochemical knowledge that when a gene is switched on at the wrong time or switched off abnormally, significant adverse effects are seen. Among the chemicals affecting genes are phthalates such as dibutylphthalate (DBP). DBP is described as an environmental toxin that is known to cause diseases and health concerns such as diabetes,
obesity, decreased fertility, cardiovascular diseases, mood disorders and altered testosterone levels (Skinner et al., 2010; Zimmermann et al., 2012).

The use of DBP as an insecticide dates back to World War II, when soldiers were described as being dependent on the compound for protection against ticks and lice (Frances et al., 1992; Philip, 1948). The same was the case for New Zealand soldiers who served in Malaya in the 1950s. They too had DBP painted onto their uniforms as an insecticide to kill ticks and lice, which are vectors of bush typhus (Frances et al., 1992; Likeman, 2006; SGADF, 2000).

A study conducted by Duty et al. (2003b) revealed that men with high concentrations of a metabolite of DBP, monoethyl phthalate MEP, had reduced sperm motility. The phthalates found in the men are believed to have been ingested or absorbed through the skin. Therefore, this study supports the potential role of DBP in the reproductive issues experienced by the New Zealand soldiers who served in Malaya. Recent studies have suggested that dermal absorption contributes significantly to the uptake of phthalates (Gong et al., 2014; Pan et al., 2014).

The health effects associated with the use of DBP have stimulated current research into the consequences for New Zealand soldiers and their families since the 1950s (Carran & Shaw, 2012). The findings suggest that the soldiers’ use of DBP contributed to reproductive health issues in the following generation. Carran and Shaw (2012) revealed that children sired by soldiers previously exposed to DBP had a higher chance of developing breast cancer and genital deformities. In addition, their results showed that male children born to these soldiers had an up to eight times higher chance of being born with genital deformities when compared to the general public.
Several studies revealed that male laboratory animals were feminized when exposed to DBP over a long period. Further study of these results revealed that hormone metabolism was either interfered with and/or destroyed (Liu et al., 2005; Mylchreest et al., 1998; Shultz et al., 2001; Thompson et al., 2005). These studies also stated that phthalate DBP causes feminization of male rats after in utero exposure by repressing expression of genes required for testicular steroidogenesis. Caran and Shaw (2012) made similar suggestions in respect to DBP’s impact on hormone metabolism. The study found some evidence of the presence of chemicals in the environment that disrupt the endocrine system, with certain chemicals tending to mimic oestrogen once in the body. As a result, Carran and Shaw (2012) equated the increase in oestrogen levels to increased feminisation in men, a reduction in the average age at which girls reach puberty, and increased cases of breast cancer. Specifically, Carran and Shaw (2012) noted that a sharp increase in breast cancer cases was witnessed in families of the veteran soldiers who served in Malaya. Although the evidence is accumulating, it is not yet possible to say definitely that DBP is the lead agent in the increase of human reproductive health complications, and further research is needed.

1.2 Phthalates in the environment

Phthalates (Fig 1.1) are widely used in industrial processes and household products, and are thought to be amongst the most commonly encountered environmental pollutants worldwide (Feige et al., 2007; Frederiksen et al., 2007; Ghisari & Bonefeld-Jorgensen, 2009; Gunnarsson et al., 2008; Kobrosly et al., 2012). Phthalates including DBP are found virtually everywhere in the environment including in food (Birnbaum & Schug, 2013; Griffiths et al., 1985). Phthalates are widely used because of their plastic softening properties and also as wetting agents in applications in medicine and agriculture (Table 1) (Fig 1.2). Humans face...
direct exposure to phthalates since they are an active ingredient in cosmetics and medicines, and as plasticisers in household products, medical devices and children’s toys. Exposure is also indirect through their emission as ubiquitous contaminants of the environment due to their use in some pesticides and other agrochemical formulations (Chen et al., 2008; Chou & Wright, 2006; Koo & Lee, 2004). Reports from the National Health and Nutrition Examination Survey (NHANES) show that most of the population of the US have been exposed to phthalates (Hernández-Díaz et al, 2013).

![Figure 1.1: Generalised molecular structure for phthalates (after Hernández-Díaz et al., 2013. Reprinted using ChemDraw software.](image-url)
Figure 1.2: Some phthalate esters used in plastics (after Birnbaum & Schug, 2013). Reprinted using ChemDraw software.
Table 1.1: List of common phthalates. Common phthalates and their major uses (after Wormuth et al., 2006).

<table>
<thead>
<tr>
<th>Name</th>
<th>Acronym</th>
<th>Common uses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dibutylphthalate</td>
<td>DBP</td>
<td>Used as plasticiser and in cosmetics</td>
</tr>
<tr>
<td>di-2-ethyl hexyl phthalate</td>
<td>DEHP</td>
<td>Used as plasticiser and in PVC</td>
</tr>
<tr>
<td>Benzyllbutylphthalate</td>
<td>BBP</td>
<td>Used in the manufacture of foamed PVC</td>
</tr>
<tr>
<td>diisodecyl phthalate</td>
<td>DIDP</td>
<td>Used in the production of plastic coating (cookware, pharmaceutical pills, food wrappers).</td>
</tr>
</tbody>
</table>

Most infants exposed to phthalates early in their life are believed to have acquired them through several dietary sources (Latini et al., 2004). A study conducted in Denmark revealed that phthalates were found to be present in every baby food and infant formula that was analysed (Petersen & Breindahl, 2000). These findings, given other evidence of the damaging effects of phthalates on humans, suggest there are likely to be impacts on the development of the babies who ingested this food, such as increasing the chances of sterility in males and breast cancer in females. Adults too are exposed to phthalates through dietary sources because foods such as milk, cream, butter and cheese have been found to contain quantifiable amounts of phthalates (Sharman, 1994). The migration of phthalates from plastic containers
into the drinks they contained was analysed, and the results showed that the migration was 5-40 fold higher from containers with soft drinks compared to identical containers containing mineral water because of the acid effects of soft drinks (Bošnir et al., 2007).

Moreover, food samples packaged in plastics had traces of phthalates, their presence attributed to residues from the packages and plastic gloves that were used to handle the food (Tsumura et al., 2001). Another way for infants and children to be exposed to phthalates is through mouthing of plastic toys and the use of food stored in plastic containers (Latini et al., 2004). To find whether food packages have an affinity for phthalates, packed school meals for children aged 3-10 were tested (Cirillo et al., 2011), and the results indicated the presence of some phthalates, including DBP, in the processed packed foodstuff, suggesting that manufacturing and contact with food wrap can play a major role in the daily exposure to phthalates. Phthalates are now banned from children’s toys and cosmetics like nail polish in some countries. Since 2005, the European Union (EU) has prohibited the use of phthalates in any type of toy and childcare article (EU Council Directives 67/548/EEC and 76/768/EEC).

Studies have also investigated the occurrence and concentration of phthalates in dust from homes and day care centres in several countries such as the US, Germany and Kuwait (Fromme et al., 2004; Gevao et al., 2013; Guo & Kannan, 2011; Langer et al 2010; Rudel et al., 2003). The results from these studies have all showed similar results in detecting phthalates. Langer et al. (2010) found that phthalates were detected in 75% of the 500 houses and in 90% of the 151 day care centres that were tested.
However, even though DBP is less extensively used than some other phthalates, human exposure to DBP is higher than to any other phthalate, and this is considered a cause for concern by The Scientific Committee on Health and Environmental Risk (SCHER) (Bodar, 2008). Exposure to individual phthalates, except DBP, is below the tolerable daily intake; however, exposure to individual phthalates is not the main concern in terms of their biological effects because phthalate effects are very likely to be additive. Therefore, it is exposure to a blend of phthalates that is likely to determine their biological effect (Reddy et al., 2006).

A review of the literature on exposure to some phthalates has been associated with adverse developmental and reproductive health effects (Sharpe & Irvine, 2004). One of the main negative implications of exposure to phthalates may be disruption of the endocrine system (Desdoits-Lethimonier et al., 2012; Johnson et al., 2012) that is responsible for the secretion of the various hormones that are key in the regulation of the reproductive process (Saillenfait et al., 2013). Disruption of the endocrine system is therefore discussed more fully below.

1.3 Endocrine disruption

Environmental exposure to chemicals can disrupt the endocrine system not only of those directly exposed but also that of the next generation. Environmental contaminants, pharmaceuticals, and industrial chemicals are called endocrine disrupting compounds (EDCs) due to their ability to alter the endocrine system. The Organization of Economic and Cooperative Development (OECD, Draft Guidance Document on Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption, 2012, p. 11) has defined an endocrine disrupting chemical as “an exogenous substance or mixture that alters the
function(s) of the endocrine systems and consequently causes adverse health effects in an intact organism, or its progeny or (sub) populations”. The effect caused by the EDC can be associated with disruption of the synthesis, secretion, transport, binding action, or elimination of natural hormones. EDCs can exert their effects through a number of different mechanisms including interfering with hormone biosynthesis, metabolism, or activity. Various EDCs bind to a cellular receptor by mimicking the biological activity of a hormone (agonistic effect) or by binding to the receptor but not activating it (antagonistic effect) (Colborn et al., 1993; Guillette & Guillette, 1996; Molina-Molina et al., 2013). Studies have shown that EDCs bind to the oestrogen receptor and inhibit oestrogen catabolism (Vandenberg et al., 2012) or the thyroid hormone receptor, or interfere with the normal signalling (Portigal et al., 2002).

Animal studies demonstrate that early exposure to EDCs induce epigenetic changes, such as DNA methylation patterns. Studies suggest that the transgenerational effects of EDCs may be due to modification of gene expression independent of mutation (Anway et al., 2005; Anway & Skinner, 2006b; Crews & McLachlan, 2006; Skinner, 2007; Skinner et al., 2011).

1.4 Synthetic ECDs

Man-made chemicals, such as phthalates, have the potential to reduce androgen (e.g. testosterone) production and function, and can act as agonists of the oestrogen receptor to feminize males in some species (Stahlhut et al., 2007). This class of chemicals has the ability to mimic endogenous steroid hormones or alter their metabolism, eventually influencing recruitment of transcription factors and altering gene expression (McLachlan, 2001), and therefore has been classified as EDCs (Rudel et al., 2003). Consistent results dating back several decades show that phthalates are known antiandrogens in experimental animal models (Sonnenschein & Soto, 1998; Stahlhut et al., 2007). In addition phthalates have been found in
several studies to potentially alter individual or multiple enzyme pathways and/or gene-dependent activities (Brucker-Davis, 1998; De Bosscher et al., 2003; Shanle & Xu, 2010).

What is presently unknown is the amount of DBP required to alter enzyme pathways and/or gene-dependent activities. Studies in rats have shown that the lowest observable effect level (LOEL) for a variety of testosterone synthesis gene endpoints is 50 mg.kg body weight/day (dosing period = 7 days) (Lehmann et al., 2004; Struve et al., 2009). This is a high dose and therefore its physiological relevance is questionable.

Studies have shown that exposure to DBP used as an acaricide in military personnel (Frances et al., 1992; Philip, 1948) resulted in a dose of approximately 64 mg.kg body weight/day which is greater than the LOEL (Carran & Shaw, 2012). In addition, studies in rats given a dose $\geq 50$ mg/kg body weight/day have shown that exposure to DBP results in reduced fetal testosterone levels (Lehmann et al., 2004). This dose is within the range of occupational human exposure. Further studies in rats have shown reduced fetal testosterone levels following exposure to very high (i.e. approximately 500 mg/kg body weight/day) DBP doses (Lehmann et al., 2004). Although these very high doses are not comparable to ‘normal’ human exposure, the results further support the possibility that DBP reduces testosterone synthesis, and therefore understanding the phthalates’ metabolism can provide a clear idea of how phthalates work in the body. The metabolism of phthalates is therefore discussed below.

1.4.1 Metabolism of phthalates
Most phthalates are well absorbed following oral, dermal and inhalation exposure (Kluwe, 1982; Kurahashi et al., 2005). Following an oral exposure they are absorbed across the esterase-rich intestinal mucosa and enter the circulatory system where they are likely to
encounter serum esterase and undergo ester changes (Frederiksen et al. 2007, Wittassek & Angerer 2008). Intestinal mucosal and blood esterase initiate the metabolic process by cleaving their R-groups (see Fig 1.1) and likely liberating mono-R phthalates or phthalic acid. Mixed oxidising agents in tissues, primarily the liver, oxidise the remaining R-groups to form hydroxyphthalates (Fig 1.3).

![Figure 1.3: Metabolism of dibutylphthalate (DBP) in mammals (Birnbaum & Schug, 2013). Reprinted using ChemDraw software.](image)

1.4.2 Mechanism of toxicity
The mechanism of toxicity of the phthalates is thought to be via inhibition of testosterone synthesis rather than androgen receptor antagonism, or the oestrogen mimicry observed in pesticides such as vinclozolin and procymidone, which explains their feminising biological
effect in males (Fisher, 2004; Parks et al., 2000). A possible explanation for phthalates inhibiting testosterone synthesis is that some phthalates or metabolites mimic steroid intermediates of the testosterone pathway and thus are potential competitive testosterone synthesis enzyme inhibitors (Fig 1.4). Xiao-feng et al. (2009) showed that DBP inhibits testosterone synthesis via the glucocorticoid pathway which in turn leads to the androgen (e.g. testosterone) synthesis pathway (Fig 1.5).

Interestingly there are molecular similarities between glucocorticoid pathway intermediates (e.g. pregnenelone) and mono(4-hydroxybutyl)phthalate which appear to support the DBP role as a glucocorticoid pathway inhibitor. On the other hand, such molecular similarities could lead to mono(4-hydroxybutyl) phthalate occupying the glucocorticoid receptor and affecting its activity either directly or via regulation of glucocorticoid receptor synthesis. Indeed, Xiao-feng et al. (2009) demonstrated up-regulation of 11βHSD1 expression which might change the steroid flux through the glucocorticoid pathway away from testosterone synthesis. Whether regulation of testosterone synthesis genes is physiologically relevant depends on the dose of DBP necessary to affect gene regulation.
Figure 1.4: Molecular structural analogies. Mono(4-hydroxybutyl)phthalate (A) and pregnenelone (B) with A and B superimposed to show their molecular structural analogies (C) (Carran & Shaw 2012). Reprinted with permission.
Figure 1.5: Steroid metabolism. The steroid metabolism pathway leading to testosterone showing the involvement of DBP-affected genes that code for key enzymes in the pathway (Carran & Shaw 2012). Reprinted with permission.
1.4.3 Toxicity of DPB

DBP has shown a potential risk to human health through effects on the development of the male reproductive system, working as an estrogenic or antiandrogenic and targeting mainly the testicular Leydig cells (Alam et al., 2010; Ema & Miyawaki, 2001; Wakui et al., 2013). Animal studies have shown that prenatal exposure to DBP has effects on adult and induces a testicular dysgenesis syndrome of disorders in male offspring (Mitchell et al., 2012a; Wakui et al., 2013). Other studies showed that the use of DBP in many applications, such as in cosmetics, can lead to the reduction of the quality of the sperm such as reduced concentration, altered morphology, reduced motility (Ema et al., 2000; Gevao et al., 2012; Kelley et al., 2012; Singh & Li., 2012).

Human studies, although quite limited, have suggested a link between exposure to environmental oestrogens and the increase in male reproductive disorders such as testicular cancer, cryptorchidism, hypospadias, and low sperm count (Choi et al., 2012; Dean & Sharpe, 2013; Thankamony et al., 2014). Carran and Shaw (2012) suggest that developmental changes seen later in life may have occurred because of the changes following fertilisation of ova as a result of the effect of DBP on the sperm level. They explain such a result by the possibility of an epigenetic gene regulation mechanism.

1.5 Epigenetics

Epigenetics refers to a range of cellular processes that impact on gene expression, but that are not strictly inherited (Bollati & Baccarelli, 2010; Ledford, 2008). These processes include stable chemical modifications of the DNA itself, and dynamic modification of the histone molecules which control the packaging of DNA into chromatin, as well as a range of processes mediated by noncoding RNA molecules (Dobosy et al., 2007). Chemical
modifications of the DNA can exert profound effects on gene expression, and of the various epigenetic processes known this is probably the most stable and enduring, and therefore more likely to contribute to intergenerational change (Jaenisch & Bird, 2003).

Many substances encountered in the environment leave their mark on the epigenome. The study of epigenetic changes in relation to health and disease was principally pioneered in cancer, and DNA methylation has been extensively studied as a biomarker of disease (Belinsky, 2004; Belinsky et al., 2006; Marsit et al., 2011; Yuasa, 2010a; Yuasa, 2010b) or of exposure to carcinogens, including sunlight (El-Abaseri et al., 2006), cigarette smoke (Huang et al., 2011; Phillips & Goodman, 2009), and asbestos (Tsou et al., 2007). Over recent years, interest has grown in examining methylation changes in relation to exposure to other important environmental toxins such as lead (Wright et al., 2010), and benzene (Bollati et al., 2007), and in relation to normal human diet (Feil, 2006; Mathers et al., 2000). Interest in methylation patterns in various disease processes is also growing with the widespread appreciation of the role this DNA modification plays in gene regulation and its ability to be modified by environmental factors (Docherty & Mill, 2008; Mill & Petronis, 2008; Mill et al., 2008; Ordovás & Smith, 2010; Ushijima et al., 2006). Epigenetic changes induced by the environment are being increasingly linked to human health and disease (Singh & Li, 2012).

Phthalates have the ability to mimic endogenous steroid hormones or alter their metabolism, eventually influencing recruitment of transcription factors and altering gene expression (McLachlan et al., 2006). Animal studies demonstrate that early exposure to EDCs induces epigenetic changes, such as DNA methylation patterns (Dolinoy, 2008). Experimental evidence suggests that transgenerational effects of EDCs may be due to this kind of modification of gene expression independent of mutation (Anway & Skinner, 2006a; Skinner
et al., 2010). The study of the human breast cancer cell line MCF7 treated with phthalate, $n$-butyl benzyl phthalate (BBP) showed demethylation of the oestrogen receptor (ESR1) promoter-associated CpG islands. This suggests that ESR1 mRNA expression was altered following BBP exposure due to DNA methylation (Singh & Li, 2012). Another study has demonstrated that the effect of the exposure of a mixture of plastic derived endocrine disruptor compounds bisphenol-A (BPA), DEHP and DBP at two different doses promoted epigenetic transgenerational inheritance of adult-onset disease, including testis disease, ovarian disease, pubertal abnormalities and obesity (Manikkam et al., 2013). Maternal exposure to phthalates showed smaller (feminized) anogenital distance in infant boys and an increase of DNA methylation and expression levels of DNA methyltransferases in mouse testes (Latham et al., 2012). The growing evidence has established that epigenetic alterations due to exposure to environmental toxicants can cause changes in gene expression that may persist throughout life and perhaps be transmitted to offspring.

Epigenetics is an important mechanism that holds considerable potential for developing biological markers, and developments in the epigenetic field show promise of gaining insights into the effects of endocrine disruptors upon human health. At the research level, these efforts currently aim to predict which chemicals would put exposed subjects at risk, and which individuals exposed would be more susceptible to developing disease.

1.6 Exposure to environmental toxins

There are many environmental contaminants that have the potential to interact with essential biological functions and cause adverse effects. Exposure to naturally occurring chemicals like arsenic, and manufactured chemicals including industrial and agricultural products, can occur via different pathways such as inhalation, leading to health impacts (Andrew et al., 2003;
A large proportion of these chemicals are carcinogens and they are part of our daily lives, therefore have been attracting considerable attention from research communities (Autrup, 2000). Developing new approaches for the identification of carcinogens and other environmental hazards has become one of the major challenges in the fields of genetic and toxicology. From animal studies it is clear that DBP represses the expression of genes required for testicular steroidogenesis. Alterations in gene expression may identify genes that are specifically modified by a toxin, and such changes could prove useful as biomarkers of exposure. For example, one study on the human bronchial epithelial cell line showed altered expression of 25 genes following low dose cadmium exposure (Andrew et al., 2003). These genes are possible biomarkers of metal exposure, and similar approaches may be applied to other toxins such as EDCs. The present study therefore focused on investigating whether DBP exposure altered the regulation of selected genes from the glucocorticoid (GC) pathway in the THP1 cell line.

1.7 Aim of the research

The aim of this study was to investigate the effects of DBP on several target steroid metabolism genes in the human cell line THP1. Key steps in the research were:

1. To establish a cell culture model for exploring human exposure to DBP.

2. To identify candidate genes that may show altered expression in response to exposure to DBP.

3. To expose the cell culture model to DBP and evaluate effects on expression of these genes.
2 Materials and methods

2.1 Tissue culture

The human monocytic cell line THP1 (Tsuchiya et al., 1980) was used for the examination of the effects of DBP in this study. A flask of THP1 cells was gifted from A/Prof Steven Giesig (Free Radical Biochemistry Laboratory, University of Canterbury, Christchurch, New Zealand). Cells were grown in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (FBS), 1mM sodium pyruvate, 0.05mM 2-mercaptoethanol and supplemented with Glutamax (Sigma-Aldrich MO USA) as an alternative energy source to support the growth of cells (according to the protocol described by the American Type Culture Collection (ATCC) (Manassas, VA USA). During routine culture, THP1 cells were counted in order to keep the cells between \(2.5 \times 10^5\) as other laboratories growing THP1 cells have suggested.

Cells were harvested by centrifugation at 300 x g for 5 minutes at room temperature. The supernatant was discarded and the pellets were resuspended with fresh RPMI media and incubated at 37°C degree in an atmosphere of 5% CO\(_2\) for two days until approximately doubled in number (as described in the following growth experiment). However, for starting a new passage, the cells were washed with fresh medium after thawing to remove as much dimethyl sulfoxide (DMSO) as possible, and then incubated for 5-7 days with 10mL of growth medium in a TC25 flask.
2.1.1 Freezing cells

The freezing medium was 5% DMSO to 95% FBS (both filtered). The total number of cells in the flask was calculated (see below, section 2.1.2), the cells were centrifuged at 300 x g for 5 min, and the supernatant was removed. The cells were resuspended in freezing medium at 6 - 12 x 10⁶ cells/mL and 1 mL aliquots were placed in a freezing container “Mr Frosty” (Sigma-Aldrich, MO, USA) that cooled cells at 1°C/minute, the optimal rate for cell preservation. This container was placed at -80°C overnight, and frozen aliquots were either stored at -80°C or in liquid nitrogen.

2.1.2 Cell counting

The haemocytometer and cover slip were cleaned using 70% ethanol and placed over the counting grid prior to adding the cell suspension. The cell suspension was well mixed to disperse any cell clumps, and 20 μl was added to 80 μl Trypan Blue 0.4% (Sigma-Aldrich, MO, USA) and then gently mixed by repeat pipetting. One or two drops of the well mixed cells were placed in the haemocytometer by gently resting the tip of the pipette at the edge of each chamber until the sample had filled the counting chamber by capillary action. The counting chamber was viewed under 10x and then 40x magnification to focus on the grid lines of the haemocytometer and count the number of live cells that looked viable (unstained by Trypan Blue) in the 4 sets of the 16 corner squares of each chamber. The total number of cells in one set of 16 corner squares is equivalent to the number of cells x 10⁴ / ml. Therefore, to calculate the cell density, the following formula was used:

\[
T = \frac{n \times 5}{8}
\]
Where:

\( T \) is the average number of cells in \( 1 \times 10^{-4} \) mL

\( (n) \) is the total count from the 8 sets of the 16 corner squares of the two chambers.

5 accounts for the 1:5 dilution in Trypan Blue.

8 accounts for the set of 8 squares of both chambers of the haemocytometer.

2.1.3 Time and dose experiment

In order to examine changes in the gene expression of THP1 due to DBP exposure, cells were grown in a TC75 flask for a week to maintain a good growth rate, and then seeded on a 12 well-plate. DBP was dissolved in ethanol with a total volume of 300 \( \mu \)L, and three doses were selected: the lowest DBP exposure dose (0.1 \( \mu \)g/mL), the middle dose (1 \( \mu \)g/mL) and the highest level of exposure to DBP (10 \( \mu \)g/mL), with a final DBP concentration of 0.086 \( \mu \)g/mL, 0.279 \( \mu \)g/mL and 2.44 \( \mu \)g/mL respectively. Concentrations of the high and medium doses were selected according to the level of DBP estimated by modelling to be in the blood of New Zealand soldiers (Carran & Shaw, 2012). The low dose was based on the average adult dietary exposure in Denmark (EFSA, 2005) (assuming 70kg and 3.3L serum volume).

The vehicle control was ethanol.
On the day of seeding, the cell sample was centrifuged, then resuspended in fresh medium at approximately $2.5 \times 10^5$ cells/mL. To each well, 2 mL of the freshly diluted cell suspension were added, followed by the appropriate treatment.

The treatments entailed adding 10 µl aliquots of either DBP solution (at high, medium or low concentration) or ethanol control to each well for the appropriate time (0, 24, 48, 72 or 96 h). This time course experiment was conducted “in reverse” over 96 h and every 24 h DBP solution was added to the appropriate set of wells to give the time points 96, 72, 48, and 24 h respectively. The zero time point used RNA extracted from cells at the time of seeding. This arrangement allowed cells from all time points to be harvested on one day, and for subsequent processing steps to be carried out without batch effects that might occur if cells were harvested at different times during the experiment. Four 12-well plates were used in this experiment (3 for the low, middle and high doses of DBP, and one for the ethanol control). Within each plate there were three technical replicates (i.e. three wells for each time point). The whole experiment was repeated three times, starting on different days (three biological replicates). Cell counts were measured for each experiment using a haemocytometer.

2.1.4 Mycoplasma detection

Infections with Mycoplasma species can dramatically affect virtually every aspect of cellular metabolism. To ensure the THP1 cell culture was free from mycoplasma contamination, a PCR method was applied to check for any mycoplasma infection (Uphoff & Drexler, 2002). THP1 cells were grown without any antibiotics in the medium for around a week, and cell supernatants were then assessed by this method for the presence of mycoplasma. The result showed no mycoplasma contamination in THP1 that was growing in our laboratory.
2.2 RNA Extraction and isolation

Cells were harvested by centrifuging at 12,000 x g for 3 minutes and the medium was removed. The cells were lysed by the addition of 1 mL of TRIzol® Reagent (Life Technologies, NY, USA) followed by repetitive pipetting of the mixture. The homogenized samples were incubated at room temperature for 8 minutes and 300 µL chloroform was added to the sample.

The tubes were vigorously shaken for 15 seconds and incubated at room temperature for 15 minutes. The tubes were then centrifuged at 12,000 x g for 15 minutes ~ 4°C.

The mixture separated into an aqueous colourless upper phase and red phenol-chloroform lower phase with an interphase layer in between. The aqueous phase containing the RNA was carefully transferred into a clean 1.5 mL Eppendorf tube and isopropanol was added (800 µl per 1 mL TRIzol®) and gently mixed. The samples were left at room temperature for 10 minutes and then centrifuged at 12,000 x g for 10 minutes at 4°C. RNA precipitated as a gel-like pellet on the side and the bottom of the tube. The supernatant layer was removed and the RNA pellet was washed with 75% ethanol, adding at least 1 mL ethanol per 1 mL TRIzol®. Samples were mixed by vortexing and then centrifuged for 5 minutes at 7,500 x g at 4°C. The washing step was repeated. The RNA was dried by leaving the open tubes on their side at room temperature, after removing all the ethanol, until residual ethanol had evaporated. The pellet was redissolved in 20 µL diethylpyrocarbonate (DEPC) -treated water and then incubated at 55-60°C for 10 minutes.
2.2.1 Measurement of RNA and DNA quality

The purity of total RNA and DNA was determined using the Thermo Scientific NanoDrop™ (ThermoScientific, Wilmington, DE, USA). Nucleic acid absorbed maximum at 260 nm therefore the ratio of the absorbance at 260 and 280 nm is used to determine the purity of the DNA and RNA preparations. The values of 1.8 and 2.0 are the acceptable values for DNA and RNA respectively. The ratio of absorbance at 260 nm and 230 nm was used as a secondary measurement to assess the nucleic acids’ purity. The acceptable ranges were between 2.0 and 2.2 for both DNA and RNA.

2.3 cDNASynthesis

cDNA was prepared from the total RNA using the qScript™ cDNA Synthesis Kit (Quanta Biosciences, Gaithersburg MD, USA). All components (except the enzyme) were thawed, mixed and centrifuged and then held in ice. The first step of the cDNA synthesis reaction is the priming step using oligo-dt and random primer which enable copying of all mRNA transcripts. The delicate second step was to catalyse the reaction by reverse transcriptase enzyme. The synthesized cDNA was then stored at -20°C for further use.

2.4 RT - qPCR

2.4.1 Primer design

For each gene (target and reference) the Universal Probe Library (UPL) assay design centre from the Roche website (http://www.roche-applied-science.com) was used to identify the appropriate probe for each particular gene.
2.4.2 Reference genes and target genes primer design

The name of each reference and target gene was entered into the search box of the assay design centre and the appropriate probe was designed by the software. The website generated the UPL probe number required for the assay, and sequences for the two primers needed for each amplicon. Table 2.1 provides information on the reference genes and Table 2.2 provides the information for target genes.

2.4.3 Optimizing QPCR

In order to check the quality of the RNA and cDNA used in experiments the PCR products were run using MultiNA (electrophoresis analysis platform). In order to check whether genes not expressing in THP1 was due to problems with designing the QPCR, three cell lines were used to check the expression of each of the genes. Jurkat, plasma B cells and Ishikawa cell lines were used to validate the expression of all the genes (target and reference) and compared to the expression of these genes in the THP1 cell line in the same run. A temperature gradient was established for all genes used in this study.

2.5 Gene expression

qPCR analysis was carried out on the Lightcycler LC480 real-time PCR system (Roche, Mt Wellington, Auckland, New Zealand). A standard master mix was prepared (Table 2.3) fresh for each experiment. In order to correct for possible run-to-run variation, an inter-run calibrator was used. Inter-run calibrators are identical samples which have been made using a single cDNA batch and tested before use. One gene was tested in a single run in a 96 well plate for the 5 time points (0, 24, 48, 72 & 96 h) and for the 3 doses (low, mid & high plus cells treated with the vehicle ethanol) as well as a calibrator and a negative control in
triplicate. The negative control is a no template control used to ensure the run is contamination free.

2.6 Statistical Analysis

The aim of this project was to determine if the exposure to DBP has an effect on expression of specific genes in the THP1 cell line. This question was investigated through the use of RT-qPCR and the data analysis was performed using one-way ANOVA through the general linear model procedure in the Statistical Package for the Social Sciences. Values were considered as statistically significant at P < 0.05. The statistical analysis was generated by Dr Anna Pilbrow (Christchurch Heart Institute, University of Otago, Christchurch).
Reference genes

Table 2.1: Reference genes primer design information.

<table>
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<tr>
<th>Gene</th>
<th>Primer</th>
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<th>Tm</th>
<th>%GC</th>
<th>Sequence</th>
</tr>
</thead>
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<td>Right</td>
<td>19</td>
<td>130 - 148</td>
<td>60</td>
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<td><code>gcccaatacgaccaaatcc</code></td>
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<td>23</td>
<td>168 - 190</td>
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<td>35</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Left Primer</td>
<td>Right Primer</td>
<td>Amplicon (110 nt)</td>
<td>Amplicon (107 nt)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>---------------</td>
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<td>856 – 877</td>
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<td>943 – 962</td>
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## Target genes

*Table 2.2: Target genes primer design information.*

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<th>Tm</th>
<th>%GC</th>
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<td></td>
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<tr>
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<td>22</td>
<td>404 - 425</td>
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<td>41</td>
</tr>
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<td>471 - 488</td>
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<td></td>
<td></td>
<td>tcccaagccatttctgaacgcacc</td>
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<td>633 - 654</td>
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<td>tcccccaacaaaaacc</td>
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### HSD3 probe: #17

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<tbody>
<tr>
<td>Left Primer</td>
<td>21</td>
<td>408 - 428</td>
<td>59</td>
<td>52</td>
</tr>
<tr>
<td>Right Primer</td>
<td>20</td>
<td>479 - 498</td>
<td>59</td>
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**Amplicon (91 nt)**

tcttccgggtgtcaactcagagagtctatcatgaatgtcaatgtgaaggtaccctaccgctcc
tgttagaggcctgtgtccagctagtggtc

### STAR probe: #44

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**Amplicon (67 nt)**
tacgtggtctactcagccatcgacctcaagggtggctgcccaagagatcatcatcaaccaggtcctgtcc
Table 2.3: qPCR master mix PCR reagents were manufactured by FisherBiotec (Wembley, WA, Australia).

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount per reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>buffer:10x Reaction Buffer</td>
<td>90 µl</td>
</tr>
<tr>
<td>dNTP</td>
<td>90 µl</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>72 µl</td>
</tr>
<tr>
<td>Taq:TAQ-Ti Heat-Activated DNA Polymerase</td>
<td>9 µl</td>
</tr>
<tr>
<td>UPL Probe</td>
<td>9 µl</td>
</tr>
<tr>
<td>Forward primer</td>
<td>18 µl</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>18 µl</td>
</tr>
<tr>
<td>MQH2O</td>
<td>369 µl</td>
</tr>
<tr>
<td>cDNA 1:5 dilution</td>
<td>225 µl</td>
</tr>
</tbody>
</table>
3 Results

3.1 Choosing a Model System

DBP and other phthalates are known to disrupt the production and action of androgens, so concentrating on the genes involved in the synthesis of steroids was an obvious choice. An ultimate goal of this research was to lay the groundwork for the development of blood-based gene expression markers that might indicate whether or not an individual had been exposed to high levels of phthalates. This dictated that the cell lines comprised distinct properties, including:

- Being derived from a human source.
- Being derived from blood.
- Being stable for secondary expression (allow reliable growth and gene expression in tissue culture).
- Capability of expressing the aromatase gene (aromatase is an essential enzyme in the biosynthesis of oestrogens and as such is an indicator that steroidogenesis is taking place within the cells).

The decision on the ideal cell line that would best match the aforementioned properties involved a reflection on the relevant literature. It was suggested that aromatase expression was to be found in various human cell lines, including MCF7, HeLa, HepG2, SK-BR-3, H295R (Chen et al., 2010) and THP1 (Jakob et al., 1995; Shozu et al., 1997). The literature further suggested that aromatase was articulated in human peripheral blood and bone marrow cells (Calado et al., 2009; Christmas et al., 2003; Nalbandian & Kovats, 2005; Vottero et al 2006).
However, there is disagreement on the model of expression in the peripheral blood cells. The considered articulation of aromatase gene in peripheral blood was suggested to be differential with reference to the age of the source (Vottero et al., 2006). Such eliminated peripheral blood was a source of aromatase genes for this study. The decision to use this source further highlighted the need to identify a stable cell line capable of generating a reproducible gene. **This would allow for the development of the required data. Further reflection on the possible aromatase articulating cell lines identified THP1.** The THP1 cell line was perceived to be fairly stable and was sourced from human blood (Jakob et al., 1995). It had little differential aspects of concern, hence its choice for the study.

### 3.2 The growth of THP1

The growth characteristics of THP1 under the tissue culture conditions of this study (with feeding THP1 every second day) were assessed using two separate culture flasks, and the results are presented in Figure 3.1. Figure 3.1 shows the cells requiring several days to double. Day 16 indicates the period of declining growth as the culture enters the death phase. The morphology of the cells was observed to be changing during the doubling and the death phase. At the doubling phase (Fig 3.2, A), the morphology of the cells may be described to be round and healthy during the growth. The morphology changes during the death phase where the cells become shapeless and unhealthy (Fig 3.2, B). The growth rate of THP1 at low, medium and high (0.1, 1.0 and 10 μg/mL) concentrations of DBP was examined (Fig 3.3). Plates rather than flasks were used, and the cells were not fed for the duration of the experiment. Fig 3.3 shows the growth rate was steady up until 72h for cells exposed to low and medium concentrations of DBP, but declined after 48h with the highest dose of DBP. It is
proposed that the decline in numbers seen at 72h with medium and low doses is due to the cells running out of nutrients.

The earlier decline seen in cells grown with a high DBP concentration is probably due to DBP also having a toxic effect on the cells.

*Figure 3.1:* Growth curve for THP1. THP1 cells from two independent flasks (1 and 2) were grown under the same tissue culture environment. Cells need two days to double as they were fed every second day and become unhealthy after 10-11 passages.
Figure 3.2: The morphology of THP1 changed dramatically as cells entered the death phase. A: Cells look healthy and round during the growth phase. B: cells started to look smaller and clumped during the death phase.

Figure 3.3: Growth over DBP exposure. The growth of THP1 cells exposed to different doses (high (H) blue, middle (M) red and low (L) green) in three independent experiments. The mean of the numbers of viable cells was taken at each time point (0, 24, 48, 72 and 96). The cell death occurred around 72h for low and middle doses, whereas the high dose showed more rapid cell death at an earlier time (~ 48h).

3.3 Choosing Target and Reference Genes
DBP has been suggested to be interfering with the production and the subsequent action of testosterone. It was, therefore, pertinent to investigate DBP’s effect on genes involved in steroidogenesis. The selection of the target genes was advised by studies that explored DBP exposure in both animals and human (Barlow et al., 2003; Sapone et al., 2003).

The five genes, steroidogenic acute regulatory protein (StAR), cytochrome P450 17-hydroxylase/17-20 lyase (CYP17), 3 beta- hydroxy-delta-5-steroid dehydrogenase (HSD3), 17-hydroxysteroid dehydrogenase (HSD17) and cytochrome P450 side chain cleavage enzyme (CYP11) were selected, and the choice of the appropriate isoform was further investigated, because the link between a known protein and its specific gene was not always obvious.

A search of the human genome was carried out using the genome browser facility at www.genome.ucsc. This site contains reference sequences and working draft assemblies for a large number of genomes. The search terms are entered in the Human (Homo sapiens) Genome Browser Gateway search bar. The quick search facility on the site www.genenames.org was used to assign correct gene names to the relevant genes. The summary data supporting the choice of genes is presented in Table 3.1. A literature search then used to find studies that supporting which isomer is appropriate for this project.

The endogenous control genes should be expressed at the same level in all study samples, that is, those unlikely to be affected by DBP, as they are used to normalize expression data. The genes assessed for this purpose were GAPDH, QARS, B2M and ABL1, which code for glyceraldehyde-3-phosphate dehydrogenase, glutaminyl-tRNA synthetase, beta-2-microglobulin and c-abl oncogene 1, and non-receptor tyrosine kinase, respectively. These genes are not involved in steroidogenesis but rather are essential genes in all mammalian cells.
<table>
<thead>
<tr>
<th>Names</th>
<th>Alternative names</th>
<th>EC#</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSD17B1</td>
<td>17-beta-hydroxysteroid dehydrogenase.</td>
<td>1.1.1.62</td>
<td></td>
</tr>
<tr>
<td>Estradiol 17-beta-dehydrogenase</td>
<td>20-alpha-hydroxysteroid dehydrogenase.</td>
<td></td>
<td>Homo sapiens</td>
</tr>
<tr>
<td>hydroxysteroid (17-beta) dehydrogenase 2</td>
<td>Estrogen 17-oxidoreductase.</td>
<td>1.1.1.62</td>
<td></td>
</tr>
<tr>
<td>HSD17B2</td>
<td>17-beta-hydroxysteroid dehydrogenase.</td>
<td>1.1.1.62</td>
<td>N/A</td>
</tr>
<tr>
<td>hydroxysteroid (17-beta) dehydrogenase 4</td>
<td>20-alpha-hydroxysteroid dehydrogenase.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hydroxysteroid (17-beta) dehydrogenase 4</td>
<td>17-beta-hydroxysteroid dehydrogenase.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hydroxysteroid (17-beta) dehydrogenase 4</td>
<td>20-alpha-hydroxysteroid dehydrogenase.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

This isoform of 17 beta-hydroxysteroid dehydrogenase is expressed predominantly in the testis and catalyzes the conversion of androstenedione to testosterone.
The protein encoded by this gene is a bifunctional enzyme that is involved in the peroxisomal beta-oxidation pathway for fatty acids. It also acts as a catalyst for the formation of 3-ketoacyl-CoA intermediates from both straight-chain and 2-methyl-branched-chain fatty acids.

**HSD17B4**
hydroxysteroid (17-beta) dehydrogenase 4

**HSD17B6**
hydroxysteroid (17-beta) dehydrogenase 6 homolog (mouse)

**HSD17B7**
3-KSR.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
<th>Enzyme ID</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSD17B8</td>
<td>17-beta-hydroxysteroid dehydrogenase. 20-alpha-hydroxysteroid dehydrogenase.</td>
<td>1.1.1.62</td>
<td>N/A</td>
</tr>
<tr>
<td>HSD17B12</td>
<td>17-beta-hydroxysteroid dehydrogenase. 20-alpha-hydroxysteroid dehydrogenase.</td>
<td>1.1.1.62</td>
<td>N/A</td>
</tr>
<tr>
<td>HSD17B14</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

This gene encodes a very important 17-beta-hydroxysteroid dehydrogenase (HSD17B12) that converts estrone into estradiol in ovarian tissue. This enzyme is also involved in fatty acid elongation.
dehydrogenase 14


Cholesterol side-chain cleavage enzyme.

Cytochrome P-450(scc). Cytochrome p450(scc).


Cholesterol side-chain cleavage enzyme.

Cholesterol side-chain-cleaving enzyme.

Cytochrome P-450(scc). Cytochrome p450(scc).


Cholesterol side-chain cleavage enzyme.

Cholesterol side-chain-cleaving enzyme.

**CYP11A1**

Cytochrome P-450(scc).

cytochrome P450, family 11, subfamily A, polypeptide 1

Steroid 20-22 desmolase.

Steroid 20-22-lyase.

1.14.15.6


**CYP17A1**


Homo sapiens chromosome 10 open reading frame 26 (C10orf26), transcript variant 1,
<table>
<thead>
<tr>
<th>Gene Abbreviation</th>
<th>Description</th>
<th>Enzyme Activity</th>
<th>OMIM Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSD3B1</td>
<td>hydroxy-delta-5-steroid dehydrogenase, 3 beta- and oxosteroid isomerase.</td>
<td>Delta(5)-3-ketosteroid isomerase. Delta(5)-steroid isomerase.</td>
<td>1.1.1.145</td>
</tr>
<tr>
<td>StAR</td>
<td>steroidogenic acute regulatory protein</td>
<td>Hydroxysteroid isomerase. Steroid isomerase.</td>
<td>5.3.3.1</td>
</tr>
<tr>
<td>STARD3</td>
<td>StAR-related lipid transfer (START) domain</td>
<td>Cholesterol homeostasis is regulated, at least in part, by sterol regulatory</td>
<td></td>
</tr>
</tbody>
</table>

Cholesterol homeostasis is regulated, at least in part, by sterol regulatory element (SRE) binding proteins (e.g., SREBP1; MIM.
containing 3

184756) and by liver X receptors (e.g., LXRA; MIM 602423).

Cholesterol homeostasis

in part, by sterol regulatory element (SRE) binding proteins (e.g., SREBP1; MIM 184756) and by liver X receptors (e.g., LXRA; MIM 602423). Upon sterol depletion, LXR

LXRs are inactive and SREBPs are cleaved, after which they bind promoter SREs and activate genes involved in cholesterol biosynthesis and uptake.

STARD4:

StAR-related lipid transfer (START) domain

containing 4 N/A N/A

STARD7

StAR-related lipid transfer (START) domain N/A N/A

Although the function of this gene is not known, its existence is supported by mRNA and EST data. The predicted gene product contains a region similar to the STAR-related

containing 7 lipid transfer (START) domain, which is often present in proteins involved in the cell signaling mediated by lipid binding.

Table 3.1: Target and reference genes database information. Descriptions are as reported on the HGNC database of human gene names website www.genenames.org and the UCSC genome browser website www.genome.ucsc. N/A means no information available. Genes in bold and underlined are the ones that “Enzyme Commission (EC)” - a repository of information relative to the nomenclature of enzymes”. 
3.4 Designing qPCR Assays

The ProbeFinder software available at www.roche-applied-science.com enabled the selection of a suitable Universal Probe Library (UPL) probe and a set of matching target-specific PCR primers for each of the genes named above. The sequences of the left and right primers and amplicons for reference and target genes are given in Tables 2.1 and 2.2 (Materials and Methods).

Standard curves (Figs 3.3-3.5) were created to check the efficiency of all genes. This involved preparing serial dilutions (1 in 4) of cDNA from each gene, running PCR assays in triplicate and plotting threshold cycle number (Ct) against the log of the nanograms (ng) of total cDNA in each sample. The efficiency of all genes was then calculated from the standard curves using the following formula:

\[
\text{Efficiency} = (10^{\frac{1}{\text{slope}}} - 1) \times 100
\]

The efficiency numbers of all genes are shown in Table 1. The efficiency numbers for the target genes, CYP17 and HSD17, are very high, while that of HSD3 is low. StAR, with 91% efficiency, was the only target gene within the desirable efficiency range of 90-110%. PCR assays outside of this range may not be sufficiently sensitive. Hence StAR was the gene selected to test the effects of DBP. The reference genes GAPDH, QARS and B2M replicated with efficiencies in the 90-110% range. On the other hand, genes that showed an efficiency number lower than 90% and greater than 110% were excluded from the experiment. For
example, *ABL1* (efficiency number 118) was not used in further studies. Table 3.2 shows the efficiency numbers of all genes for which a standard curve was generated.

*Figure 3.3*: Standard curve for *GAPDH* (blue) and *StAR* (green). Standard curve shows threshold cycle (Ct) on the y-axis and the starting quantity (the log of nanograms) of total cDNA target on the x-axis. The blue standard curve has an efficiency of 94%. The green standard curve has an efficiency of 91%.
Figure 3.4: Standard curve for B2M (green) and HSD3 (blue). Variation of C\textsubscript{t} with PCR efficiency. The blue standard curve has an efficiency of 24.8%. The green standard curve has an efficiency of 109%.
Figure 3.5: Standard curve for QARS (blue) and CYP17 (green). Variation of $C_t$ with PCR efficiency. The blue standard curve has an efficiency of 90.60%. The green standard curve has an efficiency of 232.1%.
Table 3.2: Efficiency numbers for all genes. Genes that were selected for the expression experiments in the current work indicated by *. Target gene (StAR) and the three reference genes (GAPDH, B2M & QARS) were showing strong expression level in THP1.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Efficiency</th>
<th>Reference (R)/ Target (T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>StAR*</td>
<td>91</td>
<td>T</td>
</tr>
<tr>
<td>GAPDH*</td>
<td>94</td>
<td>R</td>
</tr>
<tr>
<td>QARS*</td>
<td>90</td>
<td>R</td>
</tr>
<tr>
<td>Cyp17</td>
<td>232.1</td>
<td>T</td>
</tr>
<tr>
<td>B2M*</td>
<td>109</td>
<td>R</td>
</tr>
<tr>
<td>Hsd3</td>
<td>24</td>
<td>T</td>
</tr>
<tr>
<td>ABL</td>
<td>117.8</td>
<td>R</td>
</tr>
<tr>
<td>Hsd17</td>
<td>221.1</td>
<td>T</td>
</tr>
</tbody>
</table>

3.5 Preliminary Gene Expression Studies

3.5.1 Confirmation of RNA and cDNA Quality and optimizing PCR

Reverse transcriptase PCR followed by microchip electrophoresis using the MultiNA system was used to non-quantitatively check the expression of all genes used in this study under the THP1 cell line. This procedure also helped confirm the identity of the genes being studied, based on an appropriate amplicon size.

The reference gene GAPDH showed strong bands around the expected size. StAR also produced a band at the correct size, while other genes showed very weak amplification (Fig
3.6). The occurrence of apparent primer dimers could also affect the result of the PCR, by reducing the amplification efficiency, or by causing inaccurate quantification of PCR product.

Since there is usually a problem amplifying a specific DNA fragment using the PCR, optimizing the PCR conditions can be achieved by using a temperature gradient in the thermal cycler block, which allows the empirical determination of the optimal annealing temperature.

In order to check for the optimum annealing temperature for all selected genes (reference and target) a temperature gradient was performed by normal PCR method using the MultiNA electrophoresis system. Clear bands of appropriate size were seen for StAR and GAPDH, across the entire range of annealing temperature, and this was consistent with the observations in Fig. 3.6.

For those assays which did not generate a clear band of appropriate size there are two possible explanations. Either the assay failed for some technical reason, such as an issue with primer design or probe specificity, or the gene was simply not expressed and therefore undetectable in THP1. In an attempt to distinguish these two possibilities, the gene assays were tested on three other cell lines (Jurkatt, Ishikawa, plasma B cells), some of which may have expressed the genes in question (Fig 3.8). The result showed that StAR, GAPDH and B2M were expressed in all cell lines. Other genes such as HSD17, CYP17, HSD3 and ABL1
did not express or show weak bands at the expected size. *CYP11* did not express at any of the cell lines. ABL1 showed strong bands at different size.

*Figure 3.6:* Non-quantitative RT-PCR of all genes. Electropherogram generated by the MultiNA solid state electrophoresis system, illustrating that *StAR* and *GAPDH* were amplifying around the correct size. Other genes showed very weak amplification or none (*CYP11*) and some have a primer dimer. Lines represent the gene tested in this run as follows: 3-5 (*HSD*), 6-8 (*CYP17*), 9-11 (*HSD3*), 12-14 (*CYP11*), 15-17 (*B2M*), 18-20 (*QARS*), 21-23 (*StAR*), 24-26 (*GAPDH*).
Figure 3.7: Temperature gradient. The Electropherogram for the temperature gradient generated by the MultiNA solid state electrophoresis system showed that genes failed to express (CYP11) or showed weak bands (HSD3) during the non-quantitative RT-PCR (see Fig3.6) still showing the same along the set of temperatures. The determination of optimal annealing temperature for target and reference genes StAR and GAPDH showed strong bands across the temperature gradient. Lane 1 shows the molecular size marker (ladder) with lower marker (LM) and lower marker (UM).
Figure 3.9: Gene expression assays in four cell lines. MultiNAelectropherogram for the four cell lines (Jurkat A& E), plasma B cell (B& F), Ishikawa (C& G) and THP1 (D& H). Eight different (target and reference) genes were used in this run. HSD17 (1), CYP17 (2), HSD3 (3), CYP11 (4), GAPDH (5), B2M (6), StAR (7) and ABL1(8). GAPDH, B2M and StAR were expressed in four cell lines. CYP11 did not express in any cell line. HSD17, CYP17 and HSD3 show some weak bands on some cell lines.

3.6 Expression analysis of DBP effects on StAR gene in THP1 cell line

The correlation between reference genes was, first, examined by plotting the R^2 values of each pair of housekeepers (GAPDH, QARS and B2M). Data is shown only for replicate 1; however, the correlation was tested in all three biological replicates. (Figs. 3.10-3.12). The correlation between GAPDH and B2M was very much higher than the correlation between GAPDH and QARS or between QARS and B2M in the first two experiments. Regarding the
basis of results, $GAPDH$ and $B2M$ were chosen as the housekeeping genes to be used for normalizing the level of $StAR$ expression in the current study. The expression of $StAR$ was normalized to $GAPDH$ and $B2M$ and the result obtained from the linear model applied using the SSPS software analysed.

Figure 3.13 shows variation in expression of $StAR$ with time of exposure to DBP. The level of $StAR$ expression in treated cells differed significantly between experiments ($p=0.001$), and all gene expression analyses were adjusted to account for differences in the overall level of $StAR$ expression between replicates.

The result of the three biological experiments showed a statistically significant effect ($p=0.021$) of dose on $StAR$ levels. The high dose showed up-regulation of $StAR$ expression.

There was no association between $StAR$ levels and time ($p=0.213$) and there was no significant interaction between time and dose ($p=0.064$).

The effect of dose on the level of $GAPDH$ ($p=0.047$) and $B2M$ ($p=0.009$) was also significant, with the high dose associated with lower levels of the two housekeeping genes.
**Figure 3.10:** The coefficient of correlation between GAPDH and B2M. $R^2$ is 0.8.

**Figure 3.11:** The coefficient of correlation between B2M and QARS. $R^2$ is 0.7.
Figure 3.12: The coefficient of correlation between GAPDH and QARS. $R^2$ is 0.6
Figure 3.13: Alterations in StAR gene expression. Graph represents relative expression of StAR mRNA (normalized to the two reference genes GAPDH and B2M) due to DBP exposure at three different concentrations: 0.1 μg/mL (blue), 1 μg/mL (red) and 10 μg/mL (green). Numbers 1-5 indicate the five time points used (0, 24, 48, 72 & 96 hours respectively). The level of StAR mRNA changed around the 48h mark in THP1 cells exposed to a high dose of DBP. The medium and low doses showed no effect on StAR level. Each of the three coloured bars shows average measurements based on three biological replicates of the experiment, each with three technical replicates. The error bars represent the standard deviation of the measurements.
4  : Discussion

4.1 Choice of a Model System

The effect of DBP exposure on animal cells has been studied both \textit{in vivo} (Wakui et al., 2013; Mitchell et al., 2012) and \textit{in vitro} (Chen et al., 2013). This study set out to investigate the effects of DBP on the expression of certain genes in cells grown \textit{in vitro}, and for this reason needed a stable cell line that could be grown reliably in tissue culture. The model system needed to be a human cell line, because of the focus on the effects of phthalates in humans. It was also hoped this work would eventually lead to the development of a simple blood screening test that would indicate exposure to DBP, therefore it was important to choose a human \textit{blood} cell line. Since DBP is a known EDC, it was important that the cell line expressed the gene for aromatase (\textit{CYP19}).

Aromatase, which catalyses the conversion of testosterone to estradiol, and of androstenedione to estrone, is a key enzyme in oestrogen biosynthesis. Therefore, it would be expected that a cell line susceptible to genetic effects following DBP exposure would express aromatase. Various human cell lines are known to express aromatase, including THP1 (Jakob et al., 1995) MCF7, HeLa, SK-BR-3 and H295R cells (Chen et al., 2010). However, out of these THP1 is the only one to be derived from blood (it is a human monocyticleukemia cell line (Auwerx, 1991).

The use of in-culture gene expression as compared to whole organism articulation was advised by several factors, including:
The ability to retain cell products within the system due to the closed-system characteristic of tissue culture.

The simplicity that is associated with studying a phenomenon in a single stable cell line as compared to undertaking the same in a whole organism, or even in an organ with many types of cells being expressed.

The ease of controlling various variables in a closed system. The variables include temperature, nutrients and exposure times to various chemicals.

The evaluation or study of the effects of toxins on human cells does not promote \textit{in vitro} studies due to ethical concerns.

However, the results obtained from a cell culture system cannot necessarily be extrapolated to the whole organism. A critical problem, associated with the extrapolation problem, is that tissue culture is unable to replicate the complex metabolic, endocrine and excretory systems that exist within the whole organism. In the whole organism a steady supply of nutrients and constant removal of waste products is maintained, whereas this is not the case for a cell culture system. These are points that will be addressed later in the discussion.

4.2 THP1 growth

Tissue cultures require regular feeding in order to maintain a progressive growth rate. The growth of the DBP exposure cell lines was structured to benefit from the nutrient demand associated with a tissue culture. When the experiments of DBP exposure were conducted, 12-well plates were used, and the cells could not be provided with additional nutrients during the 96 hour time course of the experiment. Additional concerns were related to the nature of growth associated with the THP1 exposure cells lines in a tissue culture setting. THP1 cells
grow in suspension, thus feeding them during the experiment would require removal and replacement of the media containing the cells. This would significantly impact on the environment of the cells, and may introduce biases. The second limitation for providing additional nutrients was that cells were supplemented with fresh media on the first day of the experiment and plated at the same time (0h) to each well. Treatment with DPB was applied at different times, that is, DBP was introduced to the 24h a day before the 72h mark. Re-feeding the cells after 48h (routine feeding) would put the cells under the same nutritional conditions as the 0h but not the 24h.

However, the growth curves for cells exposed to DBP (Figure 2.2) show that cell numbers began declining after 72 h with low or medium concentrations of DBP. This is probably because there were insufficient nutrients for the cells to continue dividing. This means the data for gene expression at 96 h is unreliable. However, cells treated with a high dose showed rapid death at an earlier time ~48h, and this presumably resulted from a dose toxicity effect rather than starvation. Either feeding or not feeding the cells during the experiment would have impacted on cell culture conditions, and a repeat of the same experiment with more regular feeding could strengthen the result. However, the toxicity effect is probably unavoidable, and measurements for the high dose exposures must be considered inaccurate.

4.3 RT-qPCR assay method

Quantitative real-time PCR (qPCR) is a powerful technique for gene expression studies and has become the method of choice for rapid and reliable quantification of mRNA transcription in a large number of clinical and scientific fields. Real-time qPCR is the prime technique to measure gene expression due to its high sensitivity; it is fast and efficient, and provides
simultaneous measurement of gene expression in many different samples for a limited number of genes (Mestdagh et al., 2009; Nygard et al., 2007). Variation in total RNA content, RNA stability, enzymatic efficiencies, or sample loading have an effect on the result of the qPCR. A crucial step of this method is the normalization of the results to correct the differences in the purity and concentration of the samples that were introduced during the sample preparation procedure. Reference genes are frequently used to normalise mRNA levels between different samples (Gilsbach et al., 2006; Hruz et al., 2011; Watson et al., 2007). In general genes will not maintain constant expression levels under all environmental conditions, therefore it is important when designing qPCR experiments to choose appropriate reference genes against which to normalise qPCR data.

4.3.1 Choice of reference genes
Reference genes are used on the basis that they should be adequately expressed in the tissue of interest, show minimal variability in expression between samples, and their expression is not influenced by the experimental conditions (e.g. drugs applied in the assay) (Gilsbach et al., 2006). However, studies have reported the unreliability of conventionally used reference genes (Glare et al., 2001; Hruz et al., 2011; Radonić et al. 2005). Silver et al. (2006) studied the expression of seven reference genes in the human reticulocytes. The study indicated that the most suitable reference gene was GAPDH and the least suitable reference gene was B2M (Silver et al., 2006). Other studies indicated that GAPDH and ACTB vary substantially and are not reliable as reference genes for normalisation of gene expression analysis in some cases (Deindl et al., 2002; Nygard et al., 2007; Radonić et al. 2004).

Maeß et al. (2010) examined the expression of 21 genes during differentiation of THP1 monocytes into macrophages induced by phorbol 12-myristate 13-acetate (PMA). Maeß et al. (2010) stated that expression levels were distributed over a large range (Fig. 4.1), and showed that the most commonly used reference genes, such as GAPDH (glycereraldehyde-3-
phosphate dehydrogenase) and \(G6PD\) (glucose-6-phosphate dehydrogenase), were not suitable for this purpose. This study suggested that \(ACTB\) (beta-actin) and \(RPL37A\) (ribosomal protein L37a) were the most stable genes in THP1 differentiated into macrophages (Maeß et al., 2010).

The choice of the reference genes for this research was based on Maeß et al. (2010) study, as reference genes were selected to represent different levels of expression. The selected genes were tested, and the qPCR result showed that \(GAPDH\), \(B2M\) and \(QARS\) were the most stable and useful reference genes for this work.

**Figure 4.1:** The distribution of expression for 21 potential reference genes. The Cq value of the 21 genes was examined during the differentiation of THP1 into macrophage (Maeß et al 2010). Arrows indicate the selected genes. Only \(GAPDH\), \(B2M\) and \(QARS\) were used in the current work.
4.3.2 Choice of target genes

The selection of genes involved in the testosterone pathway was required in order to investigate the effects of DBP on the THP1 human cell line. Changes in gene expression following DBP exposure have been widely studied in animal models (Hannas et al., 2011; Howdeshell et al., 2007; Lahousse et al., 2006; Shultz et al., 2001).

One study exposed fetal rats to DBP and found deficits in testicular steroidogenesis (Lahousse et al., 2006). In contrast, there are few studies on the effect of phthalates on human steroidogenesis. The study by Lambrot et al. (2009) was the first to investigate the effects on development of the human fetal testis exposed to mono-2-ethylhexyl phthalate (MEHP) using an organ culture system. This work showed that this phthalate has no effect on the mRNA expression of P450c17, P450scc, or StAR (Lambrot et al., 2009). Barlow et al. (2003) examined expression of a number of critical genes, such as scavenger receptor class B-1 (SRBI), steroidogenic acute regulatory protein (StAR), P450 side-chain cleavage enzyme (P450scc), 3-hydroxysteroid dehydrogenase (3B-HSD), P450c17 (CYP 17), and 17-hydroxysteroid dehydrogenase (17B-HSD), which are involved in androgen biosynthesis (Barlow et al., 2003). Barlow et al.’s (2003) aim was to confirm that DBP induces alterations in fetal testicular (mRNA) expression as found by Shultz et al. (2001). Barlow et al. (2003) observed that SRBI, StAR, P450scc and P450c17 were downregulated following DBP exposure. This conclusion was similar to the findings of a study by Plummer et al. (2007) which showed that StAR, cytochrome P450 side chain cleavage enzyme (CYP 11a) and cytochrome P450 17-hydroxylase/17-20 lyase (CYP17) were all downregulated by DBP treatment (Plummer et al., 2007). Five genes were selected for the current project: StAR,
CYP11, CYP17, HSD 17 and HSD3. During the course of the work it became apparent that only StAR was expressed in THP1, so it was not possible to examine DBP effects on the other genes.

4.4 Gene expression analyses

Many studies have shown that DBP exposure resulted in significant dose-dependent reductions in mRNA and protein concentration of several steriodogenesis genes such as StAR (Howdeshell et al., 2007; Lahousse et al., 2006; Plummer et al., 2007). The result obtained from the current study (see Fig 3.13) contrasts with other studies in that expression of the genes involved in cholesterol uptake and conversion into testosterone were downregulated. The level of StAR expression was upregulated in THP1 cells treated with high dose DBP. However, the study by Xi–feng et al. (2009) showed up regulation of 11B-HSD1 expression.

The effect of a high dose on StAR (p=0.021) was significant, whereas an earlier study has shown the opposite result, with StAR expression reduced due to DBP exposure (Plummer et al., 2007). Variability between the biological replicates (the three separate experiments) has significant (p=0.001) effect on the StAR level. As there was a correlation between only two of the three biological replicates, this has reduced the strength of this result. The difference in the result obtained from this non-correlated replicate may have been due to a technical issue such as mislabeling, pipetting error, or mixed samples. The variability in the level of the StAR gene could be also related to the cell death. The result for THP1 growth (see Fig 3.3) has indicated that cells were dying after 72 h and this could have affected the expression of the StAR gene. However, the small number of biological replicates meant it was not reasonable to discard this replicate as an outlier.
4.5 Conclusion and future work

This study, aiming to develop a blood biomarker for DBP exposure, was stimulated by the findings of Carran and Shaw (2012) on the effects of DBP on soldiers who served in Malaya in the 1950s, and also by growing concern about the impact of exposure to phthalates in the environment on human health and reproduction. However, although the result obtained from this study did not lead to a biological marker of potential use in the field, the research could be used as a base for future research in this area.

There were several significant limitations that affected the results of this study. There was limited choice of cell line because of the need to use one that was derived from human blood, and that expressed aromatase. However, THP1 proved a difficult cell line to grow and maintain, and most target genes selected and examined were not expressed in this cell line. Choosing a different blood cell line could improve the result, as few studies have looked at the effect of DBP in particular and phthalate in general on a human blood cell line. Cultured white blood cells may have provided a better model, although they could show more variability in gene expression due to the inter-individual variation. Another possibility would be to choose an animal model, which many studies have used (Ema & Miyawaki, 2001), but for this specific marker the disadvantage would be that animals have some differences from humans in their metabolism and maybe the steroidogenic pathway. Mitchell et al. (2012b) used human fetal testis Xenografts to determine the effects of DBP exposure on testosterone production, and possibly using a similar technique instead of THP1 would produce a more successful result. For this project the choice of the cell line or the animal model needs to be very precise in order to establish a useful biological marker.
Examining different genes in the steroidogeneses pathway may be useful and worthwhile. There are several genes involved in the testosterone pathway, and the alteration of the expression of these genes would have an effect on the mechanism of action of the testosterone production.

Another limitation was the time and the cost of doing more biological replicates, as having only three replicates appeared insufficient given the experimental variability. To ensure the comparisons between replicates are correct, 6-8 biological replicates would be sufficient to provide a more accurate result.

Expression microarray or RNA-Seq data (Li et al., 2012) would allow a whole genome approach to exploring more extensive gene expression changes in response to phthalate exposure, and this could generate new insights and potential novel biomarkers.

As the effect of phthalates on humans, especially on human reproductive health, is a matter of increasing concern, it is important that a means of testing for exposure to these chemicals is developed. The results of this study confirm that there is an effect from a high dose of DPB exposure, but there is no significant effect from the medium and low doses. As our daily exposure to phthalates is normally within the low to medium range, it is suggested that only a high dose of DPB is required to damage human reproductive health.

This study has focused on developing a biological marker for one of the major phthalates, DBP, and although the results are negative, it is now known that additional investigation of the upregulation of the StAR gene would be valuable. StAR expression has shown an
interesting result in the THP1 cell line due to high dose exposure of DBP, and whether this upregulation might change the steroid flux through the glucocorticoid pathway away from the testosterone synthesis needs further research.
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