New Zealand Malayan war veterans’ exposure to dibutylphthalate is associated with an increased incidence of cryptorchidism, hypospadias and breast cancer in their children

Matthew Carran, Ian C Shaw

Abstract

It is well known that the endocrine-disrupting chemical (EDC) dibutylphthalate (DBP) inhibits testosterone synthesis and can lead to feminisation in male laboratory animals. Moreover, it has long been speculated that human exposure would result in the similar effects, but this is difficult to study because specific human exposure cohorts are rare.

We report increases in the incidences of hypospadias (p <0.05), cryptorchidism (p<0.05) and breast cancer (p<0.05) in the children of New Zealand soldiers who served in Malaya (1948–1960) and were exposed to DBP applied daily to their clothing as an acaricide to prevent tick-transmitted bush typhus. In addition, we modelled absorption of DBP from the soldiers’ clothing and using published data for skin absorption, and calculated a large theoretical absorbed dose of 64 mg/kg body weight/day which is similar to DBP’s lowest observed adverse effect level (LOAEL) of 50 mg/kg body weight/day and thus indicates a biological effect is possible.

This is the first report of a multigenerational developmental effect following DBP exposure in human males.

Endocrine-disrupting chemicals (EDCs) either have structural analogies to hormones and can occupy and activate hormone receptors (e.g. human estrogen receptors hERα and hERβ) or interfere with the metabolic production or destruction of hormones.1-3

Dibutylphthalate (DBP) and/or its metabolites reduce the activity of enzymes of the testosterone synthesis pathway4 possibly because of their structural analogy to testosterone’s steroid precursors (e.g. pregnenolone; Figures 1 and 2).

The decrease in testosterone concentration upsets the androgen:estrogen ratio and introduces significant cellular feminising pressure and therefore could result in effects on growth and development in males. Indeed, at the lower end of the evolutionary spectrum, namely amphibia, studies in frogs (Rana rugosa) have shown that exposure to DBP affects testicular differentiation during metamorphosis5. The effects of DBP in mammals are very similar; the offspring of DBP-exposed female rats mated with unexposed males results in a dose-related decrease in testis weight in the offspring.4

Thus, DBP exposure affects gonad growth and development across the evolutionary spectrum and such changes are likely to have far reaching effects on fecundity. It is likely that DBP will have the same or very similar effects on growth, development and reproduction in humans.
Figure 1. Molecular structures of dibutylphthalate (DBP), its major metabolites (mono(3-hydroxybutyl)phthalate and mono(4-hydroxybutyl)phthalate) and the testosterone precursor pregnenelone

Note: The structural analogy of the DBT metabolites to testosterone precursors is likely to explain their inhibition of testosterone synthesis enzymes.

It is widely believed that there is a link between exposure to EDCs and their effects, but there are few human exposure/effect data to substantiate this, particularly in relation to individual compounds. For example, it is known that the human sperm count is in decline\textsuperscript{5,7} worldwide; similarly, the age of onset of puberty in girls is declining\textsuperscript{8} and both have been associated with exposure to EDCs in some studies (e.g. bisphenol A [BPA]\textsuperscript{9}), but it is difficult to prove cause and effect without definitive exposure data. In addition, increasing rates of cryptorchidism and hypospadias have also been linked to EDC exposure in some studies.\textsuperscript{10}

It is widely accepted that increasing exposure to myriad EDCs is having effects on sexual development and function in humans, but the complexity of the exposure profile and the large number of individual EDCs to which we are all exposed makes unravelling cause and effect relationships almost impossible.
There is still some controversy about the cause of the observed changes in sexual development; EDCs might not be the sole cause and it is possible that, for example, changes in dietary status might also influence sexual development. This is particularly the case for precocious puberty where better diet might, at least in part, explain the phenomenon. It is, however, difficult to explain how dietary changes have caused declining sperm count in countries in the developed world. It is likely that the effects are multifactorial with multiple risk factors (e.g. diet and exposure to EDCs) leading to a common sexual development endpoint.

Figure 2. Molecular structures of mono(4-hydroxybutyl)phthalate (a metabolite of DBP) (black) and pregnenelone (grey) superimposed to show their structural analogies

Note: They have aliphatic hydroxyl groups in similar spatial arrangements; keto groups in similar positions and a significant central region of hydrophobicity.

Considering EDCs as one risk factor, they can either have direct effects (e.g. bind to and activate ERs) on the individual receiving the EDC dose or they might have multigenerational effects. The latter could involve exposure of a pregnant female to EDCs in which case the foetus might also receive a dose of the EDC if it, or its active metabolites, cross the placental barrier or are absorbed by the foetus from amniotic fluid.

In addition, it is possible that exposure of males to EDCs could cause genetic or epigenetic effects on sperm DNA that could, in theory, affect the offspring produced from the modified sperm—this is a different mechanism to the direct EDC interaction with ERs following direct exposure of adults to EDCs or indirect exposure of the foetus following maternal exposure to EDCs.

The possibility that sperm effects, following paternal exposure to EDCs, might lead to effects in offspring is, to some extent, theoretical; however, a recent review\textsuperscript{11} has explored this and presents compelling evidence that paternal exposure effects on offspring is a distinct possibility.
In this paper we present data on the incidence of cryptorchidism, hypospadias and breast cancer in the children of New Zealand veterans of the Malayan (now Malaysia) Emergency (1948–1960) who were exposed to DBP during their military service. DBP is an insecticide and acaricide and was used by the military to reduce insect and mite infestation in troops.\textsuperscript{12,13}

New Zealand troops deployed in Malaya during the 1950s and 1960s painted the seams of their uniforms with a proprietary liquid DBP concentrate preparation before undertaking operations in the jungle to prevent them being bitten by trombiculid mites (chiggers, e.g. \textit{Eutrombicula hirstii}) which carry the scrub typhus pathogen (\textit{Orientia tsutsugamushi}).\textsuperscript{14}

The New Zealand/Malaysia veterans present an interesting DBP exposure cohort in which to investigate cause and effect relationships following known exposure to this potent EDC. Our findings provide further evidence that paternal exposure to EDCs can lead to developmental changes in offspring.

\section*{Materials and Methods}

\subsection*{New Zealand Malaysian veteran questionnaire study}

\textbf{Setting & study design}—This is a retrospective cohort questionnaire study of NZ Malaysia veterans of the Malayan Emergency (1948–1960) known to have been exposed to DBP who currently live in the Canterbury province of NZ.

\textbf{Ethics committee approval}—Approval for the study was given by the University of Canterbury Human Ethics Committee on 2nd December 2009 (approval reference HEC2009/165).

\textbf{Data collection}—Specially designed data collection forms were sent to 252 NZ Army veterans who were known (from military records) to have served in the Malayan Emergency between 1948 and 1960. They were contacted via their membership of the Canterbury branch of the Malayan Veterans’ Association (Inc.), New Zealand. Data collection forms were sent out to the veterans in December 2009 and the recipients were asked to return the completed questionnaires within 2-weeks of receipt.

\textbf{Data collection forms}—Prior to designing the data collection forms we met with several members of the New Zealand Malayan Veterans Association Inc. to determine whether they, or their colleagues, were likely to remember events of some 50 years ago. We were particularly interested in their recollection of whether they had used DBP or not and how often they had applied it.

The discussion unequivocally demonstrated that the application of DBP was a memorable event since it involved painting the viscous liquid onto their uniforms in a pre-excursion military order setting and that it was applied whenever they were on military operations. The frequency of the latter was quite clearly a memorable event for the soldiers.
Specially designed data collection forms were used to collect data, including dates the veterans were stationed in Malaysia, whether or not they used DBP, whether they had children during their time in Malaysia or after returning to NZ, whether they, their children or grandchildren suffered from any of the following disorders:

- Cryptorchidism.
- Defects of the penis (respondents were asked to specify, e.g. hypospadias).
- Precocious puberty (female offspring only).
- Low sperm count.
- Reduced fertility.
- Disorders of the ovary or uterus.
- Breast cancer.

Some respondents who indicated that one of the above disorder criteria applied to them were followed up to ascertain the reliability of the diagnoses. In these cases, we talked to the respondent personally about the diagnosis, who had made the diagnosis and what the name of the disorder was. We used this process as a means of confirming the diagnosis and its reliability.

**Data analysis**—Data from veterans who had not used DBP (n=13; 14.3% of total) were discarded. Recipients whose answers to the data collection form questions were not clear were followed up by telephone to clarify the uncertainties.

The incidences of each of the disorders were calculated in the study cohort and were compared with incidence statistics for the general population. Published data for the New Zealand incidence of breast cancer are not available and therefore USA population data were obtained from the scientific literature. Binomial distribution statistics were used to compare the DBP-exposed veteran cohort data with the general population incidence of a particular disorder.

**Statistics**—A binomial distribution was used to determine the probability of the observed disorder incidence in the offspring of DBP-exposed veterans occurring in the general population. The Binomial Test was applied to the data to determine statistical significance. A particular disorder incidence was considered significantly different from its incidence in the control population if \( p \leq 0.05 \).

**Absorption of DBP through clothing**

An army uniform (trousers and shirt) used during the Malayan Emergency was obtained and its fabrics identified as 100% cotton by light microscopy. In these experiments, Cotton Drill cloth was used to represent the military uniform trousers and cotton Homespun Bedford cloth was used to represent the shirts (materials purchased from Haralds, Christchurch, New Zealand) in the DBP permeation studies as follows:

Cloth squares (5cm × 5cm) were positioned in contact with 3 layers of Whatman No 1 chromatography paper squares (5cm × 5cm) and 1mL DBP (density=1.04 g/mL; May & Baker, Dagenham, UK) was applied to the cloth with a 3cm 100% pure-bristle brush (i.e. the same method used by military personnel during the Malayan Emergency).

The cloth was left in contact with the chromatography paper for 5 hours to mimic the approximate period of time that the soldiers were exposed to DBP during their jungle activities.
Following the 5-hour diffusion period the chromatography paper was extracted with acetonitrile (10 mL × 3) in a centrifuge tube on a reciprocating shaker for 30 minutes. The extracts were combined and diluted 1/1,000 v/v with acetonitrile.

The diluted extracts were analysed for DBP by ultra violet (UV) absorption spectrophotometry (CARY®-100-Bio Spectrophotometer, Varian Inc.; 1cm quartz cuvette) at wavelength 222 nm using the molar absorption coefficient calculated from a linear calibration graph (calibration range=5–40 µg/mL; R²=0.9943) using Beer’s Law (ε=7793/M/cm). DBP absorption from the cloth was calculated.

Results

Questionnaire study

Response rate—Of the 252 data collection forms sent out 85 (33.7%) completed forms were returned. The low response rate is likely to be due to the age (expected to be ≥80 years) of the veterans and the fact that men of this age are often reluctant to discuss matters of a personal sexual nature. In addition, despite remaining on the Malaya Veterans’ Association (Inc.), New Zealand membership list, a considerable number of the veterans are likely to have died or be incapable of responding.

Cohort demography—Of the 71 veterans included in the study, 58 (81.7%) had children after serving in Malaysia, of these 155 offspring 79 (51%) were male and 76 (49%) were female. The number of children per family was 2.2±1.5 (mean ±SD). All of the children were born after their fathers had returned to New Zealand.

Findings from the questionnaire study—Table 1 shows the incidences of disorders in the children of the DBP-exposed veteran cohort compared with statistics for the general population.

Table 1. Comparison of incidences of diseases associated with exposure to estrogenic compounds in children of veterans of the Malayan Emergency who were exposed to dibutylphthalate and the incidence of the same disorders in the general population

<table>
<thead>
<tr>
<th>Disorders</th>
<th>Incidence in children of DBP-exposed veterans (number of cases)</th>
<th>General population incidence</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cryptorchidism</td>
<td>5.1% (4)‡</td>
<td>2000: 1.09% 2005: 0.91%</td>
<td>&lt;0.05*</td>
</tr>
<tr>
<td>Hypospadias</td>
<td>2.5% (2)‡</td>
<td>2000: 0.33% 2005: 0.30%</td>
<td>&lt;0.05*</td>
</tr>
<tr>
<td>Breast cancer</td>
<td>4.0% (3)‡</td>
<td>0.48%</td>
<td>&lt;0.05*</td>
</tr>
</tbody>
</table>

Note: Data for hypospadias and cryptorchidism are given for 2 years to show that the incidence statistics vary little with time. Birth statistics used to calculate population incidence were taken from http://www.stats.govt.nz/browse_for_stats/population/births/births-tables.aspx. The data for hypospadias also includes a small number of epispadias cases because they are not separated in the New Zealand Birth Defects.
All of the other disorders included in the questionnaire showed either very low incidence or incidences that were not statistically different from the incidence in control populations. For these reasons these data are not included here.

Absorption of DBP through clothing

Absorption across the shirt material (Bedford Homespun) was 25±3.2% (mean±SD, n=5) of the DBP applied. The corresponding value for the trouser material (Cotton Drill) was 35±14.2 (mean±SD, n=6). These values correspond to 0.22–0.30 g DBP and 0.22–0.56 g DBP absorbed across 25 cm² cloth following application of 1 mL DBP respectively.

Discussion

Response rate—The low response rate (33.7%) for this questionnaire study is likely to be due to the age of the New Zealand Malaysian veterans. Questionnaires were sent to all veterans on the Christchurch, New Zealand membership list of the New Zealand Malayan Veterans Association Inc. The Association updates its membership list when it is informed of members’ deaths. However, if the Association is not informed, or is not informed immediately, of members’ deaths, the members’ names remain on the membership list.

The current members of the Association are in their mid-seventies to late eighties and therefore it is very likely that a considerable number of those to whom questionnaires were sent were deceased (life expectancy for New Zealand non-Maori males is 79.0 years and for Maori males is 70.4 years¹⁷). This is likely to explain the poor response rate.

Effects of DBP—Developmental abnormalities and sex hormone-related cancers have been linked to exposure to EDCs in some human studies and in animal studies, including breast cancer, cryptorchidism and hypospadias¹,¹⁰

Effects on the developing foetus as a result of passage of EDCs and their metabolites across the placenta into the embryo/foetus are thought to account, at least in part, for these developmental disorders.¹⁴ In addition, epigenetic effects on ova and sperm might initiate gene regulatory changes (e.g. DNA methylation) that lead to developmental abnormalities that are not manifested until later in life.

In the present study, only males were exposed to DBP and therefore any multigenerational effects can only be explained by a sperm-based mechanism. In addition, all of the men in this study had returned to New Zealand before their children were born; therefore, they were not exposed to DBP immediately prior to conception. This suggests that any effects of DBP on their sperm were long lasting.

Much of the experimental work on DBP’s developmental effects has been carried out in DBP-exposed female animals (e.g. rats⁴) and thus focuses on in utero exposure. The present study shows that occupational exposure to DBP in men might lead to abnormalities in their children. In addition, the abnormalities observed (cryptorchidism, hypospadias, breast cancer)
have been associated with exposure to EDCs in animal and human studies\textsuperscript{1,10} but not specifically via exposure of males. Recent studies have shown that exposure of human sperm to phthalate esters (including DBP) concentrations in the range found in semen of exposed individuals results in decreased motility and viability.\textsuperscript{19} This suggests that phthalate esters can act directly on sperm.

If macrobiological changes (e.g. decreased motility and viability) occur, this must mean that there are underlying biochemical changes that, if exposures are low enough, might not preclude a sperm fertilising an ovum, but might have caused changes that will affect the make-up and development of the resulting zygote.

Our results suggest that DBP can affect sperm in such a way that leads to changes that, following fertilisation of ova, result in developmental changes that are manifested later in life. A possible explanation for this is epigenetic gene regulation. The mechanism of endocrine disruption is via an epigenetic mechanism; EDCs bind to the ER hormone binding domain, cause a conformational change in the receptor which leads to a sequence of events that results in the receptor-ligand complex migrating to DNA where binding to a specific site leads to DNA postsynthetic modifications (e.g. methylation) and concomitant gene regulation\textsuperscript{20}.

If testosterone levels are low the male hormone response will be concomitantly reduced while the estrogen response will remain constant. The overall effect of this activity ratio change leads to an over-expression of estrogenicity resulting in cellular feminisation. We speculate that DBP changes the balance of activity of specific genes in the sperm DNA and that this gene regulation is introduced to the zygote when the ovum is fertilised.

The outcome is a degree of biochemical feminisation of male offspring that leads to sex organ developmental abnormalities (e.g. hypospadias and cryptorchidism) or promotion of estrogen-mediated non-genotoxic carcinogenesis (e.g. breast cancer) later in life. Clearly, the DBP dose determines the magnitude of the effect; the higher the DBP dose the greater the inhibition of testosterone synthesis and therefore the greater the feminising effect.

Our study is unique because it investigates high DBP exposure individuals and thus establishes, without doubt, that the study cohort received a dermal DBP dose. It is, however, important to consider other possible exposures to EDCs that could, at least in part, explain our findings.

Dietary EDCs are important in this context because Asian diets are high in phytoestrogens (e.g. genistein in soy beans) and therefore it is possible that the NZ Malaysian veterans were exposed to higher doses of dietary estrogens than the control groups with which they were compared in this study and that this exposure accounted for the higher than control incidence of hypospadias, cryptorchidism and breast cancer.

Discussions with one of the New Zealand Malaysian veterans confirmed that the soldiers received British army rations. This suggests that the veterans did not receive a high phytoestrogen intake consistent with a typical Asian diet. It is therefore unlikely that dietary EDC intake explains our findings.

Studies on the passage of DBP across military uniforms gives an estimate of the skin exposure to DBP following its application during military operations. Combining these data
with skin absorption data in animal model systems gives an indication of circulating levels of DBP (and its metabolites) resulting from its use as an acaricide in a military operations. Comparing the received dose with the Lowest Observable Adverse Effect Level (LOAEL) for DBP indicates whether there might be a biological effect in the exposed soldiers.

In our experiments the dose delivered via clothing following an application volume of DBP similar to that used in the military field was approximately 6.6 g per person (see below). Dermal absorption of DBP in a hairless guinea pig model is 62±2% of dose\(^1\) and in a rat dermal absorption model is 73.2% of dose.\(^2\)

The similarity between these two absorption experiments in two different species suggests that absorption in humans is likely to be of the same order. Despite this, studies on fat-stripped post-mortem skin from human cadavers shows very much lower penetration than seen in ex vivo animal skin models.\(^3\) However, in our view ex vivo animal skin is likely to more accurately reflect the in vivo human situation than dead human skin and, for this reason, 68% (mean of the two animal model results) was used in our calculations to estimate the absorbed dose of DBP in the soldiers.

Clearly, this is only an approximation and does not take account of important factors such as climatic conditions, skin surface temperature and perspiration; all of which will affect absorption. Nevertheless, the estimated human DBP dose thus obtained can be compared to the DBP LOAEL to determine whether there is likely to be a human biological effect following exposure. If the DBP dose and LOAEL are of the same order of magnitude it is possible that sufficient DBP was absorbed in the soldiers to cause a biological effect and, consequently, it is possible that the observed effects in the offspring of the soldiers might be explained by DBP exposure.

Frances et al 13 reported that the DBP application rates used in military operations were 23 mL for trousers and 7 mL for shirts. Using the lowest cloth absorptions determined in our experiments (i.e. 0.22 g/mL DBP applied for both Drill and Bedford Homespun cloths) this means that the lowest total skin DBP exposure is approximately 6.6 g. Assuming 68% skin absorption the absorbed DBP dose is approximately 4.5 g.

The LOAEL [rat] for a foetal testosterone reduction end point is 50 mg/kg body weight/day\(^2\). The mechanism of testosterone reduction following exposure of rats to DBP is thought to involve gene expression with changes in genes that code for enzymes of testosterone synthesis or carrier proteins for testosterone precursor (e.g. cholesterol) uptake by cells.\(^2\)

The dose received by the New Zealand veterans (assuming an average body weight of 70 kg) was approximately 64 mg/kg body weight/day; this is close to the LOAEL and suggests that the dose received by the soldiers could have had a biological effect.

The results of our study show statistically significant effect in the incidence of hypospadias, breast cancer and cryptorchidism in the offspring of DBP-exposed soldiers (Table 1). These findings combined with the exposure estimate being close to the LOAEL point to DBP having an effect in exposed men that leads to effects in their children. It is, however, important to consider the potential for confounding effects.
It is possible that other factors could, at least in part, have contributed to our findings. The most likely is diet; however the New Zealand soldiers stationed in Malaysia received British army rations and thus they were not exposed to EDCs (e.g. genistein) associated with Asian diets. It is possible that military activity and its associated stress could have had an effect on spermatogenesis; this cannot be ruled out. It must, however be stressed that all of the veterans included in this study were exposed to high doses of DBP and that DBP has been shown to reduce testosterone synthesis possibly via epigenetic mechanisms in rats. This is a key point that must be considered with the fact that all of the children of the DBP-exposed fathers were born after their fathers returned to New Zealand from Malaysia.

Epigenetic changes (e.g. gene control by methylation) could last for a considerable time after exposure which offers a reasonable explanation for our findings; men were exposed to DBP for long periods and at high doses during their military service, this exposure led to epigenetic changes in sperm DNA which remained until conception. The epigenetically changed sperm DNA fertilised an ovum to generate a zygote with modified gene expression which led to developmental changes that resulted in higher incidences of hypospadias, cryptorchidism and breast cancer in the exposed men’s offspring.

The gene expression-modified zygote might lead to an embryo that synthesised less testosterone which, in turn, could result in developmental errors in growth and development of the reproductive cleft. Reduced testosterone levels would lead to feminisation of the genitalia which might be manifested as hypospadias and cryptorchidism. The increased incidence of breast cancer is very much more difficult to explain, but it is possible that reduced testosterone levels in the developing embryo means that the estradiol:testosterone ratio is raised which might up regulate breast cancer genes and thus increase the risk of breast cancer in later life.

These are interesting hypothetical mechanisms that might explain our data. Clearly, much work is necessary to investigate the genetic phenomena that might be at play.

Competing interests: None known.

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Author information: Matthew Carran, Postgraduate Research Student; Ian C Shaw, Professor of Toxicology; Department of Chemistry, University of Canterbury, Christchurch

Correspondence: Professor I C Shaw, Department of Chemistry, University of Canterbury, Private Bag 4800, Christchurch, New Zealand. Email: ian.shaw@canterbury.ac.nz

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