Environmental Toxicants and Sperm Production in Men and Animals

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Introduction

Endocrine disrupting chemicals (EDCs) are ubiquitous in the environment and have the ability to interfere with, amongst others, hormone-dependent physiological processes through the interaction with hormone receptors (Bergman et al. 2013). Thus, EDCs can bind to receptors and elicit direct or indirect agonist or antagonist action, binding onto allosteric sites to yield unfavourable outcomes even at very low concentrations and hindering hormone production, transport, metabolism and degradation (Zoeller et al. 2012). Numerous studies are focused on the effects that both acute and chronic exposure to EDCs may have on humans and wildlife. These studies have shown that exposure to EDCs potentially impacts the growth and development of various bodily organs, bodily processes and fertility (Bornman et al. 2010; Colborn et al. 1993). Exposures to various EDCs have been reported to influence embryonic development, especially at the androgen-sensitive sex-determining programming windows during early gestation (Sharpe 2009). Changes in endogenous hormone regulation during embryonic development may result in impaired functioning of bodily systems, such as the male urogenital system (Schug et al. 2011). The impact of embryonic exposure may be identifiable at birth, as in the case of

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urogenital abnormalities, or later in adult life, such as poor semen quality or other reproductive disorders such as tumors. Humans are exposed to numerous known EDCs including polychlorinated biphenyls (PCBs), bisphenol A (BPA), used in the production of some plastics, and insecticides used for vector control (Diamanti-Kandarakis et al. 2009).

In 2018, the World Health Organization (WHO) estimated that 228 million cases of malaria occurred worldwide, with 93% of these malaria-related deaths occurring within the African region (WHO 2019). Although faced with climates conducive for malaria transmission and being susceptible to cross border transmission, South Africa (SA) has been able to control the spread of malaria through various initiatives since the 1930s and now malaria is currently endemic in three provinces, KwaZulu-Natal, Mpumalanga and Limpopo (Raman et al. 2016). The Vhembe district in the Limpopo province has consistently reported the highest malaria incidence rates in SA and vector control measures are essential to alleviate the malaria burden.

In 2002, SA ratified the Stockholm Convention and is therefore permitted to use organochlorines 1,1,1-trichloro-2,2-bis(4-chlorophenyl) ethane (DDT) and pyrethroids such as deltamethrin and cypermethrin DDT for malaria vector control (Bouwman 2004) through IRS programs (WHO 2006). The major concern with IRS programs stems from incorrect storage, application and contamination of the surrounding areas (Van Dyk et al. 2010) which poses a health concern for both animals and humans. In light of the use of DDT in IRS, the main exposure routes in humans include inhalation and ingestion of contaminated food and water sources (Van Dyk et al. 2010). Technical-grade DDT consisting of 65–80% of the active insecticidal ingredient *p*,*p*′-DDT and 15–21% of the less insecticidal *o*,*p*′-DDT (Turusov et al. 2002) is used for IRS. Both *o*,*p*′-DDT and to a lesser extent *p*,*p*′-DDT component have estrogenic properties (ASTDR 2002). Dietary and environmental exposures to *p*,*p*′-DDT and its metabolites result in bio-accumulation of these chemicals in adipose tissue and serum in the human body (Kirman et al. 2011). The DDT from the circulation is metabolized into 1,1-dichloro-2,2-bis(*p*-chlorophenyl) ethylene (*p*,*p*′-DDE) which is the persistent metabolite that bio-accumulates in fatty tissue. DDT and *p*,*p*′-DDE have the ability to cross the placenta with concentrations in cord blood being similar to concentrations in maternal blood (ASTDR 2002). *p*,*p*′-DDE is a potent inhibitor of androgen binding to the androgen receptor (Danzo 1997), androgen-induced transcriptional activity and androgen action in males during development and in adulthood (Kelce et al. 1995). This suggests that abnormalities in male sex development induced by *p*,*p*′-DDE may be mediated at the level of the androgen receptor (Kelce et al. 1995).

Genes dependent on androgens are subject to altered expression as a result of the competitive agonist behaviour of *p*,*p*′-DDE (Phillips and Tanphaichitr 2008). It has been reported that foetal, neonatal or pubertal exposure to DDT/DDE may result in impaired reproductive function as well as semen and sperm quality (Martenies and Perry 2013). Various studies have investigated associations between serum concentration levels of DDT and DDE, on semen and sperm quality parameters including sperm count, morphology, motility, concentration and semen appearance, volume,

pH (Aneck-Hahn et al. 2007). Although studies have yielded inconsistent results, there is growing evidence of the negative impact DDT/DDE exposure has on male reproductive function.

While exposure to EDCs have been associated with adverse effects on semen and sperm quality very few studies have focused on the effects of EDCs on sperm chromatin integrity. Sperm chromatin integrity is imperative for the successful transfer of paternal genetic information from one generation to the next (Sadeghi et al. 2009). The sperm chromatin structure assay (SCSA) evaluates DNA fragmentation as a result of low pH treatment (Evenson 2016). and provides two valuable results, DNA fragmentation index (DFI) and High DNA stainability (HDS), both measures are indicators of abnormal chromatin indices (Spano et al. 2005). DFI provides a measure of damaged DNA and HDS measures impaired chromatin condensation (Evenson 2016; Spano et al. 2005).

A limited number of studies have focused on the effects of EDCs on male reproductive parameters, hormonal disruption and sperm chromatin integrity, which is essential for the optimal pre-conception environment. Thus, the aim of the study was to investigate the impact of exposure to a complex mixture of EDCs, DDT pyrethroids and other agricultural pesticides, on seminal parameters, hormonal regulation and sperm chromatin integrity.

Methods

Study Design and Population

A cross-sectional study was conducted between 2003 and 2008 ($n = 544$, from three DDT-sprayed villages—$n = 310$, three non-DDT sprayed villages—$n = 234$) and 2012–2017 ($n = 431$ from three DDT-exposed—$n = 236$; three non-DDT exposed—$n = 195$). The Limpopo Province is situated in the north-eastern corner of South Africa and is divided into five districts, including the Vhembe District. The Vhembe district is a malaria-endemic area where housing comprises traditional mud dwellings with thatch (straw grass) roofs or brick and cement houses.

Recruitment and Sampling

The participants were from rural IRS villages or nearby non-IRS villages in the Thulamela Local Municipality recruited from 2003–2008 to 2012–2017. Participants from sprayed and non-sprayed villages volunteered, but we excluded men who had lived in study villages for less than a year, those younger than 18 or older than 40 years, those with neuropsychiatric disorders or those who appeared intoxicated. All participants provided informed consent and were interviewed using a structured questionnaire, which detailed their general history, personal use of insecticides,

diet, smoking and drinking habits, illegal substance use, exposure to other insecticides and fertility history. Physical measurements included participants' weight and height and the body mass index (BMI) was calculated.

Exposure Assessment

We used a Shimadzu GCMS-QP2010 gas chromatograph/mass spectrometer to measure 1,1,1-trichloro-2,2′bis(*p*-chlorophenyl)ethane (DDT) and 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (DDE) as reported (Aneck-Hahn et al. 2007). We could not detect *o*,*p*′-DDD, *p*,*p*′-DDD, *o*,*p*′-DDE in 66–75% of the men, and this report focuses, therefore, on DDT and DDE concentrations. Total cholesterol and triglycerides were determined by enzymatic methods (Aneck-Hahn et al. 2007) and the total plasma lipid level was calculated according to Rylander et al. (2006). The lower limit of detection (LOD) for DDT and DDE was 0.02 μg/g lipid.

Biochemical Analysis

We collected venous blood samples from participants between 08:00 and 10:00. The samples were centrifuged at 670 × *g* for 10 min at room temperature and stored in 500 μL aliquots at −20 °C on-site and during transport. At the University of Pretoria laboratory, samples were stored at −80 °C until analyzed. Hormones measured, in serum with the Cobas® 6000 analyser (Roche Products (Pty) Ltd Diagnostics Division) using ECL (ElectroChemi-Luminescence) immunometric detection, were: total testosterone (t-T; 05200067160) and human sex hormone-binding globulin (SHBG; 03052001160). We measured serum albumin on the general automated platform to calculate bioavailable testosterone (b-T) and free testosterone (f-T) using the calculator available at http://www.issam.ch/freetesto.html, following Vermeulen's formula.

Semen Analysis

Semen samples were collected into a sterile wide-mouthed container after the prescribed 72 h sexual abstinence period. The semen sample was incubated at 37 °C until liquefied and semen analysis and additional andrological tests were conducted following the World Health Organization's (WHO) 1999 standards and procedures (WHO 1999) while adhering to the quality control procedures of the European Society of Human Reproduction and Embryology (ESHRE 1998). After liquefaction, semen characteristics including appearance, ejaculate volume and semen pH were assessed (Mortimer 1994). Sperm motility was assessed on a wet preparation

following WHO (1999) motility classification using the classes "a" through "d" sperm progression rating, where "a" indicates rapid progressive motility and "d" indicates immotile sperm (NAFA 2002). Sperm morphology slides were stained by the Papanicolau method and scored according to the WHO (1999) classification.

Sperm Chromatin Structure Assay

Samples with concentrations of sperm greater than 20×10^6 cells/mL were used to conduct the SCSA, using previously described methods (Evenson and Jost 2000). A BD Accuri C6 Plus™ (Beckton Dickinson, New Jersey, USA), fitted with an air-cooled argon ion laser and standard optical filters to collect red and green fluorescence, was used for flow cytometry analysis. Prior to any analysis on each day, BD CS&T RUO beads (BD, Gauteng, SA), dyed with fluorochromes, were used to calibrate the instrument and the software. To visualize the analysed sample, a forward scatter (FSC) and side scatter (SSC) dot plot was generated with a linear scale. The FSC axis provided a measure of the size of the cell and the SSC axis provided a measure of the complexity of the cell. Flow cytometric data were analysed off-line using the FlowJo version 10 (FlowJo, LLC, Ashland, Oregon, USA). The DFI frequency histogram was calculated using the ratio between the red and total (red plus green) fluorescence. The DFI histogram provided the percentage of damaged sperm with detectable DFI (%DFI). High DNA sustainability (%HDS) was determined using the percentage of sperm with high levels of green fluorescence.

Animal Study

The animal study aimed to investigate the effects of in utero-, lactational- and direct exposure to select EDCs, found in a malaria area, in South Africa, on male reproductive health and parameters, testicular histology, associated hormonal changes and apoptosis in Sprague-Dawley rats. The study design was based on the Organization for Economic Cooperation and Development (OECD) One-generation reproductive toxicity study 415 protocol (OECD 1983). Pregnant females (P1) were randomly allocated to the four experimental groups and dosed with either cottonseed oil, DDT, DDE or a mixture of EDCs throughout the duration of their gestation and lactation period. The P1 females in each group were continually dosed with either cottonseed oil, DDT, DDE or a mixture of EDCs throughout the pregnancy. Following birth, the P1 females were dosed during the lactation period of 3 weeks. Thus, the pups (F generation) were indirectly exposed to the EDCs during lactation. Following the lactation period, the male (F1) pups from each of the experimental groups were kept in their respective groups. The F1 pups were directly dosed daily for 10 weeks until reaching sexual maturity at 13 weeks of age. The anogenital distance, which is the length of the perineum from the base of the genital tubercle to

the center of the anus (Fielden et al. 2002), was measured and recorded. Body weight was recorded and the testes, seminal vesicles, the right epididymis, prostate and the liver were removed and weighed. Any macroscopic abnormalities were recorded before organs were fixed in the relevant fixatives for further analysis. The left epididymis was used for determining the epididymal sperm count. Blood was used to determine total testosterone.

Statistical Analysis

To determine median differences between measured variables in IRS and unsprayed groups, Wilcoxon rank sum tests were used. General linear models were used to determine associations between exposures to malaria vector control pesticides *p*,*p*′-DDT, *p*,*p*′-DDE and pyrethroids and SCSA parameters. Logistic regressions tested for relationships between hormone concentrations (response variables) and DDT and DDE concentration (predictor variables) expressed as a dichotomous variable (uptake vs. no uptake) and as a categorical variable as described earlier. Multivariate logistic models were adjusted for age, BMI, personal use of other insecticides, and smoking. F1 males from the same litter share a common mother, a P1 female, and hence data analysis employed the survey command in STATA 12 (StataCorp, TX, USA) to deal with the dependence of data within litters (i.e. clusters). In total, 16 clusters of F1 males were analyzed using Survey Linear Regression. The exposed groups (groups B–D) were compared to the control group (Group A), at the 0.05 level of significance. Additionally, group B was compared to group D to assess the possible effect of exposure to a single chemical compared to exposure to the same chemical in a mixture. Furthermore, differences among the exposed groups were assessed using the adjusted Wald Test at the 0.05 level of significance. Groups were compared with respect to endpoint values of the study parameters. In the analysis of the endpoint AGD, the value at baseline and the body weight was adjusted for.

Results and Discussion

Changes in DDT/DDE Concentrations

In sprayed villages *p*,*p*′-DDE lipi adjusted exposure levels were significantly lower between 2012 and 2017 (mean ± SD: 5.80 ± 6.6 μg/g) compared to the 2003–2008 (216.9 ± 210.6 μg/g) period ($P < 0.001$). In the non-sprayed villages *p*,*p*′-DDE exposure levels were significantly lower between 2012 and 2017 (mean ± SD: 1.47 ± 3.68 μg/g) compared to the 2003–2008 (2.81 ± 4.26 μg/g) period ($P < 0.001$) (Table 1). The levels of DDT in the study population in the 2003–2008 sampling period was 3.5times higher than occupational spray workers in Limpopo, South

Table 1 Changes in DDT/DDE concentrations in non-sprayed versus sprayed villages in Limpopo Province, South Africa

	Non-sprayed villages		Sprayed villages	
	p′,p′-DDT	*p′,p′*-DDE	*p′,p′*-DDT	*p′*,p-DDE
2003–2008	2.9 (9.3)	2.8 (4.3)	90.2 (102.5)	216.9 (10.6)
2012–2017	0.21 (0.45)	1.5 (2.8)	1.8 (3.8)	6.3 (6.8)

Africa (Dalvie et al. 2004) and 5times higher where DDT was phased out in Chiapas, Mexico (de Jager et al. 2006a, b). Despite the lower exposure levels in the 2012–2017 cohort compared to the 2003–2008 cohort, significant effects were seen on seminal parameters (sperm viability, morphology and counts) when comparing the DDT-sprayed and non-sprayed villages. Changes in the spray program are evident in the lower levels of DDT/DDE found in the 2012–2017 group. This change can be as a result of various factors, for example, the severity duration of a malaria outbreak. The type of housing in the villages has changed from the traditional thatch and clay to brick houses. DDT is sprayed on traditional houses while pyrethroids are sprayed on brick houses for malaria vector control. With the fluctuation in malaria cases in South Africa, mainly attributed to changes in rainfall and cross-border movement (WHO 2019), IRS spray patterns have changed. We anticipate that the sudden increase in malaria cases from 2016 (4323) to 2017 (22,061) (WHO 2019) may result in the IRS vector control strategies being intensified.

Hormonal Changes in Young Men

Crude linear regression results comparing the highest category of exposure to the lowest three showed that men whose DDE concentrations were in the highest category (173–997 μg/g lipid), had mean total testosterone concentrations that were 4.8 (CI 95% 3.3, 6.3) nmol/L higher than men in the three lower categories (BDL—172 μg/g lipid). Likewise, men whose DDT concentrations were in the highest category, (77–519 μg/g lipid), had mean total testosterone concentrations, 5.9 (CI 95% 4.4, 7.4) nmol/L higher than men in the three lower categories ("BDL" – 172 μg/g lipid). Similarly, men with DDE concentrations in the highest category had significantly higher log transformed estradiol concentrations, and lower log transformed FSH concentrations (Bornman et al. 2018). Furthermore, DDT and DDE exposure were significantly positively associated with total-, free and bioavailable testosterone, nonsignificantly associated with lower FSH and LH, whereas little effect was seen on estradiol. Low LH and FSH concentrations in the current study will result in impaired spermatogenesis. It seems possible that both androgen receptor antagonism by DDE and diminished LH and particularly FSH can account for the low sperm count and function (Moline et al. 2000; Mortimer et al. 2013). Sperm motility seemed to be additionally impaired by direct toxicity,

possibly due to DDE exposure representing a non-genomic mechanism (Tavares et al. 2015).

Urine Metabolites

Analysis showed that 3,5,6-trichloro-2-pyridinol (TCPY), 1,2,3-benzotriazine-4-one (BTA) a herbicide and 3-(2,2-dichlorорvinyl)-2,2-dimethyl-cyclopropane-1-carboxylic acid (Trans/DCCA) were the most common metabolites. Unlike DDT/DDE, pyrethroids are metabolized rapidly in the environment and have short half-lives (Saillenfait et al. 2015). Mammals metabolize pyrethroids through oxidation and ester hydrolysis and then by conjugation, which yields hydrophilic metabolites that are readily excreted in urine (Kaneko 2011). Consequently, the metabolite, 3-phenoxybenxoic acid (3-PBA), is estrogenic (Mnif et al. 2011) and is a common metabolite of deltamethrin, permethrin, fenvalerate, cypermethrin and cyhalothrin (Dalvie et al. 2004). Studies have determined possible associations between 3-PBA and decreased sperm concentration and motility (Martenies and Perry 2013; Meeker et al. 2008; Saillenfait et al. 2015). Furthermore, 3-PBA is associated with increased concentrations of follicle stimulating hormone (FSH), luteinizing hormone (LH) and decreased levels of inhibin B in men (Meeker et al. 2009), adversely affecting spermatogenesis.

SCSA

From the general linear model used to test associations between exposures to malaria vector control pesticides, an increased relative risk of increased %DFI was found when participants were exposed to *p′*,*p*-DDT and *p′*,*p*-DDE in the 20122016 sampling period. The findings, however, were not statistically significant. This finding follows a trend with the most recent study conducted in the Vhembe district, which found a significant positive association between exposure to *p′*,*p*-DDE and %DFI (de Jager et al. 2009). The lipid adjusted concentrations of both *p′*,*p*-DDT, and *p′*,*p*-DDT detected in participants from this study were significantly lower than concentrations found in other studies interested in similar outcomes. During the 2003–2009 sampling period, conducted in the Vhembe district, concentrations of 83.9 μg/g of DDT and 177.8 μg/g of DDE were found (de Jager et al. 2009). Other studies focusing on environmental exposures to DDT/DDE have reported lipid adjusted concentrations ranging from 41 μg/g of DDE, reported in Chiapas Mexico (de Jager et al. 2006a, b), to 134 μg/g and 46 μg/g of DDT and DDE respectively, in Limpopo (Aneck-Hahn et al. 2007). In the 2012–2016 study period, concentrations of DDT and DDE, were approximately 645 times lower (0.13 μg/g *p′*,*p*-DDT) and 78 times lower (2.26 μg/g *p′*,*p*-DDE) compared to the 2003–2009 sampling period (de Jager et al. 2009). The nonsignificant associations may be attributed to the

significantly lower DDT/DDE exposure levels found more recently. Although the effect of EDCs is thought to cause significant effects at low concentrations, the very low concentrations of DDT and DDE suggest that perhaps these pesticides have not been sprayed in the area as assumed. This may suggest that other pesticides are used, as the participants reported that their homes had been sprayed at least once in the past year. A study included participants from various European countries and failed to show an association between DDE and SCSA parameters, despite mean DDE concentrations of 790 ng/g (Spano et al. 2005). Suggesting that a focus should also be placed on determining the associations between pyrethroids and other EDCs as sperm DNA fragmentation.

Animal Study

The mean anogenital distance (AGD) was significantly shorter in the mixture group ($P = 0.005$) when compared to the controls. The AGD is a sensitive marker of prenatal disruption of the development of the male reproductive system (Swan et al. 2015). The AGD in males is longer than in females—generally double the distance in females measured in multiple mammalian species, thereby suggesting that the AGD is under the hormonal influence (Hsieh et al. 2008). The mixture group (group 4) received technical grade DDT, DM, *p*-NP and phytoestrogens, all of which have estrogenic properties. Synergistic activity between chemicals (Silva et al. 2002) could have further enhanced the total additive estrogenicity, resulting in a shorter AGD of the mixture group (group 4) (Patrick et al. 2016).

After prenatal exposure to the anti-androgen DDE, the mean AGD was also shorter, but not significantly. A shorter anogenital distance in males signifies feminizing changes (Palanza et al. 2001), which might be related to lower/impaired androgen function during the hormone-sensitive male programming window (Sharpe 2009). However, not only androgen plays an important role during masculinization, but an optimal androgen-estrogen balance is also involved. Maintaining the appropriate androgen–estrogen balance is crucial for the normal development of the structure and function of the male reproductive tract. Disruption of the balance during early fetal development may lead to abnormal development of the male reproductive tract (Rhind et al. 2001; Svechnikov et al. 2014). Although the shorter AGD may seem a 'minor' phenotypic variant of a normal male, the longer-term implications may be more serious.

The mean prostate mass was significantly higher in the DDT group (group 2; 1.02 g, $P = 0.018$), compared to the control group (group 1; 0.83 g). Technical grade DDT has estrogenic properties mainly due to the *o*,*p*′–DDT isomer (Metcalf 1995). Estrogens have direct effects on the adult prostate gland and have been implicated in the etiology of the prostatic disease (Sikka and Wang 2008). It seems plausible that exposure to both endogenous and exogenous estrogenic and/or anti-androgenic compounds could interfere with prostate growth (Boberg et al. 2015). Exposure to a

mixture of chemicals may exert an additive or a synergistic effect, thus, effects may exist on a histological level, which was not studied.

In the testes, testosterone plays a vital physiological role and is essential for normal spermatogenesis (Nieschlag and Behre 1998) as it promotes the differentiation of spermatogonia by stimulating genes within the Sertoli cells (Russell and Griswold 1993). The mean total testosterone concentrations were significantly higher in the DDE (group 3; 28.12 nmol/L, $P = 0.038$) and mixture (group 4; 28.62 nmol/L, $P = 0.023$ compared to the controls. These findings confirmed the previous findings of higher testosterone concentrations following exposure to 300 mg/kg *p,p′*-DDE for 15 days (O'Connor et al. 1999). In a study investigating hormonal changes associated with DDT uptake in men (Bornman et al. 2011), exposure to estrogenic- and anti-androgenic compounds increased steroid hormone binding globulin (SHBG), but it was not measured in the present study.

Where Do We Go from Here?

The concurrent exposure to both DDT and DDE seemed to result from estrogenic and/or anti-androgenic effects in men living in malaria areas. The high testosterone concentrations in both the human and animal models may result from DDE blocking the androgen receptor. Testosterone gives negative feedback to the pituitary, reducing LH and FSH secretion, which will impair testicular function. Despite the decline in exposure levels over time, seminal parameters and chromatin integrity were still affected. Utero or early life exposures, as seen in the animal model, may therefore adversely affect reproductive health, due to the potential synergistic and additive effects of known EDCs in malaria areas. While still dependent on DDT for malaria vector control, a more sustainable approach is needed towards malaria elimination, involving innovation, education, communication and health promotion strategies targeting affected populations.

The United Nations 17 Sustainable Development Goals (SDGs) (Morton et al. 2017) aim to enhance access to basic services, promote environmental sustainability and support inclusive growth. The third goal (SDG 3) aims to provide health and well-being for all and is a key factor in measuring the progress and success of the SDGs. More specifically, SDG3.3: which states that by 2030, amongst others, to end the epidemics of malaria and neglected tropical diseases. Although there are various malaria control strategies in place, it has become evident that integrative approaches are required to achieve the malaria elimination agenda. The University of Pretoria Institute for Sustainable Malaria Control's (UP ISMC) vision and mission is anchored in using an integrative transdisciplinary approach towards safe malaria control and elimination. Current initiatives include: safer alternatives (innovation)—the use of new materials and environmental friendly larviciding; development of study material (education)—Sibo Fights Malaria; launching a mobile application (communication)—Malaria Buddy and health promotion (music, drama and nutrition)—to communicate malaria messages and create awareness amongst affected communities,

these are all used as sustainable approaches towards malaria elimination. The UP ISMC is geared towards education, health promotion, communication and innovative high impact research, which are key to address the SDGs, including the elimination of malaria, while reducing reliance on potentially harmful EDCs (SDG3.9) and limiting the effect environmental toxicants have on sperm production in men and animals.

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