Simultaneous exposure to low concentrations of dichlorodiphenyltrichloroethane, deltamethrin, nonylphenol and phytoestrogens has negative effects on the reproductive parameters in male Spraque-Dawley rats

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Summary

Many reports suggest that male reproductive health has deteriorated over the last decades, possibly due to environmental contaminants that act as endocrine disruptors. This hypothesis was tested in Sprague-Dawley rats using a modified Organization for Economic Cooperation and Development 415 one-generation test. Group A received cottonseed oil as control, and Groups B, C and D received deltamethrin (DM); DM and dichlorodiphenyltrichloroethane (DDT); and DM, DDT, phytoestrogens and *p*-nonylphenol, respectively. Rats were exposed *in utero* and then received the substances for 10 weeks. The seminal vesicle mass (Group B; $P = 0.046$) and sperm count [Groups C ($P = 0.013$) and D ($P = 0.003$)] were lower and the anogenital distance [Group B ($P = 0.047$) C ($P = 0.045$) and D ($P = 0.002$)] shorter compared with the control group. The seminiferous tubule diameter [Groups B ($P = <0.001$), C ($P = <0.001$) and D ($P = <0.001$)] and epithelium thickness [Groups B ($P = 0.030$), C ($P = <0.001$) and D ($P = <0.001$)] were smaller compared with the control. The histology of the testes showed signs of apical sloughing and vacuolisation. Liver weights [Groups C ($P = 0.013$) and D ($P = 0.005$)] and liver enzymes [Group D ($P = 0.013$)] were also affected. These findings may indicate that simultaneous exposure to endocrine disrupting compounds contributes to the deterioration observed in male reproductive health.

Introduction

A variety of oestrogenic compounds exist in the environment, both natural (phyto- and myco-oestrogens) and synthetic (e.g. pesticides, industrial contaminants and sewage effluent by-products). Many of these compounds possess endocrine disrupting effects and it has been suggested that the apparent decline in male reproductive health over the last decades may be caused by exposure to excessive amounts of these oestrogenic compounds used daily in industry, agriculture and in the home (Jensen *et al.*, 1995; Toppari *et al.*, 1996).

Malaria is one of the world's most serious tropical diseases and imposes significant economic burden on some of the poorest nations. Synthetic pyrethroids such as deltamethrin (DM) were developed to limit the use of dichlorodiphenyltrichloroethane (DDT) (Sharp & le Sueur, 1996). It has been proven that pyrethroids are endocrine disruptors, which can increase the oestrogenic load in the body (Gray *et al.*, 1989) and increase the levels of oestrogen in breast cancer cells (Go *et al.*, 1999; Chen *et al.*, 2002). DM exposure studies showed decreased semen quality and impaired reproductive function in laboratory animals at 1.0 and 2.0 mg kg^{-1} (El-Aziz *et al.*, 1994). Synthetic pyrethroids, like DM, are currently also being used in agriculture, particularly for cotton-production and in small-scale farming.

Dichlorodiphenyltrichloroethane has been shown to have a negative reproductive effect on the biota (Facemire *et al.*, 1995; Sumpter, 1995). In addition to DDT exposure, exposure to its metabolites also contributes to the detrimental effects on reproduction. Technical-grade DDT (used as an indoor spray) consists of approximately 85% of *p,p'*-DDT, 15% *o,p'*-DDT and trace amounts of *o,o'*-DDT (Turusov

et al., 2002). Both *p,p'*-DDT and *o,p'*-DDT can bind to nuclear oestrogen receptors (ERs) (Charles *et al.*, 2005). *p,p'*-dichlorodiphenyl dichloroethylene (DDE), a persistent metabolite of *p,p'*-DDT, has been shown to have anti-androgenic activity and to exert developmental toxicities in male rats (Kelce *et al.*, 1995; Gray *et al.*, 2001).

The hypothesis was that *p,p'*-DDE may interact in an additive or multiplicative way with other endocrine disrupting chemicals (EDCs). This is particularly important for South Africa, as the vector control programme for malaria uses both DM and DDT for indoor residual spraying, after one of the mosquito vectors that was previously eliminated by DDT, returned and has pyrethroid resistance (Hargreaves *et al.*, 2000). The acceptable daily intake (ADI) for DDT in human milk was set at 20 μg kg^{-1} body weight (bw) and the ADI for DM is set at 10 μg kg^{-1} bw (Bouwman *et al.*, 2006). In their study Bouwman *et al.* (2006) calculated that the ADI of DDT for infants is exceeded by 1.9 times via breast milk, while the ADI for DM was exceeded slightly at 13.3 μg kg^{-1} bw. It is also important to take into account that the infants are exposed to these levels for up to 2 years, as breast milk constitutes the main food source to these infants (Bouwman *et al.*, 2006).

Humans are also exposed daily to many other EDCs. *p*-Nonylphenol (*p*-NP) belongs to a group of alkylphenols polyethoxylates and is used in detergents, paints, herbicides, pesticides and cosmetics. *p*-NP was reported to have oestrogenic properties and it was evident from reproductive toxicology studies that it exerted negative effects on the testis and epididymis of rodents (De Jager *et al.*, 1999a,b; Lee *et al.*, 1999). In South Africa, drinking water tested positively for *p*-NP and significant levels were found in sediment and in fatty tissue of eland and fish (Barnhoorn *et al.*, 2002; De Jager *et al.*, 2002). This confirmed that wildlife in South Africa is being exposed to environmental EDCs.

Phytoestrogens are natural compounds present in plants and are ingested daily in milligram quantities. The active substances are isoflavones (genistein), coumestans (coumesterol) and fungal metabolites (mycotoxins), such as zearalenone. Phytoestrogens possess oestrogenic effects *in vitro* and *in vivo* and have been responsible for reproductive disorders in some animal species (Toppari *et al.*, 1996; Santti *et al.*, 1998). Neonatal exposure of genistein is reported to inhibit the growth and proliferation of testicular cells in rats (Diaka *et al.*, 1998).

These reports support the concerns that EDCs and combinations of EDCs may have implications for the general and reproductive health of humans and animals or its progeny as a result of changes in endocrine function. In this study, potential adverse effects of DM and combinations of DM, DDT, *p*-NP and phytoestrogens on the male reproductive function and liver, after maternal and lifetime exposures were investigated.

Materials and Methods

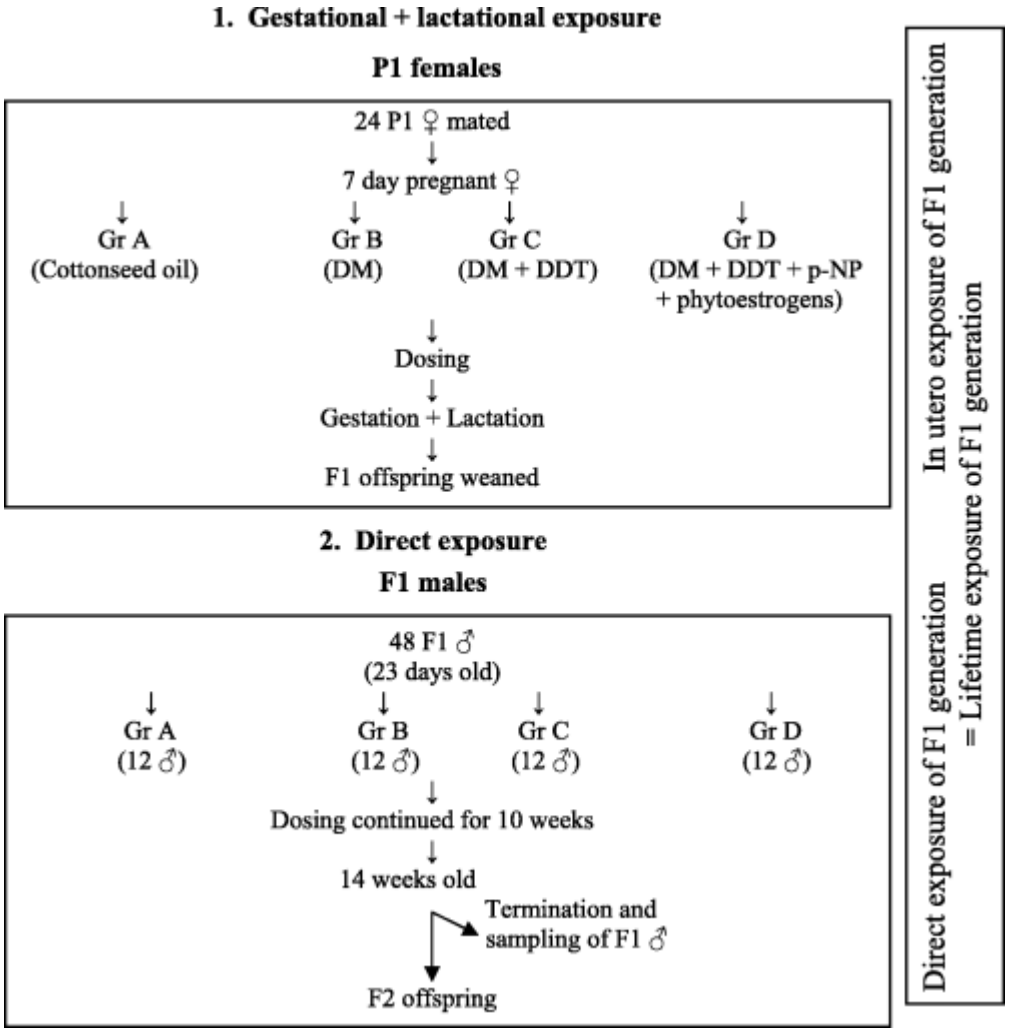
Test system and experimental design

The study was performed after approval by the Ethics Committee of the University of Pretoria (Project no: 11/2001). The Organization for Economic Cooperation and Development (OECD) 415 protocol for Toxicity Studies was modified to accommodate one control and three experimental groups (OECD Guidelines for the Testing of Chemicals, 1983). The design allowed studying the endocrine disrupting effects of maternal exposure (P1) to known EDCs on the reproductive parameters in lifetime exposed F1 males.

Forty-eight adult Sprague-Dawley rats, 24 males and 24 females, were obtained from The National Health Laboratory Service. They were housed in standard polycarbonate rat cages in rooms with constant temperatures (22 ± 2 °C), constant humidity (55 ± 10%) and 12-h day/night cycles. They were maintained on a stock pellet diet (Epol mice cubes, Pretoria, South Africa) and had free access to food and tap water. The animals were given a 2-week acclimatisation period before the study started.

Animals were randomly allocated into one control and three experimental groups and therefore consisted of six breeding pairs each (De Jager *et al.*, 1999a,b). The P1 females were allowed to mate and were observed for vaginal plugs and this was used as day one (D1) of pregnancy. To avoid interference with the blastocyst implantation and embryonic growth, dosing of the P1 females started only on day 7 of pregnancy and continued throughout the gestational and lactational period until the pups were weaned at 23 days.

Twelve F1 male offspring of each group were randomly selected. The individual animals were kept in separate cages and were directly exposed to the substances by oral gavage for a 10-week period. At week 14, the F1 males were terminated for sample collection.



Oral dosing

The different substances used for dosing were DM (99.5%, Chem Service, West Chester, PA, USA), technical-grade DDT (98%, Sigma-Aldrich, Steinheim, Germany), p-NP (Sigma-Aldrich) and phytoestrogens [Coumesterol: ~95% (Sigma-Aldrich), Genistein: 98% (Sigma-Aldrich), (Zearalenone) Sigma-Aldrich].

The concentration of the different substances was obtained from reports in the literature and was then calculated according to exposure levels in South Africa. Cottonseed oil (Lot 21 K0162; Sigma-Aldrich) was used for the control group (Group A) as vehicle. Experimental Group B received 1.0 mg kg^{-1} DM, Group C received a combination of 0.5 mg kg^{-1} DM and 35 mg kg^{-1} DDT and Group D a combination of 0.5 mg kg^{-1} DM, 35 mg kg^{-1} DDT, 2.5 µg kg^{-1} *p*-NP, 2.5 µg kg^{-1} genistein, 2.5 µg kg^{-1} coumesterol and 2.5 µg kg^{-1} zearalenone. All the substances were administered intragastrically at a dose volume of 1 ml kg^{-1}. The dosage volume for each animal was adjusted according to body mass.

Observations and procedures

Throughout the experiment, the animals were observed daily for clinical health, behavioural changes, signs of toxicity and mortality. They were weighed and dosed at the same time every day to exclude any external variables.

The weights of the F1 males were recorded before anaesthesia. The males were anaesthetised with Halothane (Safeline Pharmaceuticals (PTY) LTD, Wadeville, South Africa). Cardiac puncture was used for blood sample collection. The blood collection, together with an overdose of Halothane vapour was used for termination. The blood was spun down and the serum was stored at −20 °C for liver function analysis (n = 6) and chemical analysis (n = 6).

After termination, the anogenital distance (AGD) was measured; the testes, epididymides, seminal vesicles and the liver were weighed and the right testis fixed for histological evaluation.

Anogenital distance

The AGD, the length of the perineum from the base of the genital tubercle to the centre of the anus when the skin was naturally extended without stretching, was measured with a ruler in millimeters (Fielden *et al.*, 2002). The AGD was measured by the same individual to increase precision and to control for operator variation.

Testes, epididymides, seminal vesicles

The left and right testes and epididymides were removed and the epididymides were separated from the testes, cleaned and weighed individually. Both left and right seminal vesicles were removed and weighed. The mean testicular, epididymal and seminal vesicle mass were calculated. The right testis was placed in Bouins solution and was used for histology, with special reference to spermatogenesis (De Jager *et al.*, 1999a,b).

Cauda epididymal sperm count

The left cauda epididymis was used to determine the sperm concentration. The cauda epididymis was removed by the same individual to increase precision and to control for operator variation. The cauda epididymis was separated from the caput-corpus and was placed in 2 ml of phosphate buffered saline (PBS) medium (cat. no. BR14a, Oxiod, Hampshire, England) in a Petri dish. The cauda epididymis was cut into very small pieces to free the sperm. The PBS with sperm was transferred to a Falcon tube (De Jager *et al.*, 1999a,b). The Neubauer method was used to determine the sperm concentration, expressed as million per millilitre (WHO, 1999)

Testicular Histology and Staging

The testicular samples were fixed in Bouins fixative and were washed with 70% ethanol to remove the fixative. Fixed cross-sections of the testes were embedded in paraffin wax and the testicular tissue was dehydrated in a graded series of ethanol. Thin sections, 3 µm, were cut on a microtome and stained with a modified periodic acid-Schiff's reaction (PAS) and counterstained with Alum haematoxylin.

These slides were used to do staging of spermatogenesis using a Nikon Optiphot photomicroscope with 10× and 100× objectives. A computer software program on spermatogenesis, stages™ 2.1 (Vanguard

Media Inc., IL, USA), aided in the staging process together with the histology atlas of Russell *et al.* (1990).

For each individual rat, 30 randomly selected seminiferous tubules were staged to identify and classify all 14 stages of spermatogenesis. The tubular diameter, seminiferous epithelium and lumen diameter for all 30 tubules were measured both horizontally and vertically. The mean values of the horizontal and vertical measurements for each parameter were used for statistical analyses of the tubular diameter, seminiferous epithelium and lumen diameter.

Statistical analyses

The Kruskal-Wallis one-way nonparametric analysis of variance (AOV) was used for the comparison of total body and liver weights. For between group comparisons of all other variables one-way anova (Analysis of variance) was performed using ranks followed by pair-wise comparisons with Fisher's LSD (least significant difference method). Pair-wise comparisons between the control group and the treatment groups were performed at the Bonferroni adjusted level of significance (0.05/4 = 0.012) with the Wilcoxon Rank Sum test.

Results

No statistically significant differences were observed in total body weight between the groups. The general condition of the F1 males appeared to be unchanged. The parametric one-way anova performed on the ranked data followed by pair-wise comparisons with Fisher's LSD showed that the AGD of Groups A and B, and the AGD of Groups B, C and D were not significantly different from one another. Pair-wise comparison between the experimental groups and the control group, using the Wilcoxon Rank Sum test, indicated that the AGD of Groups B ($P = 0.047$), C ($P = 0.045$) and D ($P = 0.002$) were significantly shorter compared with the control group (Table 1).

Table 1 The effect of various admixtures on reproductive parameters of the F1 males of the different treatment groups

Variables	A	B	A/B	C	A/C	D	A/D
	Median (range)	Median (range)	*P*-value	Median (range)	*P*-value	Median (range)	*P*-value
Body mass (g)	543.7 (488.8–649.3)	538.7 (472.0–679.4)	ns	526.7 (475.7–587.3)	ns	561.2 (500.8–630.9)	ns
Anogenital distance (mm)	55 (50–60)	52 (38–56)	0.047	51 (42–58)	0.045	50 (42–56)	0.002
Seminal vesicle mass (g)	0.637 (0.404–0.815)	0.580 (0.394–0.654)	0.046	0.615 (0.464–0.724)	ns	0.667 (0.481–0.936)	ns
Epididymal mass (g)	0.661 (0.615–0.813)	0.627 (0.477–0.710)	ns	0.653 (0.589–0.668)	ns	0.653 (0.593–0.786)	ns
Testicular mass (g)	1.986 (1.777–2.055)	1.896 (1.777–2.055)	ns	1.955 (1.676–2.143)	ns	1.928 (1.805–2.329)	ns
Seminiferous tubule diameter (μm)	607.2 (507.0–695.1)	544.6 (440.7–587.0)	<0.001	513.7 (479.7–550.7)	<0.001	518.1 (393.8–595.1)	<0.001

Variables	A	B	A/B	C	A/C	D	A/D
	Median (range)	Median (range)	*P*-value	Median (range)	*P*-value	Median (range)	*P*-value
Seminiferous epithelium thickness (μm)	57.39 (43.63–64.70)	50.78 (41.38–54.16)	0.030	42.36 (36.23–50.74)	<0.001	40.34 (32.50–55.69)	<0.001
Lumen diameter (μm)	88.32 (63.71–120.87)	78.64 (55.09–105.48)	ns	84.72 (72.10–113.78)	ns	93.77 (48.78–123.88)	ns
Total sperm count ($\times 10^6$)	70.40 (53.53–103.13)	64.53 (27.00–90.00)	ns	54.16 (27.00–106.33)	0.013	48.76 (12.53–68.80)	0.003

A, control; B, deltamethrin (DM); C, DM + dichlorodiphenyltrichloroethane (DDT); D, DM + DDT + *p*-nonylphenol (NP) + Phytoestrogens; ns, nonsignificant.

The absolute testicular mass was reported rather than mass relative to body mass (testis ratio), as testicular mass and body mass are reported to be largely independent variables (Ettlin & Dixon, 1985). The testicular masses of the experimental groups were not significantly different compared with those of the control group.

The mean epididymal mass of the experimental groups compared with the control group were also not significantly different.

Between groups comparison with the anova performed on ranked data showed that the seminal vesicle mass of Group B differed significantly from Group A and Group D. The Wilcoxon Rank Sum test indicated that the median seminal vesicle mass of Group B ($P = 0.046$) was significantly lower compared with Group A. Seminal vesicle masses of Groups C and D did not differ significantly from Group A (Table 1).

The spermatogenesis of most of the animals in the experimental groups was normal and all 14 stages of spermatogenesis were present. The rat in Group C with the undescended testis had normal spermatogenesis in the right testis, but no spermatogenesis could be identified in the undescended testes. One rat in Group D had what looked like crystals inside the tubules and again no spermatogenesis could be observed.

Although the stages of the experimental groups were normal, there were signs of vacuolisation and cell necrosis with sloughing of the epithelium of the tubules of the experimental groups. The degree of vacuolisation and sloughing increased from Group B to Group D (Fig. 1).

When the anova was performed on the data using ranks followed by pair-wise comparisons with Fishers LSD, the diameters of all three experimental groups were observed to be smaller than those of the control group. Diameters of Groups C and D were also smaller than those of Group B. Groups C and D were not significantly different from one another. The Wilcoxon Rank Sum test showed that the tubule diameters of all experimental groups were significantly smaller compared with those of the control group (Table 1).

Application of the anova on ranked data of the seminiferous epithelium showed that all groups differed significantly from one another with the exception of Groups C and D, which had similar epithelial thicknesses. Paired comparisons using Wilcoxon Rank sum test between the experimental groups and

the control group showed that the epithelial thickness of Groups B (P = 0.030), C (P = < 0.001) and D (P = < 0.001) was significantly thinner compared with that of Group A (Table 1). No statistically significant difference was observed between the groups in measurements of the lumen diameter.

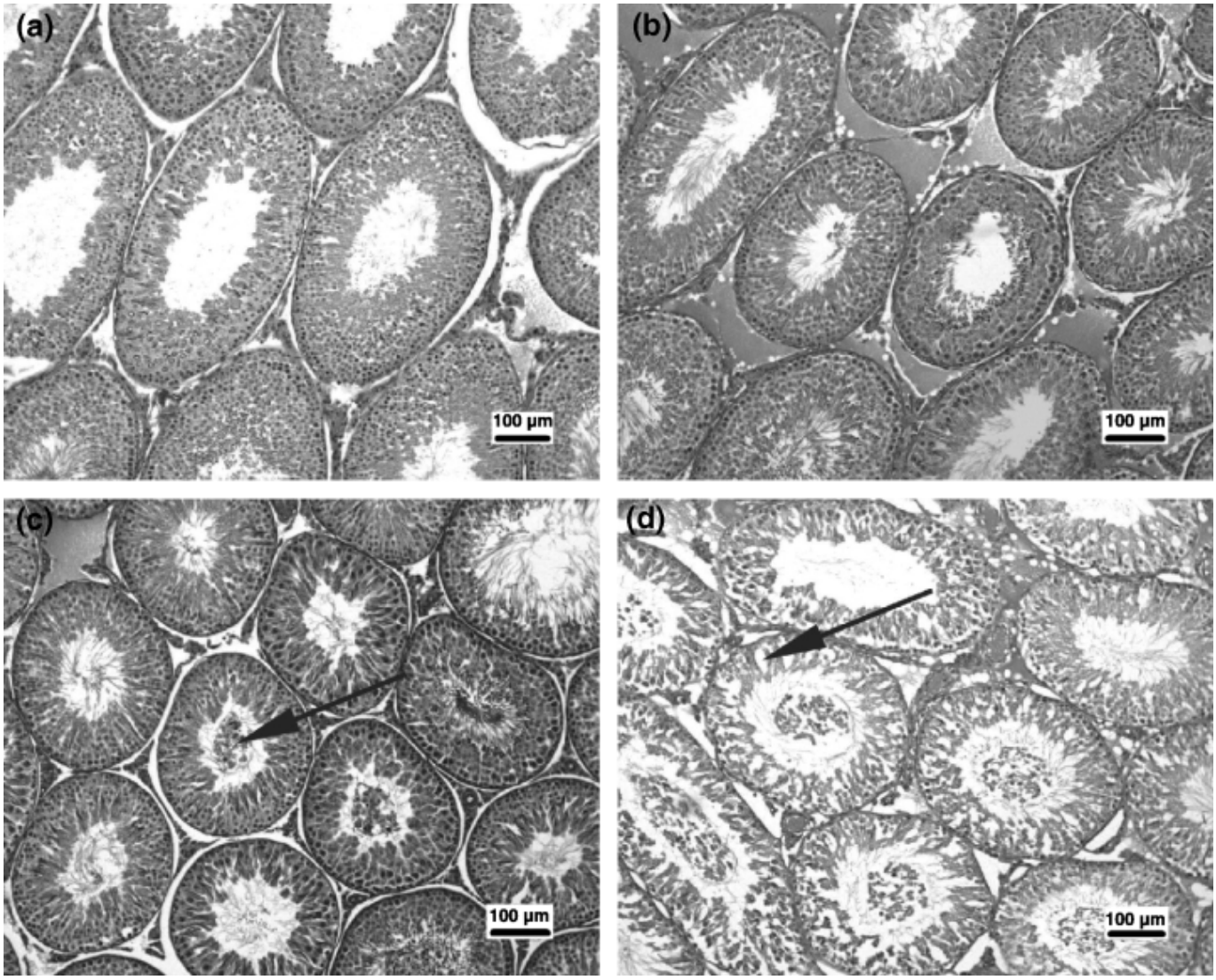


Fig. 1 Testicular histology. Note the increase in apical sloughing [indicated by the arrow in (c)] and vacuolisation [(indicated by die arrow in (d)] in the experimental groups. Group A = control; Group B = deltamethrin (DM); Group C = DM + dichlorodiphenyltrichloroethane (DDT); Group D = DM + DDT + *p*-nonylphenol (NP) + phytoestrogens modified periodic acid-Schiff's reaction (PAS) staining and counterstained with Alum hematoxylin; magnification, ×10

The sperm count of Groups C and D were significantly lower compared with that of Group A. The total sperm count of Group A and B, and the total sperm count of Groups B, C and D were not significantly different from one another when comparisons were performed with the anova following ranking of the data. The Wilcoxon Rank Sum test indicated that the total sperm count of Group A was significantly higher than the total sperm count of Groups C (P = 0.013) and D (P = 0.003) (Table 1).

Comparison of liver function enzymes with one-way AOV applied on ranked data showed that serum ALT levels of Groups A, B, and C did not differ significantly, as was the case for Groups C and D (Table 2). Comparison with control group, using the Wilcoxon Rank Sum Test showed that Group D (P = 0.013) serum alanine aminotransferase (ALT) levels were significantly lower. Serum asparate aminotransferase (AST) levels of the four groups did not appear to differ significantly from one another.

The Wilcoxon Rank Sum test indicated a significant increase in the liver mass between Groups A and C (P = 0.0019) and A and D (P = 0.0001). The liver mass of Group B was not affected.

Table 2 The effect of various admixtures on the liver

Variables	A	B	A/B	C	A/C	D	A/D
	Median (range)	Median (range)	*P*-value	Median (range)	*P*-value	Median (range)	*P*-value
Serum ALT (IU/l)	58.35 (49.90–61.90)	57.05 (43.30–63.80)	ns	4x.15 (36.50–47.30)	ns	37.25 (26.80–50.60)	P = 0.013
Serum AST (IU/l)	7.2 (3.6–9.7)	6.10 (4.30–16.80)	ns	7.20 (5.23–9.00)	ns	9.45 (8.20–16.00)	ns
Liver mass (g)	20.68 (19.00–23.28)	20.00 (17.06–28.39)	ns	25.57 (21.92–26.96)	P = 0.013	27.20 (24.34–29.62)	P = 0.005
A, control; B, deltamethrin (DM); C, DM + dichlorodiphenyltrichloroethane (DDT); D, DM + DDT + *p*-nonylphenol (NP) + Phytoestrogens; ns, nonsignificant.							

For the chemical analysis of the blood only DDT and both its metabolites (DDE and DDD) were found in Groups C and D with the limit of detection set at 40 µg l^{-1} blood. None of the other drugs were above the detection limit.

Discussion

This study investigated the endocrine disrupting effects of *in utero,* lactational and direct exposure to DM, a combination of DM and DDT and a combination of DM, DDT, *p*-NP and phytoestrogens on the liver and on the reproductive parameters of the F1 male rats.

The AGD of all three experimental groups were significantly shorter compared with the control group. Reduced male AGD is an indication of feminisation and has been observed after treatment with anti-androgenic and oestrogenic compounds (Gray *et al.*, 1994; Kelce *et al.*, 1994). The degree by which the AGD were affected increased per group as more compounds were added to the mixture. It has not yet been proven whether DM has an anti-androgenic effect (Andrade *et al.*, 2002), but the persistent DDT metabolite *p,p*'-DDE is well known for its anti-androgenic activity (Kelce *et al.*, 1995; Gray *et al.*, 1999). On the other hand, DDT as well as the phytoestrogens and *p*-NP exerts oestrogenic activity and it has been shown that low doses of *o,p'*-DDT are associated with a significant decrease in the AGD (Palanza *et al.*, 2001). This might therefore be an oestrogenic effect rather than an anti-androgenic effect.

Although the epididymal mass of the experimental groups were not affected, it is possible that the histology and cell function of this androgen-dependent organ were perturbed. The seminal vesicle mass of Group B was significantly lower compared with that of the control group. During developmental stages, adequate concentrations of androgens are necessary for the accessory sex organs to develop normally (Mably *et al.*, 1992). DM is known to have oestrogenic properties and El-Aziz *et al.* (1994) also found impaired reproductive function in laboratory animals at 1 mg kg^{-1}.

The absolute testicular masses of the experimental groups did not differ statistically significantly from those of the control group. Although the testicular masses of the experimental groups do not appear to be affected, the testicular histology demonstrates significant effects. Except for the lumen diameter, both

the seminiferous tubule diameter and the seminiferous epithelium thickness were significantly different compared with the control group. As with the AGD, Groups C and D appeared to be more affected than Group B. This may be indicative of testicular toxicity at the dose levels tested (De Jager *et al.*, 1999a; Andrade *et al.*, 2002).

All three experimental groups showed signs of Sertoli cell toxicity as became evident in some animals as germ cell necrosis, apical sloughing and vacuolisation (Russell, 1993). EDCs can reduce the size of the seminiferous tubules and thereby decrease the number of Sertoli cells. The number of Sertoli cells present during the prepubertal period is responsible for orchestrating and regulating spermatogenesis later in adulthood (Sharpe & Skakkebæk, 1993). Furthermore, Sertoli cell multiplication is controlled to a large extent by follicle-stimulating hormone (FSH) (Gustafson & Donahoe, 1994). Because oestradiol exerts negative feedback on FSH secretion, exposure to oestrogenic compounds during male sexual development could substantially reduce FSH secretion, reducing Sertoli cell multiplication (Cheek & McLanchlan, 1998). The consequences for the adult male would include decreased sperm production and, potentially, cryptorchidism (Sharpe & Skakkebæk, 1993). It is therefore not surprising that the mean total cauda epididymal sperm count of the experimental groups was lower compared with that of the control group. Although the sperm count of Group B was not significantly lower than the control group, those of Groups C and D were. Reduced cauda epididymal sperm number is a sensitive and persistent indicator of *in utero* and lactational anti-androgen exposure. DDT was used in both Group C and D and the anti-androgenic activity of the persistent DDT metabolite, *p,p'*-DDE has been demonstrated by Kelce *et al.* (1995) and Gray *et al.* (1999).

The substances and combinations of the substances used in Groups C and D are clearly hepatotoxic as indicated by the increased liver weights, which were significantly higher than that in the control group. DDE induces cytochrome P450 in the liver (You *et al.*, 1999). This induction of enzymes may account for the increased liver weight in the exposed animals (You *et al.*, 1999; Leavens *et al.*, 2002). Nagao *et al.* (2001) found that long-term administration of *p*-NP at 50 mg kg^{-1} day^{-1} has a toxic effect on the liver of adult rats. However, at lower concentrations (2 and 10 mg kg^{-1} day^{-1}) they found no effect on the liver. In this study, the concentrations of DDT and *p*-NP that were used in Groups C and D, were lower than concentrations that were used by the above-mentioned investigators; it is however the group that was affected the most. The decrease in serum ALT in Groups C and D may possibly be due to oxidative stress (Lee & Jacobs, 2006).

The effect of mixtures and low doses became evident in this study. If the results of Group C to Group D are compared, it is alarming to see the difference in testicular histology, AGD, sperm count and liver weight when phytoestrogens and *p*-NP were added to the mixture of DDT and DM in µg kg^{-1}. These results confirmed Payne *et al.* (2000) finding that substances in mixtures act additively even when they are present at concentrations that would have individually produced no detectable effects. This study further provides evidence that exposure of rats to a relevant environmental mixture of known endocrine disrupting substances during gestation, lactation and direct exposure up to 14 weeks, has a profoundly detrimental influence on male reproductive parameters.

People living in malaria stricken areas in South Africa and other countries, where spraying with DDT forms part of the malaria prevention protocol, may readily be exposed to all of the substances tested in this study. Although none of the animals died as a result of exposure to the combination of these substances, the reproductive system of the males were adversely affected. It is of the utmost important to realise that DDT in combination with low doses of other EDCs may be harmful to wildlife and to humans. These findings are also consistent with what was found in epidemiological studies in Mexico (De Jager *et al.*, 2006) and South Africa (Aneck-Hahn *et al.*, 2007) where young men were exposed to DDT. It is imperative to investigate the effects of these EDCs at different concentrations and in other combinations on male reproductive health in animals and in humans.

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