Toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in the developing male Wistar(Han) rat I: no decrease in epididymal sperm count after a single acute dose

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ABSTRACT

It has been reported that fetal exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) causes defects in the male reproductive system of the rat. We set out to replicate and extend these effects using a robust experimental design. Groups of 75 (control vehicle) or 55 (50, 200 or 1000 ng of TCDD kg^{-1} bodyweight) female Wistar(Han) rats were exposed to TCDD on Gestational Day (GD) 15, then allowed to litter. The high dose group dams showed no sustained weight loss compared to control, but four animals had total litter loss. Pups in the high dose group showed reduced body weight up till day 21, and pups in the medium dose group showed reduced body weight in the first week *post partum*. Balano-preputial separation was significantly delayed in the high dose group male offspring. There were no significant effects of treatment when the offspring were subjected to a functional observational battery, or mated with females to assess reproductive capability. 25 males per group were killed on post natal day (PND) 70, and ~60 animals per group (~30 for the high dose group) on PND120 to assess seminology and other endpoints. At PND120, the two highest dose groups showed a statistically significant elevation of sperm counts, compared to control; however, this effect was small (~30%), within the normal range of sperm counts for this strain of rat, was not reflected in testicular spermatid counts nor PND70 data, and is therefore postulated to have no biological significance. Although there was an increase in the proportion of abnormal sperm at PND70, seminology parameters were otherwise unremarkable. Testis weights in the high dose group were slightly decreased at PND 70 and 120, and at PND120, brain weights were decreased in the high dose group, liver to body weight ratios were increased for all three dose groups, with an increase in inflammatory cell foci in the epididymis in the high dose group. These data show that TCDD is a potent developmental toxin after exposure of the developing fetus, but that acute developmental exposure to TCDD on GD15 caused no decrease in sperm counts.

Keywords: Dioxin, Sperm, developmental, toxicity

INTRODUCTION

2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is a ubiquitous toxin, and prototypical representative of a series of chemicals which effect toxicity through a common mechanism, binding to the Ah Receptor (Poland and Knutson 1982). Investigation has focussed on the toxicity of TCDD, on the basis that other chemical congeners will show the same toxicity as TCDD, but with altered potency determined by their relative agonism of the Ah Receptor, and pharmacokinetics (Haws *et al.* 2006; Van den Berg *et al.* 1998). One of the most potent toxic effects of TCDD occurs after exposure of the developing rat fetus by dosing of the pregnant dam on GD15, leading to a spectrum of effects in the reproductive system of the male offspring, principally decreased sperm count in the cauda epididymis, but including decreased weight of the seminal vesicles, prostate and epididymis (Faqi *et al.* 1998; Gray *et al.* 1995; Gray *et al.* 1997a; Mably *et al.* 1992a; Mably *et al.* 1992b; Mably *et al.* 1992c). These effects occur at remarkably low doses, with statistically significant effects after a single maternal dose of 64 ng TCDD kg^{-1} bodyweight. In view of the consistent reports of developmental effects of TCDD on male epididymal sperm counts from three laboratories, these data have been used to set a Tolerable Daily Intake of 2pg kg^{-1} day^{-1} for TCDD and related compounds by the UK Committee on Toxicity (COT 2001), the WHO (JECFA 2001) and the EU Scientific Committee on Food (SCF 2001).

It is noteworthy that the mouse shows a profound resistance to these effects (Theobald and Peterson 1997) compared to rat, and the basis for this species difference in toxicity is unclear. Aspects of the original study by Mably (*e.g.* TCDD-dependent decreases in seminal vesicle weight) have been found to be irreproducible (Roman *et al.* 1995) in the same laboratory, and further, the potent developmental effects of TCDD on rat epididymal sperm levels are in dispute (Ikeda *et al.* 2005; Ohsako *et al.* 2001; Ohsako *et al.* 2002; Simanainen *et al.* 2004; Wilker *et al.* 1996; Yonemoto *et al.* 2005). However, it is difficult to reconcile these studies on account of differences in (*inter alia*) group size, rat strain, coefficient of variation and methodology in sperm enumeration, use of peripubertal animals or adults (Creasy 2003) and concurrent measurements of TCDD dose: hence a robust experimental design, coupled with improved statistical power, is required to resolve this controversy.

The developmental effects of TCDD on the male reproductive system, and particularly on epididymal sperm counts, are crucial to assessments of TCDD in the UK, Europe and the US. In view of the importance of this endpoint, it is imperative to determine if developmental exposure to TCDD does indeed cause effects on the male reproductive system. We have therefore undertaken a study to address this issue, with considerable attention to establishing a robust GLP experimental design, including Computer-Assisted Sperm Analysis for seminology, increased group size for greater statistical power, concurrent analysis of biological samples for TCDD concentration and a clear prior hypothesis that TCDD would reduce F_1 epididymal sperm levels. A single acute dose of TCDD was used, as this experimental design has previously yielded the largest effect on epididymal sperm levels (Gray *et al.* 1997a; Mably *et al.* 1992a; Mably *et al.* 1992b; Mably *et al.* 1992c). An outbred Wistar rat strain (CRL:WI(Han)) was used to ensure comparability with (1) previous use of Wistar

rats (Faqi *et al.* 1998) (2) extensive in-house experience with this strain. While developmental exposure to TCDD has toxic effects, our data have shown no potent adverse effects on epididymal sperm levels or the weights of accessory sex organs.

MATERIALS AND METHODS

Materials.

TCDD was obtained from Cambridge Isotope laboratories, Mass, USA, and purity was verified by HR-MS. All other chemicals were of the highest quality available.

Animal Study.

The animal studies were performed at Covance (Harrogate, UK), and were GLP-compliant; the full report on this study is published as supplementary material. CRL:WI(Han) rats were housed at a temperature of 19-25ûC, with one brief excursion to 17ûC. Animals were provided food (SQC rat and mouse breeder diet No. 3, expanded; Special Diets Services Ltd., Witham) and water *ad libitum*, and were housed singly for the parental generation, or in groups of five males for the F1 generation, with a 12 hour light/ darkness cycle. Animals of 16-18 weeks of age (204-294g) were time mated, with the day after mating designated as day 0 of gestation (GD0), delivered to Covance by GD 9, and assigned to treatment groups on GD 12 using a randomisation procedure based on body weight. 75 animals were treated with control vehicle (corn oil) by oral gavage, and 55 animals with 50, 200 and 1000 ng TCDD kg^{-1} bodyweight on GD15; the concentration of TCDD in the dosing vehicle was verified by GC-MS (104.6-106.1% of target concentration). 25 vehicle-treated rats and 15 TCDD-treated rats were killed on GD16 and GD21 for tissue sampling prior to TCDD analysis and mRNA analysis; the remaining females were allowed to litter and rear their offspring until weaning (PND21), and killed on PND21. The first day that pups were noted was designate day zero: litters were reduced to a maximum size of eight on PND4, and to five males on PND21. Males were then maintained untreated, until killed (25 per group) at PND70, and all remaining animals at PND120. Although kill days are referred to as PND70 and 120, the number of animals involved required that the kills were conducted during post-natal weeks 10 and 17. During post-natal weeks 12 and 13, twenty animals from each group were tested for learning ability (swimming maze), motor activity (every two minutes for thirty minutes), and in week 13, a functional observation battery. During post-natal week 16, twenty males per group were paired with untreated virgin females for up to seven days; mated females were killed on GD14, and examined for terminal body weight, pregnancy status, number of corpora lutea, number and intrauterine position of implantations, which were subdivided into live embryos, and early and late intrauterine deaths.

Necropsy and seminology.

At necropsy, animals were weighed and weights of seminal vesicles (with coagulating glands), brain, epididymes (total), liver, prostate, thymus, spleen, kidneys and testes recorded. Testis, epididymis, liver, thymus and prostate from control and high dose groups were fixed, embedded, sectioned at 5µm, stained with haemotoxylin and eosin, and examined by a pathologist. Sperm counts and viability were assessed from one epididymis from each male killed in post-natal weeks 10 and 17, and samples examined microscopically for morphology. Briefly, the cauda epididymis is dissected free, and the mid-distal cauda pierced two/three

times with a scalpel blade. The cauda is placed into 5mls of phosphate buffered saline containing 0.57% (w/v) BSA, preheated to 37ûC. Samples were incubated for 90-180 minutes, and a sample taken for sperm counting. The left testis of males was frozen, pending enumeration of homogenisation resistant spermatids. Sperm number, motility and velocity were recorded by CASA (Computer Assisted Sperm Analysis) with a Hamilton-Thorne TOX-IVOS examining n=10 fields per sample. 500 sperm per animal were examined microscopically and the number of morphologically abnormal sperm was recorded to give the % abnormal sperm.

TCDD analysis.

Samples were stored frozen until analysed. Adipose tissue and liver samples were analysed individually and fetus samples from individual females were combined, but the volumes of blood samples were too low for individual analysis, and were pooled. The tissue samples were homogenised, and an aliquot taken for analysis. Sample aliquots were fortified with ^{13}Carbon labeled dioxins, and exhaustively extracted using mixed solvents. The extracts were initially purified by acid hydrolysis, fractionated on activated carbon and further purified using adsorption chromatography, on alumina. The eluent was concentrated under nitrogen and sensitivity standardised for measurement using additional ^{13}Carbon labeled dioxins. TCDD was measured using high resolution gas chromatography with high resolution mass spectrometric detection at a resolution of ~10000 (defined at 10% of peak height). Instrument performance was monitored during the measurement interval by the use of a calibrant (perfluorokerosene) lock mass and ions corresponding to native and [^{13}C]-labelled dioxins were recorded. Data was processed using Masslynx™ and Microsoft Excel software to provide tissue concentration data. The analytical data met published acceptance criteria (Ambidge *et al.* 1990) for dioxins. The method used is accredited to the ISO17025 standard and has been validated and published after peer review (Fernandes *et al.* 2004). Each batch of samples analysed incorporated a full reagent blank, and analytical results were validated by the analysis of an in-batch reference material (RM) (Maier *et al.* 1995), for which results were compared with certified or assigned data. The contribution from the batch blanks was found to be negligible.

Statistical analysis.

Data were first analysed at Covance, as they were collected, with their standard statistical package. Continuous outcomes were analysed using one-way analysis of variance (ANOVA) or analysis of covariance (ANCOVA), after log transformation where necessary. Pairwise comparisons with control were made using Dunnett's test and a linear trend test was applied. Data measured as proportions of animals were analysed using the Cochran-Armitage test for dose-response and Fisher's exact test for pairwise comparisons. These tests were interpreted with one sided risk for increased incidence with increasing dose.

Further analyses of selected variables carried out in the package GenStat (Payne 2004) and included terms for random variation between litters. F_1 body weights were analysed by a

mixed model ANOVA, with a one-way treatment group structure, and a normally-distributed random term for litters. In most analyses litter effects were significant, with the effect that estimated standard errors were larger than in the simple ANOVA model. Comparisons between treated groups and control used Williams' test (Williams 1972). Early body weights were analysed with a two-way (dose group × day) ANOVA mixed model with random litter effects. From PND21 onwards, when the pups were individually identified in the data, a repeated measures model was applied, with random terms for litter and pup differences. Time to balano-preputial separation (BPS) was analysed by a proportional hazards mixed model with a random term for litter effects (Lee *et al.* 2006), and with body weight as a covariate.

RESULTS

The experimental dose range was chosen to be comparable to those used previously (Faqi *et al.* 1998; Gray *et al.* 1997a; Mably *et al.* 1992a). The concentration of TCDD in the dosing vehicle was verified by HR-MS to be at 104.6-106.1% of the target concentration, and the total dioxin TEQ (Toxic EQuivalents) in the feed was determined at 60pg kg^{-1} (see Supplementary data). One animal dosed with 1000 ng TCDD kg^{-1} was killed on GD19, showing hunching, piloerection and body weight loss. There was no significant effect of treatment on animals during gestation, but a subset of the high dose group (animals killed on GD21) showed significantly less weight gain than controls between GD 19-21. Animals in the high dose group gained significantly less weight than controls during days 1-4 of lactation, but there was no significant difference over the whole of the lactation period (see Supplementary Data).

Littering and offspring

At littering, four females in the high dose group showed total litter loss (versus one in the control group); the ratio of live births to implant sites was lower in the high dose group than in the control, but this difference was not statistically significant. More pups in the high dose group were found dead on PND1, and between PND11 and 14, compared to control, so that mean litter size in the high dose group was significantly lower than control throughout lactation; at PND21, the numbers of offspring per litter in the high dose group were 12% lower than control (Table 1). There was no statistically significant effect of maternal TCDD treatment on the proportion of males offspring (Table 1).

F_1 body weight gain and BPS

Males derived from the group given 1000 ng kg^{-1} TCDD started the F_1 generation phase of the study lighter than the controls and gained less weight than the controls throughout the study (Fig. 1). Body weight of offspring was found to be significantly affected by post-natal day, by dose group, and there was found to be a significant interaction between post-natal day and group (see Fig. 1B). The pups in the 1000 ng kg^{-1} group were lighter than the controls on PND 1 and remained lighter throughout the lactation period. Mean pup weight in the 200 ng kg^{-1} group was also slightly lower than control on PND 1 and the male pups in this group were lighter until PND 7. The high dose group showed a pronounced dip in body weight relative to control immediately after weaning; the ratio of high dose to control body weights remained consistently depressed, showing a persistent effect on depression of weight gain in this group. The other two dose groups did not differ markedly from controls after PND7.

The incidence of balano-preputial separation was markedly slowed, compared to controls, in the males derived from the group given 1000 ng kg^{-1} TCDD ($P<0.001$) (Fig. 2); the incidence rate in this group was estimated as 67% slower than in the controls (95% CI 48% to 79% slowing). Without adjustment for litter variation, development in the 200 ng kg^{-1} - derived group was significantly ($p<0.05$) about 30% slower than control, but after adjustment for litter

the larger standard error gave a 95% CI (4% acceleration to 53% slowing) that fell just short of statistical significance at 5%. In view of the effect of TCDD on body weight of the F_1 males, additional BPS analyses were performed with body weight at either PND21 or 42 as a covariate. Although the PND21 weight had a significant effect, adjusting for PND21 or 42 body weights as covariates did not materially affect the differences between the treatment groups. Hence there was no evidence that delay in BPS was due to a decrease in body weight at PND21 or 42.

Learning and Motor Activity

There were no adverse effects of maternal treatment on the results of the functional observation battery of tests (see Supplementary Data). In the motor activity tests during post-natal week 12, the treatment-derived males were initially less active in the automated activity recorder compared to the controls. However, there was no dose-relationship and after 6 minutes, the activity patterns were similar in all groups (see Supplementary Data). In the learning ability tests, animals in the 50 and 200 ng kg^{-1} - derived groups took significantly longer than the controls to escape when the ramp was changed from right to left (switch response) but those in the 1000 ng kg^{-1} -derived group showed no such delay (see Supplementary Data). In view of the absence of a clear dose-response relationship and the inherent variability in the data, it is unclear if these data can be interpreted as an adverse effect caused by maternal TCDD exposure.

Analysis of Reproductive Capacity of F_1 Males

20 F_1 males per group were mated during post-natal week 16. Although two females mated to males in the high dose group were not pregnant in spite of positive evidence of mating, the relevant males had macroscopically normal reproductive organs, and normal values in the seminological investigations. There were no significant differences between groups in pre-coital time, fertility index or mating index. The females were killed on GD14, and there were no significant differences between groups in number of corpora lutea per female, implantations per female, pre- or post- implantation loss, intrauterine deaths or number of embryos per female (Table 2).

Among the F_1 generation males killed in post natal Week 10, total epididymal sperm counts were similar in all groups. The percentage abnormal sperm in the 1000 ng kg^{-1} - derived group was higher than in the other groups, and the average path and straight line velocity of sperm in this group were reduced by <10%, compared to control levels. The mean numbers of spermatids in the testes in the 1000 ng kg^{-1} - derived group were not significantly different from control values. In the males killed in post natal Week 17, seminology data showed a statistically significant increase in mean epididymal sperm count of ~30% in the two highest dose groups; both control and treated values were within the range of historical background data (SC, unpublished data). The mean abnormal sperm count was slightly higher than control in the low and medium groups, and this difference was statistically significant; the high dose group counts were close to control levels, and so this endpoint fails to show a dose-response.

There were no other remarkable findings in seminology parameters, or testicular spermatid counts (Table 3).

Body weight and pathology

Terminal body weight for the high dose group was significantly lower than control in the PND70 kill, but not at the PND120 kill. There was a significant decrease (~7%) in the absolute (but not relative) testis weight of the high dose group at PND70, and a decrease in testis weight by ANCOVA at PND120 (Table 4). Although the liver-to-body weight ratio of treated versus control animals was not signficantly affected at post-natal week 10, all three treated groups were significantly greater than control (~3.5%) at post-natal week 17. Kidneys, spleen, brain, thymus, prostate, epididymes and seminal vesicle weights showed no signicant, dose-dependent effects, with the exceptions that the brain weight in the high dose group was decreased by 2.2% at PND120, the absolute epididymis weight in the medium dose group was significantly elevated and there was a significant dose-response relationship in epididymis to body weight ratio (in the absence of any significant pairwise comparisons) compared to control at PND70. Histological examination of the epididymes showed that two out of fifty-eight control animals had inflammatory cell foci, while fourteen out of thirty-three high dose group animals had this lesion. This lesion is common in this strain of rat (SC, personal communication). There were no other significant microscopic findings in other organs examined.

DISCUSSION

Reports of developmental exposure to TCDD on adult epididymal sperm counts are relied upon as the most sensitive endpoint for TCDD toxicity for the purposes of acceptable intake level calculations (COT 2001; JECFA 2001; SCF 2001), but repeat studies have seen large differences in absolute and relative sperm counts (Faqi *et al.* 1998; Gray *et al.* 1997a; Ikeda *et al.* 2005; Mably *et al.* 1992a; Mably *et al.* 1992b; Mably *et al.* 1992c; Ohsako *et al.* 2001; Ohsako *et al.* 2002; Simanainen *et al.* 2004; Wilker *et al.* 1996; Yonemoto *et al.* 2005). Sperm counts are a highly variable endpoint (*e.g.* (Ashby *et al.* 2003)), yet numerous of these studies use small group sizes, and manual sperm counting in concert with a non-blinded analysis. This study was therefore implemented using GLP methodology and large group sizes to increase the statistical power and reliability of the analysis, with the explicit prior aim of measuring epididymal sperm levels. Analysis of tissue TCDD concentrations and mRNA levels confirm that TCDD was adequately dosed, and will be reported in detail elsewhere (Bell *et al.*, manuscript in preparation).

The administration of TCDD did not cause any consistent pattern of toxicity to the dams at the doses employed. However, TCDD was frankly toxic to the offspring at the top dose, causing an increase in total litter loss, and reducing the number of offspring by ~12% by PND21, compared to the control group. Thus TCDD is more toxic to the offspring, than to the adult dams. Similar effects have been seen before at equivalent doses of TCDD (Bjerke and Peterson 1994; Bjerke *et al.* 1994; Gray *et al.* 1995; Gray *et al.* 1997a; Mably *et al.* 1992c; Roman *et al.* 1995; Roman *et al.* 1998; Sommer *et al.* 1996), although these reports vary in whether the effect is seen pre- or post-parturition. This finding shows that the maximum tolerable acute dose of TCDD for the pups has been exceeded, and at this dose level, it is impossible to separate specific effects of TCDD from non-specific effects caused by the lethality of the compound to the offspring. Although there are many studies that expose rat dams to TCDD at 1 $\mu g\ kg^{-1}$ (and even higher doses), the lethality of TCDD to the offspring at this dose level must call into question whether any effects seen result directly from the TCDD, or indirectly from the lethality caused by the TCDD, and hence complicate the interpretation of such studies.

Maternal TCDD exposure caused no change in the F_1 sex ratio, but the animals from dams treated with high dose of TCDD were lighter than control throughout their life span (Fig. 1), and the medium dose group showed transient decreases in body weight, in agreement with previous studies (*e.g.* (Bjerke and Peterson 1994; Bjerke *et al.* 1994; Gray *et al.* 1995; Gray *et al.* 1997a; Ikeda *et al.* 2005; Korte *et al.* 1992; Mably *et al.* 1992c; Roman *et al.* 1995; Roman *et al.* 1998; Simanainen *et al.* 2004; Wilker *et al.* 1996; Yonemoto *et al.* 2005)). This reduction in body weight is accompanied by a delay in balano-preputial separation in the offspring from high dose dams, with 2.8 days average delay. This developmental delay is consistent among the previous studies that have measured this parameter (Bjerke and Peterson 1994; Bjerke *et al.* 1994; Faqi *et al.* 1998; Gray *et al.* 1997a; Roman *et al.* 1995; Roman *et al.* 1998; Sommer *et al.* 1996; Yonemoto *et al.* 2005). Depression in body weight is often a sensitive parameter for toxicity, and decreased body weight arising from feed restriction can itself cause a variety of

adverse endpoints (*e.g*. (Carney *et al.* 2004)). However, the statistical analysis showed that the decreased body weight in the high dose group at PND21 or 42 was not responsible for the delay in BPS.

Measurements of organ weights showed that there was a decrease in testis weight by ~7% in the high dose group at PND70, and at PND120, but no findings were visible on histopathological examination of the PND120 testes. Liver-to-body-weight ratios were elevated in all three dose groups at PND120, but not PND70, and brain weight was slightly decreased in the high dose group at PND120 (but not PND70). Our data show no significant decrease in organ weight for seminal vesicles or prostate; although we measured total prostate weight, rather than ventral prostate weight, we can calculate that our experiment has a >95% power of detecting a significant difference at $P<0.05$ in two groups differing in prostate weight by only 20%. Given that the ventral prostate is ~50% of the prostate mass, it would be likely that our experiment is adequately powered to detect the 40% decrease in ventral prostate weight described by (Mably *et al.* 1992c). It is of note that the published experiments with Sprague-Dawley and the related Holtzmann strain rats dosed at 800-1000 ng TCDD kg^{-1} show a significant decrease in ventral prostate weight at PND120 of ~16 to 40% (Ikeda *et al.* 2005; Mably *et al.* 1992c; Ohsako *et al.* 2001; Ohsako *et al.* 2002; Wilker *et al.* 1996), whereas experiment with Long-Evans or Wistar lines fail to show this reponse (Table 4, (Bell *et al.* 2007; Faqi *et al.* 1998; Yonemoto *et al.* 2005)); thus there is a possibility that this effect on prostate may reflect a strain difference between rats in response.

The analysis of seminology shows an increase in the proportion of abnormal sperm at PND70 for all treatment groups. Best practice recommendations for analysing sperm counts (Creasy 2003; Lanning *et al.* 2002) note the undesirability of analysing sperm counts in immature animals, specifically that peripubertal animals (for rats 8-10 weeks) will have a high incidence of abnormal sperm as a result of the normal process of starting spermatogenesis, and analyses at this time period are prone to artefactual variation. In view of the effects of TCDD on developmental delay and weight loss, this critique must be particularly relevant. While the % abnormal sperm at PND120 was slightly (statistically) increased in the low and medium dose groups, the high dose group was close to control, and these small variations are likely to be without biological significance. There was no decrease in epididymal sperm numbers, or sperm production, at PND70 (Table 3), but at PND120, there was a statistically significant increase (of ~30%) in epididymal sperm numbers in the high and medium dose group, in the absence of any change in sperm production. In view of the absence of effects on testicular spermatid production at PND120, and the absence of an effect at PND70, and the fact that these sperm counts are within the expected control range for this strain of rat in this laboratory (SC, unpublished data), we believe that the statistical significance for these samples arises from random variation, and that there is no biological significance to this result. Indeed, in a functional test of mating (Table 2), the offspring of TCDD-treated animals showed no differences from control animals. Although it has been reported that developmental exposure of male Long-Evans rats to 1 μg TCDD kg^{-1} reduces their fertility by ~50% , as measured by number of implants per mated female (Gray *et al.* 1995), this finding has not been repeated in other studies, *e.g.* (Faqi *et al.* 1998; Ikeda *et al.* 2005; Mably *et al.* 1992a) (Table 2).

Under any circumstances, our data show that there is no decrease in epididymal sperm numbers, in marked contrast to the data of (Faqi *et al.* 1998; Gray *et al.* 1997a; Mably *et al.* 1992a). Given the epididymal sperm counts and sample variation of the control animals (Table 3), this study has a power of ~95% for detecting a 30% difference in sample means with a probability of $P<0.05$. This would clearly be adequate to detect the 50% decrease in sperm levels at PND120 reported by Mably (Mably *et al.* 1992a), and there is still ~70% power for detecting the ~18% decrease in sperm number in 15 month old rat reported by Gray (Gray *et al.* 1997a), or the ~21% decrease in PND170 sperm numbers reported by Faqi (Faqi *et al.* 1998). This study therefore has the statistical power to detect the decrease in sperm number, and given the wide range of TCDD doses tested, this cannot be due to an insufficient dose of TCDD being administered; we conclude that we have been unable to replicate the findings of (Faqi *et al.* 1998; Gray *et al.* 1997a; Mably *et al.* 1992a). There are many possible biological explanations for our inability to detect a developmental effect of TCDD on adult epididymal sperm levels, and we have been unable to test the effects of potentially confounding variables, such as, rat strain drift, differences in diet or housing conditions, and age of the dam when exposed to TCDD.

To establish a context for examining the developmental reproductive effects of TCDD, published data of epididymal sperm levels after TCDD exposure are plotted as a percentage of concurrent control against acute TCDD dose for studies prior to (Fig. 3A) and post-2000 (Fig. 3B). (Faqi *et al.* 1998) used a sub-chronic dosing protocol, and this was related to the equivalent acute dose on the basis of the liver TCDD concentration on GD21 (DRB *et al.*, unpublished data). This comparison reveals that the results of Faqi and Gray show a flat dose-response curve; for example, at dosing regimes of 25/5 or 300/60 ng TCDD kg^{-1} $week^{-1}$ (*i.e.* a 12-fold increase in dose), the PND170 sperm levels are 83 and 79% of control values (Faqi *et al.* 1998). This flat dose response relationship is in contrast to the initial report (Mably *et al.* 1992a), and to the developmental toxicity of other agents that affect the male reproductive system, such as phthalates (*e.g.* (Mylchreest *et al.* 1998)). In addition to (Wilker *et al.* 1996), Fig. 3B adds data from five studies (Ikeda *et al.* 2005; Ohsako *et al.* 2001; Ohsako *et al.* 2002; Simanainen *et al.* 2004; Yonemoto *et al.* 2005) published since 2000, Table 3, and (Bell *et al.* 2007), none of which shows a response to TCDD on epididymal sperm levels at doses below 300 ng kg^{-1}. These differences are difficult to explain by appealing to strain differences, since, Holtzmann rats (Ikeda *et al.* 2005; Mably *et al.* 1992a; Ohsako *et al.* 2001), Long-Evans rats (Gray *et al.* 1997b; Yonemoto *et al.* 2005), and Wistar/ Wistar(Han) (this study, (Faqi *et al.* 1998)) have all been used with markedly divergent results. There has been a failure to reproduce the potent and dose-dependent pleiotropic effects of developmental exposure to TCDD on adult spermatogenesis (Mably *et al.* 1992a), and subsequent attempts to confirm these initial papers shows no significant, potent effect of TCDD on adult spermatogenesis. It is worth noting the statistical basis of empirical criticism for hypothesis-generating studies with small effect size and small sample size (Ioannidis 2005a, 2005b). In view of this failure to satisfy the basic requirement of replication in a number of laboratories, the use of this endpoint when conducting human risk assessment may be subject to criticism.

It has been shown that a small Han(Wistar) colony (H/W(Kuopio)) of rats are resistant as adults to the acute lethality of TCDD (Pohjanvirta *et al.* 1987), although this resistance does not extend to some other aspects of TCDD toxicity, such as induction of CYP1A1, thymotoxicity or fetotoxicity (Pohjanvirta and Tuomisto 1994). Moreover, the particular resistance of the H/W(Kuopio) strain to TCDD-induced acute lethality is distinct from other strains of Han(Wistar) rat (Pohjanvirta and Tuomisto 1990). Our data show that developmental exposure of the CRL:WI(Han) rat to 1 μg kg^{-1} of TCDD yields substantial perinatal lethality (Table 1; (Bell *et al.* 2007)), and this stands in stark contrast to the lack of perinatal lethality of AhR^{hw} rats under a comparable regime and dose of TCDD (Simanainen *et al.* 2004), or the resistance of H/W(Kuopio) rats to lethality at doses in excess of 10 mg kg^{-1} (Pohjanvirta *et al.* 1987). Thus there is little reason for believing that the CRL:WI(Han) strain of rats is insensitive to TCDD, or that it is substantively different from the Wistar rats used by (Faqi *et al.* 1998). We are currently undertaking molecular characterisation of the AhR in CRL:WI and WI(Han) rats, with a view to determining whether the AhR segregates with phenotype.

In summary, the data show that TCDD is a potent toxin in CRL:WI(Han) rat, causing a transient reduction in body weight gain in offspring after a single maternal dose of 200 ng kg^{-1} on GD15, and that a dose as low as 1μg TCDD kg^{-1} induces frank lethality and a delay in puberty. Our findings on TCDD-induced fetal loss, increased pup lethality, reduced weight gain and delay of puberty are consistent with other published studies, but our data fail to confirm reports that maternal exposure to TCDD can cause defects in spermatogenesis or associated sexual organs in the offspring of treated animals. Together with the accompanying paper, there are now eight studies which find no potent developmental effect of TCDD on spermatogenesis. In view of the failure to reproduce the potent and dose-dependent pleiotropic effects of developmental exposure to TCDD on adult spermatogenesis (Mably *et al.* 1992a; Mably *et al.* 1992b; Mably *et al.* 1992c) in highly-powered and robust studies that are performed to GLP, it is untenable to rely on these data as a basis for human risk assessment.

SUPPLEMENTARY DATA

The full study report with individual animal data is provided as an appendix.

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FIGURES

FIG. 1. Body weight of F_1 males. Pregnant dams were dosed on GD15 with 0 (black circle), 50 (white inverted triangle), 200 (black square) or 1000 (white diamond) ng TCDD kg^{-1} bodyweight, and allowed to litter. Offspring from ~25 animals per group were allowed to litter, and pups maintained as described in materials and methods. A. The body weight of male offspring is recorded, and shown as mean (symbol) ± Standard Error. Body weights prior to day 21 are not matched to animal, whereas individual animals are identified after PND21, and a break in the axis is included to show this. B. Geometric mean weights were calculated for each group, and the differences on the log scale of treated groups from the control group are plotted as a function of post-natal day. The error bars represent one standard error, based on a pooled variance estimate.

FIG. 2. Balano-preputial separation in F_1 offspring from dams treated with TCDD. Pregnant dams were dosed on GD15 with 0 (black circle), 50 (white inverted triangle), 200 (black square) or 1000 (white diamond) ng TCDD kg^{-1} bodyweight, and allowed to litter; BPS was determined in male offspring by daily inspection. Cumulative % of animals undergoing BPS is shown. Dose groups that are significantly different from control (proportional hazards model with adjustment for litter) at $P<0.05$ are indicated by an asterisk.

FIG. 3. Comparison of studies of maternal dosing of TCDD on epididymal sperm levels. Epididymal sperm levels were normalised to a concurrent control value of 100% for each experiment, and data are presented as ± Standard Error of the Mean; where the SEM is not visible, it is smaller than the symbol size. The maternal dose of TCDD is shown on the X-axis. Data from PND 62-70 are presented with dotted lines and white symbols, and data from adult rats (PND120+) are shown with solid lines and black symbols. Samples that are statistically significantly different from their concurrent control ($P<0.05$) are marked with an asterisk. A. Studies published pre-2000. (Faqi *et al.* 1998) square, (Gray *et al.* 1997a) triangle, (Mably *et al.* 1992a) circle, (Wilker *et al.* 1996) diamond. (Faqi *et al.* 1998) used a chronic dosing schedule, and this data set is plotted as the equivalent acute dose that would yield the concentration of hepatic TCDD found by (Faqi *et al.* 1998) (DRBell, unpublished calculation). Data above 1000 ng TCDD kg^{-1} are not shown. B. Studies published post-2000. (Ohsako *et al.* 2001) circle, (Ohsako *et al.* 2002) circle with dot, (Ikeda *et al.* 2005) triangle, (Yonemoto *et al.* 2005) inverted triangle, (Simanainen *et al.* 2004) hexagon (strain A), hexagon with dot (strain B), hexagon with cross (strain C), this study Table 3 square, the accompanying paper (Bell *et al.* 2007) diamond.

TABLES

Table 1:

Group Mean Litter Data

Dose of TCDD	0 ng kg^{-1}	50 ng kg^{-1}	200 ng kg^{-1}	1000 ng kg^{-1}
Number of females with live pups at Day 21 post-partum	21	20	21	15
Mean duration of gestation (days)	22.2±0.4	22.3±0.4	22±0.2	22.3±0.5
Mean number of implantation sites	10.6±1.5	10.9±1.4	11.1±1.1	10.6±2.7
Number of dams with total litter loss	1	0	0	4
Mean number of pups born	9.5±1.2	9.8±1.4	9.9±1.4	8.4±2.7
Mean number of pups alive Day 1	9.5±1.2	9.7±1.5	9.9±1.3	8.3±3*
% male pups Day 1	46.5	46.9	44.9	48.7
Mean number of pups alive Day 4 before culling	9.5±1.2	9.5±1.5	9.2±2.4	8.1±4.1
Mean number of pups culled Day 4	1.5±1.1	1.6±1.3	1.7±1.2	1±1.5
Mean number of pups alive Day 4 after culling	8±0.5	7.9±0.4	8±0.2	7.1±3.3*
Mean number of pups alive Day 7	7.8±0.9	7.9±0.5	8±0.2	7.1±3.3*
Mean number of pups alive Day 11	7.7±0.9	7.9±0.5	8±0.2	6.9±3.2*
Mean number of pups alive Day 14	7.6±2	7.9±0.5	7.9±0.5	6.7±3.2*
Mean number of pups alive Day 21	7.6±2	7.9±0.5	7.9±0.5	6.7±3.2*

Time-mated CRL:WI(Han) rats were dosed on GD15 with 0, 50, 200 or 1000 ng TCDD kg^{-1} bodyweight. Gestation and littering data were recorded (excluding those animals with total litter loss), and are presented above. Results are presented as mean ± Standard Deviation. *= different from the control group at $P<0.05$

TABLE 2

F_1 mating data

Dose of TCDD	0 ng kg^{-1}	50 ng kg^{-1}	200 ng kg^{-1}	1000 ng kg^{-1}
Number of paired males	20	20	20	20
Number of males inducing pregnancy	20	20	20	18
Mean number of corpora lutea per female	12.6±1.7	12.3±1.6	13.3±2.1	12.2±1.8
Mean number of implantations per female	12±1.7	11.9±1.7	12±2.7	11.1±2.5
Pre-implantation loss: mean %	5.1±6.5	3.3±5.8	9±17.2	8.6±15.5
Post-implantation loss: mean %	5±8.2	6.3±7.3	6.9±6.7	6.5±10.2
Mean number of embryos per female	11.4±1.8	11.1±1.5	11.2±2.7	10.4±2.5

Time-mated CRL:WI(Han) rats were dosed on GD15 with 0, 50, 200 or 1000 ng TCDD kg^{-1} bodyweight, and F1 males were allowed to survive until post-natal week 16, when 20 animals per group (each from a different litter) were mated with one untreated, virgin female rat (see Materials and Methods). Pregnant females were killed for necropsy on GD14. Results are presented as mean ± Standard Deviation. *= different from the control group at P<0.05

TABLE 3

Seminology Data

Post-Natal Day 70				
Dose of TCDD	0 ng kg^{-1}	50 ng kg^{-1}	200 ng kg^{-1}	1000 ng kg^{-1}
Number of males examined	25	25	25	25
Mean total epididymal sperm count (10^6/ mL)	3.8±3.2	4.5±2.9	5.7±3.6	4.1±2.6

Mean % motile	84±13	85±12	83±11	85±9
Mean average path velocity (µm/s)	182±19	181±25	172±22	166±22*
Mean curvilinear velocity (µm/s)	345±31	341±35	332±34	323±35
Mean straight line velocity (µm/s)	130±12	130±16	124±13	119±15*
Mean straightness (%)	71±2	71±3	72±3	71±3
Mean abnormal sperm (%)	3.2±2.5	5.8±3.4*	5.7±2.3*	10.1±4.3*
Mean total homogenisation resistant spermatid count (10^6/mL)	27±7.4	ND	ND	23.6±6.6
Post-Natal Day 120				
Number of males examined	58	61	60	33
Mean total epididymal sperm count (10^6/mL)	14.2±6	14.8±5.1	19.6±7.2*	18.6±7.7*
Mean % motile	73±10	71±10	73±12	75±11
Mean average path velocity (µm/s)	163±16	159±13	168±14	160±16
Mean curvilinear velocity (µm/s)	313±34	311±26	329±26*	307±27
Mean straight line velocity (µm/s)	120±13	118±9.4	123±9.9	118±11
Mean straightness (%)	73±3	74±3	73±2	74±2
Mean abnormal sperm (%)	1.8±2.6	3±2.8*	3±2.8*	1.8±2
Mean total homogenisation resistant spermatid count (10^6/mL)	24.8±8	ND	ND	24±6.3

The indicated number of animals were killed on PND70 or 120, and sperm taken from the cauda epididymis for analysis, or homogenisation resistant spermatids from the testis, as described in the Materials and Methods. Results are presented as mean ± Standard Deviation. Samples that are different from control at P<0.05 are indicated with a *. ND= not determined.

TABLE 4

Terminal F_1 Body and Organ Weights

	PND 70				PND120			
	TCDD (ng kg^{-1})				TCDD (ng kg^{-1})			
	0	50	200	1000	0	50	20	1000
Body weight (g)	306±16	302±22	309±16	278±27*	381±35	369±34	386±38	363±35
Kidneys (g)	1.92±0.18	1.98±0.18	1.94±0.14	1.73±0.21	2.14±0.18	2.15±0.23	2.15±0.23	2.02±0.23
ratio	0.63±0.06	0.65±0.04	0.63±0.04	0.62±0.04	0.56±0.04	0.58±0.04	0.56±0.04	0.56±0.04
Spleen (g)	0.7±0.09	0.65±0.07	0.67±0.1	0.63±0.1	0.75±0.13	0.72±0.12	0.76±0.12	0.68±0.09
ratio	0.23±0.02	0.21±0.02	0.22±0.03	0.23±0.03	0.2±0.03	0.2±0.02	0.2±0.02	0.19±0.03
Liver (g)	12±0.91	11.8±1.14	12.1±1.08	10.7±1.43	11.8±1.2	11.9±1.38	12.5±1.74*	11.7±1.38
ratio	3.93±0.23	3.9±0.24	3.9±-.23	3.85±0.25	3.11±0.17	3.22±0.2*	3.2±0.21*	3.22±0.17*
Brain (g)	1.85±0.06	1.87±0.08	1.85±0.07	1.8±0.08	1.96±0.08	1.97±0.09	1.96±0.08	1.91±0.09*
ratio	0.61±0.03	0.62±0.04	0.6±0.03	0.65±0.06	0.52±0.04	0.54±0.05	0.51±0.04	0.53±0.05
Thymus (g)	0.57±0.08	0.5±0.1	0.54±0.09	0.51±0.13	0.37±0.31	0.36±0.08	0.37±0.1	0.35±0.07
ratio	0.19±0.03	0.17±0.03	0.17±0.03	0.18±0.04	0.1±0.02	0.1±0.02	0.09±0.02	0.1±0.02
Testes (g)	3.3±0.26	3.33±0.35	3.4±0.18	3.1±0.18*	3.76±0.31	3.79±0.43	3.71±0.26	3.49±0.28*
ratio	1.09±0.11	1.1±0.12	1.1±0.07	1.12±0.09	0.99±0.11	1.03±0.12	0.97±0.09	0.96±0.07
Epididymes (g)	1.07±0.14	1.03±0.09	1.17±0.11*	1.05±0.11	1.78±0.23	1.81±0.23	1.76±0.21	1.66±0.2
ratio	0.35±0.05	0.34±0.03	0.38±0.03	0.38±0.04	0.47±0.07	0.49±0.06	0.46±0.05	0.46±0.05
Prostate (g)	0.45±0.11	0.49±0.09	0.49±0.11	0.42±0.08	0.9±0.18	0.83±0.16	0.9±0.14	0.82±0.16
ratio	0.15±0.03	0.16±0.03	0.16±0.04	0.15±0.02	0.24±0.05	0.22±0.04	0.23±0.03	0.23±0.05
Seminal vesicles (g)	0.59±0.16	0.68±0.15	0.67±0.16	0.56±0.12	1.1±0.32	1.08±0.3	1.09±0.26	1.01±0.25
ratio	0.19±0.05	0.22±0.05	0.22±0.05	0.2±0.04	0.29±0.08	0.3±0.08	0.28±0.07	0.28±0.07

Animals were killed at the indicated time; the number of animals is set out in Table 3. Animals were necropsied and body weight and organ weights analysed using ANCOVA, or one-way ANOVA on absolute organ weights and organ:necropsy body weight ratios (shown as "ratio"), as set out in the Materials and Methods. Results are presented as mean ± Standard Deviation. *= different from control, $P<0.05$.

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A

Body Weight (g)

400
300
200
100
0

Control
Low
Medium
High

0 20 40 60 80 100 120

Post-natal day

B

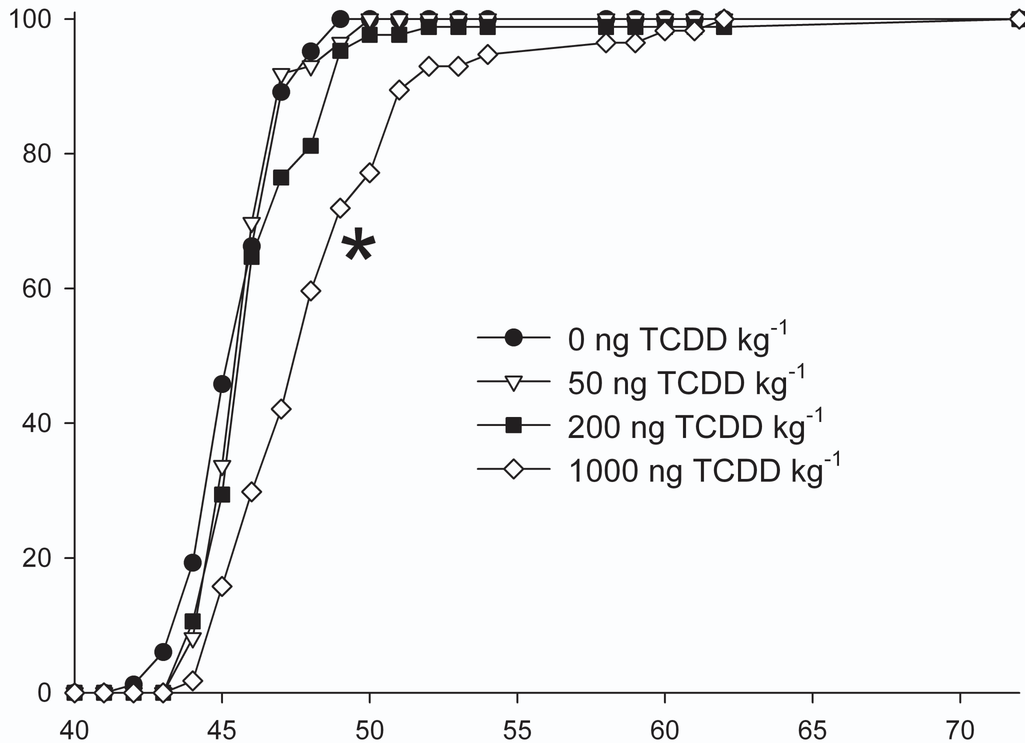
Difference from control body weight (log grammes)

0.10
0.05
0.00
-0.05
-0.10
-0.15
-0.20

Low
Medium
High

0 14 20 40 60 80 100 120

Post-natal day


Cumulative % of animals with BPS
100
80
60
40
20
0
40
45
50
55
60
65
70
Post-natal day
*
0 ng TCDD kg-1
50 ng TCDD kg-1
200 ng TCDD kg-1
1000 ng TCDD kg-1

A

Cauda epididymal sperm
% of control

- Mably et al PND120
- Mably et al PND 70
- Faqi et al PND170
- Faqi et al PND70
- Gray et al 15 months
- Gray et al PND63
- Wilker et al PND62

TCDD (ng/kg)

B

Cauda epididymal sperm
% of control

- Ohsako et al. 2001 PND120
- Ohsako et al. 2002 PND 70
- This paper PND120
- This paper PND70
- Ikeda et al PND120
- Yonemoto et al PND63
- Simanainen et al 2004 A PND70
- Simanainen et al 2004 B PND70
- Simanainen et al 2004 C PND70
- Bell et al PND70
- Bell et al PND120

TCDD (ng/kg)