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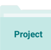
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Can resveratrol attenuate testicular damage in neonatal and adult rats exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin during gestation?

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Abstract. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is considered one of the most toxic dioxins. The effects of TCDD are exerted via binding to the aryl hydrocarbon receptor (AhR). The aim of the present study was to evaluate the possible protective effects of resveratrol, an AhR antagonist, against testicular damage caused by TCDD exposure during pregnancy. Pregnant female Sprague-Dawley rats were divided into four groups: a control group; a group treated with 1 $\mu g\,kg^{-1}$, p.o., TCDD on Gestational Day (GD) 15; a group treated with 20 $\mu g\,kg^{-1}$, p.o., resveratrol on GD10–21; and a group treated with both TCDD and resveratrol. Rats were weighed and killed, and neonatal testes were collected for histopathological analysis on Postnatal Day (PND) 1. At PND90, adult male rats were killed and the testes collected for histopathological analysis and determination of sperm count. Resveratrol had a protective effect against the effects of TCDD on Sertoli cell number in adult and neonate testes, as well as against the effects of TCDD on abnormal seminiferous tubules in adults. Combined administration of TCDD and resveratrol altered the kinetics of spermatogenesis and the proportion of neonatal testicular compartments compared with the control group In addition, combined TCDD and resveratrol treatment decreased seminiferous tubule diameter in adult male rats compared with the control group. In conclusion, resveratrol may protect against some TCDD-induced testicular damage, but, based on the parameters assessed, the administration of resveratrol and TCDD in combination may result in more severe toxicity than administration of either drug alone.

Additional keywords: postnatal developmental, Sertoli cell, testis.

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Introduction

The environment is believed to greatly affect male fertility. Several environmental pollutants responsible for the global decline in male reproductive health have been suggested (Chen *et al.* 2017; García *et al.* 2017). These pollutants can interfere with the production, metabolism or release of natural hormones responsible for the maintenance of homeostasis and regulation of developmental processes (Kavlock *et al.* 1996; Rhind 2009). Some pollutants can result from industrial effluents. Animals, including humans, may be exposed to these environmental pollutants via ingestion of contaminated food, inhalation of contaminated air or skin contact (Nash *et al.* 2004).

Gametogenesis is established during fetal development and any insult during this period may affect the quality of gametes produced (Campagna *et al.* 2001). The perturbation of organogenesis by endocrine disruptors may induce permanent changes leading to disruption of germ cell formation (Stouder and Paoloni-Giacobino 2010; Schug *et al.* 2011). Endocrine disruptors include the dioxin class of compound.

Dioxins are a subset of polyhalogenated aromatic hydrocarbons that act via the same mechanism of action. Dioxins induce toxicity by binding to the aryl hydrocarbon receptor (AhR). Dioxins are by-products of many industrial processes, primarily pulp and paper bleaching, as well as the incineration of medical

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waste and plastics (Anderson and Fisher 2002; Hewitt *et al.* 2006). Dioxins are resistant to biological and environmental degradation (Van den Berg *et al.* 1998, 2006). Thus, humans are primarily exposed to dioxins through contaminated drinking water, soil, air (Mandal 2005) and food, with high-fat animal food being the major source of exposure (van Leeuwen *et al.* 2000; Hoogenboom *et al.* 2007).

Among the dioxins, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is considered the most toxic (Van den Berg *et al.* 1998), causing adverse effects in humans (Schecter *et al.* 2006). TCDD has a broad spectrum of biochemical and toxicological effects, including teratogenesis (Peters *et al.* 1999), immunosuppression (Vos *et al.* 1997) and tumour promotion (Poland and Knutson 1982; Chen *et al.* 2014). Of the many body systems affected by TCDD, the male reproductive system is considered to be the most sensitive. Male reproductive toxicity results from an changes in androgenic status caused by TCDD exposure (Mably *et al.* 1992). Maternal exposure to TCDD may affect the sexual maturity of the offspring. A previous study showed that *in utero* exposure of rats to a single dose of TCDD (1 $\mu g\,kg^{-1}$ administered on Gestational Day (GD) 15) decreased daily sperm production (DSP) and plasma testosterone concentrations in male rats at 63 days of age (Bjerke and Peterson 1994). In addition, reduced ejaculated and epididymal sperm counts were observed in Wistar rats exposed to 0.5 $\mu g\,kg^{-1}$ TCDD from GD6 to 15, even though these offspring had a normal androgenic status (Gray *et al.* 1995).

TCDD produces its toxic effects via binding to the AhR (Fernandez-Salguero *et al.* 1996). This mechanism of action, although not completely understood, results in changes in key biochemical and cellular functions, such as increased transcription of many genes encoding drug-metabolising enzymes and activation of tyrosine kinases (Fujii-Kuriyama and Mimura 2005). Tyrosine kinase activation results in endocrine and paracrine disturbances, as well as changes in cell functions, such as cell growth (Yarden and Ullrich 1988) and differentiation (Hunter 1998). The AhR is present during fetal development in several organs, including the testis, suggesting that these organs may be more susceptible to TCDD (Jiang *et al.* 2010). The absence of an AhR during development of male mice led to changes in absolute and relative testicular weights determined at three time points (i.e. Postnatal Day (PND) 21, 35 and 90; Lin *et al.* 2001).

Resveratrol is the most active phytoalexin synthesised by plants (Langcake and Pryce 1976). Major sources of resveratrol include grapes (Langcake and Pryce 1976), wine, peanuts and soy (Cassidy *et al.* 2000). Resveratrol has been shown to have many beneficial effects on health, such as inhibition of low-density lipoprotein oxidation (Frankel *et al.* 1993). It also possesses antioxidant (Leonard *et al.* 2003), anticancer, chemopreventive and chemotherapeutic effects (Signorelli and Ghidoni 2005), as well as oestrogenic activity, which was demonstrated by its competition with oestradiol for binding to the oestrogen receptor (Gehm *et al.* 1997). Furthermore, Casper *et al.* (1999) suggested that resveratrol acts as a prophylactic agent against pathology induced by aryl hydrocarbon. Singh *et al.* (2011) showed that resveratrol (100 $mg\,kg^{-1}$ per day from GD1 until GD19) protects the pregnant mother and fetus against TCDD immunotoxicity (10 $\mu g\,kg^{-1}$, administered on GD14). Regarding the male reproductive system, Juan *et al.* (2005) showed an improvement in sperm production and an increase in serum testosterone concentrations in adult rats treated with resveratrol (20 $mg\,kg^{-1}$) for 90 days. Another study showed that resveratrol (1 and 10 $mg\,kg^{-1}$) administered to adult rats improved sperm motility and prevented lipid peroxidation (Ourique *et al.* 2013).

Therefore, based on the toxic effects of TCDD and the mode of action of resveratrol, the aim of the present study was to evaluate, in rats, the effects of resveratrol on damage to the male reproductive system induced by TCDD exposure during pregnancy.

Materials and methods

Animals

Adult female (60 days old) and male (90 days old) Sprague-Dawley rats were obtained from the Multidisciplinary Center for Biological Investigation in Laboratory Animals Science, State University of Campinas. Rats were housed in polypropylene cages (41 cm × 34 cm × 16 cm) with laboratory-grade pine shavings as bedding. Rats were maintained under controlled temperature (22 ± 2°C) and relative humidity (55 ± 10%), and a 12-h light–dark cycle. Rat chow and filtered tap water were provided *ad libitum*. The experimental procedures were performed in accordance with the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation. The study protocol was approved by the Biosciences Institute of São Paulo State University (UNESP) Ethics Committee for Animal Research (Protocol no.: 477-CEUA). Two non-gravid female rats were mated with a male rat per cage, and the day spermatozoa were detected in the vaginal smear was considered GD0. Pregnant rats were allocated into one of the four experimental groups: control, TCDD, resveratrol, and TCDD + resveratrol (T+R).

Experimental design and treatment

Pregnant rats in the TCDD and T+R groups were treated with 1 $\mu g\,kg^{-1}$ TCDD diluted in dimethylsulfoxide (DMSO) by gavage on GD15. Wilker *et al.* (1996) demonstrated that 1.0 $\mu g\,kg^{-1}$ TCDD is the lowest dose that effectively induces reproductive disorders. The oral LD_{50} for TCDD in male Sprague-Dawley rats is 43 $\mu g\,kg^{-1}$ (Agency for Toxic Substances and Disease Registry (ATSDR) 1998). Thus, the dose used in the present study is considered to be a low dose for rats, although it is sufficient to cause adverse effects. Most evidence regarding the effects of TCDD in humans is based on the Seveso accident (Bertazzi *et al.* 2001; Warner *et al.* 2013). Reports from the accident showed that for adults residing in one of the contaminated areas of Seveso, TCDD serum concentrations were approximately 100 $ng\,kg^{-1}$ (Needham *et al.* 1997).

Rats in the resveratrol and T+R groups were treated with 20 $\mu g\,kg^{-1}$ resveratrol diluted in carboxymethyl cellulose (CMC) by gavage (Juan *et al.* 2005) from GD10 to GD21. This period during which resveratrol was administered is considered a critical period for reproductive tract development (Wolf *et al.* 2000). Human consumption from commercial dietary supplements ranges between 50 and 2000 mg resveratrol (Williams *et al.* 2009). The dose of resveratrol chosen for the present study

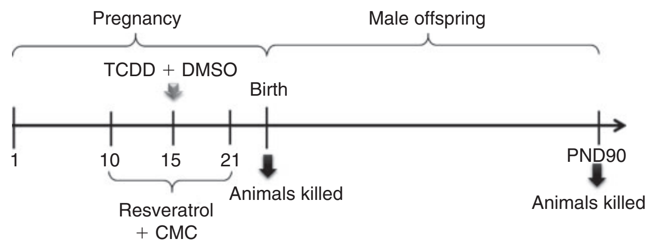

Fig. 1. Experimental design. CMC, carboxymethyl cellulose (vehicle); Resveratrol (20 μg kg^{-1}, p.o.); DMSO, dimethylsulfoxide (vehicle); TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (1 μg kg^{-1}, p.o.)..

was based on data from Juan *et al.* (2005), who showed that 20 mg resveratrol was enough to improve reproductive parameters in adult rats. Rats in the control group received equivalent volumes of DMSO (TCDD vehicle) and CMC (resveratrol vehicle) according to the same dosing schedule. Fig. 1 illustrates the study protocol. Both TCDD and resveratrol of high purity were purchased from Sigma-Aldrich.

After birth, on postnatal day (PND) 1, pups were weighted and the anogenital distance (AGD) was determined to differentiate males and females. The litter size was standardised to eight pups (the gender ratio was kept as close to 1 : 1 as possible). In litters >10 pups, extra pups were randomly removed. Litters with less than eight pups were not considered for the experiment. Five males per experimental group from different litters (to keep the litter as the unit of measure in the experimental groups) were evaluated at two different time points, PND1 and PND90, to evaluate early and late effects respectively. At both PND1 and PND90, male rats were weighed before they were killed.

Organ collection and determination of bodyweight and testis

On PND1, some rats were killed by decapitation, and neonatal testes were collected for histological evaluation, including stereology and determination of Sertoli cell numbers. On PND90, the remaining rats were anaesthetised using CO_2 and then decapitated. The testes were collected, trimmed free of fat and their weight (absolute weight and testis weight relative to bodyweight) were determined. The testes were fixed in Metacarn (60% methanol, 30% chloroform, 10% acetic acid) or frozen at −20°C until use for histological evaluation or determination of the sperm count respectively.

Hormone analysis

On PND90, blood was collected in plain Falcon tubes and used to determine serum testosterone levels. Serum was obtained by centrifugation (3000*g*, 15 min, 4°C) of blood samples in a cooling centrifuge and frozen at −20°C until use for hormone analysis. Testosterone concentrations were determined by double-antibody radioimmunoassay, using specific kits provided by MP Biomedicals at the Laboratory of Neuroendocrinology of Reproduction, School of Dentistry of Ribeirao Preto, University of Sao Paulo. All samples were processed in the same assay, to avoid interassay errors. The lowest limit of detection of the assay was 0.064 ng mL^{-1} testosterone, with a 4% intraassay CV.

Morphometric and histopathological analysis

The left testes were removed and fixed in methacarn solution (10% acetic acid, 60% methanol and 30% chloroform) for 3 h at 30°C. The testes were then embedded in Paraplast wax and cut into 5-μm sections. The testis sections were stained with haematoxylin and eosin (H&E) and examined by light microscopy for general histopathological and morphometric analyses.

Histological evaluation of neonatal male offspring testis

Number of Sertoli cells

The number of Sertoli cell nuclei was determined in 20 cross-sections of seminiferous cords per testis in each rat under a light microscope at ×400 magnification.

Stereology

To determine the proportion of the seminiferous cord and interstitial compartment in each testis, 10 photomicrographs were captured per animal using a light microscope (Leica; ×400 magnification) equipped with a digital camera. According to the method described by Favareto *et al.* (2011), a graticule containing 168 points was superimposed on the images. By counting the overlapping dots in the seminiferous cord or interstitial compartment, it was possible to establish the respective proportions of each of these components in the testis for each experimental group.

Histological evaluation of adult male offspring testis

Number of Sertoli cells

The number of Sertoli cell nuclei was determined in 20 cross-sections of the seminiferous tubules per testis in each rat, under a light microscope at ×400 magnification (Nassr *et al.* 2010).

Seminiferous tubule diameter and seminiferous epithelium height

Seminiferous tubule diameter and epithelium height were measured using BELView software version 6.2.3.0 (BEL Engineering) for Windows. To this end, 10 random testicular cross-sections (Stage IX of the seminiferous epithelial cycle) per animal were selected at random and examined under a photomicroscope at ×400 magnification. For each seminiferous tubule, the mean of four values was calculated and used in statistical analyses (Siervo *et al.* 2015).

Kinetics of spermatogenesis

Random tubular sections ($n = 100$ per animal) in three non-consecutive testis cross-sections were classified into four categories, namely Stages I–VI, VII–VIII, IX–XIII and XIV of the seminiferous epithelial cycle, according to Leblond and Clermont (1952) using a photomicroscope at ×400 magnification (Fernandes *et al.* 2011).

Histopathology

Random tubular sections ($n = 100$ per animal) in three non-consecutive testis cross-sections were analysed to detect the presence of abnormalities in the tubules, such as immature germ cells in the lumen, acidophil cells, vacuoles and tubular degeneration.

Daily sperm production

To evaluate daily sperm production (DSP), the right testes were decapsulated, weighed and homogenised, as described previously (Robb *et al.* 1978), with some modifications as suggested by Fernandes *et al.* (2007). After dilution of the homogenate, a small sample volume was transferred to a Neubauer chamber (four fields per animal) for counting of homogenisation-resistant spermatids (Stage 19 of spermatogenesis). To calculate DSP, the concentration of spermatids per testis was divided by 6.1, which refers to the number of days for which the mature spermatids are present in the seminiferous epithelium.

Statistical analysis

Data were compared using analysis of variance (ANOVA) followed by Tukey's post hoc test, or by the non-parametric Kruskal–Wallis test followed by Dunn's post hoc test, depending on data distribution. Differences were considered significant for two-tailed $P < 0.05$. Statistical analyses were performed using GraphPad InStat (version 5.0).

Results

Bodyweight and testis weights

Bodyweight and testis weight are given in Table 1. At PND1, exposure to resveratrol significantly increased bodyweight compared with the control group. However, at PND90, both the TCDD and T+R groups had increased bodyweight compared with the control group ($P < 0.01$ and $P < 0.05$ respectively). At PND90, there were no significant differences in absolute testis weight between the experimental groups.

Neonatal testis assay

Treatment with TCDD or resveratrol during gestation did not result in significant changes in the proportion of the seminiferous cord and interstitial tissue compartments in the neonatal testis ($P > 0.05$; Table 2). However, in the T+R group, there was an increase in the proportion of the seminiferous cord compartment and a decrease in the interstitial tissue compartment in neonatal testes.

No abnormal cells were observed in the seminiferous cord or interstitial tissue of neonatal rat testes in any of the experimental groups.

Sertoli cell count

Neonatal and adult testes from the TCDD group exhibited a reduced Sertoli cell count. Moreover, the number of Sertoli cells was higher in adult male offspring in the resveratrol group compared with the control group. However, there was no significant difference in the number of Sertoli cells between the T+R and control groups (Fig. 2).

Reproductive parameters in adult male offspring

Sperm count parameters and plasma testosterone concentrations did not differ among the groups (Table 3). The diameter of the seminiferous tubule was significantly smaller in the T+R

Table 1. Body and testis weight in neonate and adults rats

Data are the mean ± s.e.m. ($n = 5$ per group). Within rows, different superscript letters indicate significant differences ($P < 0.05$, ANOVA with post hoc Tukey's test). Rats were from dams in the control group, a group treated with 1 μg kg^{-1}, p.o., 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on Gestational Day (GD) 15, a group treated with 20 μg kg^{-1}, p.o., resveratrol on GD10–21 or a group treated with both TCDD and resveratrol (T+R). PND, postnatal day

	Control	TCDD	Resveratrol	T+R
Bodyweight (g)				
PND1	6.66 ± 0.14^{a}	6.68 ± 0.12^{a}	7.36 ± 0.13^{b}	6.92 ± 0.07^{a}
PND90	361.48 ± 6.33^{a}	406.08 ± 6.97^{b}	386.91 ± 7.01^{ab}	391.73 ± 6.99^{b}
Testis weight on PND90 (g)	1.60 ± 0.03	1.63 ± 0.02	1.62 ± 0.03	1.67 ± 0.03

Table 2. Morphometric parameters and neonate gonad stereology

Data are the mean ± s.e.m. ($n = 5$ per group). Within rows, different superscript letters indicate significant differences ($P < 0.05$). For comparisons of the proportion of the seminiferous cord and interstitial tissue compartments within neonatal testes, ANOVA with Tukey's post hoc test was used; for comparisons of parameters of adult testes, the Kruskal–Wallis test with Dunn's post hoc test was used. Rats were from dams in the control group, a group treated with 1 μg kg^{-1}, p.o., 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on Gestational Day (GD) 15, a group treated with 20 μg kg^{-1}, p.o., resveratrol on GD10–21 or a group treated with both TCDD and resveratrol (T+R)

	Control	TCDD	Resveratrol	T+R
Neonates				
Seminiferous cord (%)	67.64 ± 0.92^{ab}	66.27 ± 0.76^{a}	70.10 ± 0.64^{bc}	71.80 ± 0.75^{c}
Interstitial tissue (%)	32.36 ± 0.92^{ab}	33.73 ± 0.76^{a}	29.89 ± 0.64^{bc}	28.20 ± 0.75^{c}
Adults				
Seminiferous epithelium height (μm)	80.81 ± 1.80^{ab}	81.61 ± 1.52^{ab}	86.13 ± 1.54^{a}	78.41 ± 1.71^{b}
Seminiferous tubule diameter (μm)	287.38 ± 4.67^{a}	278.41 ± 4.05^{ab}	281.47 ± 4.40^{a}	262.40 ± 5.37^{b}

compared with control group. However, the height of the seminiferous epithelium in all the treated groups did not differ significantly from that in the control group (Table 2).

Histopathological analysis revealed that there was a significantly increased number of abnormal seminiferous tubules in the TCDD compared with control group. However, there were no significant differences among the other groups (Fig. 3).

Results regarding the kinetics of spermatogenesis are given in Table 4. Rats in the T+R group had the highest number of seminiferous tubules at Stages VII–VIII and XIV, whereas the number of seminiferous tubules at Stages VIX–XIII was decreased compared with the control group.

Discussion

Changes in the programming of normal reproductive development may result in deregulation of reproductive function and reduced fertility in adulthood (Bellingham *et al.* 2012). Humans are exposed to dioxins on a daily basis, and those living in more industrialised countries have an increased exposure (Schecter *et al.* 2003). Exposure to dioxins, especially during pregnancy, had adverse effects in rats, as indicated by both behavioural (Takeda *et al.* 2014) and reproductive parameters (Bjerke and Peterson 1994).

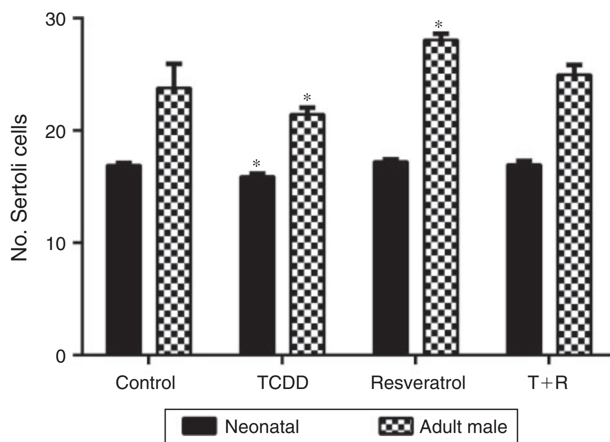

Fig. 2. Sertoli cell count in testes from neonatal and adult rat testes. Rats were from dams in the control group, a group treated with 1 μg kg^{-1}, p.o., 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on Gestational Day (GD) 15, a group treated with 20 μg kg^{-1}, p.o., resveratrol on GD10–21 or a group treated with both TCDD and resveratrol (T+R). Data are the mean ± s.e.m. ($n = 5$). *$P < 0.05$ compared with control (Kruskal–Wallis test with post hoc Dunn's test).

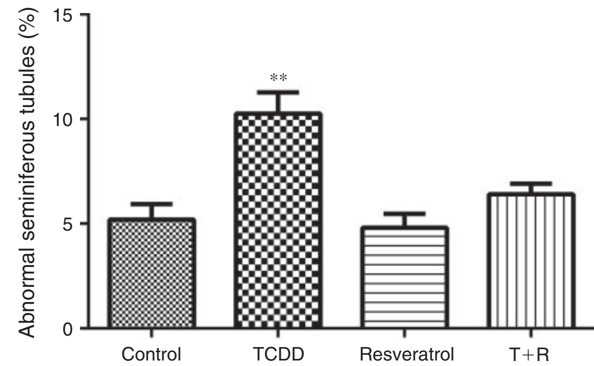

Fig. 3. Histopathological analysis of adult testis. Rats were from dams in the control group, a group treated with 1 μg kg^{-1}, p.o., 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on Gestational Day (GD) 15, a group treated with 20 μg kg^{-1}, p.o., resveratrol on GD10–21 or a group treated with both TCDD and resveratrol (T+R). Data are the mean ± s.e.m. ($n = 5$). **$P < 0.01$ compared with control (ANOVA with post hoc Tukey's test).

Table 4. Kinetics of spermatogenesis

Data are the mean ± s.e.m. ($n = 5$ per group). Within rows, different superscript letters indicate significant differences ($P < 0.05$, ANOVA, with Tukey's post hoc test). Rats were from dams in the control group, a group treated with 1 μg kg^{-1}, p.o., 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on Gestational Day (GD) 15, a group treated with 20 μg kg^{-1}, p.o., resveratrol on GD10–21 or a group treated with both TCDD and resveratrol (T+R)

	Control	TCDD	Resveratrol	T+R
Stages I–VI (%)	31.0 ± 0.7	33.0 ± 1.9	31.0 ± 1.9	28.0 ± 2.4
Stages VII–VIII (%)	37.4 ± 1.8^{a}	33.7 ± 0.7^{a}	36.0 ± 1.1^{a}	43.2 ± 1.6^{b}
Stages IX–XIII (%)	29.2 ± 1.0^{a}	29.5 ± 1.4^{a}	32.0 ± 1.5^{a}	24.0 ± 1.1^{b}
Stage XIV (%)	2.4 ± 0.7^{ab}	3.7 ± 0.7^{bc}	1.0 ± 0.3^{a}	4.8 ± 0.4^{c}

Table 3. Sperm count in the testis and testosterone concentrations

Data are the mean ± s.e.m. ($n = 5$ per group). There were no significant differences among groups ($P > 0.05$, ANOVA). Rats were from dams in the control group, a group treated with 1 μg kg^{-1}, p.o., 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on Gestational Day (GD) 15, a group treated with 20 μg kg^{-1}, p.o., resveratrol on GD10–21 or a group treated with both TCDD and resveratrol (T+R). DSP, daily sperm production; PND, postnatal day

	Control	TCDD	Resveratrol	T+R
Sperm count				
DSP (×10^{6} testis^{-1} day^{-1})	18.70 ± 2.53	17.08 ± 1.29	19.98 ± 1.50	18.27 ± 1.62
No. mature spermatids (×10^{6} testis^{-1})	114.09 ± 15.41	104.21 ± 7.91	121.86 ± 9.14	111.44 ± 9.90
No. mature spermatids (×10^{6} g^{-1} testis)	102.99 ± 16.68	85.24 ± 6.46	93.24 ± 6.92	85.52 ± 6.71
Plasma testosterone (ng/mL)				
Neonates (PND1)	0.58 ± 0.11	0.41 ± 0.14	0.56 ± 0.35	0.47 ± 0.24
Adults (PND90)	1.92 ± 0.37	2.09 ± 0.72	2.58 ± 0.75	1.60 ± 0.60

In the present study, the increased bodyweight at birth in the resveratrol-treated group represents its early systemic effect, which does not appear to be harmful because bodyweight gain was not observed in adulthood. However, increased adult bodyweight was observed after exposure to TCDD alone or in combination with resveratrol, demonstrating a late effect of TCDD. In accordance with the present study, Simanainen *et al.* (2004) reported that there was no significant change in bodyweight at PND1; however, there was a significant increase at PND70 in two rat lineages exposed to a single dose of TCDD ($1\,\mu g\,kg^{-1}$ on GD15). Moreover, Bjerke and Peterson (1994) reported decreased bodyweight on PND1 for rats treated with TCDD ($1\,\mu g\,kg^{-1}$ on GD15), whereas no weight loss was observed at PND133. Wilker *et al.* (1996) showed that male rat offspring exposed to TCDD ($1\,\mu g\,kg^{-1}$ on GD15) had decreased bodyweight on PND1 compared with the control group. Therefore, we can suggest that TCDD affects fetal programming of adult life, and its effect may be persistent.

In the present study, testicular weight was maintained during adulthood, which corroborates the results of a previous study using the same dose of TCDD and duration of exposure (Wilker *et al.* 1996). The results regarding testicular weight can be correlated with those regarding testosterone concentrations, which were similar among the experimental groups. In previous studies, TCDD (lower doses at GD15) did not have a significant effect on serum testosterone concentrations in male Sprague-Dawley rats (Wilker *et al.* 1995; Rebourcet *et al.* 2010). However, Juan *et al.* (2005) reported that testosterone was significantly higher in male rats treated with resveratrol for 90 days. Under the current experimental conditions, the combination of TCDD and resveratrol did not have any effect on testicular development.

In the present study, the results of sperm count analyses were associated with testosterone concentrations. A previous study reported a significant decline in sperm count in Long-Evans hooded rats following exposure to TCDD ($1\,\mu g\,kg^{-1}$ on GD15; Gray *et al.* 1997). In contrast with these results, in the present study there were no changes in DSP and number of mature spermatids in male offspring Sprague-Dawley rats exposed to either TCDD or resveratrol. Wilker *et al.* (1996) reported similar results using 0.5, 1.0 and $2.0\,\mu g\,kg^{-1}$ TCDD at GD15. This can be explained by the findings of Simanainen *et al.* (2004), who reported that sperm parameters differed based on resistance to AhR alleles, which varies in different rat strains. Therefore, the strain used in the present study (Sprague-Dawley) appears to be more resistant to the effects of TCDD on testicular sperm count.

TCDD acts by binding to the AhR in the cytosol. After TCDD binding, AhR translocates to the nucleus, where it triggers a cell signalling pathway that results in protein synthesis, consequently leading to biochemical changes (Mandal 2005). Cytochrome P450 (CYP) 1A1, one of the xenobiotic metabolising enzymes, is potentially induced by TCDD (Mimura and Fujii-Kuriyama 2003). Induction of CYP 1A1 in the placenta during pregnancy due to AhR ligands has been reported previously (Stejskalova and Pavek 2011). Moreover, TCDD has been shown to form a stable complex with α-fetoprotein, which, in turn, may favour the transport of TCDD across the placenta, consequently contributing to its teratogenic effects (Sotnichenko *et al.* 1999).

In the present study, the reduction in the number of Sertoli cells in the TCDD group in both neonatal and adult testes demonstrates the early and persistent effects of TCDD. However, when TCDD was administered in combination with resveratrol, the number of Sertoli cells was similar to that in the control group. These results confirm that resveratrol has a protective role against the adverse effects of TCDD on Sertoli cell proliferation because it completely prevented the impairment of cell proliferation. However, resveratrol alone resulted in an increase in the number of Sertoli cells without changing other reproductive parameters assessed in the present study. Wilker *et al.* (1995) reported a 13% reduction in the number of Sertoli cells in rats exposed to TCDD ($0.40\,\mu g\,kg^{-1}$ on GD15). Further, an *in vitro* study (Aly and Khafagy 2011) suggested that TCDD may reduce the expression of the Müllerian-inhibiting substance (*MIS*) gene (now also called anti-Müllerian hormone (*AMH*)) and induce the expression of aromatase and 17β-oestradiol (E2). Both MIS and E2 are responsible for the regulation of certain male reproductive functions, including Sertoli cell function (Aly and Khafagy 2011). Thus, *in vitro* exposure to TCDD interferes with the development of the male reproductive system (Lai *et al.* 2005). Aly and Khafagy (2011) reported an increase in oxidative stress due to TCDD exposure, which can explain the altered function of Sertoli cells. Although oxidative stress was not analysed in the present study, the antioxidant capability of resveratrol could explain its protective effect against TCDD. However, this antioxidant property of resveratrol could reduce levels of reactive oxygen species to below the minimum amount essential for maintaining homeostasis.

The decrease in the number of Sertoli cells could disrupt the seminiferous epithelium of the testis (Sharpe *et al.* 2003), as evidenced by the increase in abnormal seminiferous tubules in the TCDD group at adulthood. Notably, resveratrol completely prevented this effect when administered in combination with TCDD. According to Kim *et al.* (1999), the testes of guinea-pigs (4–5 weeks old) exposed to TCDD ($1\,\mu g\,kg^{-1}$) exhibited smaller tubules, maturation arrest at various stages and disruption of the seminiferous epithelium. Although different histopathological parameters were analysed in that study, both the present study and that of Kim *et al.* (1999) identified abnormalities in seminiferous tubules following TCDD exposure. In contrast, no histopathological evidence for the degeneration of seminiferous tubules in Long-Evans hooded rats was observed at lower doses of TCDD (0.05, 0.20, and $0.80\,\mu g\,kg^{-1}$ TCDD on GD15; Gray *et al.* 1997). Rebourcet *et al.* (2010) did not report any abnormalities in the seminiferous tubules of Sprague-Dawley rats treated with lower doses of TCDD (10, 100 or $200\,ng\,kg^{-1}$ on GD15). Thus, it seems reasonable to believe that doses $<1\,\mu g\,kg^{-1}$ do not impair testicular tissue. Intraperitoneal administration of resveratrol (2, 8 and $20\,mg\,kg^{-1}$) to adult mice for 2 weeks resulted in changes in testicular histology, probably because of its pro-oxidant effect on the testis (Ranawat *et al.* 2014). According to Ishida *et al.* (2009), different routes of administration could explain the apparent discrepancies in results among studies. Ishida *et al.* (2009) also showed that resveratrol is capable of combating TCDD toxicity.

Stereological analysis of neonatal rats in the present study showed that the combination of TCDD and resveratrol affected

the proportion of different testicular compartments. These results suggest that the reduced seminiferous tubule diameter in adulthood was due to the abnormal development of seminiferous tubules, which may be similar to the atrophy process during the growth and differentiation of tubules. Therefore, the combination of these compounds was found to be harmful, whereas the administration of resveratrol on its own is not associated with any toxic effects. Haavisto *et al.* (2006) evaluated infant rats (PND14) exposed to TCDD ($0.04\,\mu g\,kg^{-1}$ on GD13) and observed an increase in the diameter of seminiferous cords. In contrast with the results of the present study, Kim *et al.* (1999) showed that seminiferous tubules from guinea-pigs exposed to a single dose of TCDD ($1\,\mu g\,kg^{-1}$) during adulthood were smaller than those of the control group.

The kinetics of spermatogenesis were altered after exposure to the combination of TCDD and resveratrol. However, a similar change was not observed after exposure to TCDD alone. Similarly, there is no evidence that TCDD may adversely affect spermatogenesis when administered alone in the literature (Foster *et al.* 2010). Although resveratrol was previously shown to protect or re-establish the spermatogenesis process in other experimental models (Jiang *et al.* 2008; Bitgul *et al.* 2013), it was not able to exert this protective effect when used in combination with TCDD in the present study. However, changes in the kinetics of spermatogenesis did not impair DSP, and it is important to note that resveratrol alone did not alter the stages of spermatogenesis.

Conclusion

In conclusion, the present study shows the protective effect of resveratrol against TCDD on reproductive parameters, such as Sertoli cell numbers and the number of abnormal seminiferous tubules in both neonate and adult Sprague-Dawley rats. However, the combination of resveratrol and TCDD does not appear to be the best option because it may impair some testicular structural parameters, which was evidenced in the present study by disruption of normal testicular development.

Conflicts of interest

The authors declare no conflicts of interest.

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