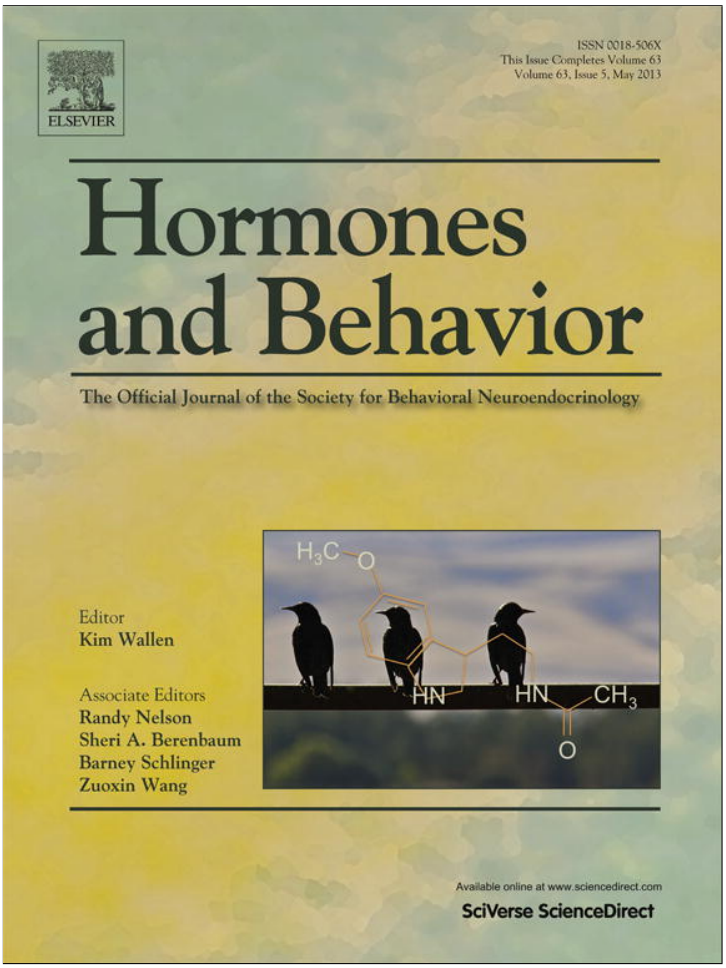

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Antiandrogenic effect of perinatal exposure to the endocrine disruptor di-(2-ethylhexyl) phthalate increases anxiety-like behavior in male rats during sexual maturation

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ABSTRACT

Di-2-ethylhexyl phthalate (DEHP) is the most widely used phthalate to convey flexibility and transparency to plastic products made of polyvinyl chloride. It has been recognized as endocrine disruptor and associated with reproductive toxic effects. We examined the effects of perinatal exposure to DEHP on anxiety-like behavior, using the Elevated Plus Maze (EPM) test, in male and female rats at different stages of sexual development. Anxiety-like behavior was expressed as a) frequency of open arm entries over the total arm entries (% FEO); b) time spent in them compared with total time the animal stayed in the EPM (% TSO) and c) time spent in closed arms (TSC). Because DEHP has anti-androgenic action we also tested control and exposed immature male rats pretreated with testosterone. We found sex differences in behavior induced by DEHP; while male rats of 45 and 60 days of age showed a significant decrease in FEO and TSO percentages, as well as an increase in TSC, no changes were observed in anxiety-like behavior in perinatal DEHP exposed females at these ages of sexual maturation. In 60-day-old male rats, DEHP exposure produced a significant decrease in serum testosterone levels. Testosterone replacement was able to antagonize the adverse effects of DEHP exposure on LH, activating the negative feed-back mechanism of this steroid on reproductive axis, as well as increasing FEO and TSO percentages to similar values observed in the control group. These findings suggest that the anti-androgenic action of this chemical could be one possible mechanism underlie anxiogenic-like behavior produced by perinatal DEHP exposure in 60-day-old male rats.

Introduction

The plasticizer di-(2-ethylhexyl) phthalate (DEHP) has been identified as endocrine disruptor (EDC) and associated with developmental and reproductive toxic effects (NTP-CERHR, 2006) in laboratory animals (Akingbemi et al., 2001, 2004; Borch et al., 2006; Foster et al., 2001; Gray et al., 2000) and in humans (Latini et al., 2003; Skakkebaek et al., 2001; Swan, 2008; Swan et al., 2005). DEHP is widely used in every day consumer products and medical devices; however the potential risk of the exposure to this substance is high because it can leach from plastic products readily into foods, beverages or directly into body fluids (Gartner et al., 2009; Latini, 2000; Lovekamp-Swan and Davis, 2003).

DEHP induces anti-androgenic action by an androgen receptor-independent mechanism (Akingbemi et al., 2004; Howdeshell et al., 2008; Moore et al., 2001; Parks et al., 2000), as well as reduction in the expression of steroidogenesis related-factors and interaction with peroxisome proliferator-activated receptors (Borch et al., 2006; Latini et al., 2006; Kambia et al., 2008). The toxicity of DEHP can begin during gestation and lactation periods and it is attributable to the action of its primary metabolite, mono-(2-ethylhexyl) phthalate (MEHP), which crosses the placental barrier and passes into breast milk (Latini et al., 2003; Main et al., 2006; Stroheker et al., 2005). Pre and perinatal exposure to DEHP produces reproductive abnormalities in androgen-dependent processes (Akingbemi et al., 2001; Albro, 1987; Borch et al., 2005; Gray et al., 2000; Parks et al., 2000), alters sexual differentiation (Andrade et al., 2006), suppresses testosterone production (Culty et al., 2008) and affects sexual behavior (Dalsenter et al., 2006) in male rats offspring. In this critical stage of development, DEHP exposure induces an increase in the number of ovarian atretic tertiary follicles, (Grande et al., 2007) hypoestrogenic anovulatory cycles and polycystic ovaries (Davis et al., 1994; Lovekamp-Swan and Davis, 2003) in adult female offspring rats. Recently, we have demonstrated that gestational and postnatal DEHP exposure modify serum gonadotropin levels and

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the hypothalamic content of amino acid neurotransmitters in male and female rats during sexual maturation (Carbone et al., 2010, 2012).

There is growing concern that, endocrine-active compounds may disrupt hormone-dependent events during nervous system development, affecting a variety of sexually dimorphic behaviors (Patisaul and Polston, 2008). However, experimental studies of DEHP effects on behavior are limited, they have shown significant associations between prenatal exposure to phthalates and adverse effects on pup learning, memory, and behavior in rodents (Arcadi et al., 1998; Li et al., 2009; Tanaka, 2002, 2005). Epidemiological studies have associated the concentrations of phthalates in maternal urine during pregnancy with adverse child cognitive, motor and behavioral development (Engel et al., 2009, 2010; Miodovnik et al., 2011; Swan et al., 2010; Whyatt et al., 2012). Detrimental effect on behavior and possible sex differences in the sensitivity of the infants to prenatal phthalates has been suggested (Kim et al., 2011). Also, clinical symptoms of attention deficit hyperactivity disorder (ADHD) have been identified in school-aged children exposed to environmental phthalates (Kim et al., 2009) and a significant association between this behavioral disorder and the severity of anxiety symptoms in children, and adolescents has been reported (Liu et al., in press). However, little information concerning the effects of perinatal exposure to DEHP in anxiety-like behavior is presently available.

On the other hand, a reduction of testosterone (T) production by phthalates during development has been hypothesized as a probable mechanism to explain the changes in play behavior observed in boys (Swan et al., 2010). This androgenic steroid is well known for its function in reproduction, sexual differentiation and sexual behavior as well as for its modulating effect on anxiety. Animal studies have demonstrated that testosterone's mediation of anxiety-like behavior and cognitive processes may be through the actions of its metabolites. It has been reported that T and its 5α reduced metabolite, dihydrotestosterone, as well as the systemic administration of anabolic steroids reduce anxiety-like behavior in rodents (Bing et al., 1998; Bitran et al., 1993; Edinger and Frye, 2005; Frye and Edinger, 2004; Frye and Seliga, 2001; Osborne et al., 2009; Rojas-Ortiz et al., 2006). While removal of testes – the primary source of endogenous androgens – through gonadectomy results in increased anxiety-like behavior (Bitran et al., 1993; Frye and Seliga, 2001), T replacement is able to abolish this effect (Edinger and Frye, 2004, 2005; Frye and Seliga, 2001).

The purpose of the present work was to study the effect of perinatal exposure to DEHP on the anxiety-like behavior, in male and female rats at different stages of sexual maturation. In order to ascertain if the antiandrogenic action of DEHP could be involved in probable changes in the behavior induced by this chemical, we also examined the effect of the pretreatment with T in DEHP-exposed immature male rats. An oral route in DEHP administration was chosen for this study to mimic the most likely route.

Materials and methods

Animals and drug

Wistar rats used for this work were provided by the Department of Physiology, School of Medicine, Universidad de Buenos Aires, Argentina. Animals were maintained under a controlled environment (temperature 22°–24°; lights on from 7.00 am to 7.00 pm) and had free access to food and filtered water, until time of sacrifice. All animals were fed with balanced food for laboratory rodents (Cooperation, ACA-16014007, Argentine Cooperative Association, Animal Nutrition Division, Argentina Industry). The diet contains 15% of soy, but as the food used and the quantity of food intake by control and DEHP treated groups were similar, we assumed that all animals were exposed to equivalent levels of food-borne phytoestrogens. Moreover, the same lots of diet were provided to animals from all groups at the same time during the course of the study to control across groups for possible variation in the content of the diet. We used ultrapure filtered water (obtained from EDS-Pack, Millipore Merck, installed in the Milli-Q water system) that was presumed to be free of phthalates and other EDC. To minimize additional exposures to endocrine-disrupting chemicals, rats were housed in stainless steel cages with wood bedding and water was supplied in glass bottles. All animal procedures were performed in accordance with protocols of the National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals. Approval to conduct the study was granted by the Animal Care and Ethics Committee of the School of Medicine-UBA (CICUAL).

DEHP (99% pure, Cat D 20,115-4, Aldrich Chemical Company, Inc. Milwaukee, Wisconsin, USA) was administered in drinking water. The estimated average dose of exposure was 30 mg/kg/bw/day of DEHP. The amount of DEHP added to each glass bottle was adjusted daily to reach this dose, according to the body weight and the volume of liquid consumed by the corresponding animal. Surrogate dams and pups receiving DEHP drank the same amount of liquid as those which did not receive this chemical. It was assumed that all DEHP solution missing in the bottle had been consumed by the animals. Assessments do not account for possible leakage or evaporation of the solution or for potential loss of DEHP activity during the 24-hour period. DEHP solution was made up fresh daily by sonicating for 30 min, which ensures a permanent and homogenized solution. Similarly to what was observed by other authors (Vandenberg et al., 2012), we found no reference in DEHP dose administered to animals, which may cause similar blood levels in humans. For this reason, we administered a dose of DEHP previously tested that is capable of producing neuroendocrine and reproductive effects in rat (Carbone et al., 2010, 2012; Tanaka, 2002, 2005). This dose is in the range of the no-observable-adverse-effect level (NOAEL 28.9–36.1 mg/kg/day) (David et al., 2000).

Testosterone propionate (Sigma Chemical Co., Saint Louis Mo.) diluted in sesame oil (vehicle) was injected subcutaneously (1 mg/0.1 ml). This is the minimal dose which is capable to return gonadotropin levels to normal levels in antiandrogenized male rats (Justo et al., 1989).

Experimental design

After acclimatization for 1 week, female (250–300 g) and male (300–350 g) were co-housed (1:1). The animals were examined daily for copulatory plug. Mating was confirmed by the presence of a copulatory plug, and this day was recorded as first day of gestation (GD1). Pregnant dams (n = 10) were each placed in an individual metallic cage. Upon delivery, pups were sexed according to anogenital distance and were cross fostered, distributing four males and four females pups per cage with one surrogate dam. These actions allowed us to minimize the use of siblings to avoid potential litter effects. Surrogate dams (n = 10) with their pups were randomly assigned into Control (C) and DEHP exposure groups (n = 5 dams per group), receiving water DEHP free or water containing DEHP (30 mg/kg/bw/day) from postnatal day (PND) 1 to weaning, respectively. Pups from control and DEHP groups were weaned at 21 days of age and housed by sex (n = 8 male or female per cage) according to the treatment they had received during breastfeeding. Pups of DEHP groups continued to receive the same dose from PND 21 to the day of sacrifice. The exposure to endocrine disruptors (e.g. bisphenol-A) during fetal and perinatal periods is an important determining factor of their effects on behavior (Xu et al., 2012). In the present study, we chose the postnatal exposure from birth to pubertal development to test the hypothesis chronic exposure to DEHP during this period could cause behavioral changes in different stages of sexual maturation in which there are also important modifications in the levels of gonadal steroids and gonadotropins (Dohler and Wuttke, 1974), as well as in brain concentration of inhibitory and excitatory neurotransmitters and its receptors (Moguilevsky et al., 1995).

The pups were randomly divided for two experiments. In the first experiment, we studied the anxiety-like behavior in C and DEHP male and female pups of 30, 45 and 60 days of age (n = 8 males or females for C and DEHP groups of each age). In the second one, we evaluated anxiety-like behavior in C and DEHP males which were injected

subcutaneously with 0.1 ml of vehicle (V) or testosterone (T) consecutively every 48 h from PND 45 to PND 60. These last groups of male rats were named: 1) C+V (n=8): controls that were given water and injected with vehicle; 2) DEHP+V (n=8): animals that were given DEHP and injected with vehicle; 3) C+T (n=8): pups that were given water and injected with this hormone; 4) DEHP+T (n=8): animals that were given DEHP and treated with testosterone (see Fig. 1).

Elevated Plus Maze (EPM) test

Before sacrifice, all groups of animals (n=8 males or females for control and DEHP groups of 30, 45 and 60 days old) were submitted to EPM to study the anxiety-like behavior. This test is a widely used behavioral assay for rodents and it has been validated to assess the anti-anxiety effects of pharmacological agents and steroid hormones, and to define brain regions and mechanisms underlying anxiety-related behavior (Walfi and Frye, 2007). The EPM apparatus consists of two open arms (30×7 cm), alternating in right angles with two closed arms (30×7×30 cm). The surface of the central area delimited by the four arms was 49 cm^2. The whole maze was elevated 60 cm above the floor. Before the start of the test, animals were individually placed in a rectangular plastiglass arena (40×40 cm) for 5 min in order to habituate them to the test environment. After that, the rats were placed in the central area of the maze, facing one of the closed arms, and were allowed to explore it for 5 min as described previously (Czerniczyniec et al., 2011; Taylor et al., 2009). Behavioral tests were performed from 12:00 to 14:00 h. Based on Czerniczyniec et al. (2011), we used 10% ethanol to clean each arm of the maze and to remove olfactory cues every time between trials. Each rat was tested only once. The animal's behavior was videotaped and the number of entries and the time spent in both open and closed arms were measured. These parameters were calculated following a four-paw criterion; entry into the arm of the EPM was defined as the animal placing all four paws in that particular part of the maze. Based on these measures, the percentages of entries in the open arms (% FEO) and the time spent in the open arms (% TSO) were calculated as entries or time in the open arms over the total entries or time, respectively, as described previously (Debatin and Barbosa, 2006). The amount of time spent in closed arms of the maze was expressed in seconds. It is well known that rodents show natural aversion to open surfaces (Czerniczyniec et al., 2011; Martínez et al., 2002; Pallarés et al., 2007). Based on this fact, the EPM test estimates the anxiety-like behavior of the animal by the reduction in % FEO and % TSO, as well as by increase in TSC (Czerniczyniec et al., 2011; Debatin and Barbosa, 2006; Jones and Watson, 2012).

Serum hormone assays

Immediately after the EPM test, animals (n=8 males or females for C and DEHP groups of each age) were sacrificed in another room by decapitation. Trunk blood was collected and the samples centrifuged for 10 min at 2500 rpm, the serum was separated and stored at −70 °C until hormone determination.

Total serum T concentrations were measured by a competitive immunoassay provided by VITROS, (Immunodiagnostic Products Testosterone Reagent Pack, Ortho Clinical Diagnostics by Johnson & Johnson Company). Duplicate volumes of 50 μl serum were used for each sample. Intra and inter assay coefficients of variation were 3.1% and 7.0% respectively. Values were expressed as nmol/ml serum.

LH was determined in duplicate by using the double antibody radio immunoassay technique (Niswender et al., 1968). The material for this assay was provided by the National Hormone and Peptide Program of the National Institutes of Diabetes and Digestive and Kidney Diseases (NIDDK) (Harbor-UCLA Medical Center, Torrance, California, USA). Results were expressed in serum ng/ml, in accordance with the referenced preparation (rat LH RP-1). All samples were analyzed in the same assay and intra-assay coefficient of variation was 6%.

Statistical analysis

The data were expressed as the mean±S.E.M. (n=8 pups per group). Because we did not keep track of the surrogate dam for each of the pups, the surrogate dam was not used as the unit. Therefore, we did not consider the potential effects of the surrogate dam in the statistical analysis, as the pups were exposed to this chemical through her milk. The behavioral data were analyzed by univariate 3-way Analysis of Variance (ANOVA) to asses interactions such as

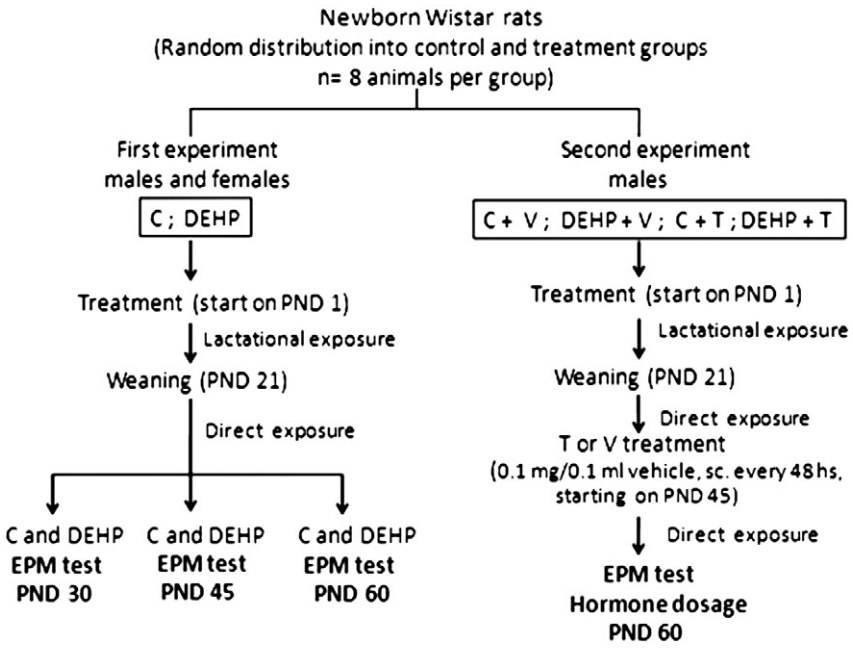

Fig. 1. Schematic representation of experimental design. C: control; DEHP: di-(2-ethylhexyl) phthalate; V: vehicle (oil); T: testosterone; PND: postnatal day.

sex (male or female) × treatment (control or DEHP) × age (30, 45 or 60 days), treatment × age; treatment × sex; age × sex; and sex, treatment and age. Statistical analysis with post-hoc Student's *t*-test was used to compare treatment within each sex and age. The effects of T replacement were analyzed by one-way ANOVA with post-hoc Tukey–Kramer Multiple Comparisons Test. Hormonal data were performed nonparametrically with Kruskal–Wallis Test followed by Dunn's Multiple Comparisons Test. SPSS (version 13.0) statistical software was used to realize the statistical analysis. Differences were considered to be statistically significant at a probability level of 5% ($p<0.05$).

Results

Effects of postnatal exposure to DEHP on anxiety behavior

The results are expressed as % TSO and % FEO (calculated as it was indicated in Materials and methods section). No significant interaction of sex × treatment × age was found in % TSO ($F(2,69)=0.523$, $p=0.595$), % FEO ($F(2,69)=0.405$, $p=0.668$) and TSC ($F(2,69)=1.911$, $p=0.158$); sex × treatment in % TSO ($F(1,69)=1.148$, $p=0.288$), % FEO ($F(1,69)=1.102$, $p=0.298$) and TSC ($F(1,69)=1.325$, $p=0.255$); sex × age in % TSO ($F(2,69)=1.483$, $p=0.234$), % FEO ($F(2,69)=2.965$, $p=0.058$) and TSC ($F(2,69=3.115$, $p=0.052$); treatment × age in % TSO ($F(2,69)=1.900$, $p=0.157$), % FEO ($F(2,69)=0.405$, $p=0.668$) and TSC ($F(2,69)=1.374$, $p=0.262$). In addition, there were no direct sex differences in % TSO ($F(1,69)=1.301$, $p=0.329$), % FEO ($F(1,69)=1.102$, $p=0.298$) and TSC ($F(1,69)=0.506$, $p=0.480$). However, age differences between groups were observed in % FEO ($F(2,69)=3.485$, $p=0.036$) and TSC ($2,69)=4.610$, $p=0.014$). Treatment significantly affected % TSO ($F(1,69)=10.085$, $p=0.020$); % FEO ($F(1,69)=6.914$, $p=0.011$) and TSC ($F(1,69)=9.662$, $p=0.003$). Compared with their respective same-sex control group, exposure to DEHP significantly reduced % TSO (DEHP vs. C: 1.6 ± 0.58 vs. 7.85 ± 1, $p<0.01$) and % FEO (DEHP vs. C: 29.52 ± 2.68 vs. 14.74 ± 2.73, $p<0.05$) and also increased TSC (DEHP vs. C: 271.2 ± 9.71 vs. 198.1 ± 15.3, $p<0.05$) in 45-day-old male rats. DEHP produced a significant decrease in % TSO in 60-day-old male rats (DEHP vs. C: 1.90 ± 0.67 vs. 10.28 ± 2.20, $p<0.01$) and % FEO (9.96 ± 3.38 vs. 30.77 ± 5.38, $p<0.05$), while increased TSC (279.75 ± 5.81 vs. 224.36 ± 11.54, $p<0.05$) (Fig. 2). However no significant changes ($p>0.05$) in % TSO (DEHP vs. C: 10.82 ± 1.32 vs. 10.98 ± 2.25), % FEO (DEPH vs. C: 42.32 ± 5.29 vs. 32.12 ± 4.7) and TSC (DEHP vs. C: 202.17 ± 14.46 vs. 196.75 ± 22.40) were detected in 30-day-old male rats. In females at 30, 45 and 60 days of age, no significant differences ($p>0.05$) were observed in % TSO (30 days DEHP vs. C: 5.56 ± 1.94 vs. 6.16 ± 2.24; 45 days DEHP vs. C: 7.47 ± 2.85 vs. 7.41 ± 4.2; 60 days DEHP vs. C: 4.93 ± 1.58 vs. 7.17 ± 3.38), as well as in % FEO (30 days DEHP vs. C: 25.64 ± 6.3 vs. 37.07 ± 5.28; 45 days DEHP vs. C: 33.6 ± 4.35 vs. 36.7 ± 3.81; 60 days DEHP vs. C: 22.74 ± 7.09 vs. 27.18 ± 5.88) and TSC of these animals (30 days DEHP vs. C: 217.15 ± 17.72 vs. 204.45 ± 11.80; 45 days DEHP vs. C: 237.95 ± 10.49 vs. 225.21 ± 7.98; 60 days: 228.33 ± 12.37 vs. 213.87 ± 16.59) (Fig. 2).

The interactions of sex × treatment × age ($F(2,69)=4.764$, $p=0.012$), sex × age ($F(2,69)=3.491$, $p=0.036$), treatment × age ($F(2,69)=9.076$, $p=0.001$) influenced the number of total entries in the open and closed arms. DEHP treatment markedly reduced the number of total entries in 45-day-old male rats when it was compared with the controls of the same sex (DEHP vs. C: 3.66 ± 0.47 vs. 9.03 ± 1.07, $p<0.01$); but no significant changes were observed in males of 30 days (DEHP vs. C: 8.4 ± 1.46 vs. 6.4 ± 2.3, $p>0.05$) and in males of 60 days of age (DEHP vs. C: 4.4 ± 0.6 vs. 5.42 ± 0.75, $p>0.05$) (Fig. 2).

Effects of T replacement on anxiety behavior

The results of the second experiment are shown in Fig. 3. Similarly to those observed in the previous assay, in male rats, postnatal exposure to DEHP produced a significant decrease in % TSO (DEHP + V vs. C + V: 2.00 ± 0.68 vs. 9.84 ± 2.73, $p<0.01$) and % FEO (DEHP + V vs. C + V: 16.37 ± 5.97 vs. 40.08 ± 5.09, $p<0.01$); also an increase in TSC

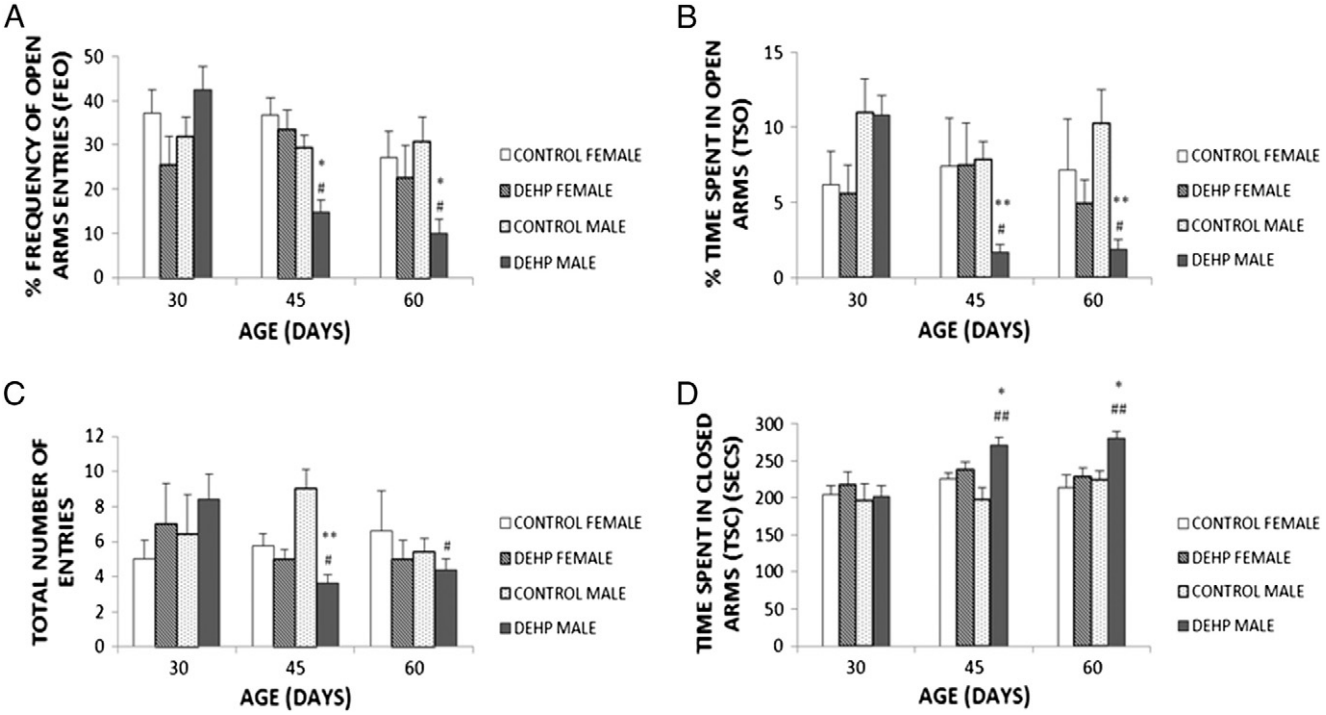

Fig. 2. Effects of postnatal DEHP (30 mg/bw/day) on the anxiety behavior in male and female rat offspring at different stages of sexual maturation. Values are percentage of frequency of open arms entries (A) percentage of time spent in the open arms (B) total number of entries into arms (C) time spent in the closed arms (D). Means ± SEM. *$p<0.05$ (DEHP vs. control); **$p<0.01$ (DEHP vs. control); #$p<0.05$ (DEHP 45 and DEHP 60 vs. DEHP 30); ##$p<0.01$ (DEHP 45 and DEHP 60 vs. DEHP 30) ($n=8$ male rats per group).

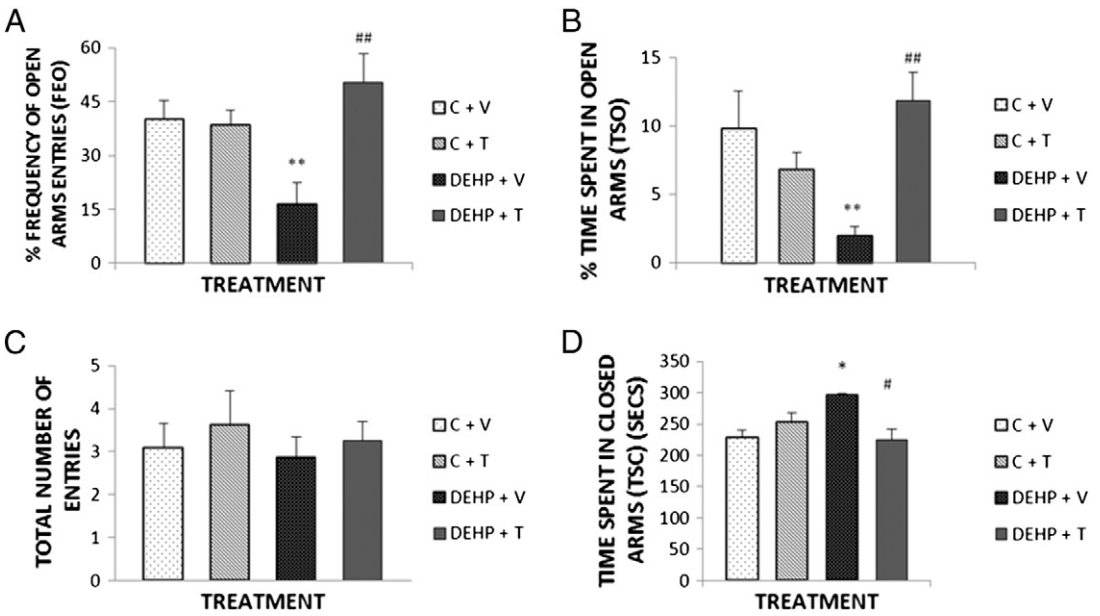

Fig. 3. Effects of testosterone supplementation (1 mg/0.1 ml) in 60 day old male rats exposed to DEHP (30 mg/bw/day). Values are percentage of frequency of open arms entries (A) percentage of time spent in the open arms (B)(C) total number of entries into arms (C) time spent in the closed arms (D). Means ± SEM. C: control; DHEP: di-(2-ethylhexyl) phthalate; V: vehicle; T: testosterone; **p<0.01 (DEHP + V vs. C + V); #p<0.01 (DEHP + T vs. DEHP + V) (N = 8 animals per group).

was observed (DEHP + V vs. C + V: 275.46 ± 3.57 vs. 227,534.36 ± 12.12, p<0.05). Testosterone injected to males from PND 45 to PND 60 was able to abolish the effect provoked by perinatal DEHP exposure in 60-day-old male rats, increasing % TSO (DEHP + T vs. DEHP + V: 11.84 ± 2.10 vs. 2.00 ± 0.68, p<0.01) and % FEO (DEHP + T vs. DEHP + V: 50.33 ± 8.00 vs. 16.37 ± 5.97, p<0.01) and lead these parameters to be equivalent to values observed in the control group injected with T (% TSO DEHP + T vs. C + T: 11.84 ± 2.10 vs. 6.89 ± 1.21, p > 0.05; % FEO DEHP + T vs. C + T: 50.33 ± 8.00 vs. 38.59 ± 3.94, p > 0.05). T replacement also reduced TSC in 60-day-old male rats exposed to DEHP (DEHP + T vs. DEHP + V: 224.04 ± 17.59 vs. 275.46 ± 3.57).

Effects of postnatal exposure to DEHP and T replacement on serum LH and serum T levels

As can be seen in Table 1, comparing with the respective controls we found a significant decrease in serum testosterone in 60 day old male rats subjected to perinatal DEHP (DEHP + V vs. C + V: 0.77 ± 0.08 vs. 1.60 ± 0.28, p<0.001). When we compared the difference between animals exposed to DEHP receiving vehicle or T, we observed that T treatment was able to increase the endogenous level of this hormone (DEHP + T vs. DEHP + V: 2.10 ± 0.22 vs. 0.77 ± 0.08, p<0.001). As we expected serum T levels in control rats receiving T treatment for a period of 15 days were above control animals without T treatment (C + T vs. C + V: 2.68 ± 0.24 vs.1.60 ± 0.28, p<0.01). No significant differences in serum T were detected between control and DEHP groups treated with T (DEHP + T vs. C + T: 2.10 ± 0.22 vs. 2.68 ± 0.24, p > 0.05). On the other hand, DEHP exposure significantly increased serum LH level in 60-day-old male rats with respect to unexposed animals of the same age (DEHP + V vs. C + V: 171.7 ± 20.8 vs. 17.9 ± 6.1, p<0.001). Treatment with exogenous T was able to reverse this effect, significantly reducing serum LH (DEHP + T vs. DEHP + V: 3.4 ± 0.4 vs. 171.7 ± 20.8, p<0.001) and lead hormone levels to be similar to values detected in unexposed males injected with T (DEHP + T vs. C + T: 3.4 ± 0.4 vs. 4.4 ± 0.7, p > 0.05). In control male rats of 60 days of age T treatment produced the characteristic negative feed-back on LH secretion (C + T vs. C + V: 4.4 ± 0.7 vs.17.9 ± 6.1, p<0.01).

Table 1
Effect of T replacement (1 mg/0.1 ml, every 48 h from 45 to 60 days) on serum LH and T levels of 60 day old male rats subjected to postnatal DEHP exposure.

Treatment	LH (ng/ml)	T (nmol/ml)
C + V	17.9 ± 6.1	1.60 ± 0.28
C + T	4.4 ± 0.7[a,b]	2.68 ± 0.24[a,b]
DEHP + V	171.7 ± 20.8[c]	0.77 ± 0.08[c]
DEHP + T	3.4 ± 0.4[d]	2.10 ± 0.22[d]

C: control; V: vehicle; DEHP: di-(2-ethylhexyl) phthalate (30 mg/bw/day); T: testosterone. N = 8 male rats per group.
[a] LH: p < 0.01 C + T vs. C + V.
[b] LH: not significant between DEHP + T and C + T.
[c] LH: p < 0.001 DEHP + V vs. C + V.
[d] LH: p < 0.001 DEHP + T vs. DEHP + V.

Discussion

In order to investigate if early exposure to endocrine disruptor DEHP can induce changes in the anxiety-like behavior, we compared the performance in the EPM of male and female rats exposed or not to DEHP perinatally, at different stages of sexual maturation: early (PND 30), middle (PND 45) and late adolescence (PND 60), based on Tirelli et al. (2003).

Parameters recorded were the time spent in the open and closed arms, the number of entries into open and closed arms and total arm entries. The EPM measures both anxiety-like and exploratory behavior based on the amount of time the animal spends in the open and closed arms of the maze. The amount of time spent in the open arms is an index of the animal's motivation to investigate their surroundings. The amount of time spent in the closed arms correlates with anxiety levels of the animal, as those that are anxious seek the security of the closed arms. Total arm entries made in the EPM are indicative of locomotion activity.

In the present work, we have not found age and sex-related differences in the total number of entries into arms and in the percentages of FEO and TSO, as well as in TSC in control rats of 30, 45 and 60 days of age. Therefore, no differences in anxiety-like behavior were found when we compared between controls of the same sex at different ages, as well as between male and female controls of the same age.

These findings are in agreement with previous papers which have shown similar anxiety behavior in male and female rats across adolescence (Estanislau and Morato, 2006; Imhof et al., 1993).

On the other hand, our results showed that postnatal DEHP exposure was unable to induce anxiogenic effect yet in 30, 45 and 60 day old female rats. Nevertheless, in 45 and 60 day old male rats DEHP produced a significant decrease in both percentages of FEO and TSO, as well as an increase in TSC, indicating an anxiogenic-like effect in these animals. A significant decrease in the total number of entries into arms was observed in the 45-day-old male rats but not in female rats or males of other ages exposed to DEHP. These results suggest that postnatal exposure to DEHP may influence on the locomotor activity of male rats of 45 days of age. It is generally agreed that the locomotor activity is related to the anxiety behavior in animals, and lower locomotor activity is associated with a greater anxiety-like state (Johnston and File, 1991). On this basis, our findings suggest that DEHP may affect the anxiety-like behavior by changing the locomotor activity in 45-day-old male rats. Because DEHP did not modify locomotor activity, in male rats of 30 and 60 days of age, it is difficult to reconcile these results. We hypothesize that this sporadic effect may be related to an age dependent interaction among DEHP, sexual hormones and the excitatory neurotransmitters involved in anxiety behavior (Barkus et al., 2010; Pickard et al., 2000; Xiang et al., 2011), as well as in the neuroendocrine control of the reproductive axis during sexual maturation (Carbone et al., 2010, 2012). However, this short lived effect could be self-correcting across development and therefore not be an important risk factor for disease or the overall health of the animal. Taking into account this possibility, the results of DEHP effect on locomotor activity in 45-day-old male rats need to be interpreted with caution.

A significant decrease in serum T in 60-day-old male rats submitted to postnatal DEHP was detected. On this basis, we hypothesize that the recognized antiandrogenic action of DEHP could be involved in the anxiogenic-like effect induced by this chemical in 60-day-old male rats. With the aim to test our hypothesis, we studied the effect of T injected from PND 45 to PND 60 in controls and DEHP exposed male rats. T replacement was able to increase the endogenous level of this hormone in both control and DEHP exposed animals leading to equivalent values. As we expected, T levels in serum of control rats receiving T treatment for a period of 15 days were higher than in control animals not receiving T treatment. The dose of T used in our experiments (Justo et al., 1989), was able to induce negative feed-back mechanism of this hormone on LH secretion by the pituitary, producing a significant decrease in plasmatic LH level in control male rats at PND 60. These results indicate that the dose of T employed physiologically suppressed LH secretion. T treatment also antagonized the stimulatory effect of DEHP exposure on LH, leading the hormone levels to be similar to values detected in unexposed male rat injected with T. In line with previous findings (Akingbemi et al., 2001), we also detected an increase in LH level in 60-day-old male rats exposed to DEHP, which was linked to lower endogenous level of T, indicating an alteration in the normal function of the pituitary–gonadal axis. Moreover, our results showed that testosterone treatment (at dose of 1 mg/kg s.c. for 2-week period) reversed the effect of perinatal DEHP exposure, leading plasmatic LH to similar level to unexposed male rats treated with testosterone.

On the other hand, in 60-day-old control male rats treated with T, we did not find changes in the total number of entries into arms, as well as in FEO, TSO and TSC with respect to the control. It may suggest a similar anxiety-like behavior in control and treated animals. In contrast, other authors (Bitran et al., 1993; Frye and Seliga, 2001) though, have reported that in adult male rats one week of testosterone propionate exposure (3.5–5.0 mg/kg per day, via subcutaneously implanted capsules) increase blood testosterone at supraphysiological levels producing an anxiolytic behavior. The anxiolytic-like behavior in adult male rats produced by T may be dose dependent (Roohbakhsh et al., 2011). In spite of this contradictory result, we observed that DEHP exposed animals and treated with testosterone exhibited a significant increase in both the frequency of entries and in the time spent in the open arms, that may suggest a decrease in anxiety-like behavior. In spite of these contradictory results, we observed that T treatment was capable of antagonizing the effects of DEHP on FEO, TSO and TSC in 60-day-old male rats by increasing FEO and TSO, and decreasing TSC. Moreover, no differences in the total number of entries into arms were found between groups. Therefore, DEHP should not affect locomotor activity in 60 day old male rats.

Our results related to the anxiety-like behavior in control animals are in agreement with previous paper in which ontogenetic differences in the performance of rats in the EPM were not detected before 60 days of age (Imhof et al., 1993). In the same line, Estanislau and Morato (2006) have suggested that sex difference does no emerge until early adulthood in the EPM. In contrast to these studies, other authors have reported differences by sex in the EPM performance during sexual development. Elliott et al. (2004) have found that mid-adolescent female rats spend more time in the open arms and show greater proportion of open arm entries, than males of the same age. Lynn and Brown (2009) have found that late-adolescent female rats spent more time in open arms of the EPM than males of the same age, without sex differences in early and mid periods of adolescence. Moreover, adult female rats exhibit lower level of anxiety than adult male rats (Johnston and File, 1991), and also show behavioral differences in anxiety across the ovarian cycle, with lower anxiety levels during proestrus than during diestrus (Marcondes et al., 2001). Nevertheless, in the present work, estrous cycle phases in females of 60 days old were not determined to avoid the possible stressful effect provoked by vaginal lavage, the typical method employed to realize the vaginal cytology. Different behavior responses could also be consequence of animals' age, environmental and genetic factors that could be associated with anxious phenotypes (Clément et al., 2002).

It is well known that the brain is very sensitive to pre and perinatal exposure to different factors such as sexual hormones, stressful events and environment contaminants, which at critical stages of development can alter neuroendocrine functions and also the morphology of several brain structures related with behavior and cognitive process (Pallarés et al., 2007; Patisaul and Polston, 2008; Weinstock, 2001). There is growing concern that developmental exposure to naturally occurring and chemically manufactured endocrine compounds, such as genistein, polychlorinated biphenyls and bisphenol-A, can alter brain sexual differentiation affecting the cognitive function and the behavior in rodents (Dickerson et al., 2011; Patisaul et al., 2006; Tian et al., 2010; Xu et al., 2012).

Therefore, our results provide information of the ontogeny of anxiety-like behavior in control rats, as well as of the effect of DEHP exposure in different stages of sexual maturation designed as early, middle and late adolescence. Sex differences in the effect of DEHP could relate to different levels of sexual steroid hormones during sexual maturation. It is well known that female rodent brain develops in the relative absence of T, but the brain of the neonatal male is exposed to higher levels of T, together with the product of its aromatization, estradiol (Mc Carthy, 2008). These sex differences in the synthesis and exposure to T result in the development of distinct male and female neuroanatomical circuits, neuroendocrine functions and behavior. Indeed, it has been reported that T has anti-anxiety effects in rodents and testosterone's mediation of anxiety-like processes may be done through the actions of its metabolites (Edinger and Frye, 2005; Osborne et al., 2009). T is metabolized by 5α-reductase to dihydrotestosterone, which is then converted to 5α-androstane-3α, 17β-diol (3α-diol) and 5α-androstane-3β17β-diol (3β-diol). Thus, androgens can mediate anxiety-like processes of male rats. On the other hand, the age differences in the effect of DEHP on the anxiety behavior of male rats could be due to changes in serum T levels. It is well demonstrated that there is a significant increase in

male T levels during sexual maturation, being higher in 45 and 60 day old male rats than in males of 30 days of age. (Dohler and Wuttke, 1974; Moguilevsky et al., 1995).

Conclusions

The present findings provide new evidence indicating that postnatal exposure to DEHP can lead to neurobehavioral disorders, and also suggest a differential effect of this antiandrogenic chemical in males and female rats, showing a relationship between increase in anxiety-like behavior and testosterone decrease, in 60-day-old male rats. Taking into account that T has an important role in the development of neuronal circuits that regulate behavior, we postulate that development changes in neural function induced by the antiandrogenic action of DEHP, could be other possible mechanisms that underlie anxiogenic-like behavior of this chemical in male rats at 60 days of age.

The attention-deficit/hyperactivity disorder (ADHD) is associated with anxiety symptoms (Liu et al., in press). On the other hand, our results show a possible relation between DEHP exposure and an increase in anxiety behavior in male rats during sexual maturation. Both factors could contribute to explain the strong positive association between the presence of phthalate metabolites in urine and symptoms of ADHD and anxiety behavior observed among school-age children (Kim et al., 2009). More experiments are needed to study other possible mechanisms, such as alterations in neurotransmitter systems, which could be involved in DEHP-induced anxiety-like behaviors.

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