DOI: 10.21451/1984-3143-AR810

Anim. Reprod., v.13, n.4, p.795-805, Oct./Dec. 2016

Effects of 4-Nonylphenol on reproduction of exposed females during puberty

R. Arcanjo[1], M. Rocha[1], M. Marques[1], M.F. Almeida-Santos[2], S.N. Báo[1,3]

[1]Department of Cell Biology, Institute of Biological Sciences, University of Brasilia, Brasília, DF, Brazil.
[2]Department of Genetics and Morphology, Institute of Biological Sciences, University of Brasilia, Brasília, DF, Brazil.

Abstract

4-Nonylphenol (NP) is considered an endocrine disruptor due to its capacity to interact with endocrine system, mimicking estrogen activity. It is a ubiquitous pollutant since it has been found in many human tissue samples and its precursor identified in sewage, sediments, rivers and drinking water. Thus, this work aimed to evaluate reproductive parameters in female Balb/C mice treated with 50 mg/kg body weight of NP for 21 days, a period in which they reach sexual maturity. Body weight during treatment, day of vaginal opening, organs weight, fertility rate and proportion of different follicle types were some of these parameters. Additionally, for analysis of some effect in littermates, a group of animals underwent two attempts at pregnancy after treatment. NP had no effect on body weight during treatment, nor did it alter the day of vaginal opening and fertility rate of females. The proportion of preantral, antral and degenerated ovarian follicles was not modified by treatment with NP, and no morphological or pathological alterations were observed. The number of pups did not differ between treatments in any attempt at pregnancy. Also the weight of pups on birth and female rate in littermates was similar among experimental groups after both trials of pregnancy. It is suggested that the NP available or accumulated during the period of sexual maturation (21 days) did not reach threshold levels capable of producing significant alterations in the reproductive parameters analyzed.

Key-words: alkylphenol, endocrine disrupting chemicals, ovarian follicle, puberty, reproductive toxicology.

Introduction

In present, living organisms are exposed to a large amount and wide diversity of synthetic chemical compounds. These chemicals are found in the air (Onofrio *et al*., 2011), soil (Covaci *et al*., 2002), oceans and rivers (Josefsson *et al*., 2011) and in animal tissues (Ying *et al*., 2002; Jaspers *et al*., 2006). As a result of lack of strict regulations or proper toxicity tests when these compounds go on the market, the adverse effects of many compounds are discovered only after their widespread use in industry and by consumers (Rudel and Perovich, 2009; Knez, 2013). Some of these substances are known as endocrine disrupting chemicals (EDC), exogenous agents that can interfere with synthesis, secretion, transport, metabolism, binding, action, or elimination of natural hormones (Gore, 2008). EDC are found even in products considered inert, such as plastic (Hunt *et al*., 2003), and they are associated with some health problems in humans and animals, such as female and male subfertility (Annamalai and Namasivayam, 2015; Hond *et al*., 2015), certain types of cancers (Annamalai and Namasivayam, 2015), and neurological, developmental and reproductive disorders (Ness *et al*., 1993; Gore, 2008). These chemicals are claimed to be contributing to the fall in global fertility or be interacting with other causes for it, such as dietary factors (Petro *et al*., 2012). A worrying feature of EDC is that they usually have long half-lives, so they are persistent in environment (Bergeron *et al*., 1994). Also, the majority of them are lipophilic, accumulating in adipose tissue (Brevini *et al*., 2005). They have the capacity to cross the placental barrier (Barr *et al*., 2007) and be transmitted through suckling to the offspring of mammals, acting on developing embryos or newborns (Vos *et al*., 2003; Darbre and Harvey, 2008). Thus, EDC could affect not only exposed individuals, but subsequent generations (Dumesic *et al*., 2007).

Among EDCs, 4-nonylphenol (NP) is a chemical compound which mimics estrogen. NP is present in the environment mainly after degradation of nonylphenol ethoxylates (NPE; Soares *et al*., 2008). NPE is an alkylphenol widely utilized in the non-ionic surfactant industry, lubricants, antioxidants, detergents, paints, insecticides and herbicides, and also used as a stabilizer in plastic polymers (Oliveira-Filho *et al*., 2005; Rivero *et al*., 2008). Many studies show the presence of this pollutant in sewage, sediments, rivers and drinking water (Tsuda *et al*., 2000; Berryman *et al*., 2004; Shao *et al*., 2005). NP has been shown to be a global pollutant, frequently found in diverse human tissues. For example, it has been found in human adipose tissue samples (Lopez-Espinosa *et al*., 2009; Ferrara *et al*., 2011), breast milk (Ademollo *et al*., 2008) and blood samples from lactating women (Gyllenhammar *et al*., 2012).

Estrogenic action of NP is described for diverse animals, including birds (Razia *et al*., 2006), fish (Rivero *et al*., 2008) and mammals (Chapin *et al*., 1999; Tagaki *et al*., 2004). The median lethal dose of NP in male adult mice is 170 mg/kg body weight (b.w.) when administered intraperitoneally (El-Dakdoky and Helal, 2007), however effects can be observed at lower doses, such as 50 mg/kg b.w. (Nagao *et al*., 2001; Green *et al*., 2003). NP was capable of reducing the gene expression of the estrogen receptor in placental tissue and fetuses in pregnant female rats exposed to 50 mg/kg b.w. via oral for eight days or in a single dose intraperitoneally (Veld *et al*., 2009). Male mice treated with a lower dose of NP, 42.5 mg/kg b.w. via intraperitoneal for 35 days, showed a reduction in

[3]Corresponding author: snbao@unb.br.
Received: February 8, 2016
Accepted: August 31, 2016

epididymis and testes weights, lower concentration of stored sperm and reduced motility. They also presented signs of oxidative stress and a reduction in antioxidant enzyme levels (El-Dakdoky and Helal, 2007).

Estrogens promote growth and development of female reproductive tract, regulating cell proliferation and gene expression in reproductive organs, pituitary and mammary glands (Soto *et al*. 1991; Costanzo, 2004). Primordial follicle formation is regulated by paracrine growth factors and local concentrations of steroid hormones, with small influence of follicle-stimulating hormone (FSH) and luteinizing hormone (LH; Hunter *et al*., 2004; Uzumcu and Zachow, 2007). After the follicle enters the antral stage, it becomes dependent on FSH and LH to continue its maturation process (Hunter *et al*., 2004). From that point, growth is controlled mainly by FSH, whose production is modulated by the secretion of estradiol and inhibin A (Petro *et al*., 2012). As proper follicle and oocyte development relies on a well-orchestrated hormonal signaling, exposure to EDC in follicular environment could reduce oocyte quality or even jeopardize future embryo development (Petro *et al*., 2012).

Considering their characteristics, their ubiquitous presence in environment and the hazard posed by EDCs, especially estrogenic disruptors, it is important to carry out studies with these compounds, in order to identify those that affect reproductive function, at which concentrations, their mechanism of action and how to minimize damages. The objective of this work was to evaluate effects of NP on reproductive parameters in pubertal female mice, as well as to identify possible adverse effects on offspring.

Materials and Methods

All procedures of this work were approved by The Ethics Committee on Animal Use of the Institute of Biological Sciences at the University of Brasília, registered under number 98076/2012.

Male and female Balb/C mice were purchased from the Breeding Center of Laboratory Animals (Cecal) of the Oswaldo Cruz Foundation (Rio de Janeiro, Brazil) just after weaning, at post-natal day (PND) 21. They were maintained in the facility of the Institute of Biological Sciences with controlled temperature, food and water *ad libitum* and 12:12 h light/dark cycle.

Experimental design

4-Nonylphenol (technical grade, CAS 84852-15-3) and estradiol (17β-estradiol, CAS 50-28-2) were purchased from Sigma-Aldrich. Corn oil used as vehicle was obtained commercially (Liza, Mairinque, Brazil).

Ninety-seven females at PND 25 were divided into four experimental groups: control group with animals receiving only tap water (n = 26); vehicle control group receiving only corn oil (n = 28); NP group with animals treated with 50 mg/kg b.w. NP diluted in corn oil (n = 22; Nagao *et al*., 2001; Kimura *et al*., 2007); and E2 group, treated with 1 mg/kg b.w. 17β-estradiol (E2) diluted in corn oil (n = 21; Veld *et al*., 2009). This E2 dose was chosen since it has more estrogenic activity according to *in vitro* studies compared to NP dose (Veld *et al*., 2009).

Treatments were given orally with the aid of a gavage needle according with experimental group. Treatments were given to females daily for 21 days, the period they reach sexual maturity (Paiva *et al*., 2005). Females were weighed weekly during treatment and observed daily for identification of vaginal opening, an evidence of sexual maturity (Chorilli *et al*., 2007). At the end of treatment, females of each experimental group were divided into two subgroups: nulliparous and a group to attempt pregnancy. A schematic diagram of the whole experimental design is showed in Fig. 1.

Nulliparous females

Eight females of each treatment group (except vehicle group with n = 10) did not cohabit with males. These females were kept in collective cages for 15 days after the end of treatments (until PND 61), the time spent to obtain gestations in the other female's subgroup, and then euthanized. Liver, kidneys, spleen, uterus and ovaries were collected and weighed. From these animals, data previous to pregnancy were obtained, i.e. an overall picture of the follicles and oocytes that would have become fertilized.

Pregnant females

The other subgroup of each experimental group was put in cohabitation with males of the same age for 15 days to get pregnant. Two females from the same experimental group and one male were kept in cages with food and water *ad libitum*. This arrangement allowed monitoring of male fertility. After this period, females were put in individual cages and natural parturition was awaited. Offspring were weighed on their first day of life and had sex identified at 13th day of life. Offspring and dams were maintained until the day of sex identification.

Females that did not become pregnant were subjected to a second mating process as described above (2nd attempt at pregnancy). After this period, the occurrence of pregnancy was observed and natural delivery of offspring was awaited. Offspring were also weighed on their first day of life and had sex identified at 13th day of life. All animals were euthanized at PND 13 of litter, but dams had organs collected as described for the nulliparous females. Parameters analyzed in the litters comprised number of live offspring, weight at first day of life, female/male ratio and appearance of malformations.

The fertility rate was calculated by the proportion of females that got pregnant in relation to total females put in cohabitation with males. Different fertility rates were calculated for each attempt at pregnancy, in as much as animals were at different age brackets at the time of pregnancy assessment.

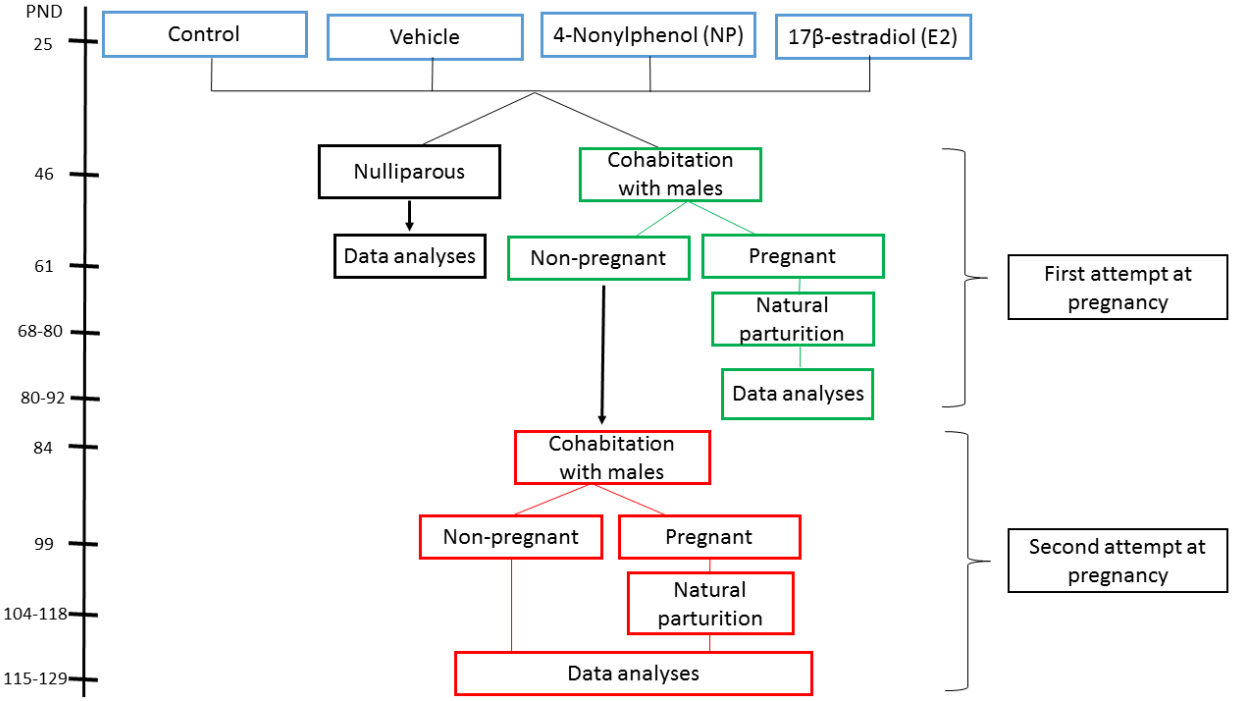

Figure 1. Experimental design with timeline representing post-natal day (PND) of treated females. Treatments are in blue, steps of the first attempt at pregnancy are in green and steps of the second attempt at pregnancy are in red.

Euthanasia

Females were anesthetized with 100 µl of anesthetic solution (20% ketamine and 12% xilazine in saline solution 0.9%), via intraperitoneal injection. After euthanasia, ovaries, uterus, liver, kidneys and spleen were collected and weighed. Males utilized in the experiment and offspring were euthanized with an overdose of anesthetic solution (200 and 50 µl, respectively).

Histological analyses

For histological analyses, the right ovary of each animal was fixed in paraformaldehyde (4%) in phosphate buffer (0.1 M, pH 7.4), dehydrated in serial baths of increasing concentration of ethanol (70-100%), diaphanized with xylene and embedded in paraffin wax with aid of automatic tissue processor Oma DM-40. Sections of 5 µm thickness were obtained serially, discarding five sections between each, with microtome Leica RM 2235. Tissue sections were stained with hematoxilin-eosin to analyze ovarian follicles, classifying their stage and identifying morphological alterations. Microscope slides were analyzed under light microscope Axioskop 2 and images were obtained with Zen lite 2011 software.

Approximately 200 ovarian follicles were examined from each animal, counting only follicles presenting oocyte and granulosa cells, presenting the oocyte nucleus or not. Follicles were classified as preantral, antral and degenerated, considering as degenerated the follicles possessing oocytes with retracted cytoplasm or without contact with granulosa cells. The proportion of each type of follicle was calculated in relation to the total number of follicles observed. These proportions were calculated for every dam, and then, the mean for specific experimental group and age was calculated.

The uteri of three nulliparous animals from the control group and of six from the NP group were processed for histological analysis as described above. Sections with 5 µm thickness were obtained from a uterine horn region next to the oviduct. Microscopic slides were stained with hematoxilin-eosin and observed under light microscope Axioskop 2. Images were obtained with Zen lite 2011 software.

Statistical analyses

Normality of the data were tested by Kolmogorov-Smirnov or Shapiro-Wilk tests and, when proved, these data were compared by one way ANOVA parametric test and Tukey's *post hoc* test. Data not normally distributed were analyzed and compared with Kruskal-Wallis non-parametric test and Dunn's *post hoc* test. Differences were considered statistically significant when $P < 0.05$. These statistical analyses were performed using GraphPad Prism 5.00 software.

Data of age when 50% of females from a particular experimental group reached sexual maturity as accessed by vaginal opening, were analyzed by Mantel-Cox's test with significance level of 5%, performed in GraphPad Prism 5.00 software. Fertility rates in different attempts at pregnancy for each experimental group were analyzed using the Logistic

procedure from SAS v.9.3 software, using the chi-square test with significance level of 5%.

Results

Females' weight and sexual maturity

Figure 2 shows the mean weight of females from all experimental groups at the beginning and during the three weeks of treatment. Control and NP groups were significantly different from E2 group only at first day of treatment, which indicates treatment does not affect animals' growth.

Vaginal opening is a common indicator of sexual maturity in females (Fig. 3). The ages at which 50% of females of the same group were already sexually mature were 31, 30, 30, and 29 days, for control, vehicle, NP and E2 groups respectively. Vaginal opening curve was analyzed by Mantel-Cox's test and did not show differences among experimental groups (Fig. 4).

Fertility rate and parameters analyzed in offspring

The fertility rates in the first attempt at pregnancy and after the second attempt at pregnancy are shown in Table 1. There was no statistical difference ($P > 0.05$) between treatments concerning fertility rates.

All pups born had normal appearance with no signs of malformation. Mean number of live offspring, mean weight on first day of life, and female/male ratio of offspring born after both first and second attempts at pregnancy are shown in Table 2. Mean number of live offspring born after first and second attempt at pregnancy did not show an alteration in the experimental group to which dams pertained. In both attempts, the mean weight of offspring was not altered by any treatment received by their mothers. The female ratio of offspring from the first attempt at pregnancy did not differ among groups. Also, female ratio of offspring from the second attempt at pregnancy did not show differences between groups.

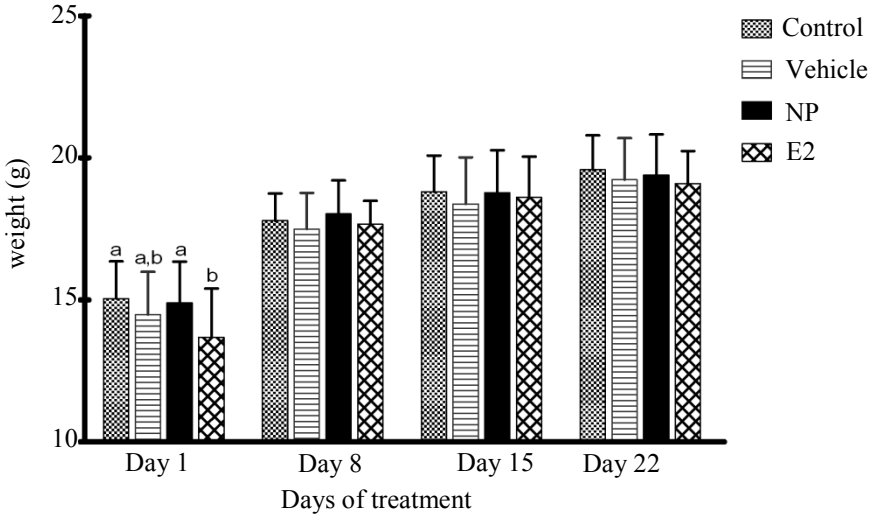

Figure 2. Mean weight of females from all experimental groups at the beginning and during three weeks of treatment. The only difference appears between control and NP group compared with E2 group, but just on the first day of treatment. This difference was rapidly surpassed, indicating that treatment did not have effect on females' growth**.** ANOVA and Tukey's post-test, $\alpha = 0.05$. Different letters indicate statistical difference between values.

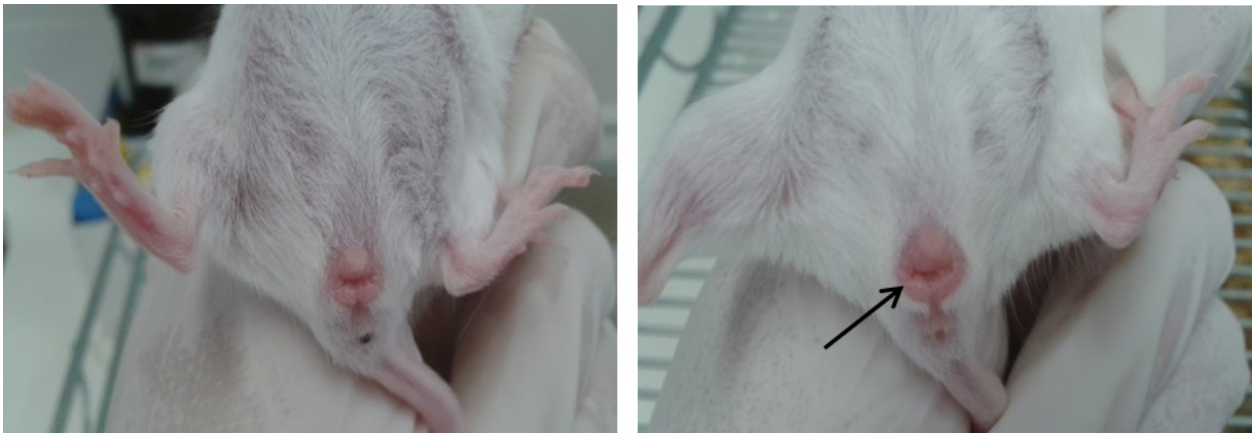

Figure 3. Identification of vaginal opening indicating sexual maturity in female mice. Left: female before vaginal opening. Right: female after vaginal opening, when protuberant external genitalia (arrow) and vaginal canal lumen can be observed.

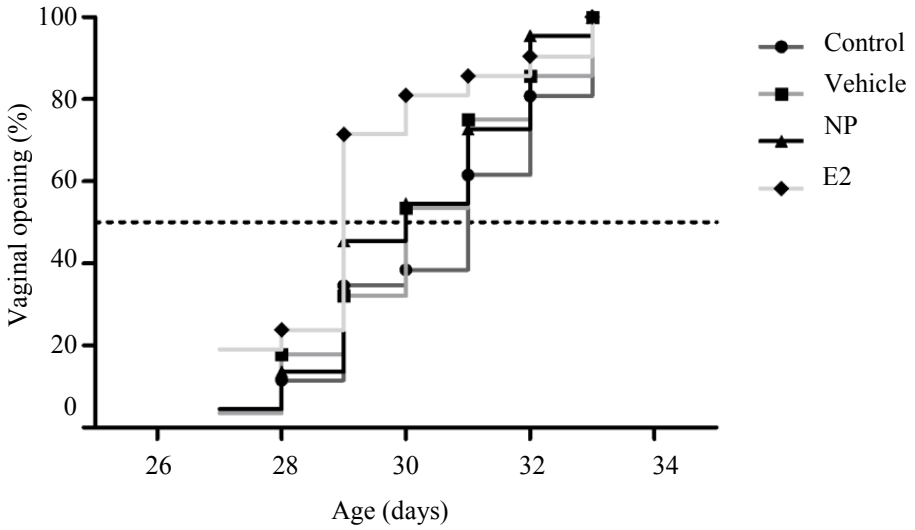

Figure 4. Vaginal opening curve for each experimental group. Age when females become sexually mature did not differ between treatments. Mantel-Cox's test, α = 0.05. Dashed line indicates vaginal opening had occurred in 50% of animals from that group.

Table 1. Fertility rate of females after first and second attempts at pregnancy.

Group	First attempt at pregnancy	Second attempt at pregnancy
Control	38.9% (7/18)	81.8% (9/11)
Vehicle	38.9% (7/18)	90.9% (10/11)
NP	57.1% (8/14)	100.0% (6/6)
E2	61.5% (8/13)	40.0% (2/5)

Number of pregnant/total number in parenthesis. α = 0.05.

Table 2. Data obtained from pups born after mating treated females in two pregnancy tries.

Group	N	Weight (g)	Female %
Pups born after first attempt at pregnancy			
Control (7)	3.19 ± 1.25	1.65 ± 0.24	54.8 ± 23.7
Vehicle (7)	5.14 ± 1.22	1.56 ± 0.08	59.8 ± 26.4
NP (8)	3.88 ± 1.46	1.57 ± 0.22	51.0 ± 32.9
E2 (8)	4.86 ± 1.95	1.56 ± 0.17	54.0 ± 27.0
Pups born after second attempt at pregnancy			
Control (9)	6.78 ± 2.44	1.51 ± 0.23	56.2 ± 22.4
Vehicle (9)	7.33 ± 2.65	1.50 ± 0.15	52.0 ± 16.6
NP (6)	8.33 ± 3.27	1.40 ± 0.05	55.0 ± 7.1
E2 (2)	8.50 ± 0.71	1.56 ± 0.03	58.3 ± 11.8

N = mean number of pups. α = 0.05. Numbers in parenthesis represent number of dams for each group.

Relative weight of organs

Mean relative weight of organs from all treatment groups of nulliparous females are shown in Table 3. Group treated with E2 had lower relative weight of spleen compared with control. It is noteworthy that the standard deviation of the relative weight of uteri from NP group was quite high, above the mean weight of the group. This fact is probably due to three animals, in a total of eight, presenting notable augmented uteri in relation to others from this group. The mean weight of the uterus of these three animals was 2.35 g, while the other five animals had a mean weight of 0.45 g.

The relative weight of organs from females of all experimental groups euthanized after first and second attempts at pregnancy were also shown in Table 3. No difference was observed in any organ between experimental groups at that age.

Table 3. Mean relative weight of organs from nulliparous and pregnant females after both tries.

	Ovary (right)	Ovary (left)	Uterus	Liver	Kidneys	Spleen
Nulliparous						
Control (8)	0.11 ± 0.02	0.15 ± 0.03	0.49 ± 0.20	5.64 ± 0.31	1.42 ± 0.11	0.90 ± 0.08 [a]
Vehicle (10)	0.12 ± 0.05	0.12 ± 0.03	0.38 ± 0.12	5.58 ± 0.49	1.37 ± 0.10	0.80 ± 0.14 [ab]
NP (8)	0.15 ± 0.02	0.15 ± 0.05	1.17 ± 1.50	5.41 ± 0.35	1.43 ± 0.14	0.73 ± 0.07 [ab]
E2 (8)	0.12 ± 0.05	0.12 ± 0.03	0.33 ± 0.13	5.59 ± 0.38	1.41 ± 0.07	0.62 ± 0.25 [b]
First attempt at pregnancy						
Control (6)	0.04 ± 0.010	0.04 ± 0.009	0.32 ± 0.19	6.76 ± 0.55	1.16 ± 0.04	0.46 ± 0.11
Vehicle (7)	0.05 ± 0.030	0.05 ± 0.020	0.33 ± 0.19	6.54 ± 0.64	1.22 ± 0.10	0.46 ± 0.11
NP (7)	0.05 ± 0.007	0.05 ± 0.020	0.33 ± 0.20	6.64 ± 0.58	1.20 ± 0.06	0.52 ± 0.08
E2 (8)	0.05 ± 0.010	0.05 ± 0.110	0.31 ± 0.14	6.54 ± 0.73	1.25 ± 0.05	0.48 ± 0.09
Second attempt at pregnancy						
Control (8)	0.04 ± 0.008	0.04 ± 0.009	0.24 ± 0.05	6.98 ± 0.30	1.24 ± 0.12	0.39 ± 0.08
Vehicle (10)	0.04 ± 0.010	0.04 ± 0.010	0.23 ± 0.08	6.76 ± 0.47	1.20 ± 0.05	0.41 ± 0.08
NP (6)	0.04 ± 0.007	0.04 ± 0.006	0.24 ± 0.03	6.89 ± 0.44	1.25 ± 0.07	0.50 ± 0.33
E2 (2)	0.04 ± 0.001	0.03 ± 0.004	0.20 ± 0.04	6.45 ± 0.23	1.12 ± 0.03	0.34 ± 0.03

Mean ± S.D. $\alpha = 0.05$. Different letters indicate statistical difference between values in same column. Numbers in parenthesis represent number of animals analyzed for each group.

Ovarian follicles

Preantral, antral and degenerated follicles were identified in every animal studied, independently of experimental group or age (Fig. 5). The percentages of these types of follicles for each experimental group are shown in Fig. 6. They were compared among different treatments and confirmed that treatments did not alter percentages of different types of follicles in nulliparous females (Fig. 6A). In females that got pregnant at the first attempt, the percentage of antral follicles was shown to be lower in E2 than in NP group (Fig. 6B). Females that got pregnant only after the second attempt at pregnancy had their ovarian follicles classified too and no difference was detected in any type of ovarian follicle for distinct treatment groups (Fig. 6C).

Six females did not get pregnant at either attempt: two from control group, one from vehicle group and three from E2 group. Ovaries of these animals were analyzed to assess percentages of follicle types. Mean percentages of each type of ovarian follicle from non-pregnant females, independently of experimental group, were compared with the control group at different ages. There were no differences in preantral, antral and degenerated percentages between control and non-pregnant group (Fig. 6D). Thus, the absence of pregnancy cannot be explained by alterations in number of ovarian follicles.

Histology of uterus

After observing anatomically augmented uteri, a histological analysis was performed to verify the occurrence of any anomaly (Fig. 7). A uterus without alterations, with regular appearance, presents a large lumen in the center (Fig. 7A). This lumen is lined by one layer of cylindrical epithelium, which joins with connective tissue layer, found just beneath, forming the endometrium, the portion that undergoes most alterations during the estrous cycle of females. The layer of connective tissue has endometrial glands and is rich in extracellular material, particularly reticular fibers. These fibers become more abundant as distance from the lumen increases, while cells become rarer. Beneath connective tissue is localized the musculature. This description is in accordance with Junqueira and Carneiro (2004) and Kierszenbaum (2008).

A nulliparous female treated with NP, whose relative uterus weight was considered regular, had morphological alterations identified compared with control group. The epithelium was shorter, the endometrium was thicker and the lumen of the organ was reduced (Fig. 7B). In contrast, animals from NP group with relative uterus weight considered higher did not show histological modifications compared with control (Fig. 7C and 7D), except for one animal that presented glands with larger lumens, which may have contributed to increasing the diameter of the organ (Fig. 7D).

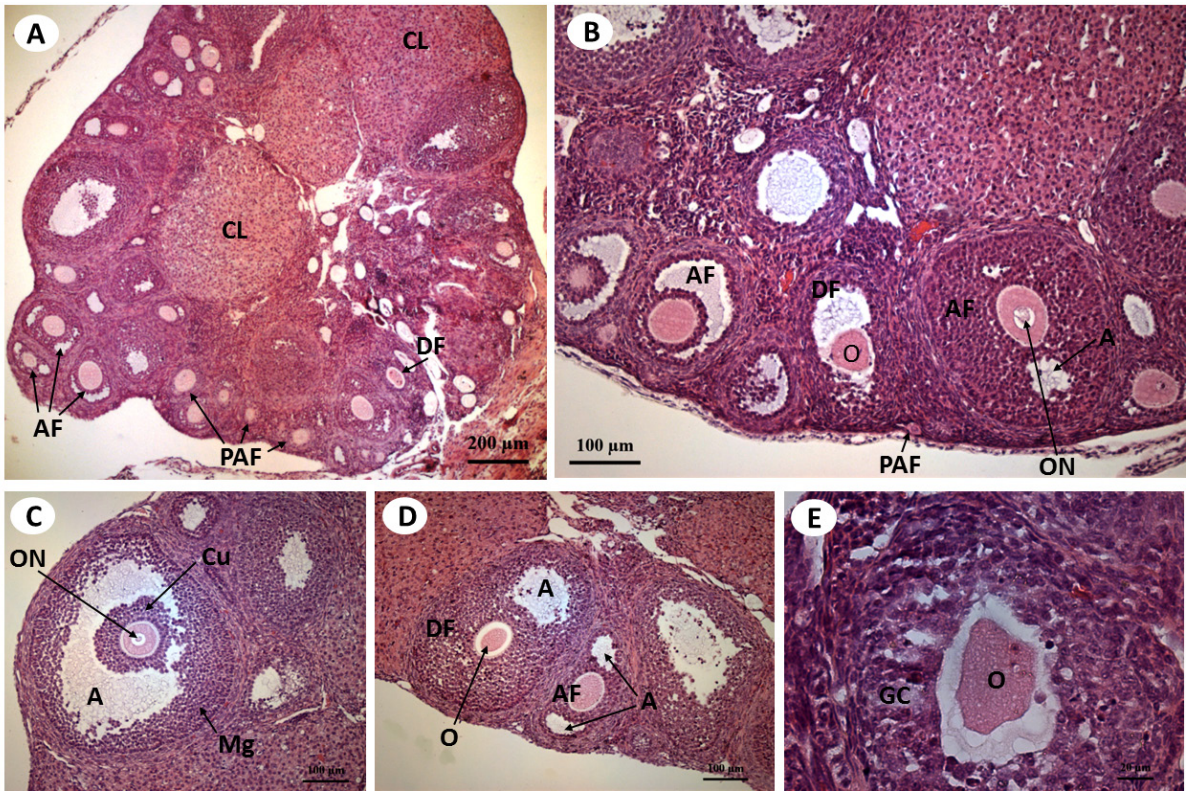

Figure 5. Diverse examples of ovarian follicle types. A: General view of ovary from a female pregnant after first attempt at pregnancy treated with NP. B: Ovary's general view of female pregnant after first attempt from control group. C: Antral follicle found in female pregnant after first attempt treated with E2. D: Degenerated follicle, near to an antral, from a female pregnant after first attempt from NP group. E: Detail of degenerated follicle showing lack of contact of oocyte with granulosa cells, found in female pregnant after second attempt treated with NP. A: antrum; AF: antral follicle; CL: corpus luteum; Cu: cumulus cells; DF: degenerated follicle; GC: granulosa cells; Mg: mural granulosa cells; O: oocyte; ON: oocyte nucleus; PAF: preantral follicle.

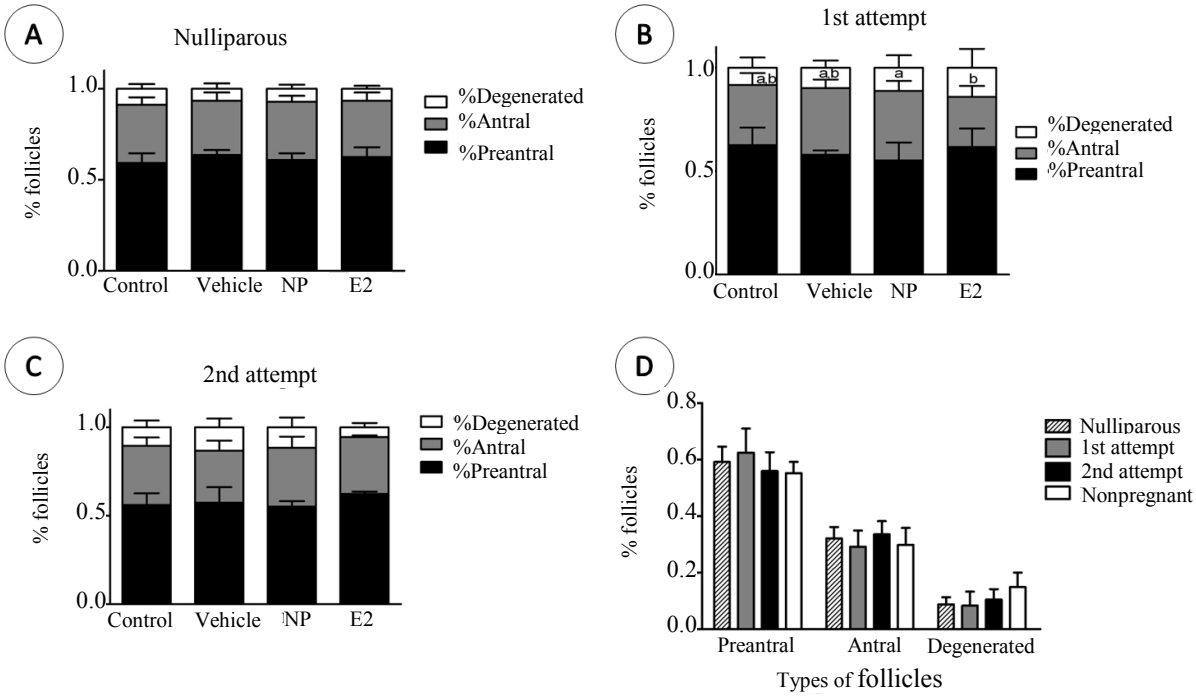

Figure 6. Percentage of follicle types identified in animals. A: Nulliparous females. B: Pregnant females after first attempt. C: Pregnant females after second attempt. D: Percentage of follicular types of control groups with different ages and non-pregnant females. ANOVA with Tukey's *post hoc* test or Kruskal-Wallis with Dunn's *post hoc* test, $\alpha = 0.05$. Different letters indicate statistical difference between values.

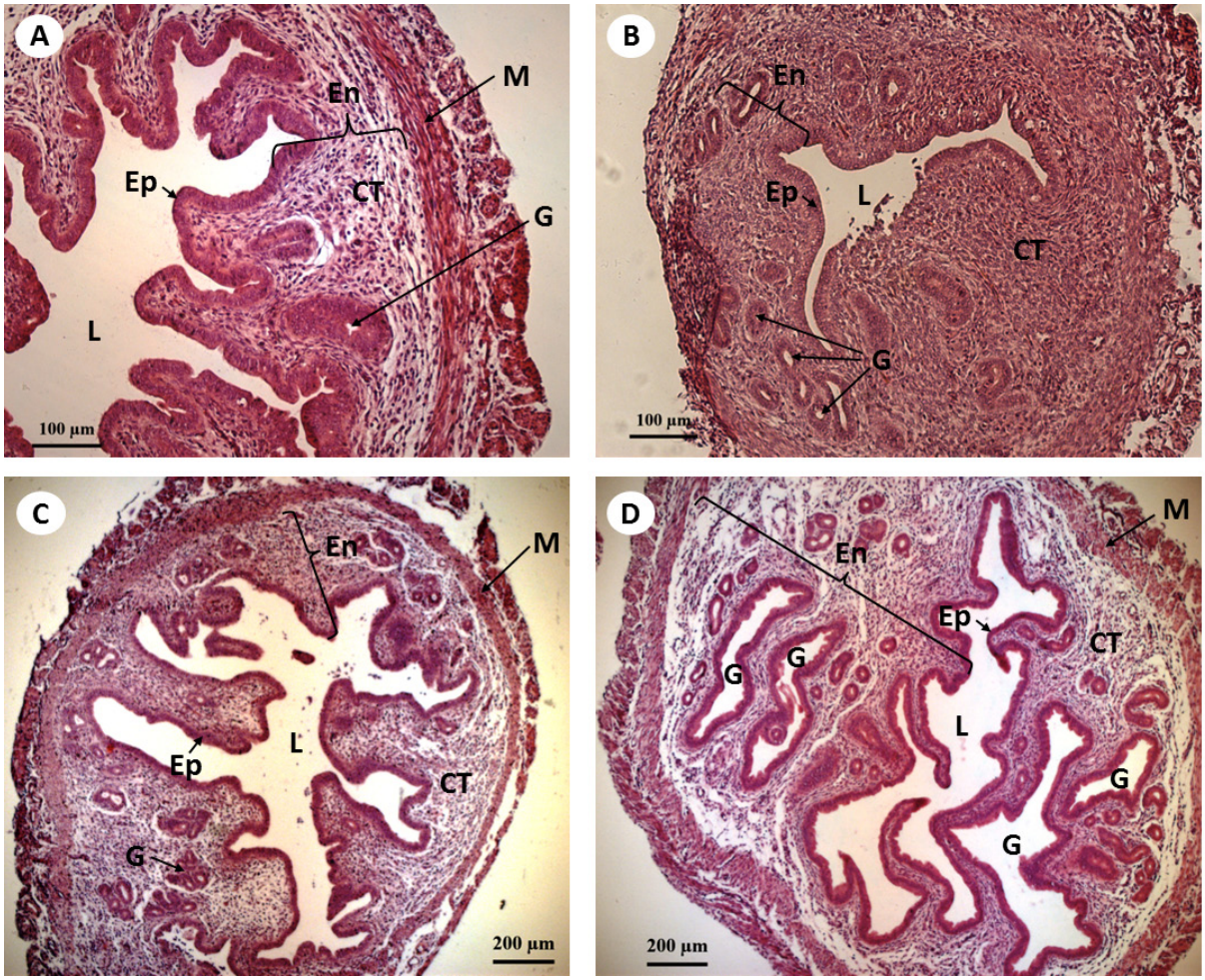

Figure 7. Histological analysis of uteri. A: Animal of control group presenting all layers of a regular uterus. B: Female of NP group whose uterus had relative weight considered normal, showing endometrium thickened. C: Animal treated with NP whose uterus was augmented, presenting histologically similar to control group. D: Animal from NP group, whose uterus was augmented, resembled control group, although it had wider lumen of glands. CT: connective tissue; En: endometrium; Ep: epithelium; G: glands; L: lumen; M: muscles

Discussion

The stage of life at which exposure to endocrine disruptors occurs is crucial to determine the extension of injury. Developing tissues are more sensitive to endocrine signals, so deregulation of these signals may cause permanent damage to structure or function of tissues, which makes exposure during development (*in utero*, neonates or young) potentially more harmful (Markey *et al*., 2003; Rudel and Perovich, 2009). Considering this fact, the present work aimed to analyze whether NP given to recently weaned females during the period of sexual maturation provokes prejudicial effects on their reproductive function.

Animals' weight was not altered compared with control and vehicle groups during the entire treatment. This indicates NP and E2 do not affect the growth of animals at doses of 50 mg/kg b.w. and 1 mg/kg b.w., respectively. Although E2 group has initiated treatment with lower weight, these animals gained weight soon in the first week and maintained it in the normal pattern. Also, no sign of general toxicity was noted in female mice.

Administration of exogenous steroids, as estradiol and testosterone, to immature rodents disrupts the progression of puberty (Pak *et al*. 2003). Kim *et al*. (2002) have affirmed that NP modifies the estrous cycle in rats. Oral exposure to NP continuously for three generations of rats showed estrogenic activity, and the hastening of the vaginal opening by two days for the group exposed to 650 ppm NP (30-100 mg/kg b.w.), and by six days for the group exposed to 2000 ppm NP (100-350 mg/kg b.w.) in all generations (Chapin *et al*., 1999). In this same work, the estrous cycle of females was deregulated only in the group treated with the higher dose. However, in the present study, carried out with a lower and more realistic dose, there was no significant difference in the day of vaginal opening between experimental groups.

NP was not shown to impair mating or fecundation as fertility rates were similar for every group. The first attempt at pregnancy was less successful than the second due to the young age of females (between 46 and 60 days of life, just after

sexual maturation), while in the second attempt at pregnancy females had between 84 and 98 days of life and were thus completely mature sexually (Chorilli *et al*., 2007). NP also did not influence the number of live-born offspring after the two attempts at pregnancy, nor their weight registered on the first day of life. Nagao *et al*. (2001), studying two generations of rats, treating males and females with 50 mg/kg b.w NP, also did not find significant differences in the fertility rate and number of offspring from the parental generation when compared with control. Similarly, males treated with 42.5 mg/kg b.w. of NP intraperitoneally for 35 days, when mated with non-treated females, obtained fertility rate, number of implantations/litter, number of live fetuses, fetal body weight and occurrence of external abnormalities equal to the control group, apart from adverse effects noted in testes and sperm (El-Dakdoky and Helal, 2007).

Genetic sex, determined by chromosomes present in cells, is considered the major factor for development of sexual differences. However, it is currently known that many of them may occur as a result of variable exposure to sexual hormones during development (Majdic and Tobet, 2011). Besides, it is also known that intrauterine environment and the concentration of sexual hormones with which fetuses are in contact may modulate sexual differences, especially brain development and behavioral characteristics (Saal *et al*., 1999). For these reasons, this work evaluated if females' exposure to NP while they are young would be capable of producing an alteration in offspring sex. This effect was not observed, as the proportion of females in the litter was the same in all experimental groups, independent of the age of dams. This result demonstrated that 50 mg/kg b.w. of NP given to females for 21 days did not have an effect upon the sex of their offspring. All data issued from offspring suggested there was not any delayed action of estrogenic compounds, given to females during sexual maturation, upon development of their offspring.

The most consistent toxic effect described in works with NP is mineralization of kidneys in male mice, although there is a variation in doses at which this effect is observed (Green *et al*., 2003). NP produced an increase in the relative weight of kidney and liver, and a reduction in the weight of thymus in males treated with 50 mg/kg b.w. (Nagao *et al*., 2001), while in greater doses, such as 30-350 mg/kg b.w., alterations in the weight and also in the structure of kidneys were identified (Chapin *et al*., 1999). However, in the present work, which analyzed only organs from females, differences in relative weight of ovaries, uterus, liver and kidneys were not found, independently of experimental group and age of females undergoing euthanasia. Only the group of nulliparous females treated with E2 had a reduction in relative weight of spleen compared with control group. Estradiol and some endocrine disrupters have the capacity to inhibit proliferation of lymphocytes isolated from the spleen (Sakazaki *et al*., 2002) which is likely the cause of the observed effect in mice spleens. This effect has also been demonstrated in dogs treated with estrogen (Hart, 1990). Older animals did not show this effect on spleen.

A reduction in the proportion of antral follicles and a resulting increase in proportion of preantral follicles were expected (Zeleznik, 1981), due to increased amount of exogenous estrogen circulating, which is able to activate the negative feedback mechanism in the pituitary. This mechanism diminishes FSH secretion and interrupts the development of immature follicles (Zeleznik, 2004). Meanwhile, NP did not alter proportions among preantral, antral and degenerated follicles. The expected effect was observed just in the E2 group that got pregnant after the first attempt and was not too substantial (percentage of antral follicles only diverged from NP group). At the same time, alterations or deformities in ovarian follicles were not identified in any group. Mehranjani *et al*. (2010), working with offspring of rats submitted to an elevated dose of NP (250 mg/kg b.w.) *in utero*, found a reduction in the number of antral and preovulatory follicles and an increase in number of atretic ones.

Histological analyses of animal uteri did not confirm any adverse effect that might have been caused by NP. On the contrary, organs with relative weight diverging from the rest of the group were seen to be histologically similar to the control uterus. The sole alteration noticed was an augmentation of the secretory glands in the endometrium, which could be described as an estrogenic effect, since estrogens play a role in the development and proliferation of the uterine endometrium (Junqueira and Carneiro, 2004; Kierszenbaum, 2008). However, more studies should be performed to confirm if this effect is prejudicial to animal's health. Augmentation in uterine weight was previously reported for rats treated with 100 and 200 mg/kg b.w. of NP. This effect was comparable to that of diethylstilbestrol (DES), a well-known endocrine disruptor (Kim *et al*., 2002). Although in the present work NP did not produce anomalies, it was capable of promoting focal mucinous metaplasia in the endometrium and uterine glands in guinea pigs exposed to 40 mg/kg b.w. for 14 days (Danzo *et al*., 2002). Incongruity between these works may be the result of using different experimental animals, since different species have variable susceptibility to this compound.

When administered at a dose of 50 mg/kg b.w. during the period of sexual maturation in female mice, available or accumulated NP did not reach levels to produce significant alterations in the analyzed parameters, a few days after ending exposure, when females effectively initiate reproductive life. NP probably reaches a balanced state in tissue accumulation and bioavailable quantity after metabolization does not achieve the threshold for action (Green *et al*., 2003). The dose used in this work is equivalent to 3.5 g in a 70 kg person and do not had effect in the main outcome of reproduction: offspring birth. For comparison purpose, daily intake for humans is estimated between 0.37 µg/kg b.w (Gyllenhammar *et al*., 2012) and 0.43 µg/kg b.w (Lu *et al*., 2007). The absence of significant effects in E2 group, treated with a more estrogenic dose, corroborates these findings.

In conclusion, NP at 50 mg/kg b.w. do not

cause damage to reproductive organs and function in female mice exposed for 21 days during puberty. Moreover, this work shows this length of treatment with NP does not have drastic effects on later fertility of females or birth weight and sex of offspring.

Acknowledgments

We are grateful to Dr. César Koppe Grisólia for the use of equipment in his laboratory, to Dr. Concepta McManus for assistance with some statistical analyses, to Dr. Cláudio Afonso Pinho Lopes for valuable considerations to this work and to Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Empreendimento Científicos e Tecnológicos (FINATEC) and Financiadora de Estudos e Projetos (FINEP) for financial support.

References

Ademollo N, Ferrara F, Delise M, Fabietti F, Funari E. 2008. Nonylphenol and octylphenol in human breast milk. *Environ Int*, 34:984-987.

Annamalai J, Namasivayam V. 2015. Endocrine disrupting chemicals in the atmosphere: their effects on humans and wildlife. *Environ. Int*, 76:78-97.

Barr DB, Bishop A, Needham LL. 2007. Concentrations of xenobiotic chemicals in the maternal-fetal unit. *Reprod Toxicol*, 23:260-266.

Bergeron JM, Crews D, McLachlan JA. 1994. PCBs as environmental estrogens: turtle sex determination as a biomarker of environmental contamination. *Environ Health Perspect*, 9:780-781.

Berryman D, Houde F, Deblois C, O'Shea M. 2004. Nonylphenolic compounds in drinking and surface waters downstream of treated textile and pulp and paper effluents: a survey and preliminary assessment of their potential effects on public health and aquatic life. *Chemosphere*, 56:247-255.

Brevini TAL, Cillo F, Antonini S, Gandofi F. 2005. Effects of endocrine disrupters on the oocytes and embryos of farm animals. *Reprod Domest Anim*, 40:291-299.

Chapin RE, Delaney J, Wang Y, Lanning, Davis B, Collins B, Mintz N, Wolfe G. 1999. The effects of 4-nonylphenol in rats: a multigeneration reproduction study. *Toxicol Sci,* 52:80-91.

Chorilli M, Michelin DC, Salgado HRN. 2007. Animais de laboratório: o camundongo. *Rev Ciênc Farm Básica Aplic*, 28:11-23.

Constanzo LS. 2004. *Fisiologia*. 2nd ed. Rio de Janeiro, RJ: Elsevier. 464 pp.

Covaci A, Manirakiza P, Schepens P. 2002. Persistent organochlorine pollutants in soils from Belgium, Italy, Greece, and Romania. *Bull Environ Contam Toxicol*, 68:97-103.

Danzo BJ, Shappell HW, Banerjee A, Hackey DL. 2002. Effects of nonylphenol, 1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene (p,p'-DDE), and pentachlorophenol on the adult female guinea pig reproductive tract. *Reprod Toxicol*, 16:29-43.

Darbre PD, Harvey PW. 2008. Paraben esters: review of recent studies of endocrine toxicity, absorption, esterase and human exposure, and discussion of potential human health risks. *J Appl Toxicol*, 28:561-578.

Dumesic DA, Abbott DH, Padmanabhan V. 2007. Polycystic ovary syndrome and its developmental origins. *Rev Endocr Metab Disord*, 8:127-141.

El-Dakdoky MH, Helal MAM. 2007. Reproductive toxicity of male mice after exposure to nonylphenol. *Bull Environ Contam Toxicol*, 79:188-191.

Ferrara F, Ademollo N, Orrú MA, Silvestroni L, Funari E. 2011. Alkylphenols in adipose tissues of Italian population. *Chemosphere*, 82:1044-1049.

Gore AC. 2008. Developmental programming and endocrine disruptor effects on reproductive neuroendocrine systems. *Front Neuroendocrinol*, 29:358-374.

Green T, Swain C, Van Miller JP, Joiner RL. 2003. Absorption, bioavailability, and metabolism of para-nonylphenol in the rat. *Regul Toxicol Pharm*, 38:43-51.

Gyllenhammar I, Glynn A, Darnerud PO, Lignell S, Van Delft R, Aune M. 2012. 4-Nonylphenol and bisphenol A in Swedish food and exposure in Swedish nursing women. *Environ Int*, 43:21-28.

Hart JE. 1990. Endocrine pathology of estrogens: species differences. *Pharmacol Ther*, 47:203-218.

Hond ED, Tournaye H, Sutter P, Ombelet W, Baeyens W, Covaci A, Cox B, Nawrot TS, Larebeke N, D'Hooghe T. 2015. Human exposure to endocrine disrupting chemicals and fertility: a case-control study in male subfertility patients. *Environ Int*, 84:154:160.

Hunt PA, Koehler KE, Susiarjo M, Hodges CA, Ilagan A, Voigt RC, Thomas S, Thomas BF, Hassold TJ. 2003. Bisphenol A exposure causes meiotic aneuploidy in the female mouse. *Curr Biol*, 13:546-553.

Hunter MG, Robinson RS, Mann GE, Webb R. 2004. Endocrine and paracrine control of follicular development and ovulation rate in farm species. *Anim Reprod Sci*, 82/83:461-477.

Jaspers VLB, Covaci A, Voorspoels S, Dauwe T, Eens M, Schepens P. 2006. Brominated flame retardants and organochlorine pollutants in aquatic and terrestrial predatory birds of Belgium: levels, patterns, tissue distribution and condition factors. *Environ Pollut*, 139:340-352.

Josefsson S, Karolsson OM, Malmaeus JM, Cornelissen G, Wiberg K. 2011. Structure-related distribution of PCDD/Fs, PCBs and HCB in a river-sea system. *Chemosphere*, 83:85-94.

Junqueira LC, Carneiro J. 2004. *Histologia Básica*. 10th ed. Rio de Janeiro, RJ: Guanabara Koogan. 488 pp.

Kierszenbaum AL. 2008. *Histologia e Biologia celular: uma introdução à patologia*. 2nd ed. Rio de Janeiro, RJ: Elsevier. 677 pp.

Kim HS, Shin JH, Kang IH, Kim TS, Kim IY, Seok JH, Pyo MY, Han SY. 2002. Comparative estrogenic effects of nonylphenol by 3-day uterotrophic assay and female pubertal onset assay. *Reprod Toxicol*, 16:259-268.

Kimura T, Kimura N, Totsuawa K. 2007. Effect of

compound exposure to bisphenol A and nonylphenol on the development and fertility of fetal mice. *J Mamm Ova Res*, 24:35-41.

Knez J. 2013. Endocrine-disrupting chemicals and male reproductive health. *Reprod Biomed Online*, 26:440-448.

Lopez-Espinosa MJ, Freire C, Arrebola JP, Navea N, Taoufiki J, Fernandez MF, Ballesteros O, Prada R, Olea N. 2009. Nonylphenol and octylphenol in adipose tissue of women in Southern Spain. *Chemosphere*, 76:847-852.

Lu Y, Chen M, Sung F, Wang PS, Mao I. 2007. Daily intake of 4-nonylphenol in Taiwanese. *Environ Int*, 33:903-910.

Majdic G, Tobet S. 2011. Cooperation of sex chromosomal genes and endocrine influences for hypothalamic sexual differentiation. *Front Neuroendocrinol*, 32:137-145.

Markey CM, Rubin BS, Soto AM, Sonnenschein C. (2003). Endocrine disruptors: from Wingspread to environmental developmental biology. *J Steroid Biochem Mol Biol*, 83:235-244.

Mehranjani MS, Noorafshan A, Hamta A, Momeni HR, Abnosi MH, Mahmoodi M, Anvari M, Hazaveh M. 2010. Effects of vitamin E on ovarian tissue of rats following treatment with p-nonylphenol: a stereological study. *Iran J Reprod Med*, 8:1-9.

Nagao T, Wada K, Marumo H, Youshimuda S, Ono H. 2001. Reproductive effects of nonilfenol in rats after gavage administration: a two-generation study. *Reprod Toxicol*, 15:293-315.

Ness DK, Scantz SL, Moshtaghian J, Hansen LG. 1993. Effects of perinatal exposure to specific PCB congeners on thyroid hormone concentrations and thyroid histology in the rat. *Toxicol Lett*, 68:311-323.

Oliveira-Filho EC, Geraoldino BR, Grisolia CK, Paumgartten FJR. 2005. Acute toxicity of endosulfan, nonylphenol ethoxylate and ethanol to different life stages of the freshwater snail *Biomphalaria tenagophila* (Orbigny, 1835). *Bull Environ Contam Toxicol*,75:1185-1190.

Onofrio M, Spataro R, Botta S. 2011. The role of a steel plant in north-west Italy to the local air concentrations of PCDD/Fs. *Chemosphere*, 82:708-717.

Paiva FP, Maffili VV, Santos ACS. 2005. *Curso de Manipulação de Animais de Laboratório*. Salvador, BA: Centro de Pesquisas Gonçalo Moniz.

Pak TR, Lynch GR, Ziegler DM, Lunden JB, Tsai PS. 2003. Disruption of pubertal onset by exogenous testosterone and estrogen in two species of rodents. *Am J Physiol. Endocrionol Metab*, 284:206-212.

Petro EML, Leroya JLMR, Van Cruchtenb SJM, Covacic A, Jorssena EPA, Bols PEJ. 2012. Endocrine disruptors and female fertility: focus on (bovine) ovarian follicular physiology. *Theriogenology*, 78:1887-1900.

Razia S, Maegawa Y, Tamotsu S, Oishi T. 2006. Histological changes in immune and endocrine organs of quail embryos: exposure to estrogen and nonylphenol. *Ecotoxicol Environ Saf*, 65:364-371.

Rivero CLG, Barbosa AC, Ferreira MFN, Dorea JG, Grisolia CK. 2008. Evaluation of genotoxicity and effects on reproduction of nonylphenol in *Oreochromis niloticus* (Pisces: cichlidae). *Ecotoxicology*, 17:732-737.

Rudel RA, Perovich LJ. 2009. Endocrine disrupting chemicals in indoor and outdoor air. *Atmos Environ,* 43:170-181.

Saal FS, Clark MM, Galef-Rj BG, Drickmer LC, Vandenbergh JG. 1999. Intrauterine position phenomenon. *Encycl Reprod*, 2:893-900.

Sakazaki H, Ueno H, Nakamuro K. 2002. Estrogen receptor α in mouse splenic lymphocytes: possible involvement in immunity. *Toxicol Lett*, 133:221-229.

Shao B, Hu J, Yang M, An W, Tao S. 2005. Nonylphenol and nonylphenol ethoxylates in river water, drinking water, and fish tissues in the area of Chongqing, China. *Arch Environ Contam Toxicol*, 48:467-473.

Soares A, Guieysse B, Jefferson B, Cartmell E, Lester JN. 2008. Nonylphenol in the environment: a critical review on occurrence, fate, toxicity and treatment in wastewaters. *Environ Int*, 34:1033-1049.

Soto AM, Justicia H, Wray JW, Sonnenschein C. 1991. p-Nonyl-phenol: an estrogenic xenobiotic released from "modified" polystyrene. *Environ Health Perspect*, 92:167-173.

Tagaki H, Shibutani M, Masutomi N, Uneyama C, Takahashi N, Mitsumori K, Hirosene M. 2004. Lack of maternal dietary exposure effects of bisphenol A and nonylphenol during the critical period for brain sexual differentiation on the reproductive/endocrine systems in later life. *Arch Toxicol*, 78:97-105.

Tsuda T, Takino A, Kojima M, Harada K, Muraki T, Tsuji M. 2000. 4-Nonylphenols and 4-tert-octylphenol in water and fish from rivers flowing into lake Biwa. *Chemosphere*, 41:757-762.

Uzumcu M, Zachow R. 2007. Developmental exposure to environmental endocrine disruptors: consequences within the ovary and on female reproductive function. *Reprod Toxicol*, 23:337-352.

Veld MGR, Zawadzka E, Rietjens IMCM, Murk AJ. 2009. Estrogenicity of food-associated estrogenic compounds in the fetuses of female transgenic mice upon oral and IP maternal exposure. *Reprod Toxicol*, 27:133-139.

Vos JG, Becher G, Van Den Berg M, De Boer J, Leonards PEG. 2003. Brominated flame retardants and endocrine disruption. *Pure Appl Chem*, 75: 2039-2046.

Ying GG, Williams B, Kookana R. 2002. Environmental fate of alkylphenols and alkylphenol ethoxylates. *Environ Int*, 28:215-226.

Zeleznik AJ. 1981. Premature elevation of systemic estradiol reduces serum levels of follicle-stimulating hormone and lengthens the follicular phase of the menstrual cycle in Rhesus monkeys. *Endocrinology*, 109:352-355.

Zeleznikz AJ. 2004. The physiology of follicle selection. *Reprod Biol Endocrino*l, 2:31. doi: 10.1186/1477-7827-2-31.