Pesticide chlorpyrifos acts as an endocrine disruptor in adult rats causing changes in mammary gland and hormonal balance

Clara Ventura[a], María Rosa Ramos Nieto[a], Nadia Bourguignon[b], Victoria Lux-Lantos[b], Horacio Rodriguez[c], Gabriel Cao[d], Andrea Randi[e], Claudia Cocca[a], Mariel Núñez[a],[*]

[a] Laboratorio de Radioisótopos, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Argentina
[b] Laboratorio de Neuroendocrinología, Instituto de Biología y Medicina Experimental (IByME), CONICET, Argentina
[c] Instituto de Salud y Ambiente del Litoral (ISAL), Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, Argentina
[d] Instituto de Investigaciones Cardiológicas, CONICET, Argentina
[e] Laboratorio de Efectos Biológicos de Contaminantes Ambientales, Departamento de Bioquímica Humana, Facultad de Medicina, Universidad de Buenos Aires, Argentina

A B S T R A C T

Endocrine disruptors (EDs) are compounds that interfere with hormone regulation and influence mammary carcinogenesis. We have previously demonstrated that the pesticide chlorpyrifos (CPF) acts as an ED in vitro, since it induces human breast cancer cells proliferation through estrogen receptor alpha (ERα) pathway. In this work, we studied the effects of CPF at environmental doses (0.01 and 1 mg/kg/day) on mammary gland, steroid hormone receptors expression and serum steroid hormone levels. It was carried out using female Sprague-Dawley 40-days-old rats exposed to the pesticide during 100 days. We observed a proliferating ductal network with a higher number of ducts and alveolar structures. We also found an increased number of benign breast diseases, such as hyperplasia and adenosis. CPF enhanced progesterone receptor (PgR) along with the proliferating cell nuclear antigen (PCNA) in epithelial ductal cells. On the other hand, the pesticide reduced the expression of co-repressors of estrogen receptor activity REA and SMRT and it decreased serum estradiol (E_2), progesterone (Pg) and luteinizing hormone (LH) levels. Finally, we found a persistent decrease in LH levels among ovariectomized rats exposed to CPF. Therefore, CPF alters the endocrine balance acting as an ED in vivo. These findings warn about the harmful effects that CPF exerts on mammary gland, suggesting that this compound may act as a risk factor for breast cancer.

© 2015 Elsevier Ltd. All rights reserved.

1. Introduction

Chlorpyrifos (CPF) is a broad spectrum organophosphate pesticide (OP) used for pest control in agriculture as well as in residential applications. It is widely employed worldwide and largely applied in Argentina [1–3]. The main mechanism of CPF toxicity is the acetylcholinesterase (AChE) inhibition [4,5]. Many studies have demonstrated that even at low levels, CPF interferes with normal development through different cellular and molecular mechanisms beyond the inhibition of AChE [6–9].

Endocrine disruptors (EDs) are molecules found both in the environment and in the diet that interfere with normal hormone regulation [10]. These substances could act as agonist or antagonist of hormone receptors, and they can also affect enzymatic pathways involved in hormone biosynthesis, bioavailability or metabolism [11]. Several studies demonstrated that CPF affects the thyroid and adrenal gland homeostasis both in human and animal models [12,13]. It has been demonstrated that CPF presents an inhibitory effect on the secretion of pituitary hormones, luteinizing hormone (LH) and follicle stimulating hormone (FSH), and a decrease in testosterone (T) biosynthesis in testes of rats [14]. Other researchers reported that CPF presents anti-androgenic activity, altering male reproductive abilities and the expression of some steroidogenic enzymes [15]. We have previously demonstrated

that environmental concentration of CPF promotes cell proliferation through estrogen receptor alpha (ERα) in hormone-dependent MCF-7 breast cancer cells [16]. In concordance, others authors found that ecologically relevant concentrations of pesticide interfere with sex differentiation and reproductive development in *Rana dalmatinavia* via endocrine-disrupting mechanisms [17]. Those results confirm the action of this pesticide as an ED.

The mammary gland is a complex organ that undergoes a serie of coordinated phases of development under the influence of cyclic hormonal stimulation from birth to senescence. Thus, it represents a useful model to evaluate the effects of ED [18]. Many hormones, such as estradiol (E_2) and progesterone (Pg) participate in the normal development of mammary gland [19,20]. The effects of steroid hormones are mediated via progesterone (PgR) and estrogen (ER) receptors, which are involved in the lobuloalveolar development and ductal elongation, respectively [21,22].

The steroid nuclear receptors activity is regulated by their binding to co-regulators. The classification of co-regulators into co-activators or co-repressors is based on general observations of their activity. Co-activators enhance ligand-induced transcriptional activation of PgR, ERα, glucocorticoid receptor, thyroid receptor, and retinoid X receptor. In contrast, the role of co-repressors is altering the chromatin structure of the promoter to an inactive state. Co-repressors such as SMRT (silencing mediator of retinoid and thyroid receptors) and REA (repressor of ER activity) recruit and activate histone deacetylases, which results in a more compact chromatin structure and, consequently, in a gene expression inhibition [23–25].

The aim of this work was to evaluate whether the pesticide CPF could act as ED on the mammary gland. Thus, we analyzed the effects exerted by CPF on breast tissue structures as well as the consequences on circulating hormone levels, steroid hormone receptors and co-repressors expression in mammary gland of rats exposed to the pesticide chronically.

2. Materials and methods

2.1. Animals

Experimental protocols were designed in accordance with the Guide for the Care and Use of Laboratory Animals issued by the U.S. National Academy of Sciences [26] and approved by the ethical committee of the School of Pharmacy and Biochemistry, Buenos Aires University. Virgin female 40-days-old Sprague-Dawley rats (from the National University of La Plata, Animal Production Division, Argentina) were housed in stainless steel cages with water and food *ad libitum*, temperature of $22 \pm 2\,^{\circ}C$, humidity around 56% and 12 h light-dark cycle. Rats were randomly separated in three groups with 6 animals each one. Three independent experiments were performed.

2.2. Dosage exposures

Chlorpyrifos (99.5% purity) was purchased from Chem. Service, Inc., West Chester, PA, USA. For administration, dilutions were made in castor oil (*Ricinus communis*). Doses were selected taking into consideration the No Observed Adverse Effects Level (NOAEL, 1 mg/kg/day) and the Acceptable Daily Intake (ADI, 0.01 mg/kg/day), reported by World Health Organization [27]. Animals were exposed to CPF or vehicle daily via oral intake during 100 days.

2.3. Sample collection

Since mammary histology and hormone serum levels are dependent on oestrous cycle state, all samples were collected during oestrous phase. The stages of the oestrous cycle were determined using vaginal smears, collected every day for at least two weeks. The animals' weight was recorded prior to sacrifice. Serum was stored at $-80\,^{\circ}C$ until biochemical determinations. Livers were quickly removed and wet weights were determined. Mammary glands were removed, a fraction was fixed in 10% (v/v) buffered formalin, and embedded in paraffin and the rest of the gland was kept at $-80\,^{\circ}C$ for western blot assay.

2.4. Cholinesterase activity

AChE activity was determined in animal blood after 100 days of CPF or vehicle administration. Red cells were separated by centrifugation and washed three times with saline solution. 100 μL were added to 2.4 mL of distilled water in order to lyse erythrocytes. AChE activity was determined according to Ellman's method [28] using acetylthiocholine as a substrate. Briefly, 20 μL of lysed erythrocytes were added to a final volume of 3 mL containing 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB, 0.25 mM) and acetylcholine (5 mM). Enzyme activity was measured by following the absorbance increase at 405 nm ($\epsilon = 13{,}600$ $mM^{-1}\,cm^{-1}$) every 1 min for 5 min, and expressed as U/L.

Butyrylcholinesterase (BChE) was determined in animals' plasma. 20 μL of plasma was added to a final volume of 3 mL containing DTNB (0.25 mM) and butyrylcholine (16.3 mM) as a substrate. Enzyme activity was measured using absorbance at 405 nm ($\epsilon = 13{,}600\,mM^{-1}\,cm^{-1}$) every 0.5 min for 1.5 min and expressed as U/L.

2.5. Biochemical parameters

Serum aspartate aminotransferase (ASAT), alanine aminotranferase (ALAT) and cholesterol were determined by standard automated techniques following the standards recommended by the International Federation of Clinical Chemistry Laboratory Medicine (IFCC), at the Clinical Biochemistry Department, José de San Martín Hospital.

2.6. Histological analysis

Ducts and lobular structures were quantified in hematoxylin and eosin-stained (HE) mammary sections at $50\times$ and $100\times$ magnifications, respectively. The percentage of hyperplastic ducts was determined. The primary criterion used for diagnosing hyperplasia was the presence of an increased number of epithelial layers within the ducts [29]; only ducts with four or more layers of epithelial cells were considered hyperplastic. Each hyperplasia was classified as moderate (until four layers of epithelial cells within the ducts) or florid (more than five layers of epithelial cells lining the ducts). To obtain the percentage of hyperplastic ducts, we evaluated three randomly selected microscope fields per sample of mammary gland, which were at least 30 μm apart from each other, and analyzed 50 ducts per section.

The presence of adenosis, characterized by an increased number or size of lobular structures was determined and classified as sclerosing or non sclerosing according to the presence or absence of stromal sclerosis surrounding the alveoli. The percentage of alveolar buds presenting adenosis was quantified on three randomly selected microscope fields per sample of mammary gland, at least 30 μm apart, and analyzed 100 alveolar buds per section.

2.7. Immunohistochemical assay

Protein expression was detected by inmunohistochemical assay using rabbit anti-ERα (1:50, Santa Cruz Biotechnology, USA), rabbit anti-PgR (1:50, Santa Cruz biotechnology, USA), mouse anti-PCNA

(1:100, Dako Cytomation, Denmark), rabbit anti-SMRT (1:50) and rabbit anti-REA (1:50) specific antibodies as previously described [30]. Both anti-SMRT and anti-REA antibodies were generated and tested in ISAL Institute [31].

2.8. Western blotting

Proteins were detected by Western blot using rabbit anti-phospho-Y537-ERα (1:200, Santa Cruz Biotechnology, Santa Cruz, USA), rabbit anti-PgR (1:200, Santa Cruz Biotechnology, Santa Cruz, USA), and mouse anti-β-actin (1:1000, Sigma Chemical Co., MO, USA) antibodies, as previously described [32].

2.9. Hormone levels

2.9.1. Serum steroid hormone levels.
E_2, T and Pg were determined by radioimmunoassay (RIA) as previously described [33,34] using specific antiserum kindly provided by G.D. Niswender (Colorado State University, Fort Collins, CO, USA). Serum steroid hormone levels were determined in serum after ethyl ether extraction. Labeled E_2 and Pg were obtained from Perkin-Elmer (Wellesley, MA, USA), and T was from New England Nuclear (Boston, MA, USA). Assay sensitivities were 11.3 pg (E_2), 12.5 pg (T), and 500 pg (Pg). Intra and inter-assay coefficients of variation were 6.8% and 11.7% for E_2, 7.8% and 12.3% for T, and 7.1% and 12.15% for Pg, respectively.

2.9.2. Serum protein hormone levels.
LH and FSH hormone were determined by RIA using kits obtained from the National Hormone and Peptide Program (Torrance, CA, USA) as previously described [33]. Results are expressed in terms of reference preparation RP3 rat LH and FSH standards. Assay sensitivities were 0.015 ng/mL for LH and 0.1175 ng/mL for FSH. Intra and inter-assay coefficients of variation, respectively, were as follows: LH, 7.2% and 11.4% and FSH, 8.0% and 13.2%.
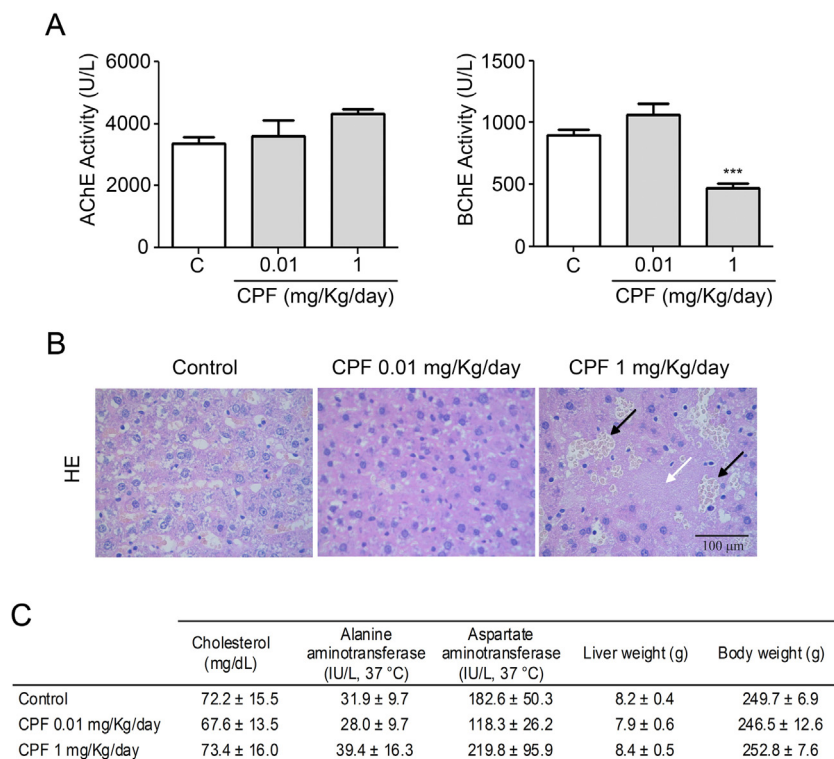
2.10. Effect of CPF on gonadotrophin release in ovariectomized rats

Virgin female 40-days-old Sprague-Dawley rats were bilaterally ovariectomized and CPF (0.01 and 1 mg/kg/day) or vehicle was daily administered for 10 days. Subsequently, blood was collected and serum was stored at −80 °C until hormone assays.

3. Results

3.1. CPF effects on cholinesterase activity and liver damage

Changes in erythrocyte and plasmatic cholinesterase activities are shown in Fig. 1A. AChE activity was not modified by CPF at the assayed doses. BChE activity was significantly reduced following CPF 1 mg/kg/day exposure (49.1% vs. C, $p < 0.001$) but no changes were observed in rats exposed to CPF 0.01 mg/kg/day. The Fig. 1B shows representative photographs of rats' livers exposed to CPF or vehicle. Histological examination revealed a normal liver histology in both control and 0.01 mg/kg/day CPF treated animals. However, the rats intoxicated with CPF 1 mg/kg/day showed a small number of necrotic and hemorrhagic foci. To study liver physiology, we also evaluated serum ALAT and ASAT enzymes activities, which constitute liver damage indicators. Additionally, total cholesterol

	Cholesterol (mg/dL)	Alanine aminotransferase (IU/L, 37 °C)	Aspartate aminotransferase (IU/L, 37 °C)	Liver weight (g)	Body weight (g)
Control	72.2 ± 15.5	31.9 ± 9.7	182.6 ± 50.3	8.2 ± 0.4	249.7 ± 6.9
CPF 0.01 mg/Kg/day	67.6 ± 13.5	28.0 ± 9.7	118.3 ± 26.2	7.9 ± 0.6	246.5 ± 12.6
CPF 1 mg/Kg/day	73.4 ± 16.0	39.4 ± 16.3	219.8 ± 95.9	8.4 ± 0.5	252.8 ± 7.6

Fig. 1. CPF effects on cholinesterase activity and liver damage. (A) Erythrocyte AChE and plasma BChE activities were determined spectrophotometrically following administration of CPF (0.01 and 1 mg/kg/day) or vehicle (C) over a period of 100 days. Graphs show the mean values ± SEM of two independent experiments. In each assay, experimental groups were composed of six animals ($N = 6$) (***$p < 0.001$; Kruskal–Wallis non-parametric analysis and Dunn's multiple comparison post test). (B) Representative photographs of the livers of rats after 100 days of administration of CPF (0.01 and 1 mg/kg/day) or vehicle (control). Magnification: 630×. Black arrows indicate red blood cells characteristic of hemorrhagic foci. White arrow shows necrosis. Scale bar: 100 μm. (C) Cholesterol concentration, ALAT and ASAT activities were determined on serum of animals. Liver and body weight were determined after administration of CPF (0.01 and 1 mg/kg/day) or vehicle (C) for a period of 100 days. Data represent mean values ± SEM of two independent experiments (p:ns; Kruskal–Wallis non-parametric analysis).

concentration as well as body and liver weights were evaluated (Fig. 1C). Our results demonstrated that CPF 1 mg/kg/day treatment slightly increased ALAT and ASAT activity levels compared to control animals; however, this increment was not statistically significant. No changes were observed in ALAT and ASAT activities when the animals were exposed to CPF 0.01 mg/kg/day. Cholesterol concentration was not found altered.
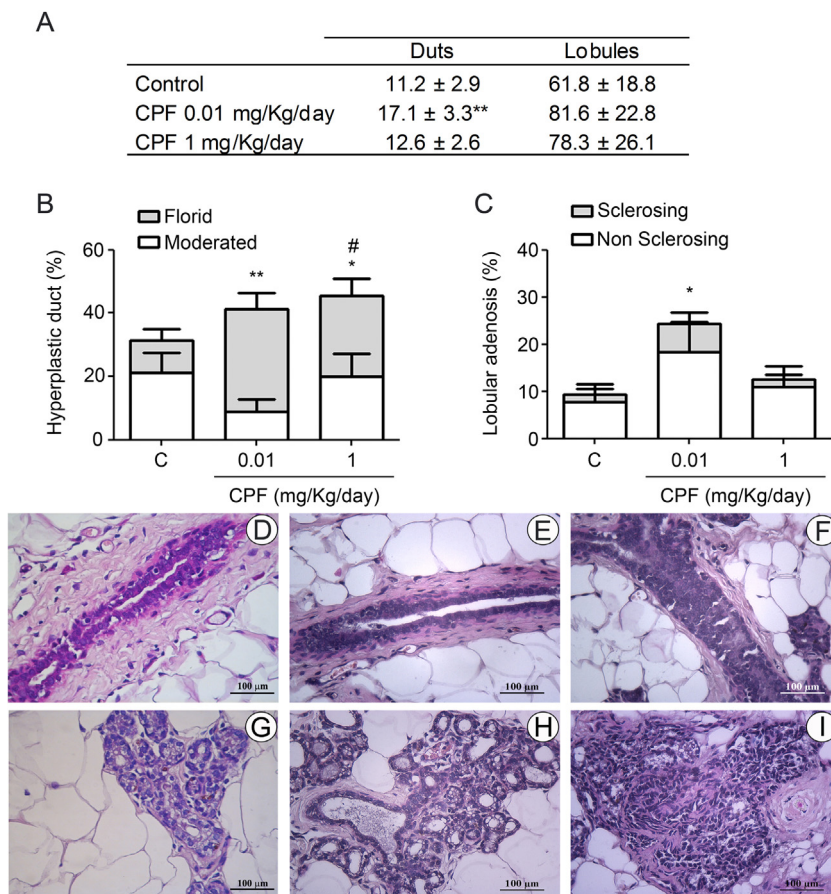
Finally, CPF did not affect the final body and liver weights respect to the control animals at any evaluated dose.

3.2. CPF effect on mammary histology

Studies from our laboratory have previously reported that low concentrations of CPF increase cell proliferation by ERα phosphorylation in MCF-7 cells. In this work we investigated the effect of CPF on the development of mammary gland in Sprague-Dawley rats exposed to the pesticide from 40 days old. Mammary gland development was assessed by quantification of ducts and lobular buds on the mammary tissue sections of animals exposed to CPF or vehicle during 100 days (Fig. 2A). We observed an increasing number of ducts in the mammary sections of the animals exposed to CPF 0.01 mg/kg/day respect to the control (52%, $p < 0.01$). No changes were observed on lobular buds at any CPF dose evaluated.

Additionally, we determined the incidence of proliferative benign lesions in mammary gland of the animals after CPF or vehicle administration. As the Fig. 2B shows, the animals exposed to CPF 1 mg/kg/day exhibited an increased percentage of hyperplastic ducts respect to the control group ($45.6 \pm 7.1\%$ vs. $35.1 \pm 8.5\%$, respectively, $p < 0.05$). We have also observed an increment of ductal hyperplasia when animals were exposed to CPF 0.01 mg/kg/day comparing to the control group, but it was not statistically significant ($41.2 \pm 4.5\%$ vs. $35.1 \pm 8.5\%$, respectively, p:ns). Hyperplasias were discriminated according to each lesion severity (Fig. 2B). We noticed a significant higher percentage of florid hyperplasias on the mammary gland of animals exposed to CPF 0.01 mg/kg/day ($32.3 \pm 8.0\%$, $p < 0.01$) and CPF 1 mg/kg/day ($28.7 \pm 9.1\%$, $p < 0.05$) with respect to the control group ($10.1 \pm 5.7\%$). However, non-significant changes were observed on moderate ductal hyperplasia.

Lobular adenosis was evaluated in animals exposed chronically to the pesticide or vehicle. The Fig. 2C illustrates a lobular adenosis percentage significantly increased in the mammary gland of the animals exposed to CPF 0.01 mg/kg/day respect to the control group ($24.3 \pm 12.0\%$ vs. $9.4 \pm 5.8\%$, $p < 0.05$). No changes were observed in animals exposed to CPF 1 mg/kg/day. Lobular adenosis was classified as sclerosing and non-sclerosing adenosis. The Fig. 2C reveals how both types of lesions contributed to the increment of lobular adenosis observed.

A

	Duts	Lobules
Control	11.2 ± 2.9	61.8 ± 18.8
CPF 0.01 mg/Kg/day	17.1 ± 3.3**	81.6 ± 22.8
CPF 1 mg/Kg/day	12.6 ± 2.6	78.3 ± 26.1

Fig. 2. CPF effect on mammary histology. (A) Number of ducts and alveolar buds per field, quantified in photographs taken at 50× and 100× magnification, respectively (**$p < 0.01$ vs. control). (B) Percentage of ductal hyperplasias (without atypia). Lesions were classified as moderate or florid according to the histology of the lesion. Percentage of ductal hyperplasia (#$p < 0.05$ vs. C), percentage of ducts with florid hyperplasia (*$p < 0.05$, **$p < 0.01$ vs. C) and percentage of ducts with moderate hyperplasia (p:ns) were compared. (C) Percentage of alveolar buds presenting adenosis. Adenosis was classified as sclerosing or non sclerosing according to the histology of the lesion. In statistical analysis percentage of alveolar buds presenting adenosis (sclerosing and non sclerosing) (*$p < 0.05$ vs. C), percentage of alveolar buds presenting sclerosing adenosis (p:ns vs. C) and percentage of alveolar buds presenting non sclerosing adenosis (p:ns vs. C) were compared. In all cases (A–C), data represent mean values ± SEM of three independent experiments, 5 randomly selected microscope fields per sample ($N = 6$) were evaluated and non-parametric data analysis was performed: Kruskal–Wallis and Dunn's multiple comparison post test. (D–I) Representative photographs of a normal duct (D), moderate hyperplasia (E), florid hyperplasia (F), normal alveolar bud (G), non sclerosing adenosis (H) and sclerosing adenosis (I). Magnification 400×. Scale bar: 100 μm.
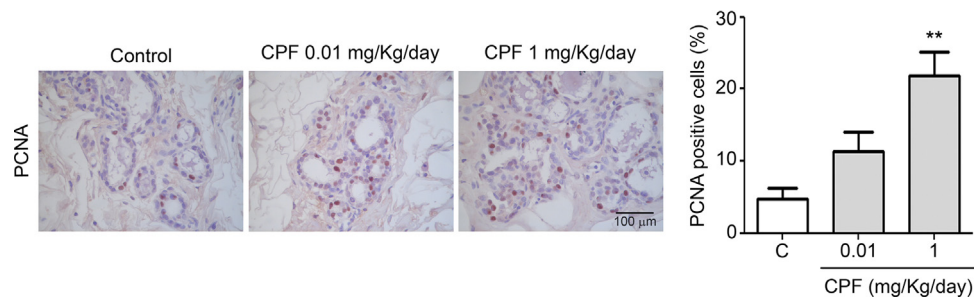
Fig. 3. CPF effect on cell proliferation. Representative photographs and graphical representation of the immunohistochemical detection of PCNA in mammary gland of rats exposed to CPF (0.01 and 1 mg/kg/day) or vehicle (control) during 100 days. The PCNA-positive nuclei are stained brown. Magnification: 630×. Scale bar: 100 μm. Percentage of PCNA-positive cells was calculated as the number of positive cells/total number of cells per field. 5 randomly selected microscope fields per sample ($N = 6$) were evaluated. Data represent mean values ± SEM of two independent experiments (**$p < 0.01$ vs. control; one-way ANOVA and Dunnett post test).

3.3. CPF effect on cell proliferation

After observing an increment of ductal hyperplasia and lobular adenosis in rats exposed to CPF, we decided to study the effect of this pesticide on cell proliferation by PCNA specific staining. Following a chronic intoxication, CPF 1 mg/kg/day significantly increased the percentage of PCNA positive cells in the mammary gland of animals ($21.8 \pm 3.3\%$ vs. $4.7 \pm 1.9\%$, $p < 0.01$), as shown in Fig. 3. We have also observed an increased percentage of PCNA positive cells when animals were intoxicated with CPF 0.01 mg/kg/day, however it was not statistically significant ($11.3 \pm 2.7\%$ vs. $4.7 \pm 1.9\%$, p:ns).
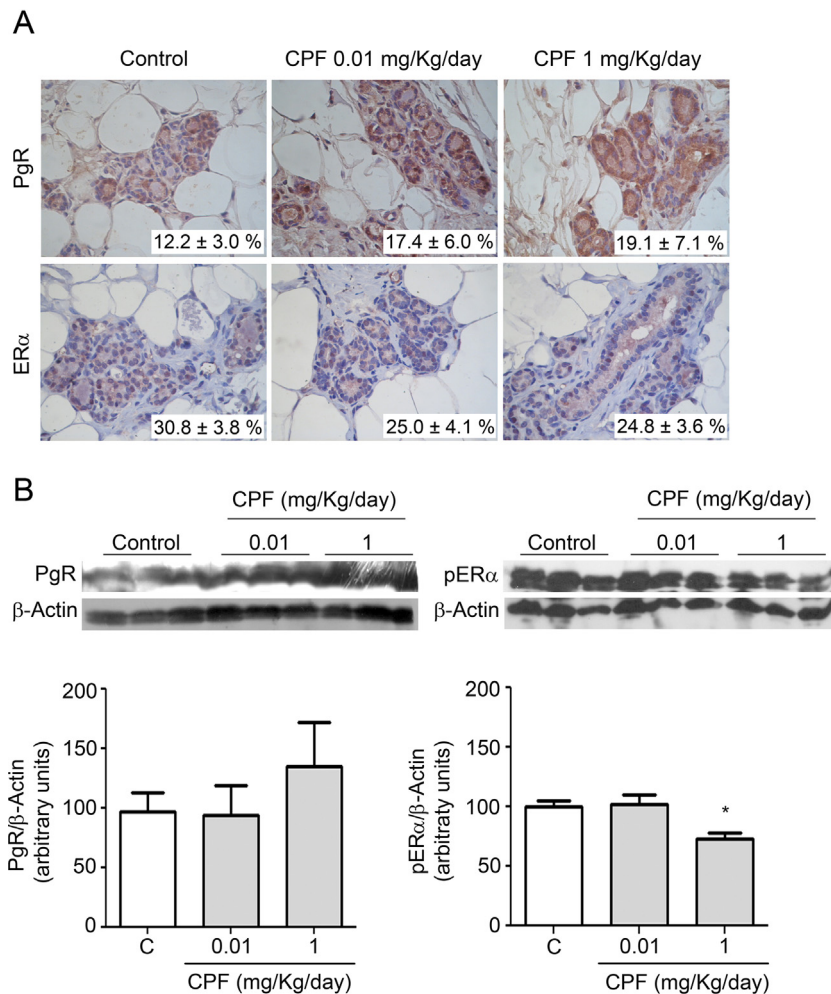
Fig. 4. CPF effect on estrogen and progesterone receptors expression. (A) Representative photographs illustrating PgR and ERα expression evaluated by immunohistochemistry in rat mammary gland after 100 days of exposure to CPF (0.01 and 1 mg/kg/day) or vehicle (control). Positive nuclei are observed brown stained. Magnification: 630×. White boxes show percentage of positive cells calculated as the number of positive cells/total number of cells per field. Five randomly selected microscope fields per sample were evaluated. Data are mean ± SEM of two independent experiments (p:ns; Kruskal–Wallis non-parametric analysis). (B) PgR and phospho-Y537-ERα (pERα) were assayed by western blot using total mammary gland homogenates. Photographs from one representative experiment are shown. Quantification of PgR/β-actin and pERα/β-actin ratios are shown in the lower panels. Data represent mean values ± SEM of two independent experiments, each one realized by triplicate ($p < 0.05$ vs. C; Kruskal–Wallis non-parametric analysis and Dunn's multiple comparison post test).
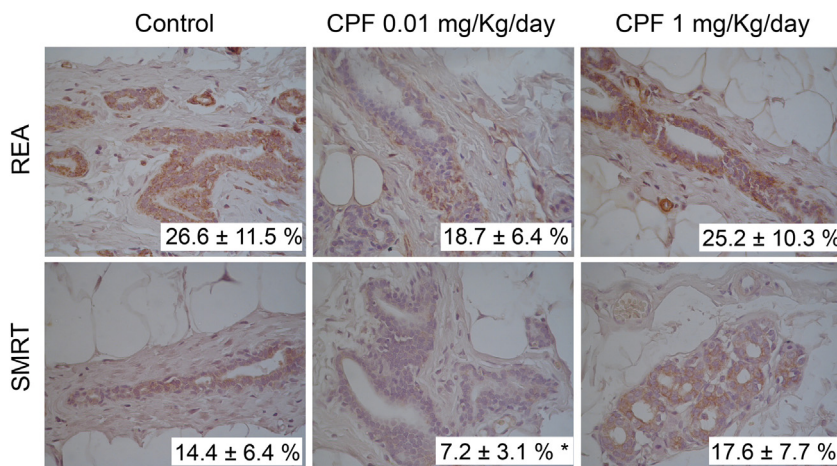
Fig. 5. CPF effect on co-repressor expression. Representative photographs illustrating REA and SMRT protein expression evaluated by immunohistochemistry in rat mammary gland exposed to CPF (0.01 and 1 mg/kg/day) or vehicle (control) during 100 days. Positive cells are observed brown stained. Magnification: 630×. White boxes show the percentage of positive cells calculated as the number of positive cells/total number of cells per field. Five randomly selected microscope fields per sample ($N = 6$) were evaluated. Data represent mean values ± SEM of two independent experiments ($p < 0.05$; Kruskal–Wallis non-parametric analysis and Dunn's multiple comparison post test).

3.4. CPF effects on estrogen and progesterone receptors expression

In order to illustrate the effect exerted by CPF on mammary tissue, we studied the expression of ERα and PgR on the mammary gland of rats exposed to vehicle or CPF (0.01 and 1 mg/kg/day) during 100 days. The expression of receptors was analyzed by immunohistochemistry and western blot. It was observed that CPF (0.01 and 1 mg/kg/day) increases the percentage of PgR+ cells in mammary gland of rats. Furthermore, the control animals presented $12.2 \pm 3.0\%$ of PgR+ cells and the percentage increased to $17.4 \pm 6.0\%$ and $19.1 \pm 7.1\%$ when they were exposed to CPF 0.01 and 1 mg/kg/day, respectively (Fig. 4). Accordingly, we observed high levels of PgR in mammary tissue homogenates of animals exposed to CPF 1 mg/kg/day, analyzed by western blot. ERα expression in rat mammary tissue did not change when animals

were exposed to the pesticide. However, the exposure of animals to CPF 1 mg/kg/day reduced phospho-Y537- ERα in the mammary gland (27% respect to the control animals) as it is shown in Fig. 4B. On the other hand, no changes were observed when using CPF 0.01 mg/kg/day (Fig. 4A and B).

3.5. CPF effect on co-repressor expression

REA and SMRT proteins contribute to the negative regulation of ERα. Since we did not observe changes on this receptor expression, we proceeded to assess the expression of co-repressors in mammary gland of rats exposed to vehicle or CPF (0.01 and 1 mg/kg/day) during 100 days. Both REA and SMRT were expressed in the epithelia of ducts and alveolar buds, with a cytoplasmic staining pattern. CPF 0.01 mg/kg/day reduced the expression of
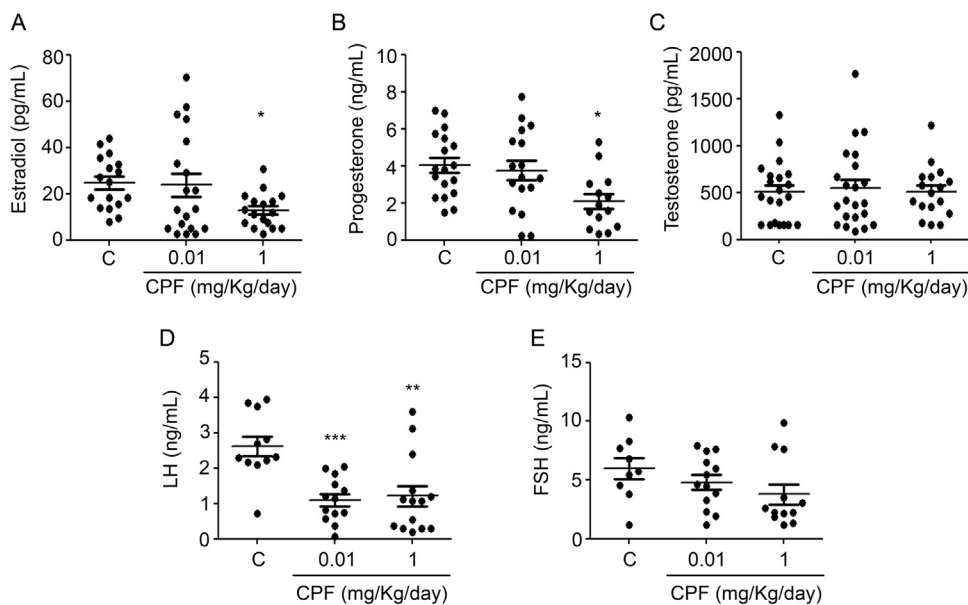
Fig. 6. CPF effects on steroid hormones and gonadotropins levels. (A) Estradiol, (B) progesterone, (C) testosterone, (D) LH and (E) FSH levels were evaluated in serum of rats exposed to CPF (0.01 and 1 mg/kg/day) or vehicle (C) during 100 days (C). The points illustrate the hormonal concentration of an individual rat. Data of three independent experiments is shown. Solid lines indicate the mean values ± SEM (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; Kruskal–Wallis non-parametric analysis and Dunn's multiple comparison post test).

Table 1
CPF effects on gonadotropin levels in ovariectomized rats. LH and FSH levels were evaluated in serum of ovariectomized rats exposed to CPF (0.01 and 1 mg/kg/day) or vehicle (C) during 10 days. Data of two independent experiments is shown. Data represent mean values $\pm$ SEM (*$p < 0.05$; Kruskal–Wallis non-parametric analysis and Dunn's multiple comparison post test).

	LH (ng/mL)	FSH (ng/mL)
Control	15.83 ± 2.75	13.31 ± 0.88
CPF 0.01 mg/kg/day	11.34 ± 2.53	17.24 ± 2.61
CPF 1 mg/kg/day	6.17 ± 1.71*	9.59 ± 1.22

REA and SMRT proteins (30.7%, p:ns, and 50%, $p < 0.05$; respectively). No changes were observed when the animals were exposed to CPF 1 mg/kg/day (Fig. 5).

3.6. CPF effect on steroid hormone and gonadotropins levels

The steroid hormone serum levels of rats exposed to vehicle or CPF (0.01 and 1 mg/kg/day) during 100 days were evaluated. As can be seen in the Fig. 6, CPF altered hormone levels in rats exposed to a dose of 1 mg/kg/day. E_2 concentration was 22.3 ± 6.3 pg/mL in serum of control animals and it was reduced significantly by CPF 1 mg/kg/day (12.8 ± 3.9 pg/mL, $p < 0.05$). The animals exposed to CPF 0.01 mg/kg/day, presented similar average level of E_2 to the control group (23.7 ± 10.9 pg/mL). However, we distinguished two populations within this later group: one presenting higher levels of E_2 than the control animals whereas the other exhibited lower levels (Fig. 6A). We have also evaluated the Pg levels in the serum of rats exposed to both doses of CPF or vehicle. Pg concentration in control animals was 4.0 ± 0.8 ng/mL and it was reduced to 2.0 ± 0.7 ng/mL ($p < 0.05$) when rats were exposed to CPF 1 mg/kg/day. We have not observed changes in Pg levels when the animals were exposed to CPF 0.01 mg/kg/day (4.2 ± 1.4 ng/mL) (Fig. 6B). Finally, no changes were observed in T concentration at any dose of pesticide assayed (Fig. 6C).

Ovarian synthesis of steroid hormones is controlled by gonadotropins LH and FSH. Circulating levels of these hormones were determined on blood serum of animals chronically exposed to CPF or vehicle (Fig. 6D and E). LH serum concentration in control animals was 2.62 ± 0.28 ng/mL and decreased to 1.10 ± 0.17 ng/mL ($p < 0.001$) and 1.20 ± 0.29 ng/mL ($p < 0.01$) after exposure to CPF 0.01 and 1 mg/kg/day, respectively. However, no changes were observed in FSH levels at any employed dose.

As we described above, CPF alters circulating levels of LH in chronically intoxicated rats. Additionally, the pesticide increases cell proliferation and PgR expression in mammary gland. Since these events are physiologically induced by estrogens, we decided to evaluate whether CPF is able to inhibit gonadotrophin releasing in ovariectomized rats like E_2. Our results demonstrated that CPF 1 mg/kg/day significantly prevents the LH increment induced by the ovariectomy, while a lower dose of the pesticide (0.01 mg/kg/day) reduces LH levels slightly (Table 1). No changes were observed in FSH levels after CPF administration in ovariectomized rats. Serum concentration of both LH and FSH of ovariectomized rats are listed in Table 1.

4. Discussion

In the present study we evaluated the effects of the pesticide CPF on mammary gland in female rats. The doses used in our experiments were 1 and 0.01 mg/kg/day according to NOAEL and ADI respectively. We demonstrated that these doses were not toxic in our experimental model. Liver weight, serum cholesterol concentration, ALAT and ASAT activities were not altered by CPF.

Furthermore, erythrocyte AChE activity, the most accepted method to evaluate OPs intoxications, was not inhibited after CPF intoxication respect to the control values. A large body of bibliography indicates that CPF induces changes in liver function. However, the doses used in those studies greatly exceeded the ones selected in our work [35–38]. We detected a significant reduction of BChE activity in plasma of animals exposed to CPF 1 mg/kg/day. This result confirms that BChE activity is more sensitive than AChE regarding the inhibition induced by CPF [4,39,40].

We have previously reported that 0.05 μM CPF promotes cell proliferation through ERα in hormone-dependent breast cancer cells MCF-7 [16]. In the present work, we demonstrate the effect of this pesticide as an ED using female rats as experimental model. We observed an increased number of ducts and alveolar structures on mammary gland of rats exposed chronically to low doses of CPF. In agreement with this, other researchers recently demonstrated that CPF 0.1 and 2.5 mg/kg increases the number of terminal end buds (TEBs), alveolar buds, the TEB diameter and the interlobular ductal thickness in the mammary glands of rats [41].

In addition, we observed an increasing incidence of benign proliferative lesions present in the mammary gland of rats exposed to the pesticide. Our results showed a high percentage of sclerosing and non sclerosing adenosis present in the breast tissue of the animals intoxicated with CPF 0.01 mg/kg/day. Several studies have shown that women with sclerosing adenosis have 1.5–2 times greater risk of developing breast cancer compared to women who do not have this injury [42–44]. We have also observed an increased percentage of moderated and florid hyperplasia in mammary gland of animals intoxicated with the pesticide. Similar results have been reported after bisphenol A (BPA) intoxication [31]. In the same way, Dolapsakis et al. [45], reported a significant increment of ductal hyperplasia on women occupationally exposed to pesticides. Finally, a recently study demonstrated a synergistic effect between the OP malathion and E_2, which indeed resulted in the presence of proliferative lesions and induction of tumors in breast tissue of animals [46]. Comparable results have been reported regarding other OPs. Malathion and parathion increased cell proliferation in the mammary gland and the development of mammary tumors after 28 months of intoxication, which were not observed in the control group [47]. Together with the morphological changes, we found an increase in the percentage of cells expressing PCNA in mammary tissue when animals were intoxicated with CPF, which confirms the proliferative effect of this pesticide.

Many studies demonstrated the role of PgR on ductal side branching and alveolar morphogenesis [48–52] and PgR involvement on paracrine induction of cell proliferation [53,54]. We found a significant increase in PgR expression levels in mammary tissue among animals exposed to CPF. However, serum levels of Pg decreased significantly due to the pesticide. PgR activation has been traditionally associated with Pg binding but this receptor may be activated by other pathways such as c-Src kinase, signaling pathways of MAPK and AKT and various growth factors secreted by fibroblasts [55]. In this sense, we observed that CPF 1 mg/kg/day induces ERK1/2 phosphorylation in the mammary gland (data not shown).

ERα is a nuclear receptor whose signaling is required for ductal elongation [56]. In our experiments, we did not observe changes in the expression of ERα, but we found a decrease in phospho-Y537-ERα levels after CPF 1 mg/kg/day treatment. Although CPF decreased ERα phosphorylation, we postulate here that this receptor is activated, since PgR expression was enhanced by the pesticide. Besides tyrosine-537 phosphorylation, ERα undergoes a hyperphosphorylation on serine residues following hormone binding, which were not evaluated in this work. Moreover, we found a reduction of SMRT and REA levels in mammary tissue of

animals exposed to CPF 0.01 mg/kg/day. These proteins are able to repress the transcription induced by ERα in the absence of ligand or presence of antagonists, such as tamoxifen [57]. Additionally, SMRT is required for full agonist-dependent ERα activation in MCF-7 cells [58]. The reduction in SMRT expression induced by CPF 0.01 mg/kg/day but not by CPF 1 mg/kg/day may be the key of the different effects produced by the pesticide according to the doses utilized. Similar results have been reported to EDs such as BPA [31]. These results indicate that CPF induces mammary gland cell proliferation and modifies both the expression and the activation of steroid receptors, altering the endocrine environment of the gland.

EDs are able to modify serum levels of hormones in exposed individuals. In this work, we analyzed steroid hormone levels after 100 days of CPF administration. Our results indicated that the CPF 1 mg/kg/day significantly reduces circulating levels of E_2 and Pg. In concordance with our results, Das et al. [59], reported a decrease in circulating levels of E_2 after CPF intoxication of rats. The authors attributed this effect to oxidative damage caused by the pesticide in the ovaries of animals. Oxidative stress induced by CPF in reproductive organs has been largely reported [41,60]. Additionally, we have previously demonstrated that CPF induce redox disbalance in MCF-7 and MDA-MB-231 cells [61].

We also observed that LH levels were significantly decreased after CPF administration. Recently, an increased oxidative damage in adenohypophysis of rats intoxicated with CPF 10.6 mg/kg/day, along with a reduction in circulating levels of LH, FSH and T were reported. Moreover, these effects were reversed after a natural antioxidant administration to the animals, indicating the importance of oxidative damage in decreasing hormone levels [62]. Finally, Gore demonstrated that CPF greatly affects GT1-7 cell morphology, and gonadotropin-releasing hormone (GnRH) gene expression and biosynthesis [63]. Additionally, 3β-HSD and CYP17 steroidogenic enzymes are positively regulated by the hormone LH [64]. These effects could be related with the decrease of E_2 and Pg levels showed in our experiments. These findings indicate that CPF has endocrine disrupting properties.

It is important to remark that many of the effects exerted by CPF are naturally induced by estradiol. Therefore, we postulate that the pesticide reproduces the estradiol induced action in a local and systemic way. To prove this hypothesis, we evaluated the gonadotrophin concentration in serum of ovariectomized rats after pesticide or vehicle administration. Our results demonstrated that both doses of the pesticide inhibited the increment of LH induced by the ovariectomy. This experiment proves that CPF presents an estrogenic action in the feedback negative regulation of pituitary LH releasing.

Our aim is to emphasize that the doses of pesticide utilized in this work are usually considered as non-toxic and safe. Taken together, our results indicate that CPF alters the endocrine environment of the mammary gland and acts as an ED in this tissue. These findings warn about the harmful effects exerted by CPF in the mammary gland development, suggesting that this toxic could act as a risk factor for breast cancer.

Acknowledgments

References

[1] S. Jergentz, H. Mugni, C. Bonetto, R. Schulz, Assessment of insecticide contamination in runoff and stream water of small agricultural streams in the main soybean area of Argentina, Chemosphere 61 (2005) 817–826, doi:http://dx.doi.org/10.1016/j.chemosphere.2005.04.036.

[2] R.M. Loewy, L.B. Monza, V.E. Kirs, M.C. Savini, Pesticide distribution in an agricultural environment in Argentina, J. Environ. Sci. Health 46 (2011) 662–670, doi:http://dx.doi.org/10.1080/03601234.2011.592051.

[3] D. Marino, A. Ronco, Cypermethrin and chlorpyrifos concentration levels in surface water bodies of the Pampa Ondulada, Argentina, Bull. Environ. Contam. Toxicol. 75 (2005) 820–826, doi:http://dx.doi.org/10.1007/s00128-005-0824-7.

[4] G. Amitai, D. Moorad, R. Adani, B.P. Doctor, Inhibition of acetylcholinesterase and butyrylcholinesterase by chlorpyrifos-oxon, Biochem. Pharmacol. 56 (1998) 293–299, doi:http://dx.doi.org/10.1016/s0006-2952(98) 00035-5.

[5] M. Marty, A. Andrus, M. Bell, J. Passage, A. Perala, K. Brzak, et al., Cholinesterase inhibition and toxicokinetics in immature and adult rats after acute or repeated exposures to chlorpyrifos or chlorpyrifos-oxon, Regul. Toxicol. Pharmacol. 63 (2012) 209–224.

[6] S.C. Gupta, M. Mishra, A. Sharma, T.G.R. Deepak Balaji, R. Kumar, R.K. Mishra, et al., Chlorpyrifos induces apoptosis and DNA damage in Drosophila through generation of reactive oxygen species, Ecotoxicol. Environ. Saf. 73 (2010) 1415–1423, doi:http://dx.doi.org/10.1016/j.ecoenv.2010.05.013.

[7] M.D. Howard, C.N. Pope, In vitro effects of chlorpyrifos, parathion, methyl parathion and their oxons on cardiac muscarinic receptor binding in neonatal and adult rats, Toxicology 170 (2002) 1–10.

[8] X. Song, F.J. Seidler, J.L. Saleh, J. Zhang, S. Padilla, T.A. Slotkin, Cellular mechanisms for developmental toxicity of chlorpyrifos: targeting the adenyl cyclase signaling cascade, Toxicol. Appl. Pharmacol. 145 (1997) 158–174, doi: http://dx.doi.org/10.1006/taap.1997.8171.

[9] D. Qiao, F.J. Seidler, T.A. Slotkin, Developmental neurotoxicity of chlorpyrifos modeled in vitro: comparative effects of metabolites and other cholinesterase inhibitors on DNA synthesis in PC12 and C6 cells, Environ. Health Perspect. 109 (2001) 909–913.

[10] E.K. Shanle, W. Xu, Endocrine disrupting chemicals targeting estrogen receptor signaling: identification and mechanisms of action, Chem. Res. Toxicol. 24 (2011) 6–19, doi:http://dx.doi.org/10.1021/tx100231n.

[11] S. De Coster, N. Van Larebeke, Endocrine-disrupting chemicals: associated disorders and mechanisms of action, J. Environ. Public Health 2012 (713696) (2012) , doi:http://dx.doi.org/10.1155/2012/713696.

[12] S. De Angelis, R. Tassinari, F. Maranghi, A. Eusepi, A. Di Virgilio, F. Chiarotti, et al., Developmental exposure to chlorpyrifos induces alterations in thyroid and thyroid hormone levels without other toxicity signs in CD-1 mice, Toxicol. Sci. 108 (2009) 311–319, doi:http://dx.doi.org/10.1093/toxsci/kfp017.

[13] S.-H. Jeong, B.-Y. Kim, H.-G. Kang, H.-O. Ku, J.-H. Cho, Effect of chlorpyrifos-methyl on steroid and thyroid hormones in rat F0- and F1-generations, Toxicology 220 (2006) 189–202, doi:http://dx.doi.org/10.1016/j.tox.2006.01.005.

[14] S.C. Joshi, R. Mathur, N. Gulati, Testicular toxicity of chlorpyrifos (an organophosphate pesticide) in albino rat, Toxicol. Ind. Health 23 (2007) 439–444. http://www.ncbi.nlm.nih.gov/pubmed/18536496.

[15] G. Viswanath, S. Chatterjee, S. Dabral, S.R. Nanguneri, G. Divya, P. Roy, Anti-androgenic endocrine disrupting activities of chlorpyrifos and piperophos, J. Steroid Biochem. Mol. Biol. 120 (2010) 22–29, doi:http://dx.doi.org/10.1016/j.jsbmb.2010.02.032.

[16] C. Ventura, M. Núñez, N. Miret, D. Martinel Lamas, A. Randi, A. Venturino, et al., Differential mechanisms of action are involved in chlorpyrifos effects in estrogen-dependent or -independent breast cancer cells exposed to low or high concentrations of the pesticide, Toxicol. Lett. 213 (2012) 184–193, doi: http://dx.doi.org/10.1016/j.toxlet.2012.06.017.

[17] I. Bernabò, L. Gallo, E. Sperone, S. Tripepi, E. Brunelli, Survival, development, and gonadal differentiation in Rana dalmatina chronically exposed to chlorpyrifos, J. Exp. Zool. A. Ecol. Genet. Physiol. 315 (2011) 314–327, doi: http://dx.doi.org/10.1002/jez.678.

[18] M.V. Maffini, B.S. Rubin, C. Sonnenschein, A.M. Soto, Endocrine disruptors and reproductive health: the case of bisphenol-A, Mol. Cell. Endocrinol. 254–255 (2006) 179–186, doi:http://dx.doi.org/10.1016/j.mce.2006.04.033.

[19] C.S. Atwood, R.C. Hovey, J.P. Glover, G. Chepko, E. Ginsburg, W.G. Robison, et al., Progesterone induces side-branching of the ductal epithelium in the mammary glands of peripubertal mice, J. Endocrinol. 167 (2000) 39–52.

[20] R.B. Clarke, Ovarian steroids and the human breast: regulation of stem cells and cell proliferation, Maturitas 54 (2006) 327–334, doi:http://dx.doi.org/10.1016/j.maturitas.2006.06.002.

[21] S.Z. Haslam, G. Shyamala, Effect of oestradiol on progesterone receptors in normal mammary glands and its relationship with lactation, Biochem. J. 182 (1979) 127–131. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1161241&tool=pmcentrez&rendertype=abstract.

[22] S. Mallepell, A. Krust, P. Chambon, C. Brisken, Paracrine signaling through the epithelial estrogen receptor alpha is required for proliferation and morphogenesis in the mammary gland, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 2196–2201, doi:http://dx.doi.org/10.1073/pnas.0510974103.

[23] A. Baniahmad, Nuclear hormone receptor co-repressors, J. Steroid Biochem. Mol. Biol. 93 (2005) 89–97, doi:http://dx.doi.org/10.1016/j.jsbmb.2004.12.012.

[24] K.M. Dobrzycka, S.M. Townson, S. Jiang, S. Oesterreich, Estrogen receptor corepressors – a role in human breast cancer? Endocr. Relat. Cancer 10 (2003) 517–536, doi:http://dx.doi.org/10.1677/erc.0.0100517.

[25] P. Mussi, L. Liao, S.-E. Park, P. Ciana, A. Maggi, B.S. Katzenellenbogen, et al., Haploinsufficiency of the corepressor of estrogen receptor activity (REA) enhances estrogen receptor function in the mammary gland, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 16716–16721, doi:http://dx.doi.org/10.1073/pnas.0607768103.

[26] U.S. National Academy of Sciences, Guide for the Care and Use of Laboratory Animals, Eighth ed., U.S. National Academy of Sciences, Washington, D.C, 2011.

[27] World Health Organization, WHO (2009). Specifications and evaluations for public health pesticides, Chlorpyrifos. Available: http://www.who.int/whopes/quality/Chlorpyrifos_WHO_specs_eval_Mar_2009.pdf.

[28] G.L. Ellman, D. Courtney, V. Andres, R.M. Featherstone, A new and rapid colorimetric determination of acetylcholineterase activity, Biochem. Pharmacol. 7 (1961) 88–95.

[29] M. Singh, J.N. McGinley, H.J. Thompson, A comparison of the histopathology of premalignant and malignant mammary gland lesions induced in sexually immature rats with those occurring in the human, Lab. Invest. 80 (2000) 221–231, doi:http://dx.doi.org/10.1038/labinvest.3780025.

[30] M. Núñez, G. Martín, C. Cocca, N. Mohamad, A. Gutiérrez, G. Cricco, et al., Effect of rosiglitazone on N-nitroso-N-methylurea-induced mammary tumors in rat, Anticancer Res. 26 (2006) 2113–2122. http://www.ncbi.nlm.nih.gov/pubmed/16827153.

[31] M. Durando, L. Kass, V. Perdomo, V.L. Bosquiazzo, E.H. Luque, M. Muñoz-de-Toro, Prenatal exposure to bisphenol A promotes angiogenesis and alters steroid-mediated responses in the mammary glands of cycling rats, J. Steroid Biochem. Mol. Biol. 127 (2011) 35–43, doi:http://dx.doi.org/10.1016/j.jsbmb.2011.04.001.

[32] C.A. Pontillo, P. Rojas, F. Chiappini, G. Sequeira, C. Cocca, M. Crocci, et al., Action of hexachlorobenzene on tumor growth and metastasis in different experimental models, Toxicol. Appl. Pharmacol. 268 (2013) 331–342, doi:http://dx.doi.org/10.1016/j.taap.2013.02.007.

[33] M. Fernández, N. Bourguignon, V. Lux-Lantos, C. Libertun, Neonatal exposure to bisphenol a and reproductive and endocrine alterations resembling the polycystic ovarian syndrome in adult rats, Environ. Health Perspect. 118 (2010) 1217–1222, doi:http://dx.doi.org/10.1289/ehp.0901257.

[34] P. Silveyra, V. Lux-lantos, C. Libertun, Both orexin receptors are expressed in rat ovaries and fluctuate with the estrous cycle: effects of orexin receptor antagonists on gonadotropins and ovulation, Am. J. Physiol. Endocrinol. Metab. 293 (2007) 977–985, doi:http://dx.doi.org/10.1152/ajpendo.00179.2007.

[35] A. Goel, D.P. Chauhan, D.K. Dhawan, Protective effects of zinc in chlorpyrifos induced hepatotoxicity: a biochemical and trace elemental study, Biol. Trace Elem. Res. 74 (2000) 171–183.

[36] A.M. Kammon, R.S. Brar, H.S. Banga, S. Sodhi, Patho-biochemical studies on hepatotoxicity and nephrotoxicity on exposure to chlorpyrifos and imidacloprid in layer chickens, Vet. Arch. 80 (2010) 663–672.

[37] P. Ma, Y. Wu, Q. Zeng, Y. Gan, J. Chen, X. Ye, et al., Oxidative damage induced by chlorpyrifos in the hepatic and renal tissue of Kunming mice and the antioxidant role of vitamin E, Food Chem. Toxicol. 58 (2013) 177–183, doi:http://dx.doi.org/10.1016/j.fct.2013.04.032.

[38] S.H. Orabi, B.E. Elbialy, S.M. Shawky, Ameliorating and hypoglycemic effects of zinc against acute hepatotoxic effect of chlorpyrifos, Global Vet. 10 (2013) 439–446, doi:http://dx.doi.org/10.5829/idosi.gv.2013.10.4.7318.

[39] D.L. Eaton, R.B. Daroff, H. Autrup, J. Bridges, P. Buffler, L.G. Costa, et al., Review of the toxicology of chlorpyrifos with an emphasis on human exposure and neurodevelopment, Crit. Rev. Toxicol. 38 (Suppl. 2) (2008) 1–125, doi:http://dx.doi.org/10.1080/10408440802272158.

[40] J. Shenouda, P. Green, L. Sultatos, An evaluation of the inhibition of human butyrylcholinesterase and acetylcholinesterase by the organophosphate chlorpyrifos oxon, Toxicol. Appl. Pharmacol. 241 (2009) 135–142, doi:http://dx.doi.org/10.1016/j.taap.2009.08.014.

[41] K. Nishi, S.S. Hundal, Chlorpyrifos induced toxicity in reproductive organs of female Wistar rats, Food Chem. Toxicol. 62 (2013) 732–738, doi:http://dx.doi.org/10.1016/j.fct.2013.10.006.

[42] M. Guray, A.A. Sahin, Benign breast diseases: classification, diagnosis, and management, Oncologist 11 (2006) 435–449, doi:http://dx.doi.org/10.1634/theoncologist.11-5-435.

[43] D.W. Visscher, A. Nassar, A.C. Degnim, M.H. Frost, R.a. Vierkant, R.D. Frank, et al., Sclerosing adenosis and risk of breast cancer, Breast Cancer Res. Treat. 144 (2014) 205–212, doi:http://dx.doi.org/10.1007/s10549-014-2862-5.

[44] R.A. Jensen, D.L. Page, W.D. Dupont, L.W. Rogers, Invasive breast cancer risk in women with sclerosing adenosis, Cancer 64 (1989) 1977–1983.

[45] G. Dolapsakis, I.G. Vlachonikolis, C. Varveris, A.M. Tsatsakis, Mammographic findings and occupational exposure to pesticides currently in use on Crete, Eur. J. Cancer 37 (2001) 1531–1536, doi:http://dx.doi.org/10.1016/s0959-8049(01)00159-9.

[46] G.M. Calaf, C. Echiburú-Chau, Synergistic effect of malathion and estrogen on mammary gland carcinogenesis, Oncol. Rep. 28 (2012) 640–646, doi:http://dx.doi.org/10.3892/or.2012.1817.

[47] G. Cabello, M. Valenzuela, a. Vilaxa, V. Durán, I. Rudolph, N. Hrepic, et al., A rat mammary tumor model induced by the organophosphorous pesticides parathion and malathion, possibly through acetylcholinesterase inhibition, Environ. Health Perspect. 109 (2001) 471–479.

[48] J.P. Lydon, F.J. DeMayo, C.R. Funk, S.K. Mani, A.R. Hughes, C.A. Montgomery, et al., Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities, Genes Dev. 9 (1995) 2266–2278, doi:http://dx.doi.org/10.1101/gad.9.18.2266.

[49] J.P. Lydon, F.J. DeMayo, O.M. Conneely, B.W. O'Malley, Reproductive phenotypes of the progesterone receptor null mutant mouse, J. Steroid Biochem. Mol. Biol. 56 (1996) 67–77, doi:http://dx.doi.org/10.1016/0960-0760(95) 00254-5.

[50] S.R. Oakes, H.N. Hilton, C.J. Ormandy, The alveolar switch: coordinating the proliferative cues and cell fate decisions that drive the formation of lobuloalveoli from ductal epithelium, Breast Cancer Res. 8 (2006) 207, doi:http://dx.doi.org/10.1186/bcr1411.

[51] W. Ruan, M.E. Monaco, D.L. Kleinberg, Progesterone stimulates mammary gland ductal morphogenesis by synergizing with and enhancing insulin-like growth factor-I action, Endocrinology 146 (2005) 1170–1178, doi:http://dx.doi.org/10.1210/en.2004-1360.

[52] M.D. Sternlicht, H. Kouros-mehr, P. Lu, Z. Werb, Hormonal and local control of mammary branching morphogenesis, Differentiation 74 (2006) 365–381, doi:http://dx.doi.org/10.1432-0436.2006.00105.x.

[53] E. Anderson, R.B. Clarke, Steroid receptors and cell cycle in normal mammary epithelium, J. Mammary Gland Biol. Neoplasia 9 (2004) 3–13, doi:http://dx.doi.org/10.1023/B:JOMG.0000023584.01750.16.

[54] R.B. Clarke, Steroid receptors and proliferation in the human breast, Steroids 68 (2003) 789–794, doi:http://dx.doi.org/10.1016/s0039-128x(03) 00122-3.

[55] S. Giulianelli, A. Molinolo, C. Lanari, Targeting progesterone receptors in breast cancer, Vitam. Horm. (2013) 161–184, doi:http://dx.doi.org/10.1016/B978-0-12-416673-8.00009-5.

[56] C. Brisken, B. O'Malley, Hormone action in the mammary gland, Cold Spring Harb. Perspect. Biol. 4 (2010) 1–15, doi:http://dx.doi.org/10.1101/cshperspect.a013086.

[57] E.K. Keeton, M. Brown, Cell cycle progression stimulated by tamoxifen-bound estrogen receptor-alpha and promoter-specific effects in breast cancer cells deficient in N-CoR and SMRT, Mol. Endocrinol. 19 (2005) 1543–1554, doi:http://dx.doi.org/10.1210/me.2004-0395.

[58] S. Peterson, M.C. Karmakar, T. Pace, C.L. Gao, The silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) corepressor is required for full estrogen receptor alpha transcriptional activity, Mol. Cell. Biol. 27 (2007) 5933–5948, doi:http://dx.doi.org/10.1128/MCB.00237-07.

[59] K. Das, K. Sarkar, P. Tarafder, P.P. Nath, G. Poul, Chlorpyrifos suppresses reproductive function in rat, Int. J. Pharm. Bio Sci. 5 (2014) 810–818.

[60] A.J. Umosen, U. Chidiebere, Effect of melatonin on chlorpyrifos-induced alterations in reproductive hormones and semen characteristics in Wistar rats, Am. J. Phytomedicine Clin. Ther. 2 (2014) 742–753.

[61] C. Ventura, A. Venturino, N. Miret, A. Randi, E. Rivera, M. Núñez, et al., Chlorpyrifos inhibits cell proliferation through ERK1/2 phosphorylation in breast cancer cell lines, Chemosphere 120 (2015) 343–350, doi:http://dx.doi.org/10.1016/j.chemosphere.2014.07.088.

[62] S. Muftau, O.O. Aisha, S.F. Ambali, I.T. Oyedepo, K.M. Umaru, P.O. Yusuf, et al., Ameliorative effect of hibiscus sabdariff a linn of subchronic chlorpyrifos-induced alterations in sex ans thyroid hormones in male wistar rats, Am. J. Pharmacol. Toxicol. 9 (2014) 96–106, doi:http://dx.doi.org/10.3844/ajptsp.2014.96.106.

[63] A.C. Gore, Organochlorine pesticides directly regulate gonadotropin-releasing hormone gene expression and biosynthesis in the GT1-7 hypothalamic cell line, Mol. Cell. Endocrinol. 192 (2002) 157–170.

[64] C. Stocco, C. Telleria, G. Gibori, The molecular control of corpus luteum formation, function, and regression, Endocr. Rev. 28 (2007) 117–149, doi:http://dx.doi.org/10.1210/er.2006-0022.