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Effects of the pesticide chlorpyrifos on breast cancer disease. Implication of epigenetic mechanisms

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ABSTRACT

Chlorpyrifos (CPF) is an organophosphorus pesticide used for agricultural pest control all over the world. We have previously demonstrated that environmental concentrations of this pesticide alter mammary gland histological structure and hormonal balance in rats chronically exposed. In this work, we analyzed the effects of CPF on mammary tumors development. Our results demonstrated that CPF increases tumor incidence and reduces latency of NMU-induced mammary tumors. Although no changes were observed in tumor growth rate, we found a reduced steroid hormone receptor expression in the tumors of animals exposed to the pesticide. Moreover, we analyzed the role of epigenetic mechanisms in CPF effects. Our results indicated that CPF alters HDAC1 mRNA expression in mammary gland, although no changes were observed in DNA methylation. In summary, we demonstrate that the exposure to CPF promotes mammary tumors development with a reduced steroid receptors expression. It has also been found that CPF affects HDAC1 mRNA levels in mammary tissue pointing that CPF may act as a breast cancer risk factor.

1. Introduction

Breast cancer is the most frequently diagnosed cancer and has the highest cancer-related mortality rate among women. In 2012, breast cancer accounted for 25.1% of all cancer cases among women´s worldwide according to the International Agency for Research on Cancer (IARC) [1]. The exposure to environmental pollutants constitutes a risk factor associated with this malignancy, because many pollutants can act as endocrine disruptors (EDs).

The influence of the estrogenic action in breast cancer disease is well known. It has been demonstrated that estradiol (E_2) induces cell proliferation in mammary gland and breast cancer cells [2–4]. In the same way, several xenobiotic compounds with estrogenic role induce cell proliferation and mammary gland alterations that may lead to breast cancer development [4–7].

Chlorpyrifos (CPF) is an organophosphorus pesticide used for pest control. Recent results of our group demonstrated that this pesticide increases the number of ducts and alveolar structures in the mammary gland of rats exposed chronically to low doses, and increases the incidence of benign proliferative lesions in the mammary gland of these animals [8]. In addition, CPF decreases circulating steroid hormones and gonadotrophins levels [8]. Moreover, in a previous report, we demonstrated that an environmentally relevant concentration of CPF induces cell proliferation through estrogen receptor alpha (ERα) in hormone-dependent breast cancer MCF-7 cells [9]. Considering these results, we postulated that the pesticide CPF acts as an ED in the mammary gland, and this effect could have an impact on breast tumorigenesis.

Epigenetic mechanisms are essential for normal development and tissue-specific gene expression, so perturbations in these processes may

Abbreviations: ADI, acceptable daily intake; ALAT, alanine amino-transferase; BChE, butyrylcholinesterase; COBRA, combined bisulfite restriction analysis; CPF, chlorpyrifos; DNMT, DNA methyltransferase; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); ED, endocrine disruptor; ERα, estrogen receptor alpha; HDAC, histone deacetylase; LINE-1, long interspersed nucleotide element; LP, latency period; NMU, N-nitroso-*N*-metilurea; NOAEL, no observed adverse effects level; PCNA, proliferating cell nuclear antigen; PgR, progesterone receptor; TI, tumor incidence

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contribute to the development of cancer [10,11]. Epigenetic changes in cancer include altered DNA methylation and histone modifications, such as histone acetylation. Altered DNA methylation has been found in breast cancer related genes, such as BRCA1, even in the absence of traditional genic mutations [12]. Similar results were reported for cell cycle regulating proteins like p27 [13]. Histone acetylation constitutes another important epigenetic mechanism in the control of gene expression. This process is performed by histone acetyltransferase enzymes, while acetyl group is removed by histone deacetylases (HDACs) enzymes. Many HDACs have been found overexpressed in breast cancer, particularly HDAC1 which is able to modify ERα regulation [14–17].

Epigenetic changes have been related to environmental pollutants, such as pesticides and EDs in mammary gland and breast tumors [18–20]. In order to investigate if CPF modifies the development of breast cancer, we studied different parameters of tumor growth, hormone receptor status, as well as epigenetic alterations in an experimental mammary tumor model induced in female rats.

2. Materials and methods

2.1. Animals

Experimental protocols were performed in accordance with the Guide for the Care and Use of Laboratory Animals issued by the U.S. National Academy of Sciences [21] and approved by the ethical committee of the School of Pharmacy and Biochemistry, University of Buenos Aires. Virgin female 40-days-old Sprague-Dawley rats were housed in stainless steel cages with water and food *ad libitum*, a temperature of 22 ± 2 °C, humidity around 56% and a 12 h light-dark cycle. Rats were randomly separated in three groups with 6 animals each one. Two independent experiments were performed.

2.2. Tumor induction

Mammary tumors were induced by three intraperitoneal (i.p.) injections of N-nitroso-*N*-methylurea (NMU). The carcinogen was injected at doses of 50 mg/kg body weight when animals were 50, 80 and 110 days old [22,23]. Rats were palpated three times per week to monitor mammary tumor appearance.

2.3. Chlorpyrifos exposure

Chlorpyrifos (99.5% purity) was purchased from Chem. Service, Inc., West Chester, PA, USA. CPF administration was performed as previously reported [8]. Briefly, pesticide dilutions were made in castor oil (*Ricinus communis*) and daily orally administered, starting at 40 days old during 150 days. For tumor evaluation experiments, the experimental groups were: 1) Castor oil (vehicle) (v.o) + NMU (i.p.); 2) CPF 0.01 mg/Kg/day (v.o) + NMU (i.p.); 3) CPF 1 mg/Kg/day (v.o) + NMU (i.p.). For mammary gland determinations, experimental groups were: 1) Castor oil (v.o.), 2) CPF 0.01 mg/Kg/day (v.o.); 3) CPF 1 mg/Kg/day (v.o.). CPF 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. We previously reported that the doses selected for the experiments were not found to significantly affect the general health status of the animals [8].

2.4. Tumor development evaluation

To evaluate mammary tumor development, the latency period (LP), tumor incidence (TI) and the number of tumors per rat (n/r) were determined. LP was defined as the number of days between the first NMU injection and the appearance of the first tumor in each rat. TI was defined as the percentage of rats that developed at least one tumor. Finally, n/r is the average number of tumors developed per rat.

2.5. Tumor doubling time

Tumor growth was determined collecting the longest two diameters of each tumor (d_1 and d_2) using a manual caliper, three times per week for 150 days. The volume of each tumor (Vt) was calculated using the formula: Vt [cm^3] = $4/3\pi$ x r[cm]^3, where r is calculated as [(d_1 + d_2)/2]/2. Tumor doubling times were obtained by nonlinear regression of tumor growth rate fitting to an exponential growth equation using the software GraphPad Prism version 7.00™.

2.6. Sample collection

All samples were collected during oestrous cycle phases determined by microscopic evaluation of the types of cells present in the vaginal smears collected every day for at least two weeks. Serum was stored at −80 °C until biochemical determinations. Mammary tumors were removed and representative specimens for each tumor were preserved in 10% (v/v) formaldehyde buffer and embedded in paraffin; 3–4 μm slices were stained with hematoxylin-eosin for microscopic examination. Fragments from mammary tissue of non NMU-injected rats and mammary tumor of NMU-injected rats were stored at −80 °C until RNA and DNA extractions were performed.

2.7. Cholinesterase activity

Butyrylcholinesterase (BChE) was assayed in animals' serum. 20 mL of serum were added to a final volume of 3 mL containing 0.25 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and 16.3 mM butyrylcholine as a substrate. Enzyme activity was measured using absorbance at 405 nm (ε = 13,600 mM^{-1} cm^{-1}) every 0.5 min for 1.5 min and expressed as U/L.

2.8. Biochemical parameters

Serum alanine amino-transferase (ALAT) was 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.

2.9. Immunohistochemical assay

Protein expression was detected by immunohistochemical assay using rabbit anti-ERα (1:50, Santa Cruz Biotechnology, USA), rabbit anti-PgR (1:50, Santa Cruz biotechnology, USA), and mouse anti-PCNA (1:100, Dako Cytomation, Denmark) specific antibodies as previously described [8]. Positive cells were visualized in brown. The percentages of positive cells were calculated as the number of brown nuclei cells/number of total cells per field. The staining intensity was not taken into account.

2.10. RNA isolation and cDNA synthesis

Total RNA was isolated using TRIZOL reagent (Life Technologies, USA) and quantified by measuring optical densities using NanoDrop™ spectrophotometer (Thermo Science, USA). cDNA was synthesized from RNA template (2 μg) via reverse transcription using random hexamer primers and MMLV Reverse Transcriptase (Promega Corporation, USA) in a final volume of 20 μl, according to manufacturer's instructions.

2.11. Real time PCR

Quantitative real-time PCR (qPCR) was performed by triplicate employing the Applied Biosystems 7500/7500 Fast Real-Time PCR

Table 1
List of Primers Sequences used for RT-qPCR.

	Primer sequence (5'-3')	Reference
HDAC1	F-AATGCTAATGTTGGGAGG R-ATTGGAAGGGCTGATGTG	[24]
ERS1	F-CGTTTCAGGGATTCGCAGAA R-TTCCCCAACACCATCTGAGAA	[25]
PgR(A + B)	F-GGTCTAAGTCTCTGCCAGGTTTCC R-CAACTCCTTCATCCTCTGCTCATTC	[26]
PgRB	F-GCATCGTCTGTAGTCTCGCCAATAC R-GCTCTGGGATTTCTGCTTCTTCG	[26]
GAPDH	F-TGCACCACCAACTGCTTAG R-GGATGCAGGGATGATGTTC	[27]

System (Life Technologies Corporation, USA) using HOT FirePol Eva Green qPCR mix according to manufacturer's instructions (Solis Biodyne, Estonia). Primer sequences are listed in Table 1. 0.1 µL of cDNA was used as template. The cDNA was amplified by 40 cycles of denaturation (30 s at 95 °C), annealing (60 s at 60 °C), and extension (60 s at 72 °C) steps. The specificity of each primer set was monitored by analyzing the dissociation curve. Relative mRNA quantification was performed by ΔΔCt method using GAPDH as the housekeeping gene.

2.12. DNA isolation

50 mg of mammary tissue were incubated with digestion buffer (50 mM Tris/HCl, pH = 8, 100 mM NaCl, 1% SDS, 50 mM EDTA) and proteinase K (150 µg/mL) 2 h at 55 °C, followed by 18 h at 37 °C. Then, 5 M LiCl (300 µL) were added. Samples were mixed by inversion during 1 min and chloroform:isoamyl alcohol (24:1) solution was added. Samples were incubated at room temperature 30 min and centrifuged at 18,000 x g for 15 min. Pellets were washed with absolute and 70% ethanol prior their dissolution in TE buffer (10 mM Tris, 1 mM EDTA pH = 8). DNA concentration was quantified by measuring optical densities using NanoDrop™ spectrophotometer (Thermo Science, USA).

2.13. CpG methylation in the CDKN1B and BRCA1 promoter regions

CpG methylation in the CDKN1B and BRCA1 promoter regions were analyzed by Methylation Specific PCR (MSP) method. Briefly, DNA (1 µg) was subjected to bisulfite modification using the EpiJET Bisulfite Conversion Kit (Life technologies, USA) according to manufacturer's instructions. 40 ng of bisulfite-modified DNA was used as template for PCR reactions using specific primers to study methylated and unmethylated CpG regions on CDKN1B and BRCA1 gene promoter regions (Table 2). The PCR amplified products were separated on a 4% of NuSieve® 3:1 agarose gel and visualized using GelRed™ (Biotium, Inc., USA) staining.

Table 2
List of Primer Sequences used for MSP and COBRA assays.

	Primer sequence (5'-3')	Ta (°C)	bp	Reference
CDKN1B-Met	F-AGTATATTTGATTATTGAAGTTTCGA R-AATTCTACGACTACACACAAAAACG	57	145	[29]
CDKN1B-Unmet	F- AGTATATTTGATTATTGAAGTTTTGA R-TTCTACAACTACACACAAAAACAAC	55	143	
BRCA1-Met	F- GCGAGAAGGTTTTTGTTGTATC R- ACCAATTCCAACATACATTACG	55	142	[12]
BRCA1-Unmet	F-GTGAGAAGGTTTTTGTTGTATT R- CCAATTCCAACATACATTACA	55		
LINE-1	F-TTTGGTGAGTTTGGGATA R- CTCAAAAATACCCACCTAAC	55	163	[28]

2.14. CpG methylation in the LINE-1 promoter region

LINE-1 promoter region methylation was evaluated by COBRA assay [28]. Briefly, DNA was first modified using a bisulfite conversion method and then a 163-bp fragment corresponding to the LINE-1 promoter region was amplified (Table 2). Subsequently, the amplicons were digested with RsaI enzyme according to manufacturer's instructions. The digested PCR products were electrophoresed in 4% NuSieve® 3:1 agarose gels stained with GelRed™. Band intensities were obtained using ImageJ 1.32 J software (NIH, Bethesda, MD, USA). The relative LINE-1 methylation level was calculated as follows: digested amplicons (115- and 48-bp bands) divided by undigested/total DNA amplicons (163-, 115- and 48-bp bands), see Fig. 6D. The fraction of methylation can vary between 0 and 1.0 (corresponding to 0–100% of methylated DNA).

2.15. Statistical analysis

Statistical methods are included in the figure legends. Data were analyzed using GraphPad Prism 7.00™ (GraphPad Software Inc., USA) and InfoStat (InfoStat version 2016. InfoStat Group, Argentina) softwares. A complete block design was performed when data of at least two independent experiments were included in the statistical analysis. p values less than 0.05 were considered statistically significant.

3. Results

3.1. CPF effect on cholinesterase activity and liver function of NMU-injected rats

We have previously reported that no significant signs of intoxication were found in rats chronically exposed to CPF at 0.01 and 1 mg/Kg/day [8]. To evaluate the toxicity of CPF on NMU-injected rats, plasmatic cholinesterase and ALAT activities were assayed. Both BChE and ALAT constitute liver damage indicators. Additionally, BChE is the most sensitive method to evaluate organophosphorus intoxications. As Fig. 1 shows no significant changes were observed in these enzyme activities after CPF administration, demonstrating that the doses employed in our study were not hepatotoxic in our experimental model (Fig. 1).

3.2. CPF effect on parameters of tumor development

The effect of CPF on mammary carcinogenesis was evaluated by determining TI, LP and n/r, in rats which received the pesticide in comparison with the animals which have received the vehicle only. As shown in Fig. 2A, CPF significantly increased TI ($p < 0.05$). After 110 days after first NMU injection, 82.2 ± 2.8% of the animals exposed to 0.01 mg/Kg/day of CPF and 79.1 ± 4.1% of 1 mg/Kg/day CPF administered animals had developed tumors. However, only 35.0 ± 15.2% of the control group had developed tumors at the same time. At the end of the experiment, TI was not found significantly different among the groups. LP was also analyzed. As Fig. 2B shows, both doses of CPF significantly reduced this parameter in comparison with the control group ($p < 0.05$). Furthermore, rats exposed to 1 mg/Kg/day CPF showed a higher number of tumors per rat compared to the control group (Fig. 2C). At the end of the experiment, 1 mg/Kg/day CPF-exposed animals presented 2.8 ± 0.5 tumors per animal, while this parameter was 1.5 ± 0.5 in the control group though these differences were not statistically significant.

3.3. CPF effect on tumor growth

Tumor growth was evaluated by tumor doubling time and the proliferating cell nuclear antigen (PCNA) tumor expression. Fig. 3A shows that tumor doubling time was not significantly affected by any dose of CPF. This result was confirmed by the analysis of PCNA

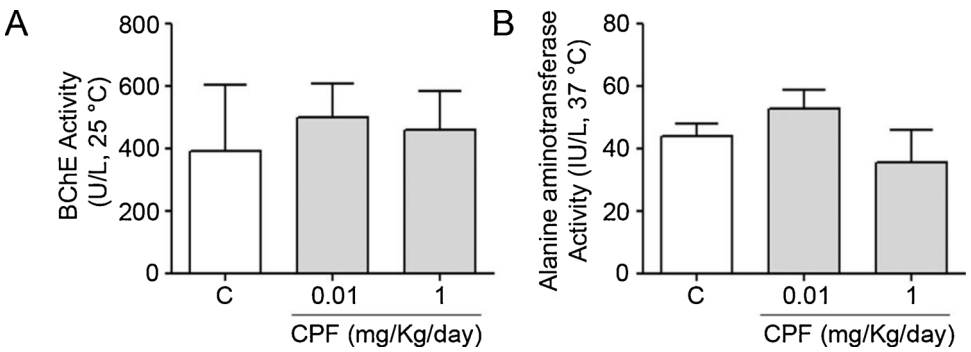


Fig. 1. CPF effects on cholinesterase activity and liver function. A) BChE and **B)** ALAT activities were determined in serum of animals following CPF (0.01 and 1 mg/Kg/day) or vehicle (C) administration over 150 days. Graphs show the mean values ± SEM of two independent experiments (N = 6) (p:ns; Kruskal-Wallis non-parametric analysis).

expression, which was not affected by the pesticide treatment (Fig. 3B).

3.4. CPF effect on steroid receptor expression in mammary tumors

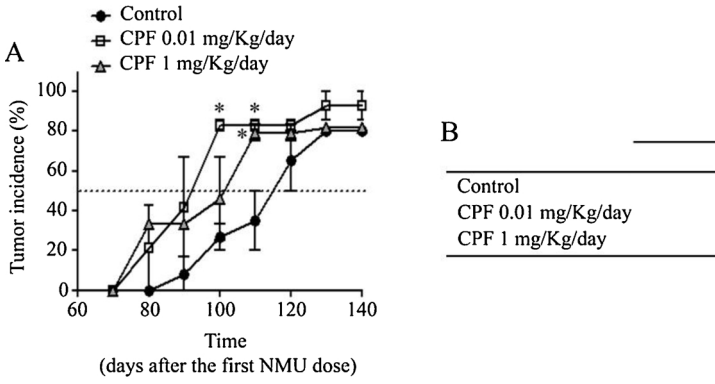
In order to analyze the effect exerted by CPF on ERα and progesterone receptor (PgR) status in the NMU-induced tumors developed in animals exposed to CPF (0.01 and 1 mg/kg/day) or vehicle during 150 days, the expression of steroid hormone receptors was analyzed by immunohistochemistry. It was observed that CPF (0.01 and 1 mg/kg/day) decreased the percentage of ERα and PgR positive cells. In this way, rat tumors exposed to the vehicle presented 10.2 ± 2.5% of ERα positive cells. However, this percentage was reduced in tumors of animals exposed to 0.01 mg/Kg/day (7.3 ± 0.7%, p:ns) and 1 mg/Kg/day (4.7 ± 0.9%, $p < 0.05$) of CPF. A tendency for decreased expression was also found in PgR, whose expression was 15.4 ± 4.2%, 9.4 ± 2.6% and 8.3 ± 3.0% in control, 0.01 and 1 mg/Kg/day CPF treated animals, respectively (p:ns) (Fig. 4).

These results were confirmed by quantification of ERα (ERS1) and PgR (PgR A + B and PgRB) mRNA levels in the mammary tumors of rats exposed to the pesticide or vehicle. As Fig. 5 shows, CPF slightly reduces ERS1 mRNA level, achieving a decrease of 40.3 and 35.8% (p:ns) in rats exposed to 0.01 and 1 mg/Kg/day of CPF, respectively. Although CPF exposure did not alter PgR (A + B) mRNA levels in mammary tumors, we observed that PgRB mRNA levels show a tendency to decrease in the animals that were exposed to CPF 0.01 and 1 mg/Kg/day (55.1 and 48.5% respectively, p:ns).

3.5. CPF effect on epigenetic regulation

Hypermethylation of tumor suppressor gene promoters is an important mechanism implicated in carcinogenesis. Therefore, we analyzed the effects of CPF on CpG methylation status for CDKN1B and BRCA1 promoters in mammary tissue using methylation specific PCR method [12,29]. As Fig. 6 shows, CpG methylation status was not altered by any dose of the pesticide neither in CDKN1B or BRCA1 promoter region (Fig. 6A and B). Because we did not observe alterations on CpG methylation of specific tumor suppressor gene promoters, we decided to evaluate the genome-wide methylation by determination of the CpG methylation status in the long interspersed nucleotide elements, LINE-1 [28]. This parameter was also unaffected in the mammary gland following the chronic exposure to the pesticide (Fig. 6C and D). Although mammary tumors presented a slight non-significant increment in LINE-1 methylation compared to the normal mammary tissues (Fig. 6C), it represents a large number of copies affected.

Covalent modification of histone is another mechanism of epigenetic regulation related to cancer. As we did not observe alterations on



B

	Latency period (days)
Control	111.6 ± 3.7
CPF 0.01 mg/Kg/day	96.3 ± 7.5*
CPF 1 mg/Kg/day	97.0 ± 5.7*

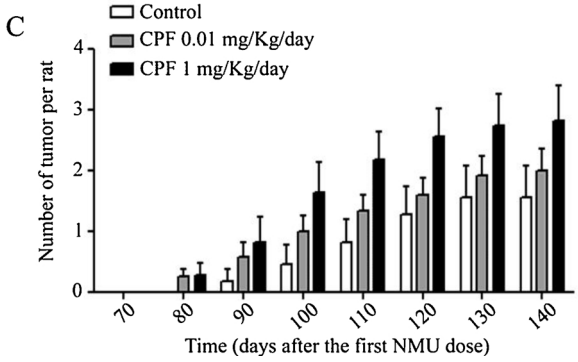


Fig. 2. CPF effects on tumor development parameters. A) The graph shows the TI of each experimental group vs. time (days after the first NMU dose). The intersection of each curve and the dotted line indicates the time at which 50% of animals had at least one tumor. Data are the mean values ± SEM of two independent experiments (N = 6) (*$p < 0.05$ vs. C at the corresponding time. Kruskal-Wallis non-parametric analysis and Dunn Multiplex comparison post test). **B)** The table shows the LP for the three experimental groups. Data are the mean values ± SEM of two independent experiments (N = 6) (*$p < 0.05$; Kruskal-Wallis non-parametric analysis and Dunn Multiplex comparison post test). **C)** The graph shows the mean number of tumor per rat of each experimental group (N = 6) vs. time (days after first NMU dose). Data are the mean value ± SEM of two independent experiments (p:ns; Two-way ANOVA).

A

	Tumor doubling time (days)
Control	7.77 ± 1.66
CPF 0.01 mg/Kg/day	6.47 ± 0.89
CPF 1 mg/Kg/day	7.58 ± 0.98

B

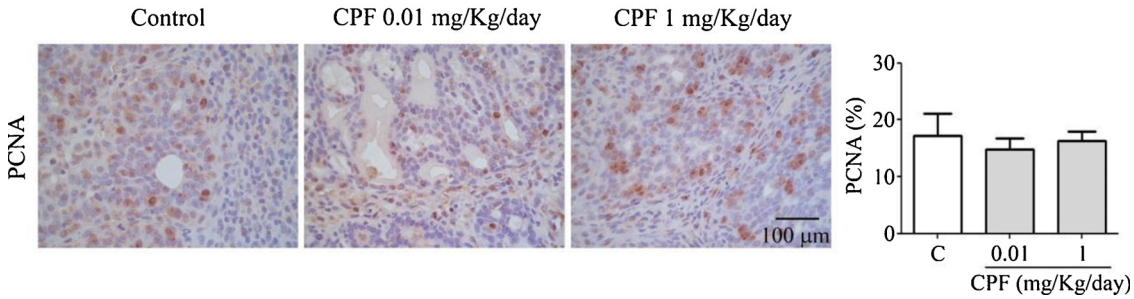


Fig. 3. CPF effects on tumor growth. A) Tumor doubling time was determined as described in Material and Methods section. Data represent mean values ± SEM of two independent experiments (N = 12–18) (p:ns; Two-way ANOVA). **B)** Representative images and graphical representation of the immunohistochemical detection of PCNA in tumoral tissue of rats exposed to CPF (0.01 and 1 mg/Kg/day) or vehicle (Control) during 150 days. The PCNA-positive nuclei are stained in 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. Five randomly selected microscope fields per sample were evaluated (N = 12–18). Data represent mean values ± SEM of two independent experiments (p:ns; One-way ANOVA).

DNA methylation after CPF exposure, we analyzed histone acetylation in mammary gland of the animals by quantification of HDAC1 mRNA levels. As Fig. 7 shows, 0.01 mg/Kg/day CPF-treated animals presented an increment of 55% in the expression of this enzyme in mammary tissue ($p < 0.001$).

4. Discussion

CPF is one of the most used pesticides in agricultural activity. We have previously demonstrated that this pesticide may act as an ED *in vitro*, by inducing the proliferation of estrogen dependent MCF-7 breast cancer cells [9]. More recently, we have reported that CPF is able to change the mammary tissue architecture and hormonal balance in chronically exposed rats, showing the susceptibility of the gland to this pesticide [8]. In this work, we evaluated the effects of environmental relevant doses of CPF in an experimental breast cancer model and in mammary gland of rats. CPF doses were selected taking into account the ADI and NOAEL limits. It can be considered that ADI levels are those 'without appreciable risk', and both, ADI and NOAEL are periodically monitored by international organizations with the aim of ensuring safety, minimizing the risk of chemical exposure. However, our results demonstrated that ADI limit use does not imply absolute safety. To know if the pesticide could affect mammary carcinogenesis, we evaluated the CPF effects using an experimental breast cancer model in female rats. Our results demonstrated that CPF exposure did not affect biochemical parameters such as ALAT and BChE in NMU-injected rats.

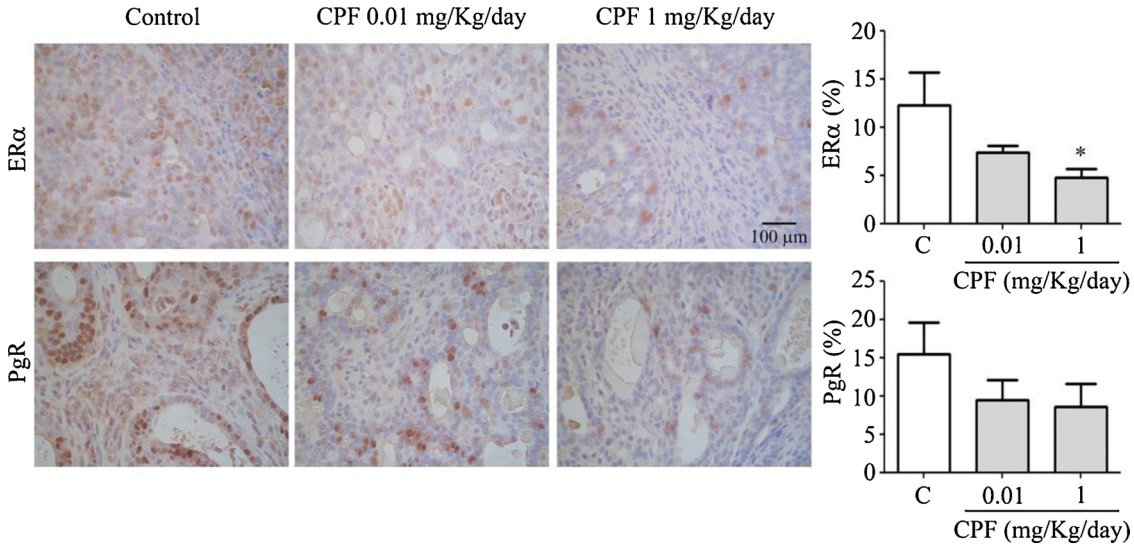


Fig. 4. CPF effect on Estrogen and Progesterone receptors expression. Representative images illustrating ERα and PgR expression evaluated by immunohistochemistry in tumor tissue of rats after 150 days of exposure to CPF (0.01 and 1 mg/Kg/day) or vehicle (Control). Positive nuclei are observed brown stained. Magnification: 630 × . Percentages of positive cells were calculated as the number of positive cells/total number of cells per field. A distribution that ranged from 20 to 200 positive nuclei per field was observed. Five randomly selected fields per sample were evaluated by microscopy (N = 12–18). Data are mean ± SEM of two independent experiments (*$p < 0.05$; One-way ANOVA and Dunnett post test).

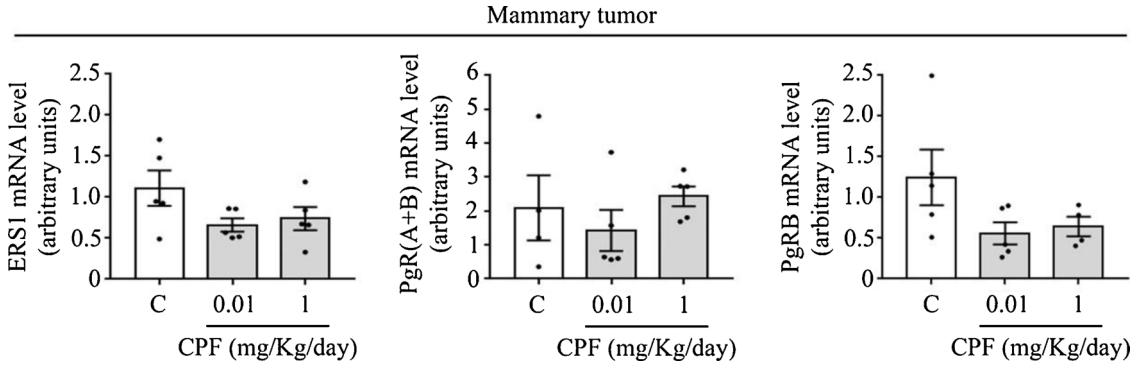


Fig. 5. CPF effect on Estrogen and Progesterone receptors mRNA levels. ERS1, PgR (A + B) and PgRB mRNA levels were quantified by qPCR on mammary tumors of rats exposed to CPF (0.01 and 1 mg/Kg/day) or vehicle (C) over a period of 150 days. Data represent mean values ± SEM (N = 5). The points illustrate the mRNA level of each individual rat analyzed (p:ns; One-way ANOVA).

Both ALAT and BChE are liver damage indicators, and BChE is also the most sensitive method to evaluate organophosphorus intoxications. In this sense, it is worth noting that CPF effects on breast cancer disease reported in this work can be present even in the commonly considered sub-toxic exposures.

Previous studies performed in NMU-induced mammary tumorigenesis demonstrated that NMU-induced tumors are strongly regulated by hormones such as E_2 and prolactin, as well as by growth factors [22,30,31], making it a suitable experimental model to evaluate the action of putative EDs such as CPF. In this work, we reported an increment in tumor incidence in animals exposed to this pesticide. It is important to note that, at the end of the experiment, all experimental groups achieved about 80% of tumor incidence, which is a characteristic of the experimental model used in this work. We have also observed a decrease in the latency tumor period and a slight increment in the number of tumors per rat when the animals were exposed to CPF. Similar results have been found using bisphenol A (BPA), a well-recognized ED, which reduced the latency period and increased the number of tumors per rat [32,33]. It was also reported that BPA is able to increment cell proliferation, triggering diverse signaling pathways such as Akt and c-Src in mammary tissue [33]. Although we observed a reduced latency period, the pesticide did not alter tumor growth parameters, such as the tumor doubling time and PCNA expression, when the tumors have been installed. Taken together, our results indicate that the effects of CPF on mammary tumorigenesis are fundamentally related to the initiation phase of this malignancy, while it does not seem to alter the subsequent tumor progression. CPF effects on carcinogenesis initiation could be related to the increment of benign proliferative lesions in the mammary gland, a finding that we had previously reported in rats [8]. In the same way, the organophosphorus pesticides parathion and malathion have been found to promote cell proliferation in the mammary gland of rats, resulting in an increased mammary carcinogenesis after 28 months of exposure, which was not observed among the vehicle-exposed animals [34]. Similar results were observed after E_2 administrations, supporting the possible role of those pesticides as EDs [35].

Breast cancer classification is usually performed on the basis of histopathological, molecular and genetic parameters, being ERα, PgR

A

	Mammary gland									
	Control			CPF 0.01 mg/Kg/day			CPF 1 mg/Kg/day			
	U	M/U	M	U	M/U	M	U	M/U	M	p
CDKN1B	1/6	4/6	1/6	2/6	3/6	1/6	2/5	2/5	1/5	0.907
BRCA1	1/3	2/3	0/3	0/4	4/4	0/4	0/3	2/3	1/3	0.823

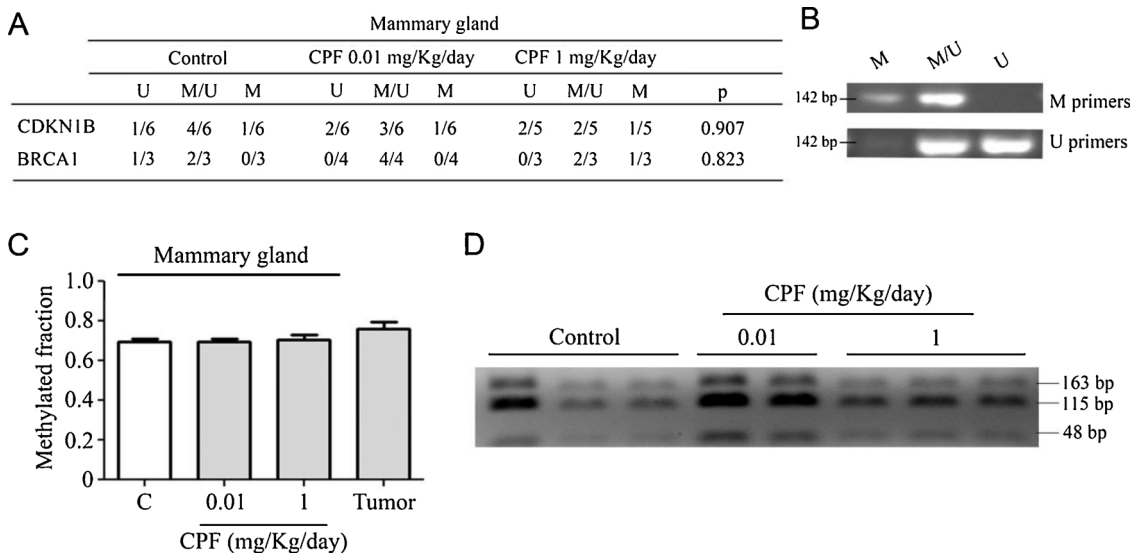


Fig. 6. CPF effect on DNA methylation. A) CpG methylation status of the CDKN1B and BRCA1 promoter regions in mammary gland was assayed by MSP. The table shows the frequency of completely unmethylated (U), methylated and unmethylated (M/U), and completely methylated (M) samples for each experimental group. Frequencies were expressed as the number of samples amplified for each primer set/total number of samples. Furthermore, the error probability (p) calculated by performing a Fisher test in a 3 × 3 contingency table for each gene is shown. **B)** Representative PCR reaction products showing CDKN1B promoter completely methylated (M), methylated and unmethylated (M/U), and completely unmethylated (U) are shown. Each sample was amplified using specific primer sets for methylated (M primers) or unmethylated (U primers) CpG. **C)** The graph shows the methylated fraction of LINE-1 retrotransposon for each experimental group. Methylated fraction was calculated by densitometric quantification of methylated DNA (48 + 115 bp)/total DNA (48 + 115 + 163 bp) in each street. Data are mean ± SEM of two independent experiments (N = 6) (p:ns. One-way ANOVA). **D)** Representative photograph showing the restriction fragments after COBRA assay.

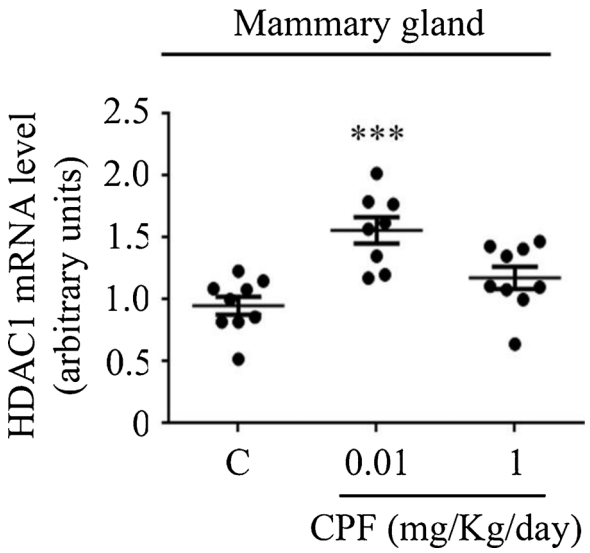


Fig. 7. CPF effect on histone acetylation. HDAC1 mRNA levels were quantified by qPCR on mammary gland tissue of rats exposed to CPF (0.01 and 1 mg/Kg/day) or vehicle (C) over a period of 150 days. The points illustrate the mRNA level of each individual rat analyzed Solid lines indicate the mean values ± SEM (N = 9). ($p < 0.001$ vs. C; One-way ANOVA).

and HER2 patterns expression essential to define the therapy [36–38]. In this sense, the worst prognoses correspond to basal type tumors, which do not express ERα, PgR and HER2 [38–40]. Here, we demonstrate that CPF exposure decreases ERα expression and slightly decreases PgR expression in mammary tumors of rats. Although diverse epidemiologic studies have evaluated a possible correlation between pesticide exposure and steroid hormone receptor expression, the results remain controversial [41,42].

Many environmental pollutants induce cancer by altering epigenetic regulation, particularly those which behave as EDs [19,43–47]. Epigenetic mechanisms include DNA methylation, histone covalent modification and microRNA regulation. DNA methylation process has been extensively studied and it is known that this mechanism plays a key role in various types of cancer [48–50]. Moreover, a recent article alerts about the effects of the EDs on DNA methylation in breast cancer [18]. In particular, it has been reported that CPF alters the methylation status of the tumor suppressor gene H19 [51]. In this work, we analyzed the effect of a chronic exposure of laboratory rats to environmentally relevant doses of CPF on CpG methylation of CDKN1B and BRCA1 promoters. Both genes are involved in breast cancer disease, and their epigenetic modulation has been related to contaminants exposure. CDKN1B is a key gene that regulates cell cycle progression, and we have previously demonstrated that p27 protein expression is altered by the ED HCB in breast cancer [52]. Additionally, it has been reported that BRCA1 promoter region is hypermethylated in the presence of an agonist of the aryl-hydrocarbon receptor (AhR) [12]. However, our results indicate that CPF does not significantly alter CDKN1B and BRCA1 promoter methylation.

In addition to the hypermethylation of the tumor suppressor gene promoter, a global genomic hypomethylation has been related to cancer progress [48,53,54]. In the present work, we analyzed this parameter by quantifying LINE-1 promoter methylation status [28,55], observing no differences between the samples of rats exposed to the pesticide and the control group. We have also compared LINE-1 promoter methylation status in the mammary gland *versus* mammary tumors. It is important to note that although we did not observe significant differences, the slight increase in this parameter could represent a relevant change in methylation level. It is due to the large number of copies of LINE-1 per genome, which is greater than 500,000–600,000 copies, representing a 15–20 % of the genome [56]. In this context, the slight difference observed between mammary gland and mammary tumor tissue represents around of 25,000 methylated copies, which means a 0.75% of the genome. Even though carcinogenesis has been associated to LINE-1 promoter hypomethylation status, it has been reported that colorectal tumors show a variable grade of LINE-1 promoter methylation, it has been found that a fraction of tumor samples studied present a higher LINE-1 promoter methylation than the adjacent healthy tissue [57].

On the other hand, we studied the effects of CPF on covalent histone modifications, particularly, histone acetylation. We found an increased expression of HDAC1 enzyme in the mammary gland of animals exposed to the pesticide. Many studies have reported that HDAC1 may be recruited to the ERα promoter in breast cancer cells, which conduces to a decreased expression of the receptor [16,58–60]. In the same way, overexpression of HDAC1 in the human breast cancer cell line MCF-7 decreases the expression of ERα and increases clonogenic capacity [15]. Interestingly, both ERα transcriptional repression and clonogenic capacity are also induced by CPF [8,9]. Moreover, it has been reported that the co-repressors SMRT and N-CoR contain repression domains associated with histone deacetylases, which could be related to gene repression associated to histone hypoacetylation [61,62]. It this sense, SMRT expression was significantly reduced by CPF 0.01 mg/Kg/day in mammary gland of rats [8]. Moreover, HDAC1 is induced by hypoxia [63], which is also associated to CPF exposure in breast cancer cell lines [9,64]. In summary, we postulate that increased expression of HDAC1 induced by the pesticide could be related to the alterations of mammary gland histology previously reported by our group, which ends up in a major mammary tumors development.

Altogether, these results strongly suggest that CPF exposure, even at sub-toxic doses, constitutes a risk factor for breast cancer disease. This effect could be related to the endocrine disruption produced by the pesticide, as well as epigenetic alterations such as promotion of HDAC1 expression.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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