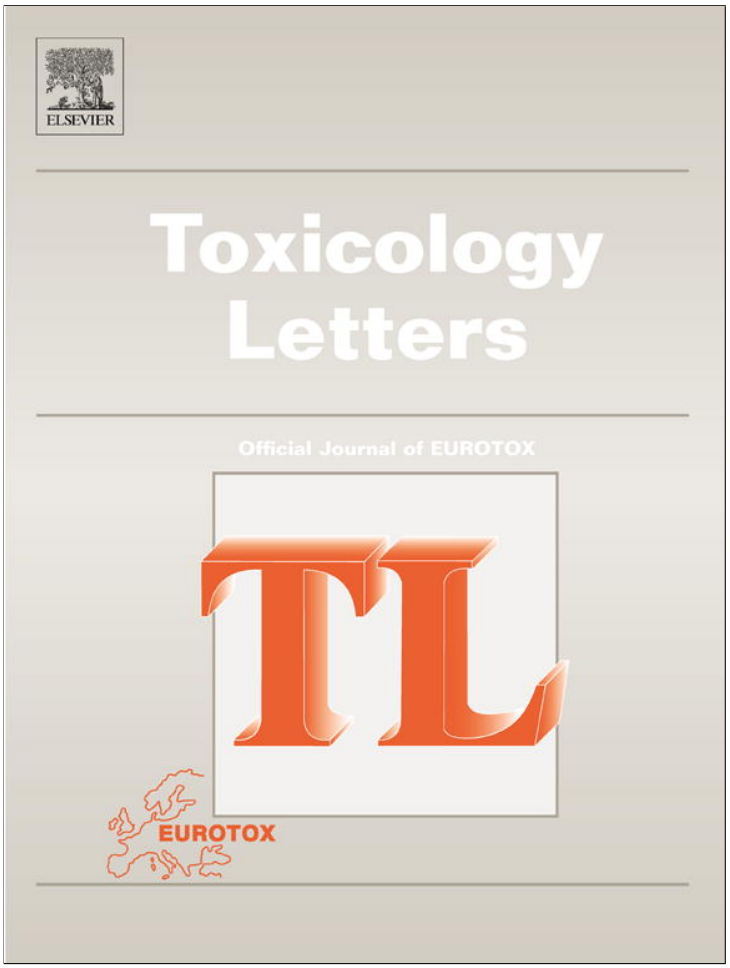

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Differential mechanisms of action are involved in chlorpyrifos effects in estrogen-dependent or -independent breast cancer cells exposed to low or high concentrations of the pesticide

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HIGHLIGHTS

- CPF is a pesticide that is widely used for insect control worldwide.
- CPF effects on cell proliferation and the role of ERα on this action was investigated in breast cancer cell lines.
- Environmental and higher concentrations of CPF were assayed.
- Dose as low as 0.05 μM of CPF induced proliferation through ERα in MCF-7 cells.
- High doses of CPF induced cell cycle arrest through changes in redox balance.

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ABSTRACT

It has reported that many environmental compounds may display estrogenic actions and these findings led to researchers to associate breast cancer risk with the use of some pesticides. The aim of this work was to investigate the effect of chlorpyrifos (CPF) on cell proliferation and the ERα-dependence of this action employing MCF-7 and MDA-MB-231 breast cancer cell lines. We have also analyzed CPF action on the cell cycle distribution and the cyclins that are implicated in G1-S and intra-S checkpoints. Finally, the action on cell death and ROS production were studied. We demonstrated the ability of CPF 0.05 μM to induce cell proliferation through ERα in hormone-dependent breast cancer cells. In contrast, CPF 50 μM induces intra-S arrest modifying checkpoints proteins, through a mechanism that may involve changes in redox balance in MCF-7. In MDA-MB-231, we have found that CPF 50 μM produces an arrest in G2/M phase which could be related to the capacity of the pesticide for binding to tubulin sites altering microtubules polymerization. Altogether, our results provide new evidences on the action of the pesticide CPF as an environmental breast cancer risk factor due to the effects that causes on the mechanisms that modulate breast cell proliferation.

1. Introduction

Organophosphate pesticides (OPs) comprise a group of toxic substances that are used in agriculture for insect and plague control. The use of insecticides represents the main environmental risk due to the high mass of product annually applied affecting aquatic species and human health (Anguiano and Pechen de D'Angelo, 2007; Loewy et al., 2011). Chlorpyrifos (CPF) is a broad spectrum organophosphate pesticide. The primary target of CPF toxicity is the central and peripheral nervous system, due to its ability to inhibit the acetylcholinesterase activity (Eaton et al., 2008). It has been suggested that alternative mechanisms of action for CPF could potentially contribute to toxic effects including cytotoxicity, effects

Abbreviations: AChE, acetylcholinesterase; BrdU, 5-Bromo-2′-deoxyuridine; CPF, chlorpyrifos; E_2, 17β-estradiol; EDs, endocrine disruptors; ERα, estrogen receptor alpha; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide; OPs, organophosphate pesticides; ROS, reactive oxygen species.

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on synthesis of macromolecule like DNA, RNA and proteins, interactions with neurotransmitter receptors and other neurochemical effects (Slotkin et al., 2008).

Breast cancer is the most frequent malignant disease in women. Exposure to estrogens throughout a woman's life is a risk factor for the development of this malignancy. Estrogen receptor alpha (ERα) is the major regulator of breast cancer tumor behavior. In the mammary gland, 17β-estradiol (E2) promotes cell proliferation in both normal and transformed epithelial cells by modifying the expression of hormone responsive genes involved in the cell cycle and/or programmed cell death (Renoir et al., 2008). ERα phosphorylation in tyrosine 537 (Y537) is required to stimulate the Src/Shc/Ras/Erk pathway in MCF-7 cells and the inhibition of this phosphorylation interferes in ER/Src association and prevents receptor-dependent Src/Erk signaling, the expression of cyclin D and the stimulation of G1-S progression of the cells (Varricchio et al., 2007). A recent analysis has revealed that high levels of phospho-tyrosine 537-ERα (p-Y537-ERα) are associated with poor overall survival in breast cancer patients (Skliris et al., 2010).

Endocrine disrupters (EDs) are defined as compounds which may be of industrial or natural origin and interfere with hormone biosynthesis, action and metabolism, resulting in a deviation from normal homeostatic control or reproduction (Waring and Harris, 2011). EDs may act via nuclear receptors such as ERα, non-nuclear steroid hormone receptors, non-steroid receptors, enzymatic pathways involved in steroid biosynthesis and/or metabolism, and several other mechanisms (Diamanti-Kandarakis et al., 2009). CPF was recognized as an endocrine disruptor since it has been demonstrated to possess the ability to interfere with the ERβ mRNA steady state levels (Grünfeld and Bonefeld-Jorgensen, 2004). Moreover, Viswanath et al. (2011) demonstrated that CPF has antiandrogenic activity and significantly decreases testosterone biosynthesis. In another hand, CPF administration alters thyroid function at concentrations that do not modify AchE in mice (De Angelis et al., 2009). A recent epidemiological study of pesticide applicators reported a significant correlation between CPF use and lung and rectal cancer (Alavanja et al., 2004; Lee et al., 2007). In addition, it has been reported that there is an interaction between CPF and E2 in the digestive gland of the marine mussel, indicating that the pre-exposure to sublethal concentrations of the pesticide affects the transcriptomic fingerprint that is induced in response to E2 (Canesi et al., 2011).

Furthermore, it is amply demonstrated that OPs are able to generate oxidative stress affecting the different antioxidant enzymatic systems (Kristoff et al., 2008; Lascano et al., 2011). Particularly, CPF may induce damage by DNA, RNA, lipid and proteins oxidation, which in turn, alters the cell physiology and provokes cell death (Gupta et al., 2010). Reactive oxygen species (ROS) are described as potent mutagens, increasing genomic instability and, thereby, contributing to the initiation as well as the progression of cancer (Carew and Huang, 2002). In addition, a moderate increase of ROS has been found to stimulate cellular proliferation (Liu et al., 2002). Although estrogens have been postulated to induce antioxidant effects in several tissues, their action on estrogen-dependent tissues are still not clear. It has been reported that estrogens may increase mitochondrial ROS production through an ER-dependent way (Sastre-Serra et al., 2010).

The aim of this work was to investigate the effect of CPF on cell proliferation and the ERα-dependence of this action employing estrogen-dependent (MCF-7) and estrogen-independent (MDA-MB-231) breast cancer cell lines. We have also analyzed CPF on the cell cycle distribution and cyclins that are implicated in G1-S and intra-S checkpoints. Finally, the action on cell death and ROS induction by CPF were studied.

2. Materials and methods

2.1. Cell culture

Estrogen-dependent MCF-7 (ERα+) and estrogen-independent MDA-MB-231 (ERα−) human breast cancer cell lines were purchased from American Type Culture Collection (ATCC, USA). Cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 0.3 g/l glutamine and 0.04 g/l gentamicin (Gibco BRL, NY, USA). Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. For the experiments, cells were exposed to CPF (Chem Service, Inc., West Chester, PA, USA). The pesticide was dissolved in ethanol and the final concentration of this solvent did not exceed 0.5% in any of the assays. Treatments were added to the cultures in phenol red free-RPMI medium (Gibco BRL, NY, USA) and 10% charcoal-treated FBS. Ethanol 0.5% (vehicle) was added to the control cells.

2.2. Clonogenic assay

3×10^3 MCF-7 cells and 1.5×10^3 MDA-MB-231 cells were treated for 10 days with CPF at 0.05, 0.5, 5 and 50 μM or vehicle. E2 10 nM was added to MCF-7 cells as positive control. When it was required, ICI 182,780 1 nM (Tocris Bioscience, MO, USA) was added to the culture 15 min before CPF treatments. Fixed cells were stained with 0.05% violet crystal in 10% ethanol. The clonogenic capacity was evaluated by counting colonies containing 50 cells or more.

2.3. Doubling time

For this experiment, 2×10^4 cells were seeded. After an overnight incubation starved cells were exposed to CPF (0.05 and 50 μM) or vehicle. Cells were counted by hemocytometer at 0, 24, 48, 72 and 96 h. The experiments were performed in the exponential phase of cell growth. Triplicate plates were analyzed for each treatment at each time. The following formula was used to calculate the doubling time: $N_t = N_0 \times e^{kt}$, where N_0 was the initial number of cells that increased exponentially with a rate constant, k. The doubling time (T_D) was calculated as: $T_D = \ln 2/k$. The GraphPad Prism 5.0 software (GraphPad Software Inc., Philadelphia, USA) was employed for equation fitting to data and parameter estimation.

2.4. Cell cycle analysis

Cells were plated and then serum-starved for 24 h. Synchronized cells were treated with CPF (0.05, 0.5, 5 or 50 μM) or vehicle for 24 h. Cells were collected by trypsinization, fixed with ice-cold methanol, centrifuged, resuspended in 0.2 mg/ml of DNase-free RNase A (Sigma Chemical Co., MO, USA) and then were stained with propidium iodide (PI) staining solution (50 μg/ml PI in PBS; Sigma Chemical Co., MO, USA) and evaluated by flow cytometry (CyFlow Pas III, Partec; Görlitz, Deutschland, Germany). Cell cycle distribution was analyzed using Cyflogic v1.2.1 software (Perttu Terho & © CyFlo Ltd, Turku, Finland).

2.5. 5-Bromo-2′-deoxyuridine (BrdU) incorporation assay

2×10^4 cells were seeded on cover slips in RPMI complete growth medium followed by overnight incubation to allow the cells to attach. Synchronized cells were treated with CPF (0.05, 0.5, 5 or 50 μM) or vehicle for 24 h. Then, BrdU 30 μM (Sigma Chemical Co., MO, USA) was added for the last 2 h of exposure to the pesticide or vehicle and proceeded as described (García et al., 2010). Cells were finally exposed to anti-BrdU primary antibody (1:200, Sigma Chemical Co., MO, USA) and stained with FITC-conjugated anti-mouse IgG (1:200, Sigma Chemical Co., MO, USA) and 4′-6-diamidino-2-phenylindole (DAPI) (1:8000, Sigma Chemical Co., MO, USA). Cover slips were mounted with Fluor-Save™ Reagent (Calbiochem, Darmstadt, Germany) and fluorescence was observed by epifluorescence using an Olympus BX50 microscope (Center Valley, PA, USA). Images were acquired using a CoolSnap digital camera (Silver Spring, MD, USA). At least 1000 cells were scored per replicates.

2.6. Necrosis and apoptosis determination

Starved cells were exposed to CPF 0.05 and 50 μM or vehicle for 24 and 48 h. Phosphatidylserine exposure on the surface of apoptotic cells was detected by flow cytometry after staining with Annexin V-FITC (BD Biosciences, USA), and PI (50 μg/ml). Data were analyzed using Cyflogic v1.2.1 software (Perttu Terho & © CyFlo Ltd, Turku, Finland). Apoptotic cells after 48 or 72 h of CPF exposure were also analyzed by DAPI nucleus staining. The morphology of the cells' nuclei was observed using a fluorescence microscope BX50 microscope (Center Valley, PA, USA) at excitation wavelength 350 nm. Nuclei are considered to have the normal phenotype when glowing bright and homogeneously. Apoptotic nuclei can be identified by the condensed chromatin gathering at the periphery of the nuclear membrane or a total fragmented morphology of nuclear bodies. More than 150 nuclei per field in 10 random fields were counted and the percentage of apoptotic nuclei determined.

2.7. Western blot analysis

Cells were treated with CPF or vehicle and proteins was measured by Bradford assay (Bradford, 1976). Proteins (50 μg) were analyzed by SDS-PAGE and immunoassay as described (García et al., 2010). Membranes were probed overnight with primary rabbit anti-ERα (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-pTyr537-ERα (1:200, Abcam Inc., Cambridge, UK), mouse anti-Cyclin D1 (1:200, Cell Signaling Tech. Inc., Danvers, MA, USA), mouse anti-Cyclin E (1:500, Sigma Chemical Company, USA) and mouse anti-β-Actin (1:1000, Sigma Chemical Company, USA). Immunoreactivity was detected by using horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG and visualized by enhanced chemiluminescence (Amersham Biosciences, Arlington Heights, IL, USA). Densitometric analyses were performed using the software ImageJ 1.32J (NIH, Bethesda, MD, USA).

2.8. Measurement of intracellular reactive oxygen species (ROS) production

Cells treated with CPF (0.05, 0.5, 5 or 50 μM) or vehicle were incubated with 5 μM DCF-2DA (dichlorodihydrofluorescein diacetate; Sigma Chemical Co., MO, USA) as a fluorescent probe for 30 min at 37 °C. Cells were then trypsinized, and suspended in PBS. Levels of intracellular ROS were measured immediately by flow cytometry (CyFlow Pas III, Partec; Görlitz, Deutschland, Germany) and data were analyzed using WinMDI 2.8 software (Scripps Institute, CA, USA). H_2O_2 contribution in ROS levels was studied by adding catalase (30 IU/ml, Sigma Chemical Co., MO, USA) before incubation in presence of DCF-2DA.

2.9. Statistical analysis

Statistical analysis performed in each study is indicated in the legends. Data were analyzed using the GraphPad Prism 5.0 (GraphPad Software Inc., Philadelphia, USA). p values less than 0.05 were considered statistically significant.

3. Results

3.1. Chlorpyrifos modifies MCF-7 and MDA-MB-231 cell proliferation

At first, pesticide concentrations to be employ in the experiments were chosen by MTT viability assay. For this purpose, cell lines were exposed to CPF at 0.05, 0.5, 5, 50 and 100 μM and the results were compared to the negative control cells exposed to vehicle (ethanol 0.5%). Concentration 100 μM CPF resulted in a decreased in cell viability greater than 50% for both cell lines, therefore this concentration was considered cytotoxic and was not further assayed in the subsequent experiments (data not shown).

To evaluate clonogenic capacity, cells were exposed to the pesticide (0.05, 0.5, 5, 50 μM) for 10 days. As shown in Fig. 1A, CPF 0.05 μM increased in 46% the clonogenic capacity with respect to negative control values in MCF-7 cells ($p < 0.05$). However, CPF at 50 μM reduced the proliferation by 40% the proliferation in this cell line ($p < 0.05$). Cells exposed to 10 nM E2 were included as positive control in MCF-7 cell proliferation assay. Furthermore, in MDA-MB-231 cell line, CPF 0.05 and 0.5 μM reduced clonogenicity by 25% ($p < 0.05$), CPF 5 μM and CPF 50 μM also inhibited clonogenic capacity by 36% ($p < 0.01$) and 65% ($p < 0.001$), respectively.

To determine if the exposure to CPF causes alterations in proliferation rate, we analyzed the effect of CPF on doubling time. In MCF-7 cells, CPF 50 μM led to a significant increase in doubling time (55 h vs. 45 h; $p < 0.001$ vs. control) whereas CPF 0.05 μM did not modify this parameter (41 h vs. 45 h; pNS). On the other hand, MDA-MB-231 cells showed a significant decrease in proliferation rate when they were exposed to CPF 50 μM (90 h vs. 57 h; $p < 0.001$ vs. control). CPF 0.05 μM slightly increased doubling time but the values were not significantly different (65 h vs. 57 h; pNS) (Fig. 1B).

3.2. CPF induces cell proliferation by ERα phosphorylation in MCF-7 breast cancer cell line

In this work we have determined that CPF 0.05 μM induced cell proliferation in estrogen-dependent MCF-7 cell line. We analyzed if CPF is capable to induce ERα Tyr 537 phosphorylation (p-Y537-ERα) as an estrogenic-like mechanism amply associated with cell proliferation induction. Our results indicated that CPF 0.05 μM induced an enhancement of p-Y537-ERα levels when MCF-7 starved cells were exposed for 15 min to the pesticide (87% over control; $p < 0.001$). In contrast, CPF 50 μM did not induce changes with respect to negative control (Fig. 2A). To prove if cell proliferation induced by 0.05 μM CPF is mediated by ERα, we analyzed this effect in presence or absence of the irreversible inhibitor of ERα activation ICI 182,780. To this aim MCF-7 cells maintained under estrogen-free conditions, were treated with CPF (0.05, 0.5, 5 and 50 μM) or E2 10 nM as positive control. The inhibitor ICI 182,780 completely abrogated cell proliferation induced by CPF 0.05 μM, as evaluated by clonogenic assay (Fig. 2B). These results highly suggest that the proliferation induction observed in MCF-7 cells exposed to the lower concentration used in the present work, is mediated by an estrogenic effect of this pesticide on estrogen-dependent breast cancer cells.

3.3. Chlorpyrifos modulates cell cycle progression and cyclin E and D1 expression

It has been described that many environmental toxic compounds are capable of modifying the cell cycle progression. This deregulation is a hallmark of tumor cells and is crucial in human cancer progression (Marc et al., 2004). Taking into account that CPF modulated the proliferation of both cell lines as described above, we wondered if this pesticide could affect cycling in breast cancer cells. To evaluate this action, flow cytometry measurements with PI were performed for both MCF-7 and MDA-MB-231 breast cancer cell lines after CPF (0.05, 0.5, 5, 50 μM) or vehicle exposure for 24 h. As shown in Fig. 3A, CPF 50 μM induced cell accumulation in S phase in MCF-7 cell line (49% over control, $p < 0.001$) whereas, in MDA-MB-231 cells, a significant increment of cells in G2/M phase was observed at the same concentration (57% over control; $p < 0.001$). However, CPF at 0.05, 0.5 and 5 μM did not affect the cell cycle distribution in either MCF-7 or MDA-MB-231 cells as can be seen in Fig. 3B. Furthermore, we studied the expression of cell cycle regulation proteins cyclins D1 and E in MCF-7 cell line. As shown in Fig. 3C, CPF 0.05 μM induced an increment in these proteins levels (25% and 42% over control, respectively; $p < 0.01$ and $p < 0.001$) whilst CPF 50 μM produced a decrease in cyclin D1 (22%; $p < 0.01$) and very significantly augmented cyclin E expression (87% over control; $p < 0.001$). Since we had previously observed by clonogenic assay that CPF 50 μM induced a decrease on proliferation in MCF-7 cells, we evaluated if the increment of cells in S phase was the consequence of cell cycle arrest in this state. To this aim, MCF-7 and MDA-MB-231 cells were exposed to 0.05 or 50 μM of CPF or vehicle, and a BrdU incorporation assay was performed. MCF-7 estrogen-dependent cells exposed to the lowest concentration assayed, showed a significant increment of BrdU positive cells (58% over control; $p < 0.01$), but no difference in BrdU incorporation was found when these cells were exposed to CPF 50 μM. Conversely, MDA-MB-231 cells did not show significant differences in the percentage of BrdU stained cells when they were treated with CPF at any concentration assayed (Fig. 4).

3.4. Effects of chlorpyrifos on cell death

We have also evaluated if cycle arrest induced by CPF at 50 μM was accompanied by apoptosis. Cell death was monitored with Annexin-V/PI stain followed by flow cytometry analysis. We observed a very significant increase in the percentage of necrotic cells stained with PI only when both cell lines were exposed at CPF 50 μM for 48 h (MCF-7: 35.3% vs. 4.6%, $p < 0.001$ vs. control; MDA-MB-231; 13.6% vs. 6.0, $p < 0.001$ vs. control). No differences were found when the cells were exposed to lower concentration of CPF

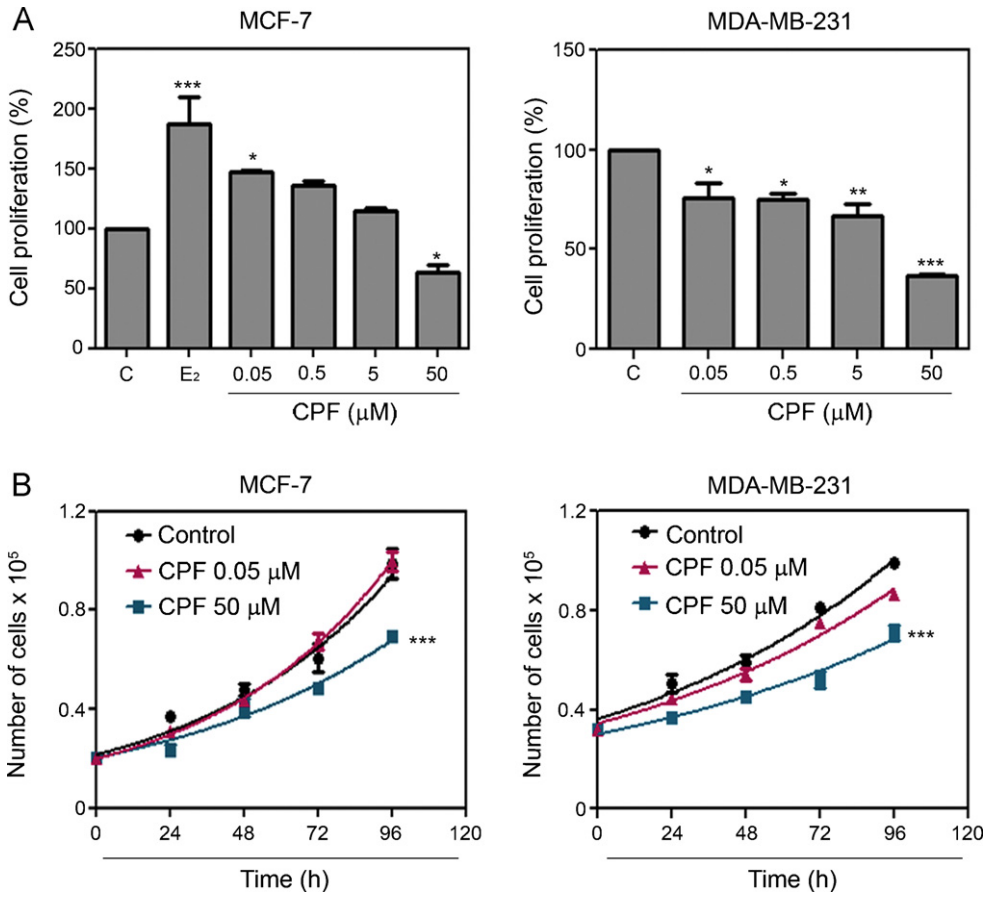


Fig. 1. CPF effects on breast cancer cell growth. (A) Cells were exposed to CPF (0.05, 0.5, 5 and 50 μM), E2 (10 nM) or vehicle (ETOH 0.5%) for 10 days. Proliferation was evaluated by counting colonies with 50 cells or more and expressed as percentage of values obtained with vehicle. Data were indicated as the mean ± SEM of three independent experiments, each one performed by duplicate. ***$p < 0.001$ vs. control; **$p < 0.01$ vs. control; *$p < 0.05$ vs. control; One way ANOVA and Dunnett's Multiple Comparison post hoc test. (B) Cells in the exponential growing phase were exposed at CPF 0.05 and 50 μM or vehicle (ETOH 0.5%) for 0, 24, 48, 72 and 96 h, and counted using a hemocytometer; the curves represent the exponential fitting to data using GraphPad Prism 5.0 software. Results are expressed as the mean ± SEM of three experiments on parallel, each one performed by triplicate. ***$p < 0.001$ vs. control; Two way ANOVA and Bonferroni post hoc test.

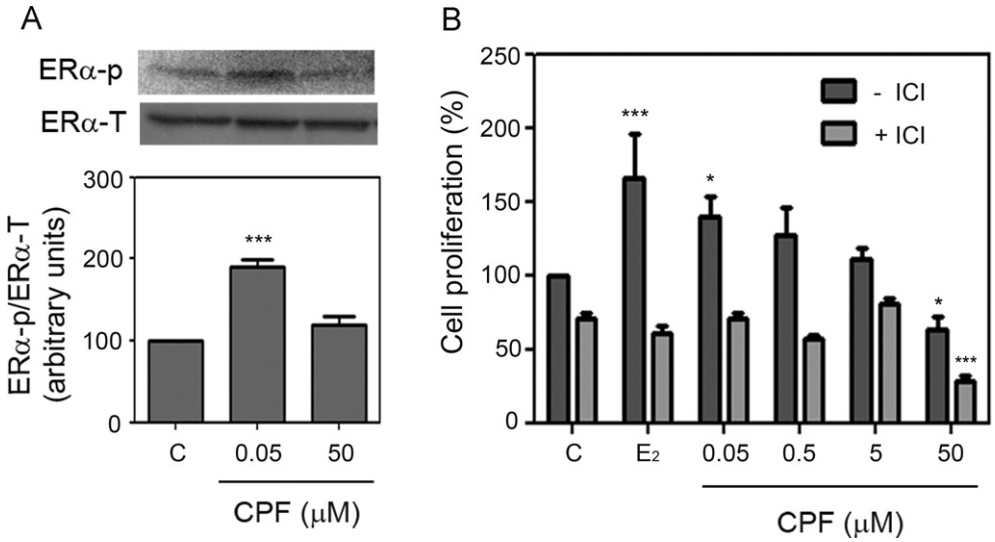
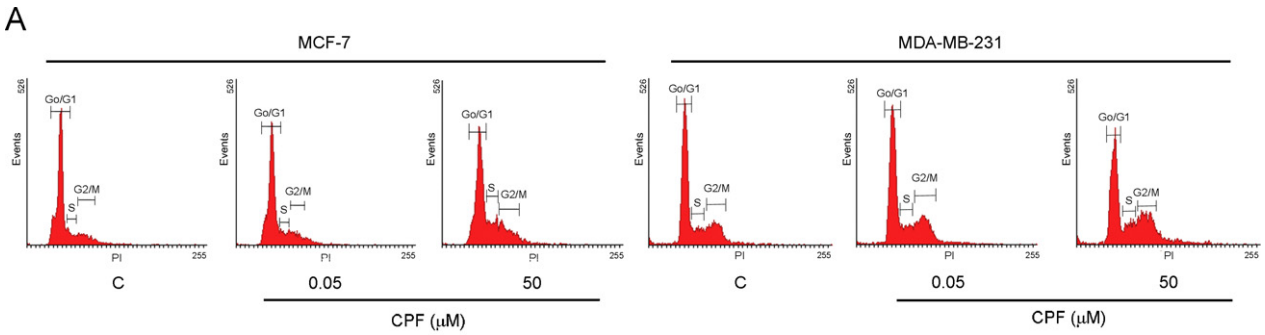


Fig. 2. CPF effects on ERα-mediated proliferation in MCF-7 breast cancer cell line. (A) Cells were exposed to CPF (0.05 and 50 μM) or vehicle (ETOH 0.5%) during 15 min. ERα phosphorylation levels were determined and a representative blot with normalized densitometric data is shown. Densities were normalized to the total ERα content. A representative experiment of three independent experiments is shown. Data were expressed as mean ± SD. ***$p < 0.001$; One way ANOVA and Dunnett's Multiple Comparison post hoc test. (B) Cells were exposed at CPF (0.05, 0.5, 5 and 50 μM), E2 (10 nM) or vehicle (ETOH 0.5%) for 10 days in presence or absence of ICI 182,780 (1 nM). Proliferation was evaluated by counting colonies with 50 cells or more and expressed as percentage of values obtained with vehicle. Data were indicated as the mean ± SEM of three independent experiments, each one performed by duplicate. ***$p < 0.001$; *$p < 0.05$ vs. its corresponding control; One way ANOVA and Dunnett's Multiple Comparison post hoc test.



B

	MCF-7			MDA-MB-231		
	Cell cycle distribution			Cell cycle distribution		
Treatment	G0/G1	S	G2/M	G0/G1	S	G2/M
C	68.6 ± 0.9	26.6 ± 0.8	4.7 ± 0.21	55.1 ± 1.3	32.5 ± 2.5	12.4 ± 0.4
CPF 0.05 μM	67.1 ± 4.0	24.5 ± 4.3	6.2 ± 2.8	48.8 ± 0.8	35.7 ± 1.0	15.5 ± 1.3
CPF 0.5 μM	66.5 ± 1.1	29.3 ± 1.2	4.2 ± 1.9	52.3 ± 3.2	38.9 ± 4.1	8.8 ± 0.2
CPF 5 μM	66.9 ± 1.8	27.9 ± 2.2	5.2 ± 0.7	49.7 ± 2.6	36.0 ± 1.5	14.3 ± 2.7
CPF 50 μM	55.9 ± 0.4	39.7 ± 2.3***	4.3 ± 2.6	48.4 ± 0.7	32.1 ± 0.6	19.5 ± 0.9***

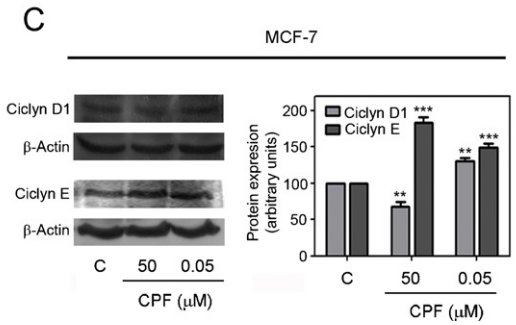


Fig. 3. CPF modifications on cell cycle distribution and cyclins expression. (A) Starved cells were exposed to CPF (0.05, 0.5, 5 and 50 μM) or vehicle (ETOH 0.5%) during 24 h. Cells were stained with propidium iodide (PI), and analyzed for DNA content by flow cytometry. (B) The numbers in the table indicate the percentage ± SD of cells in each phase of the cell cycle. Data are representative of three independent experiments. ***$p < 0.001$ vs. control; Two way ANOVA and Bonferroni post hoc test. (C) MCF-7 cells were exposed to CPF (0.05 and 50 μM) or vehicle (ETOH 0.5%) during 24 h. Cyclins D1 and E levels were determined and a representative blot with normalized densitometric data is shown. Densities were normalized to the β-Actin content. A representative experiment of three independent experiments is shown. Data were expressed as mean ± SD. **$p < 0.01$, ***$p < 0.001$; One way ANOVA and Dunnett's Multiple Comparison post hoc test.

with respect to negative control at neither 24 nor 48 h (Fig. 5). We have confirmed the absence of apoptosis by staining the nucleus with DAPI and there were not significant differences in the percentage of apoptotic nucleus observed by fluorescence microscopy studies at any concentration assayed in either MCF-7 or MDA-MB-231 cells with respect to negative control. Average percentage of apoptotic cells is shown in Fig. 6.

3.5. Chlorpyrifos alters redox metabolism in breast cancer cells

Since ROS intracellular production is one of the most important factors implicated in genomic instability and subsequent cell cycle arrest, we evaluated ROS levels in CPF treated cells after 24 h of exposure. As shown in Fig. 7, CPF 50 μM induced an increment in ROS levels in MCF-7 (58%) and MDA-MB-231 (108%). In contrast, CPF at 0.05, 0.5 or 5 μM did not induce significant changes in cell ROS production. Catalase was added to the culture in order to determine if the increment in ROS levels was attributable to H_2O_2. Moreover, catalase totally revert the increment of ROS induced by CPF 50 μM in MCF-7 cells but failed to restore ROS content to the basal levels in MDA-MB-231 cells.

4. Discussion

Many commonly used pesticides were identified as EDs because of their capacity in interfering with the response of natural hormones like estrogens or androgens. CPF is a pesticide that belongs to this group of compounds but the mechanism of action on breast cancer cell proliferation has not been established yet. In our work, we studied the effect of CPF on cell proliferation by analyzing clonogenic capacity, doubling time, cell cycle progression and BrdU incorporation when estrogen-dependent MCF-7 and estrogen-independent MDA-MB-231 cells were exposed to the chemical in concentration ranging from 0.05 to 50 μM. The lowest concentration was chosen taking into account that it is similar to environmental values that it could be found in water or soil (Del Prado Lu, 2010; Jaipieam et al., 2009; Loewy et al., 2011). Andersen et al. (2002) have reported that many pesticides currently used in both agriculture and pest control show estrogenic activity. They observed that the exposure of MCF-7 cells to several chemicals such as methiocarb, fenarimol, chlorpyrifos, deltamethrin and tolclofos-methyl induce human ER promoter transactivation. It has also been described that several pesticides, including chlorpyrifos, seem to have a stimulatory effect on aromatase activity. Kojima et al. (2004) have demonstrated that an important number of pesticides show positive response in the ERα transactivation. However, a direct estrogenic effect of CPF on cell proliferation has not been confirmed.

We have seen that CPF at 0.05 μM induces cell proliferation in MCF-7 but not in MDA-MB-231 cells as clonogenic and BrdU incorporation assays have shown. In view of these results, we evaluated the CPF action on ERα phosphorylation on tyrosine residue in position 537 (Tyr537). We observed that CPF was capable of inducing the phosphorylation of this receptor in Tyr537 and that it was significantly increased at the lowest concentration used. Since cell proliferation induction could be reverted by ICI 182,780 in MCF-7 cells, we concluded that CPF shows a clear estrogenic action in breast cancer cells. In our work we have demonstrated for the first time that ERα-dependent proliferation is induced by low concentrations of CPF. Furthermore, CPF 50 μM inhibited cell proliferation and increased the doubling time and cell death in both cell lines but BrdU incorporation assay did not show modifications in respect to negative control which may be due to the different periods of exposure to the pesticide among these experiments.

We have studied the effect of the pesticide on cell cycle and found that the CPF 50 μM induces an increment of cells in S phase in MCF-7 cells. It has been reported that different compounds may induce S arrest through redox state unbalance (Chang et al., 2005).

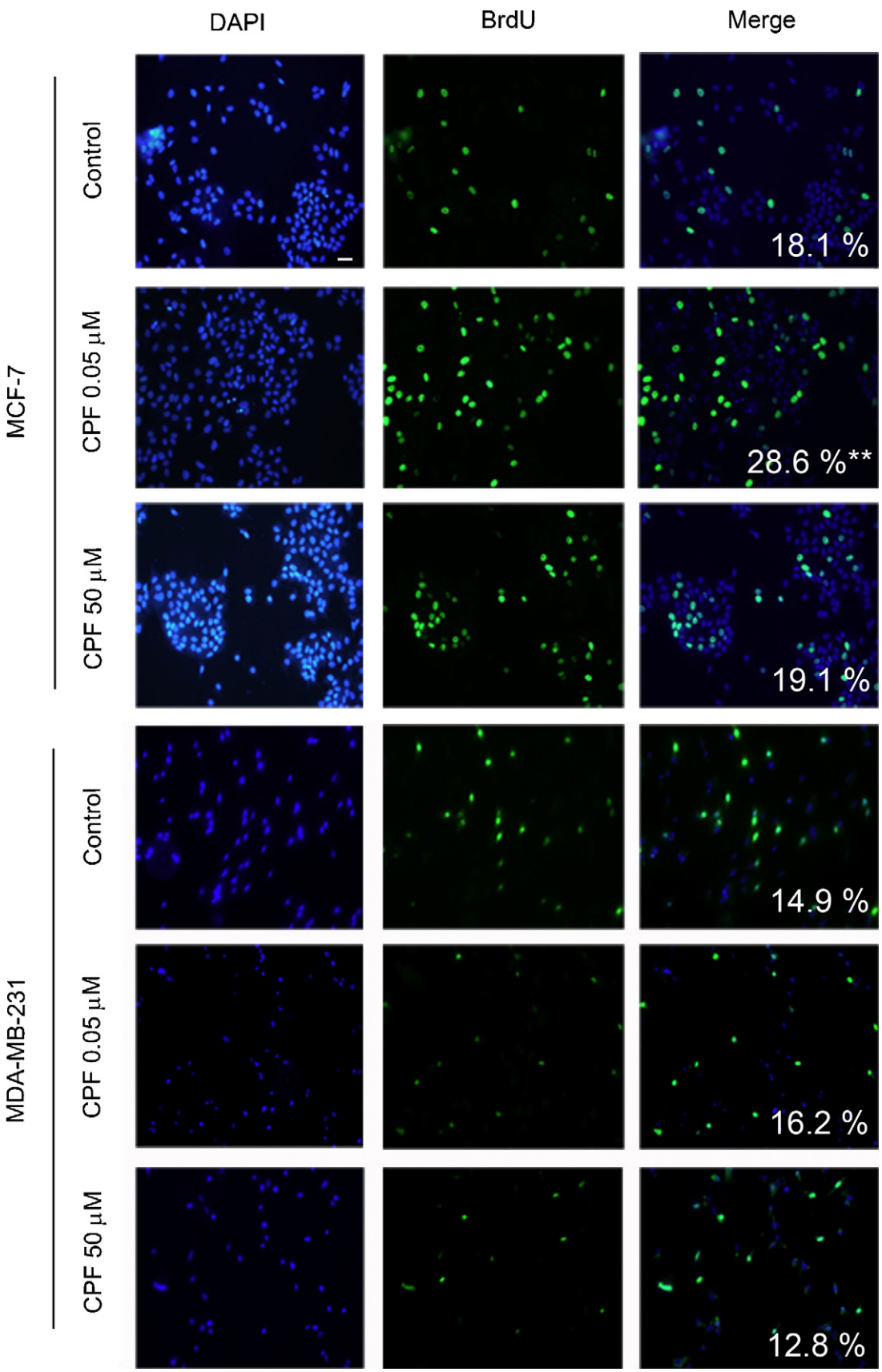


Fig. 4. Effect of CPF on BrdU incorporation. Starved cells were exposed to CPF (0.05 and 50 μM) or vehicle during 24 h. Cells were fixed and stained with anti-BrdU (green). DNA was stained with DAPI (blue). The percentages of BrdU-positive cells are indicated. Data are representative of three independent experiments. $^{**}p < 0.01$ vs. control. One way ANOVA and Dunnett's Multiple Comparison post hoc test. Magnification: 200×. Scale bar: 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

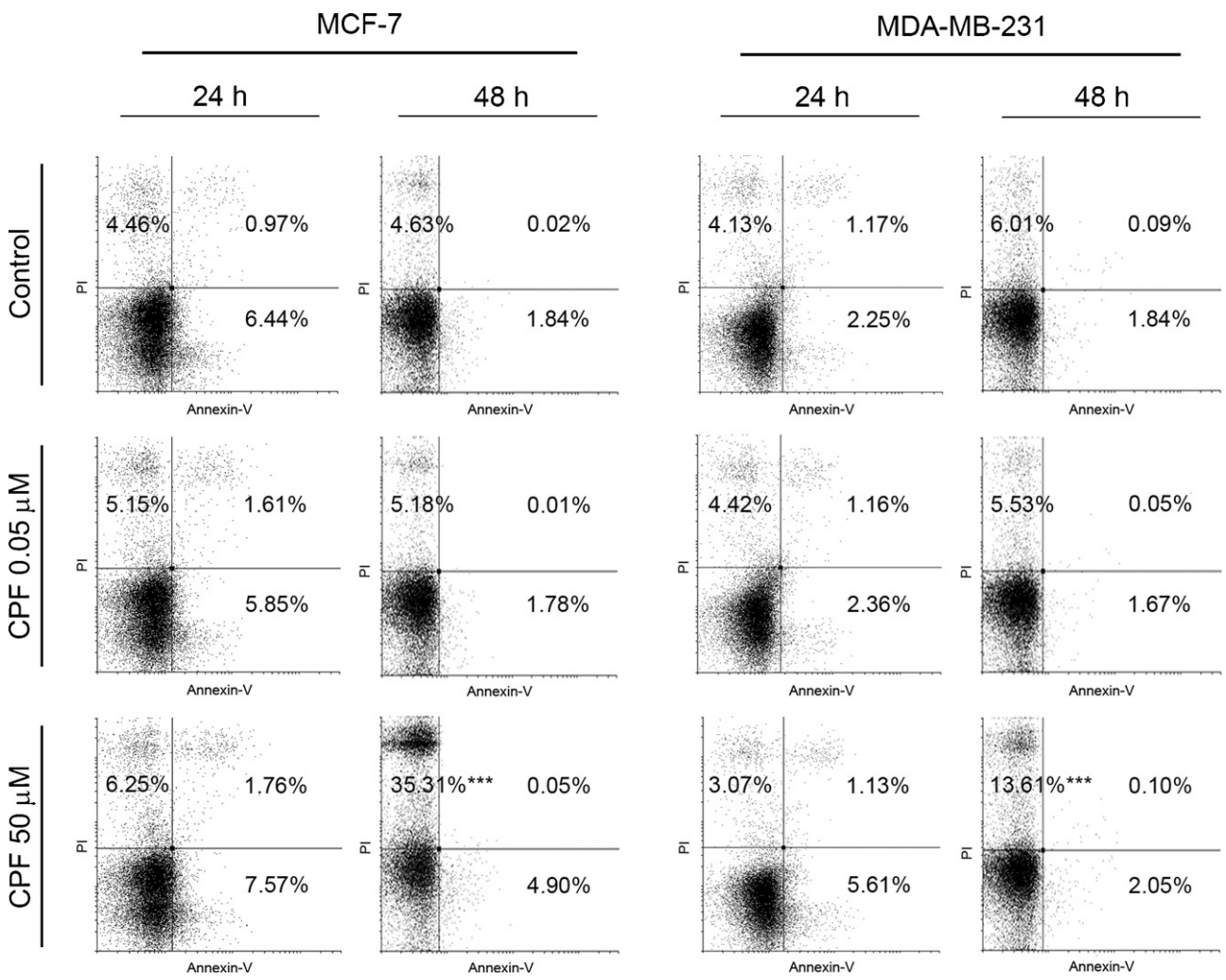


Fig. 5. CPF effects on cell death. Cell death was assessed after 24 and 48 h of exposure to CPF (0.05 and 50 μM) or vehicle (ETOH 0.5%). Cells were stained with Annexin-V/propidium iodide followed by flow cytometry analysis. The percentages of cells positive for PI and/or Annexin V-FITC are reported inside the quadrants. Lower left quadrant, viable cells (Annexin V−/PI−); lower right quadrant, early apoptotic cells (Annexin V+/PI−); upper right quadrant, late apoptotic cells (Annexin V+/PI+); upper left quadrant, necrotic cells (Annexin V−/PI+). Data are representative of three independent experiments. The percentages of cells in each quadrant are indicated. Data are representative of three independent experiments. ***$p < 0.001$ vs. its corresponding control; One way ANOVA and Dunnett's Multiple Comparison post hoc test.

These findings indicate that the increment of MCF-7 cells in S phase induced by CPF 50 μM may be related to the incapacity to progress to G2/M phase. Our results are in concordance with those reported for guggulsterone, a plant polyphenol, which suppresses cell proliferation through inhibition of DNA synthesis, producing intra S-phase arrest (Shishodia et al., 2007). A similar mechanism of action has been reported to describe the toxic effects of urethane dimethacrylate, the major component released from various dental resin materials which induces intra S-phase arrest and ROS accumulation (Chang et al., 2010). Pontano et al. (2008) demonstrated that exist an association between cyclin D1 degradation and intra S-phase arrest following DNA damage. We postulate that in our study, ROS increase induced by CPF 50 μM may trigger the cyclin D1 degradation to prevent genomic instability following genotoxic insult, in MCF-7 cells. We observed that CPF 50 μM produces a significant enhancement of cells in S-phase, due to attenuation of cell cycle progression until the damaged DNA is repaired. E2F and Myc jointly activate the key target gene cyclin E whose product activates the CDK2 kinase necessary for the actual initiation of DNA replication. Consequently, the cyclin E protein becomes detectable and accumulates only in late G1, a few hours after the passage through the restriction point (Bartek and Lukas, 2001). Hence, the increment of cyclin E expression by CPF 50 μM found in MCF-7 cells indicates that the cells are capable to progress through G1 and enter S but are incapable to progress to G2/M phase in view that the cells remain arrested. On the other hand, we have found that CPF induce G2/M arrest in MDA-MB-231 cells. In this regard, multiple tyrosine sites for CPF binding in tubulin molecule have been identified (Grigoryan et al., 2009) and the metabolite chlorpyrifos-oxon disrupts tubulin polymerization (Grigoryan and Lockridge, 2009). The results of those reports could explain the G2/M arrest that we observed in MDA-MB-231 cells exposed to CPF in this work. As cell division approaches metaphase, microtubules are disassembled thereby facilitating chromosomal alignment on the metaphase plate. In this process, tubulin subunits freely exchange on the microtubules. If such free exchange of tubulin subunits is disrupted, the mitotic spindle is compromised and the cell cannot divide. As a consequence, cells undergoing division and particularly those cells showing rapid division are arrested (Cocca et al., 2009; Jordan and Wilson, 2004). We have not detected a G2/M arrest in MCF-7 cells. In relation to this, it was previously demonstrated that MCF-7 cells and MDA-MB-231 cells may present β-tubulin isotypes profiles which could explain the different sensibility to the chemotherapeutic agents by affecting the ability of these compounds to bind to the microtubular system, thus preventing its anti-tumor effects (Shalli et al., 2005).

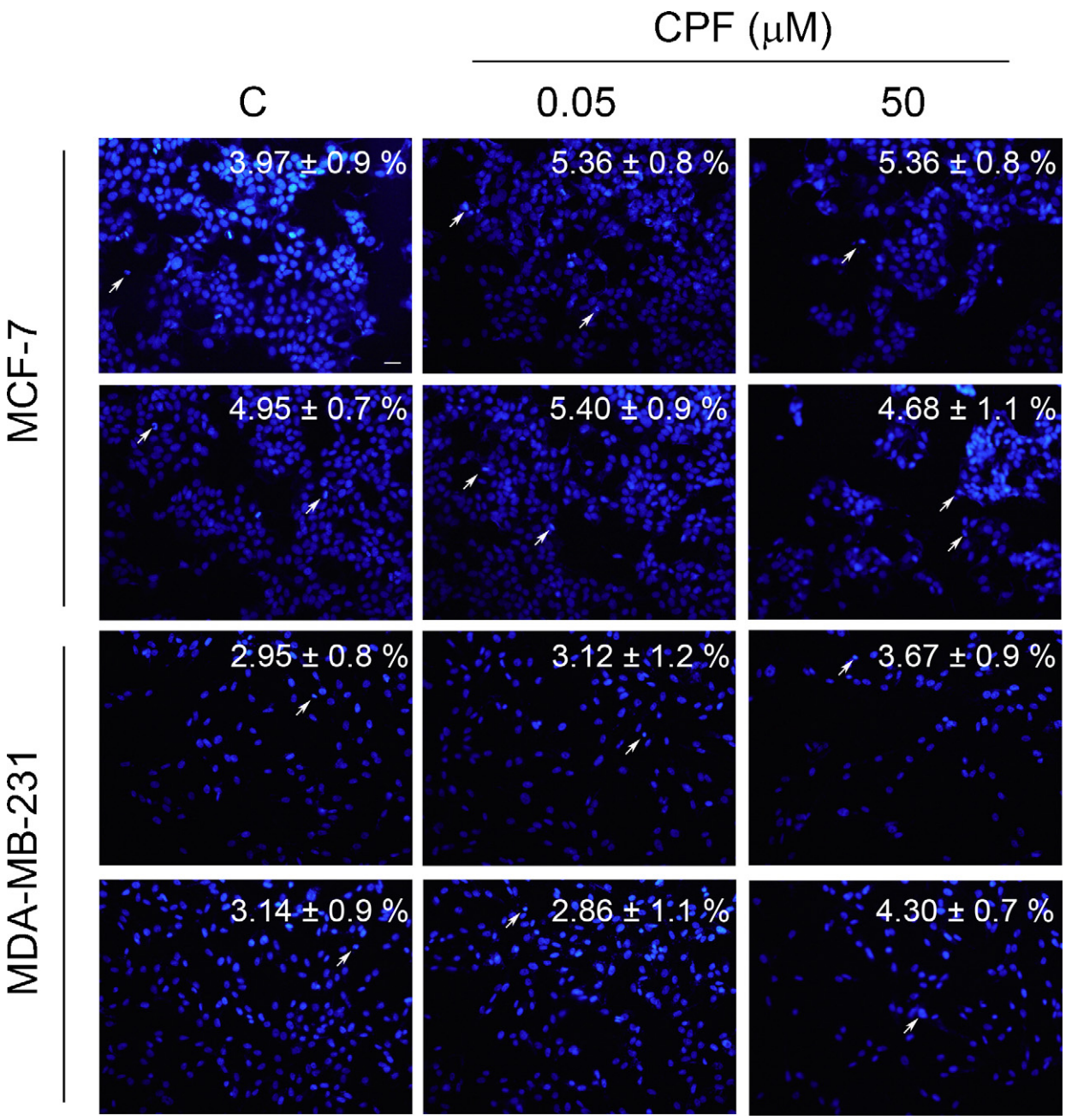


Fig. 6. Analysis of the effect of CPF on apoptosis. Visualization of apoptosis in MCF-7 and MDA-MB-231 cells treated with CPF (0.05 and 50 μM) or vehicle for 48 h or 72 h. Cells were fixed in methanol and DNA was stained with DAPI for the determination of morphological and quantitative analysis of apoptosis, and photographed by microscope (200×). Arrows represent apoptotic cells with highly condensed chromatin. pNS; Two way ANOVA. Scale bar: 20 μm.

Many environmental compounds are able to inhibit cell proliferation and induce apoptosis through modifying oxidative balance (Itziou et al., 2011). Since it has been reported that CPF may induce oxidative stress and modify DNA structure in larvae of *Drosophila* (Gupta et al., 2010), we also analyzed if this pesticide could also affect the redox balance in breast cancer cells. In this work, we have observed an increment in ROS production induced by the higher CPF concentration employed in our experiments. In MCF-7 cells, preincubation of the cultures with catalase abolished this increase, suggesting that hydrogen peroxide is a major component of those species. In turn, we postulate that this increment may produce the cell cycle arrest on S-phase and the increase of cell death that we observed in MCF-7 cells. On the contrary, in the MDA-MB-231, catalase incorporation was unable to restore the ROS levels to basal values suggesting that other ROS than H_2O_2 were produced and further experiments are needed to elucidate other reactive species that are involved.

It is essential to point out that many other researchers have reported that CPF induces cell death. However, the CPF concentrations used in this study that are capable to induce proliferation are markedly lower than those generally described (Li et al., 2009; Nakadai et al., 2006).

In conclusion, we demonstrated the ability of CPF 0.05 μM to induce cell proliferation through ERα in hormone-dependent breast cancer cells. In contrast, CPF 50 μM induced cell cycle arrest in S–phase modifying checkpoints proteins, through a mechanism that may involve changes in redox balance in MCF-7 cells. In the MDA-MB-231, we have found that CPF 50 μM produces an arrest in G2/M phase which could be related to the capacity of the pesticide for binding to tubulin sites altering microtubules polymerization.

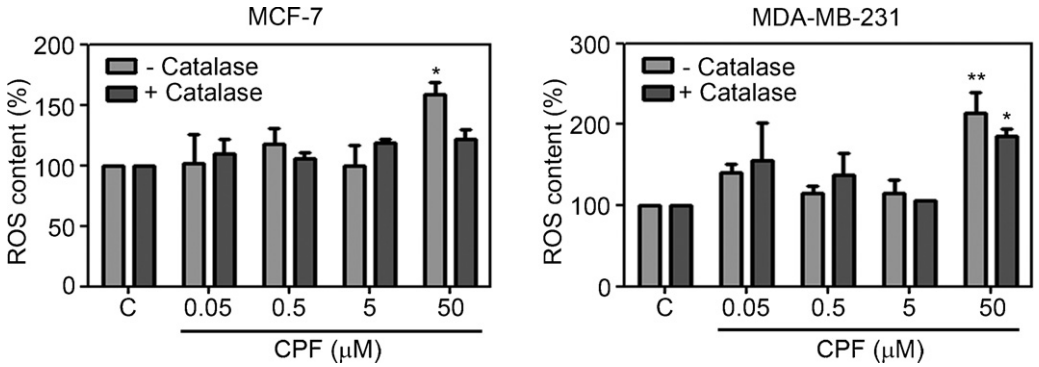


Fig. 7. Effects of CPF on ROS levels. Cells were exposed to CPF (0.05, 0.5, 5 and 50 μM) or vehicle (ETOH 0.5%) during 24 h. After CPF treatment the cells were exposed to catalase activity (0 and 30 IU/ml) for 15 min. Intracellular ROS was analyzed by flow cytometry using the fluorescent dye DCF-2DA. The graphs show the mean fluorescence intensity as percentage of values obtained with vehicle. Results are expressed as mean ± SD. Data are representative of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. its corresponding control; One Way ANOVA and Dunnett's Multiple Comparison post hoc test.

Altogether, our results provide new evidences on the action of the pesticide amply used CPF as an environmental breast cancer risk factor due its effects on the mechanisms involve in the modulation of breast cell proliferation.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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