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The organophosphate chlorpyrifos disturbs redox balance and triggers antioxidant defense mechanisms in JEG-3 cells

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A B S T R A C T

Introduction: Reactive oxygen species (ROS) are produced by a number of physiological and pathological processes which influence the function of a diverse array of cellular events. An imbalance between ROS generation and elimination was reported for different environmental xenobiotics exposure. Here, we analyzed the effect of chlorpyrifos (CPF) on the JEG-3 cell antioxidant defense in conditions where cell viability and morphology were not altered.
Methods: Acetylcholinesterase (AChE) activity, reduced glutathione (GSH) content and catalase (CAT) antioxidant enzyme activity were measured by biochemical studies. ROS production was detected using the fluorogenic probe 2′,7′-dichlorodihydrofluorescein diacetate. The transcript level of superoxide dismutase enzyme 1 (SOD1), glutathione reductase (GR), heme oxygenase-1 (HO-1), and nuclear factor E2-related factor 2 (Nrf2) as well as Nrf2 protein amount were analyzed by quantitative real time PCR and Western blot, respectively.
Results: The results showed that CPF inhibited AChE activity, induced ROS production, upregulated CAT activity, and decreased GSH concentration. In response to CPF exposure, GR and HO-1 mRNA levels were increased with no changes in SOD1 mRNA. Furthermore, CPF significantly augmented Nrf2 at both mRNA and protein levels trigging the antioxidant status by increasing nuclear Nrf2 translocation.
Discussion and conclusion: Taken together, these data indicate that JEG-3 cells are able to attenuate the oxidative stress induced by CPF through the adaptive activation of the Nrf2/ARE pathway.
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1. Introduction

Chlorpyrifos (CPF) is an organophosphorous (OP) pesticide widely used in agricultural, industrial, and household applications. The primary acute toxicological effect of OPs is associated with inhibition of acetylcholinesterase (AChE). Additionally, several studies also provide evidence that OP exposure induces oxidative stress by different mechanisms such as an increased formation of reactive oxygen species (ROS) [1,2], depletion of antioxidant defenses, and the impairment of antioxidant enzyme function [3]. ROS act as second messengers modifying gene expression by the activation of several signaling pathways, influencing normal cell growth and survival [4]. However, ROS are well recognized for playing a dual role as both beneficial and deleterious species [5]. Thus, cells have developed complex antioxidant systems (both enzymatic and non-enzymatic) to alleviate and restore the damage caused by ROS. These systems include a large battery of proteins encoded by cytoprotective genes mainly controlled by the nuclear factor erythroid 2-related factor 2 (Nrf2) that positively regulates their basal and inducible expression [6]. Alterations in these oxidant/antioxidant status in fish [7], earthworm [8], and rats [9] after CPF administration, as well as in vitro exposure studies [10,11] have been well documented.

In humans, prenatal OP exposure is associated with decreased birth weight and length [12,13], alterations in developmental and in psychomotor indices [14] and immunological abnormalities [15]. Although most studies have focused on the direct effect of OPs on the fetus, few reports have assessed their effects on the placenta.

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It has been reported that placental function may be interfered by OP compounds at many levels. For example, altered placental AChE, catalase (CAT), and carboxylesterase activity has been associated with prenatal exposure to OP pesticides [16–18]. Azinphos-methyl, phosmet and CPF modified placental phosphoinositide metabolism and phosphoinositide-4 kinase activity [19], and CPF induced apoptosis in the trophoblastic-derived JAR cell line, through a signaling mechanism not dependent on FAS/tumor necrosis factor, activation of caspases or inhibition of cholinesterase [20]. Cell apoptosis and changes in the cytokine pattern towards an inflammatory profile have been described in JEG-3 exposed to phosmet and CPF [21]. Additionally, we have reported that JEG-3·cell exposed to CPF markedly alters the expression of human chorionic gonadotropin_β-subunit (βhCG), glial cell missing-1 and ATP-binding cassette sub-family G member 2 molecules which are relevant for the maintenance of a healthy pregnancy [22].

Although placental oxidative stress is produced by toxics like 2,3,7,8-tetrachlorodibenzo-p-dioxin [23], cadmium [24] and alcohol [25], to our knowledge, there is no study that links the potential impact of CPF with oxidative stress and the antioxidant defense response of the trophoblast. Thus, the aim of the present study was to examine whether CPF modifies the oxidant/antioxidant balance in the placental-derived JEG-3 cell line. The levels of ROS, reduced glutathione (GSH) content, superoxide dismutase enzyme 1 (SOD1), CAT, glutathione reductase (GR), heme oxygenase-1 (HO-1), and the abundance of Nrf2 involved in the antioxidant defense mechanisms were determined.

2. Materials and methods

2.1. Antibodies

Polyclonal rabbit Nrf2 (C-20) antibody was purchased from Santa Cruz Biotechnology. Mouse monoclonal anti-α-actin was obtained from Sigma Chemical Co. and mouse monoclonal anti-GADPH from Ambion.

2.2. Chemicals

CPF (O,O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate) with purity of 99.5% and all other reagents (analytical grade) were purchased from the Sigma Chemical Company (St. Louis, MO, USA).

2.3. Cell culture and CPF treatment

The choriocarcinoma-derived JEG-3 cell line (ATCC, HTB-36) was purchased from the American Type Culture Collection (ATCC, Rockville, USA) and grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 0.1 mg/mL streptomycin. CPF was prepared as a 0.25 M stock solution in dimethylsulfoxide (DMSO). Twenty-four hours before CPF treatment, 1.5×10^6 JEG-3 cells were plated on a 100 mm cell dish culture in 10 mL of culture medium (used for biochemical studies) or 5.5×10^5 cells on each well of 6-well plates in 2 mL of culture medium (used for mRNA and protein determinations) and treated with CPF (0, 5, 50, 100 μM) for 24 h. Final DMSO concentrations did not exceed 0.04%. Under these conditions cell viability, measured by 3-(4,5-dimethlthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) assay, was not affected [22]. When indicated, 10 mM N-acetyl cysteine (NAC) was added 2 h before CPF exposure.

2.4. Biochemical studies

After 24 h of CPF exposure, cultured cells were centrifuged, resuspended in 50 mM sodium phosphate buffer (pH 7) and lysed in 50 mM sodium phosphate buffer (pH 7.0). The protein content was quantified by Lowry method [26] with bovine serum albumin as standard and this cellular extract was used for enzyme activity and reduced glutathione determinations. Measurements were made using a Shimadzu UV–Visible 1601 spectrophotometer fitted with a thermostatic cell holder.

CAT activity was determined recording the continuous decrease in hydrogen peroxide (H_2O_2) absorbance at 240 nm ($\varepsilon = 40$ mM^{-1} cm^{-1}) according to Beers and Sizer [27]. To this end, 125 μL of cellular extracts (100 μg total protein) were incubated in 875 μL of 50 mM sodium phosphate buffer (pH 7.0) containing 25 mM H_2O_2.

AChE activity was determined according to the method of Ellman [28] using acetylthiocholine as a substrate. Briefly, 200 μL of cellular extract (containing 500 μg of proteins) was added to a final volume of 1570 μL containing 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) (0.10 mM) and acetylcholine iodide (0.75 mM). The enzyme activity was measured by following the absorbance increase at 412 nm produced from thiocholine when it reacts with 5,5′-dithiobis(2-nitrobenzoate) ion ($\varepsilon = 14{,}150$ mM^{-1} cm^{-1}). Enzyme activities were expressed as mU/mg of protein. One U is defined as the amount of the enzyme, which decomposes 1 μmol of substrate per min at 25 °C for CAT and at 30 °C for AChE enzymes. Linear conditions were previously adjusted for each enzyme determination.

GSH was determined by the method described by Ellman [29]. GSH estimation was based upon the development of the relatively stable yellow color on addition of DTNB to compounds containing sulfhydryl group. GSH content was expressed as nmol/mg of protein.

2.5. Quantitative reverse transcription-PCR

Total RNA was extracted from cultured cells using Trizol (Invitrogen), according to the manufacturer's instructions. cDNAs were synthesized using one μg of total RNA incubated with 1.25 ng/μL of random primers (Invitrogen) in a 20 μL reaction. The reverse transcriptase reaction was performed as previously described [30]. For real-time PCR, cDNA was mixed with 1× SYBR Green PCR Master Mix (Applied Biosystems) and the forward and reverse primers were added to a final volume of 15 μL. Primer sequences and concentrations are listed in Table 1. Real-time PCR was carried out on an ABI 7500, Applied Biosystems with Sequence Detection Software v1.4. The cycling conditions included a hot start at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Specificity was verified by melting curve analysis and agarose gel electrophoresis. Each sample was analyzed in triplicate. Transcript levels were normalized to those of cyclophilin A and relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method [31]. Amplification efficiency for each set of primers was near 98%. RNA samples incubated without reverse transcriptase during cDNA synthesis, as well as PCR reactions using water instead of template showed no amplification.

2.6. Preparation of whole cell and nuclear extracts

Whole protein extracts from JEG-3 were prepared in 5× Laemmli buffer containing 60 mM Tris–HCl pH 6.8, 10% glycerol, 2% sodium dodecyl sulphate, 1% 2-β-mercaptoethanol. Nuclear and cytosolic extracts were isolated as described by Schreiber [32] with modifications. Cells were washed with cold phosphate balanced saline (PBS) and resuspended in cold buffer A (20 mM HEPES pH 8.0, 1 mM EDTA, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT) containing protease inhibitor cocktail. Cells were allowed to swell on ice for 15 min, then 7.5 μL of 10% Nonidet P-40 was added and vortex mixed vigorously for 10 s. The homogenate was centrifuged for 50 s at 16,000× *g* and the supernatant was used as the cytosolic extract. The nuclear pellet was washed with cold buffer A and resuspended in cold buffer B (20 mM HEPES pH 8.0, 1 mM EDTA, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, protease inhibitor cocktail, and 20% glycerol). All protein fractions were stored at −80 °C until use and protein concentrations were determined by Bradford method with bovine serum albumin as standard.

2.7. SDS-PAGE and Western blotting

Protein samples were loaded onto a 10% SDS-PAGE gel. After migration, proteins were electrotransferred to nitrocellulose (Amersham Bioscience). The membrane was blocked in Tris buffered saline (25 mM Tris, 150 mM NaCl, 2 mM KCl, pH 7.4) containing 0.2% Tween 20 and 5% non-fat dry milk, washed and incubated with each one of the following primary antibodies: anti-Nrf2 (1:200), anti-α-actin (1:3000), and anti-GADPH (1:2000) for 1 h at room temperature with shaking. After washing, the blots were incubated with horseradish peroxidase-conjugated donkey anti-rabbit or sheep anti-mouse secondary antibodies (1:5000) at room temperature

Table 1
Primer sequences and concentrations used for quantitative RT-PCR.

	Sequence (5′–3′)	nM
SOD1		
Sense	TCA GGA GAC CAT TGC ATC ATT	150
Antisense	CGC TTT CCT GTC TTT GTA CTT TCT TC	
GR		
Sense	TCA CGC AGT TAC CAA AAG GAA A	250
Antisense	CAC ACC CAA GTC CCC TGC ATA T	
Nrf2		
Sense	AAA CCA GTG GAT CTG CCA AC	200
Antisense	GAC CGG GAA TAT CAG GAA CA	
HO-1		
Sense	AGG CCA AGA CTG CGT TCC	150
Antisense	GCAGAATCTTGCACTTTGTTGCT	
Cyclophilin A		
Sense	GTC AAC CCC ACC GTG TTC TT	100
Antisense	CTG CTG TCT TTG GGA CCT TGT	

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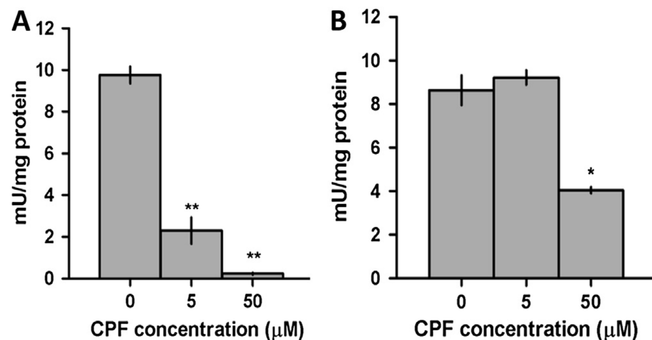

Fig. 1. Effect of CPF treatment on acetylcholinesterase activity in JEG-3 cells after 24 h of exposure in the absence (A) or presence (B) of 10 mM N-acetyl cysteine (NAC). Values are expressed as mU/mg of protein and represent the median and 25th–75th percentiles of at least three independent experiments performed in duplicate. Significantly different from the corresponding control (0) group (*$p < 0.05$, **$p < 0.01$).

for 1 h. Protein–antibody complexes were visualized by an enhanced chemiluminescence detection system (SuperSignal West Pico; Pierce).

2.8. Detection of ROS generation

In vivo intracellular ROS production was detected using the fluorogenic probe 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA). Cells were grown on coverslips for 24 h in the presence or not of CPF and then changed to media lacking both serum and phenol red. Cells were incubated with 10 μM of H2DCFDA for 30 min at 37 °C followed by rinsing with PBS 3 times. Cells were visualized under a fluorescence microscope Nikon Eclipse TE 2000U (Nikon Corporation, Japan).

2.9. Data analysis

Significant differences for control and test conditions were identified using the nonparametric paired Wilcoxon test or unpaired Mann–Whitney *U*-test. A Kruskal–Wallis with a Dunn's post-test was performed to obtain a multiple comparison of independent sample populations. Significance was taken as $p < 0.05$.

3. Results

3.1. CPF inhibits acetylcholinesterase activity

In a previous report we demonstrated that cell viability, measured by MTT assay, and cell morphology was not significantly modified when trophoblast-derived JEG-3 cells were treated with CPF concentrations up to 100 μM for 24 h [22]. Herein, we tested the hypothesis that, in these conditions, JEG-3 cells are able to attenuate the CPF insult by altering the antioxidant defense mechanisms. Initially, in order to explore whether CPF is able to produce AChE inhibition we measured the AChE activity. As shown in Fig. 1A, CPF caused a substantial AChE inhibition in JEG-3 cells in a dose-dependent manner. When cells were incubated with 10 mM of NAC 2 h before CPF treatment, AChE activity was not inhibited by 5 μM of CPF and partially inhibited at the 50 μM concentration (Fig. 1B). Thus, AchE enzyme is expressed in JEG-3 cells, its activity is inhibited by CPF exposure, and inhibition is reversed in the presence of the antioxidant compound.

3.2. CPF enhances ROS generation

Next, we ascertained whether the JEG-3 cell-AChE inhibition induced by CPF was accompanied with an increase in ROS generation as it was demonstrated in other experimental systems. To do this, cells were exposed to 50 or 100 μM CPF for 3 and 24 h and then loaded for 30 min with 10 μM H2DCFDA, a membrane permeable non-fluorescent dye which is cleaved by intracellular esterases. Once cleaved, H2DCFDA combines with reactive oxygen species to produce green fluorescence. CPF induced dose-dependent increases in H2DCFDA fluorescence intensity relative to the vehicle control (Fig. 2A and B). It should be noted that H2DCFDA fluorescence is primarily due to the interaction of the dye with hydrogen peroxide or peroxyl products. Hence, our results indicate that CPF induces generation of hydrogen peroxide or peroxyl radicals. Cells treated with 100 μM H_2O_2 were used as a positive control (Fig. 2C).

3.3. CPF alters antioxidant defense system

We next analyzed whether CAT antioxidant enzyme activity (H_2O_2 scavenger) and GSH content (an important non-enzymatic antioxidant) were modified in response to CPF exposure. In JEG-3 cells exposed to 100 μM CPF, CAT activity was increased with respect to control cells treated with DMSO ($p < 0.05$) (Fig. 3A). Conversely, GSH content was significantly decreased in comparison to control cells at both CPF doses ($p < 0.01$) (Fig. 3B). These data corroborate that CPF disturbs antioxidant defense in JEG-3 cells.

3.4. CPF triggers Nrf2 expression and nuclear translocation

In order to elucidate the mechanisms which underlie CPF induction of antioxidant CAT activity we analyzed the Nrf2 protein expression and localization in JEG-3 cells. We found that cells treated with 50 μM or 100 μM CPF for 24 h upregulated Nrf2

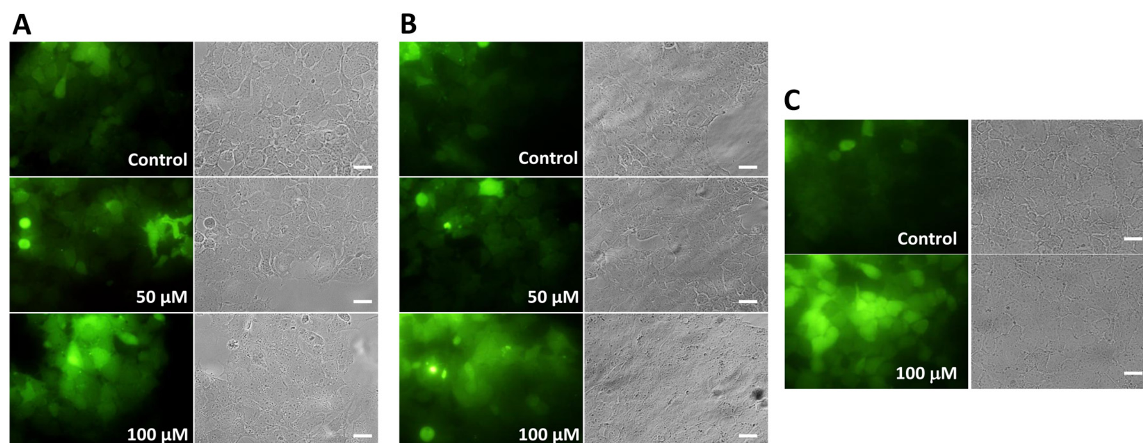

Fig. 2. Generation of ROS by CPF. JEG-3 cells were exposed to 0.04% DMSO (control), 50 or 100 μM of CPF for 3 h (A) or 24 h (B) then labeled with H2DCFDA (10 μM) for 30 min and ROS generation was examined. (C) As positive control, cells were exposed to 100 μM of H_2O_2 for 3 h. Fluorescent and the corresponding DIC images were acquired with a Nikon microscope. Scale bar: 20 μm. Original magnification: 400×. Data are representative of at least three independent experiments.

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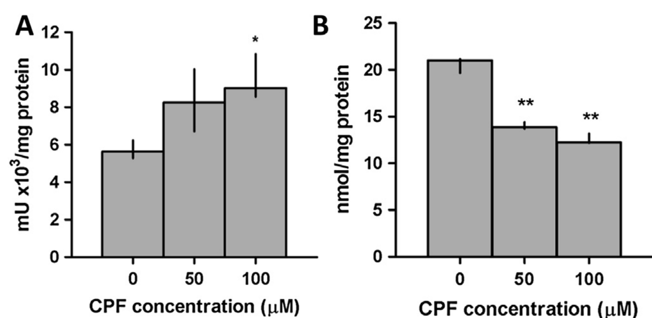

Fig. 3. Effect of CPF treatment on CAT activity (A) and GSH content (B) in JEG-3 cells after 24 h of culture. Values represent the median and 25th–75th percentiles of at least five independent experiments performed by duplicate. Significantly different from the corresponding control (0) group (*$p < 0.05$, **$p < 0.01$).

expression (Fig. 4A). A similar increase of Nrf2 expression was observed when cells were treated with 100 μM of H_2O_2 (positive control) (data not shown). In addition, quantitative PCR revealed that Nrf2 protein upregulation was accompanied with an increase in its mRNA level (Fig. 4B).

Nrf2 translocation from the cytoplasm to the nucleus is essential for the induction of several antioxidant and detoxification genes. Therefore, we examined the localization pattern of Nrf2 in protein extracts of untreated- and CPF-treated JEG-3 cells. Western blot analysis of nuclear extracts using the Nrf2 antibody confirmed that Nrf2 protein was accumulated in the nucleus after 3 h of CPF exposure (Fig. 5A). An important Nrf2 amount remained at the nuclear compartment after 24 h of CPF treatment (Fig. 5B). Absence of the cytoplasmic marker GAPDH in the nuclear fraction confirmed no cross-contamination.

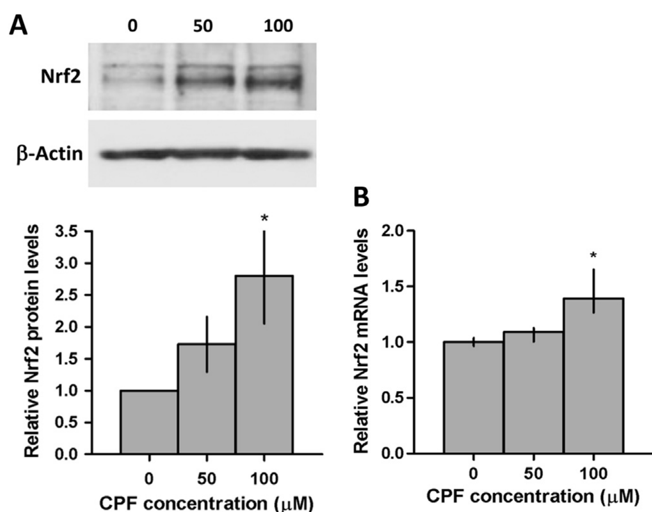

Fig. 4. Effect of CPF on the expression of Nrf2 protein and mRNA levels. (A) Western blot analysis of protein extracts prepared from JEG-3 cells exposed to CPF (0, 50 or 100 μM) for 24 h. Assays were performed using anti-Nrf2 and α-actin antibodies as described in Materials and methods. A representative blot of at least three different experiments is shown. The bar graph represents the densitometric quantification of Nrf2 protein levels relative to β-actin of three separate experiments. Significantly different from the corresponding control (0) group (*$p < 0.05$). (B) mRNA expression of the Nrf2 gene was quantified by qRT-PCR (ABI 7500, Applied Biosystems) in JEG-3 cells cultured during 24 h in the presence of the indicated CPF concentrations or vehicle alone (0). Results were normalized to cyclophilin A and expressed according to the $2^{-\Delta\Delta Ct}$ method using as a calibrator the mRNA level obtained from the control condition (0). Data are presented as the median and 25th–75th percentiles of at least three independent experiments performed by duplicate. Significantly different from the corresponding control (0) group (*$p < 0.05$).

3.5. CPF induces transcriptional activation of antioxidant enzymes

To determine whether the increased expression and nuclear translocation of Nrf2 modulate the transcription of the *HO-1*, *SOD1* and *GR*, their expression was analyzed by real time PCR in JEG-3 cells exposed to CPF (Fig. 6). We found that antioxidant *HO-1* and *GR* were significantly upregulated whereas SOD1 mRNA remained unchanged in the conditions analyzed.

These results indicate that redox imbalance induced by CPF is associated with increased expression of antioxidant enzymes.

4. Discussion

This study provides evidence that CPF disturbs redox balance and triggers antioxidant defense mechanisms in JEG-3 cells. Our findings show several interesting points. First, AChE enzyme is indeed expressed in JEG-3 cells and its activity was clearly inhibited after 24 h of CPF exposure. This inhibition was reversed in the presence of NAC, a well known antioxidant GSH pro-drug. In line with this finding, NAC recovered the diazinon-induced AChE inhibition in exposed rats [2]. It has been demonstrated that human CYP1A2, CYP2B6, CYP2C19 and CYP3A4 are responsible for the oxon formation from a number of OPs with different chemical structure [33]. Certainly, the bioactivated CPF-oxon is three orders of magnitude more potent that CPF to inhibit AChE enzyme [34]. Since CYP1A2 and CYP3A4 [35,36] are present in JEG-3 cells our result strongly suggests that JEG-3 cells metabolize CPF to its oxon derivative.

Second, CPF induced an increase in ROS production, a reduction in the intracellular GSH content, and an increase in CAT enzyme activity, suggesting that CPF alters the redox balance in JEG-3 cells. Certainly, it has been previously reported that CPF and CPF-oxon, generated through a P-450 mediated desulfuration reaction, may act as a source of ROS that could lead to cell injury [37]. CAT is one of the important antioxidant defense enzymes that is meant to scavenge H_2O_2. Therefore, an increase in the activity of this enzyme in the CPF-exposed JEG-3 cells may be an attempt by it to counterbalance the ROS generated.

Third, in response to CPF, the de novo synthesis of *HO-1* and *GR* detoxifying phase II genes was induced in parallel with an augmented Nrf2 expression and nuclear localization. The three antioxidant genes investigated in this study, *HO-1*, *GR*, and *SOD1*, have a common antioxidant response element (ARE) in their promoters and are expressed in an Nrf2-dependent manner. It is now accepted that the basic leucine zipper transcription factor Nrf2, acting via an antioxidant/electrophile response element, plays a crucial role in cellular redox homeostasis [38]. Nrf2 is a highly unstable protein, with a half-life of ~15 min [39], and its expression level is controlled acutely by a balance between new synthesis and degradation by the ubiquitin–proteasome system. Under normal conditions, Nrf2 is bound to Keap1 in the cytoplasm, where it undergoes proteolytic degradation and rapid turnover [40]. The Keap1/Nrf2 system mainly senses low intensity oxidative stress [40]. In response to this, Nrf2 translocates to the nucleus, where it binds to ARE to induce expression of multiple cellular defense genes, including *CAT*, *GR* and *HO-1* [38,41,42].

Among the antioxidant genes studied, *HO-1* was the most induced in response to CPF treatment. HO catalyzes the rate limiting steps of heme oxidation to biliverdin, carbon monoxide and free iron. Biliverdin is rapidly converted to bilirubin, a potent endogenous antioxidant. Three HO isozymes have been identified, the constitutive HO-2 and HO-3, and HO-1, which is highly induced by various stimuli such as heme, cytokines, mitogens, metals, reactive oxygen species, heat shock, UV radiations, hypoxia, or hyperoxia [43]. An increasing number of studies performed in

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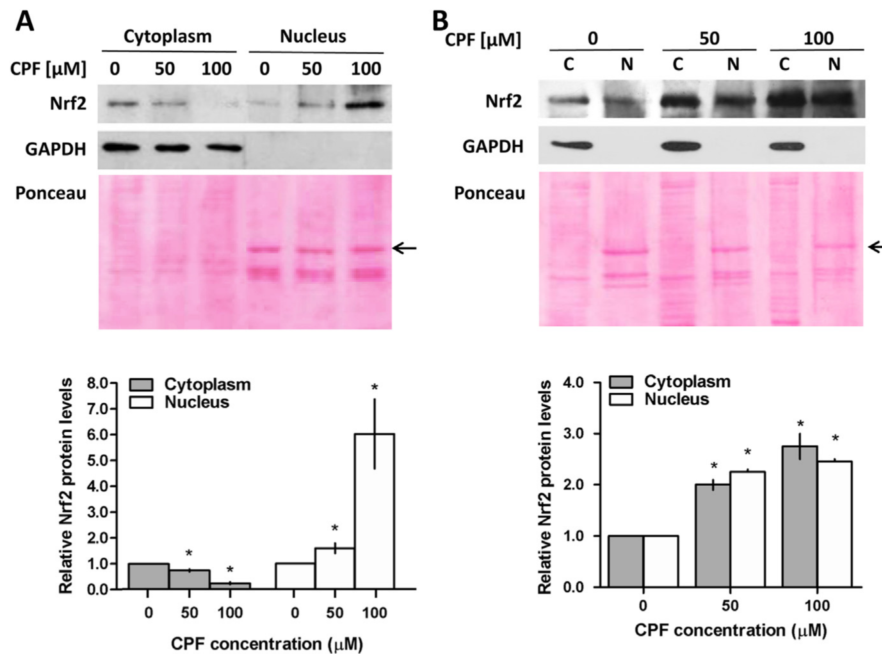

Fig. 5. Effect of CPF on Nrf2 protein translocation. Western blot analysis of protein extracts prepared from JEG-3 cells exposed to CPF (0, 50 or 100 μM) for 3 h (A) or 24 h (B). Cells were isolated and fractionated into nuclear (N) and cytoplasmic (C) extracts (see Materials and methods). Equal amounts of protein extracts were loaded in each nuclear and cytoplasmic extracts, resolved by SDS-polyacrylamide gel electrophoresis, and probed for Nrf2 by immunoblot analysis. Membranes were probed for GAPDH to normalize cytoplasmic extracts loading and to control for cross-contamination. Representative blots of three experiments are shown. Ponceau staining was used to normalize protein loading in nuclear extract. The bar graphs represent the densitometric quantification of Nrf2 protein levels relative to GAPDH (white bars) or to the intensity of the band indicated by the arrow in the Ponceau stain (grey bars) of three separate experiments. Significantly different from the corresponding control (0) group (*$p < 0.05$).

several experimental conditions implicate HO-1 in the regulation of oxidative stress [11,44–46]. Particularly, the HO protein and more specifically its by-products have been assumed to be involved in the preservation of uterine quiescence throughout gestation, regulation of hemodynamic control within the uterus and placenta, regulation of the apoptotic and inflammatory cascades in trophoblast cells, and more importantly in the maintenance of the oxidant–antioxidant balance within the placental tissues [47]. Moreover, it has been proposed that a decrease in HO expression and/or activity throughout gestation is involved in the initiation of several pathological processes implicated in the etiology of preeclampsia [47]. In this sense, immunohistochemical analysis revealed an apparent decrease in both HO-1 and HO-2 protein expressions in pathological placentas [48].

Another of the antioxidant enzyme analyzed that underwent upregulation was GR, which contributes to maintain GSH content [49]. GSH is the cell's natural antioxidant, which destroys free radicals formed in the cells. The increased expression of GR in CPF-treated JEG-3 cells suggests that this molecule is contributing to improve the antioxidant cell capacity. Collectively, these data indicate that the tolerance of JEG-3 cells to CPF exposure can be explained, at least in part, by the cell capacity to activate the antioxidant defense mechanisms.

Even though CPF maternal plasma concentrations were found in the order of pg/g [50], values that differ widely from those used in our experiments, concentrations in the micromolar range, are still within the range of potential human exposure [51]. Moreover, meconium OP values, which yield a longer-term dosimeter of prenatal exposure [52], suggest that the fetus and placenta may be exposed to high CPF doses in agricultural communities [53]. In this sense, trophoblast cells have the necessary intracellular machinery and physiological concentrations of cofactors for both phase I and

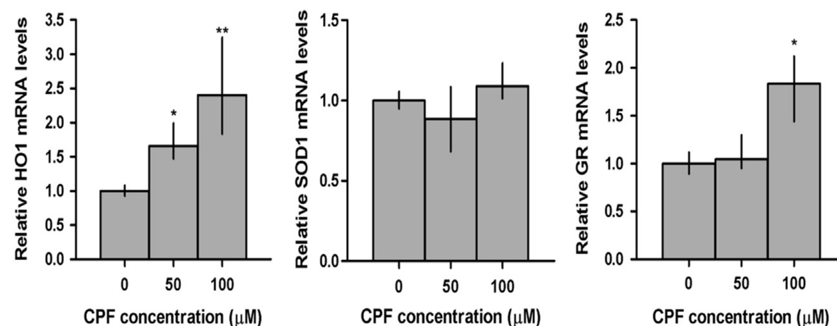

Fig. 6. Effect of CPF on the expression of HO-1, SOD1 and GR mRNA levels. mRNA expression of the indicated genes was quantified by qRT-PCR (ABI 7500, Applied Biosystems) in JEG-3 cells cultured during 24 h in the presence of the indicated CPF concentrations or vehicle alone (0). Results were normalized to cyclophilin A and expressed according to the $2^{-\Delta\Delta Ct}$ method using as a calibrator the mRNA level obtained from the control condition (0). Data are presented as the median and 25th–75th percentiles of at least three independent experiments performed in duplicate. Significantly different from the corresponding control (0) group (*$p < 0.05$, **$p < 0.01$).

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phase II reactions, the cholinergic system, as well as the complete array of transport proteins and nuclear receptors involved in the antioxidant defense response [54–57]. JEG-3 cells are a widely accepted in vitro model to evaluate mechanisms of placental drug metabolism [22,58–60]. Even though we cannot directly extrapolate our results to the in vivo placental response, it is tempting to propose that the human placenta is capable to control the oxidative insult generated by the presence of OPs like CPF in order to preserve trophoblast cell integrity and placental functions.

Pathophysiological significance of an imbalance of ROS production and elimination has been demonstrated during pregnancy [61]. It is well documented that there is a reduced antioxidant response in patients with preeclampsia and increased lipid peroxidation [62]. Certainly, oxidative stress has been implicated as a key factor in contributing to the pathogenesis of a variety of placental diseases triggering reduction of placental blood flow, which may even lead to fetal death [63,64]. Furthermore, an increased production of hCG by the syncytiotrophoblast [65], as well as a positive correlation between serum βhCG level and H_2O_2 concentration have been reported in preeclampsia [66]. These findings suggest that oxidative stress leads to an increase in the maternal circulation of H_2O_2 which triggers the secretion of hCG as a cytoprotective action against oxidation. In agreement with this, we have reported that CPF induces a significant increase of βhCG in JEG-3 cells in conditions where the morphology and viability were not compromised [22].

Herein, we demonstrated for the first time that CPF alters the redox balance inducing an adaptive activation of the Nrf2/ARE pathway with an increase in *HO-1* and *GR* phase II detoxifying genes, in trophoblast cells. Altogether, the results of this study suggest that the adaptive activation of the Nrf2/ARE pathway in placenta could confer fetal protection against xenobiotics injury.

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