Biochemical biomarkers of sublethal effects in *Rhinella arenarum* late gastrula exposed to the organophosphate chlorpyrifos

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ABSTRACT

We determined the biochemical and molecular effects of the organophosphate insecticide chlorpyrifos (CPF) in the late gastrula embryonic stage of the South American toad *Rhinella arenarum* continuously exposed from fertilization (24 h). Our objective was to evaluate these responses as potential biomarkers at low, sublethal levels of the toxicant. We first established the EC_{50} for embryo arrest in 21.3 mg/L, with a LOEC of 16 mg/L. At 4 mg/L CPF, some embryos were unable to complete the dorsal lip of the blastopore and the yolk plug became blur, probably because of abnormal cell migration. Acetylcholinesterase activity, the specific biomarker for organophosphates, was unaffected by any of the tested concentrations of CPF (2–14 mg/L). In turn, 2 mg/L CPF increased the reduced glutathione levels and inhibited glutathione-S-transferase activity, suggesting an oxidative stress and antioxidant response. Catalase was induced by CPF exposure at higher concentrations (8 and 14 mg/L). We also studied transcription factor c-Fos as a signaling event related to development in early embryogenesis. Analysis of nuclear c-Fos protein showed two bands, both enhanced in embryos exposed to 2 and 8 mg/L CPF. While nuclear Erk protein was practically unaffected, Mek protein levels were induced by the OP. Transcription factor c-Fos may be then linking oxidative stress with developmental alterations observed due to CPF exposure. These molecular and biochemical responses observed in *R. arenarum* gastrula at sublethal CPF exposures may replace non-responsive AChE as very early biomarkers in toad gastrula.

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1. Introduction

It has been reported that pesticides may alter the normal development in embryos, resulting in teratogenic effects and arrest [1–4]. We have focused our research in *Rhinella arenarum*, an autochthonous species, testing the effects of carbamate and organophosphorus (OP) pesticides during embryo development, considering that these pesticides are the most widely applied in the

irrigated valleys at North Patagonia, Argentina [5–7]. Since amphibians spend the first phase of their life cycle in aquatic environments, they are particularly sensitive to contaminants, in part due to the permeability of their skin to toxic substances [8]. In this regard, many amphibian species, including *R. arenarum*, have their offspring in spring, when great amounts of pesticides are released to the environment. Even more, applied pesticides are deposited on soils and water courses that are the natural environments of amphibian embryos and larvae.

Chlorpyrifos (CPF) is a well known OP pesticide of agriculture and domestic use. Although acute toxicity elicited by CPF active metabolite, CPF oxon, is mediated through inhibition of cholinesterases (ChE), there is evidence supporting the fact that CPF may be directly influencing brain development through cell replication and differentiation [9,10]. We recently showed the teratogenic effects of CPF in *R. arenarum* embryos at tail bud stage, linking them to alterations in the polyamine pathway through ornithine decarboxylase (ODC) activity [7]. The gene containing the ODC sequence is regulated by AP-1 transcription factor, which acts as an heteroduplex c-Fos:c-Jun or as an homoduplex c-Jun:c-Jun. CPF can directly interfere with AP-1 binding to DNA site, thus targeting nuclear events of this OP irrespective of its active metabolite conversion and

inhibition of ChE [10]. Phosphorylation of c-Fos is generally under regulation of the MAPK pathway (Raf/Mek/Erk), which may be activated in response to oxidative stress [11].

In fact, oxidative stress has been associated with OP metabolization, and CPF has been proposed to increase reactive oxygen species (ROS) [12]. Oxidative stress and antioxidant responses were also demostrated in *R. arenarum* embryos exposed to OP [3,13]. Antioxidant defenses are present throughout embryo development in *R. arenarum*, with superoxide dismutase (SOD) and catalase (CAT) being the main protecting system against ROS in the first embryonic stages. GSH and GSH-related enzymes increase their relevance in antioxidant protection when the embryos are subjected to a higher O_2 challenge [3].

The aim of this work was to analyze and compare biochemical effects, such as the activity of enzymes involved in the antioxidant response and AChE, versus molecular effects involving c-Fos protein as a member of AP-1 transcription factor, elicited by exposure to the OP CPF in order to determine early biomarkers of effect in *R. arenarum* embryos at LG stage.

2. Materials and methods

2.1. Chemicals

The insecticide chlorpyrifos (CPF,O,O-diethyl O-[3,5,6-trichloro-2-pyridyl phosphorothioate], 99% purity) was purchased from Chem Service (West Chester, Pennsylvania, USA). Reduced GSH, acetylthiocholine iodide, bovine serum albumin (BSA), 1-chloro-2,4-dinitrobenzene 97% (CDNB), 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), secondary antibody anti-Mouse, Rabbit and Rat IgG-biotin conjugate (B-1404), Avidin-Alkaline Phosphatase conjugate (A-7294), Nitro-Blue Tetrazolium (NBT S-380), 5-bromo-4-chloro-3-indolyl-phosphate (BCIP S-381), secondary antibody anti-Rabbit IgG-Peroxidase (A-6154), Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) (A8511) and *p*-coumaric acid (C9008), were purchased from Sigma Co. (St. Louis, MO). The primary antibodies, anti-c-Fos (K25, SC-253), anti-Mek (SC-436), and anti-Erk1/2 (SC-154), were purchased from Santa Cruz Biotechnology. Folin–Ciocalteu reagent was purchased from Anedra (Buenos Aires), and all the other reagents used were also of analytical grade.

2.2. Fertilization and test conditions

Adult *R. arenarum* toads were obtained from Los Barreales lake and kept in captivity outdoors in a small aquarium containing grass. Ovulations were induced by an intraperitoneal injection of 2500 units of human chorionic gonadotrophin (ELEA laboratory, Ciudad Autónoma de Buenos Aires, Argentina). The oocytes were extracted from the sacs and fertilized with a testicular homogenate in Amphibian Ringer's solution (NaCl 0.65 g/L, KCl 0.01 g/L, CaCl₂ 0.02 g/L). Recently fertilized embryos were exposed to CPF in the conditions described before [7]. Groups of 300 embryos at four-cell-stage (3 h since fertilization) were continuously exposed to 0, 2, 4, 8, 16 and 32 mg/L CPF in 300 mL of Amphibian Ringer's media and 0.3% acetone as vehicle, keeping the natural protection of the typical jelly coat surrounding the embryos during the early stages of embryo development. Solutions were prepared from a standard solution of CPF consisting of the pure chemical dissolved in acetone; the exact concentration of the insecticide was checked by gas chromatography with a nitrogen-phosphorus detector (GC-NPD). Amphibian Ringer's solution plus 0.3% acetone was used for control groups. All the treatments were performed in triplicate. The embryos were maintained at 20–22 °C in a 12-h light–12-h dark photoperiod until reaching LG stage (24 h of development).

2.3. Malformations and arrest assessment

A stereomicroscope (Wild M3, Heerbrugg) was used to follow embryonic stages [14], and to assess different kinds of malformations typified according to the *Atlas of Abnormalities* for amphibians [15]. Photographs were taken using a Sony digital camera. Data regarding number and type of malformations and number of arrested embryos were collected.

2.4. Enzymatic determinations

2.4.1. Sampling and homogenization

After 24 h of exposure, three samples of 50 embryos at LG stage were randomly taken from each replicate, and were thoroughly washed with cold Ringer's solution and briefly cooled on ice. Liquefying of the jelly coat was performed by adding thioglycolic acid (1%) neutralized with KOH 1 M in Ringer's solution and immediately washed with cold Ringer's solution. Embryos were homogenized in 1 mL of 143 mM cold potassium phosphate buffer (pH 7.5) with 6.3 mM EDTA. The homogenates were centrifuged at $10.000 \times g$ for 20 min at 4 °C and the supernatants were collected.

2.4.2. ChE determination

Cholinesterase activity was measured at 25 °C according to Ellman [16] with modifications. Each supernatant was measured by triplicate. The activity was determined using a microplate reader (Thermo Max, Molecular Devices) during 30 min. Reactions were performed using acetylthiocholine iodide (0.75 mM) in distilled water as substrate and DTNB (0.2 mM) in TrisHCl buffer 10 mM (pH 8.0). Activity was continuously recorded at 412 nm and corrected for spontaneous hydrolysis of the substrate.

2.4.3. Glutathione-S-transferase (GST)

GST activity was assayed by triplicate in a final volume of 1.0 mL of 100 mM phosphate buffer (pH 6.5) containing 0.5 mM CDNB dissolved in 1% v/v acetonitrile and 2.5 mM GSH as substrates. Baseline (non enzymatic reaction) was continuously recorded at 340 nm, and 10 μL of the supernatant was added [1]. The changes in the absorbance were recorded and corrected for spontaneous reaction, and the moles of conjugated CDNB were calculated using a molar extinction coefficient of $9.6 \text{ mM}^{-1} \text{ cm}^{-1}$.

2.4.4. Catalase

CAT activity was determined by recording the continuous decrease in hydrogen peroxide (H_2O_2) absorbance at 240 nm. The reaction was performed in 3 mL sodium phosphate buffer 50 mM pH 7.0 containing 25 mM H_2O_2. The enzymatic activity determination was carried out at the linear range of response with respect to substrate concentration. Baseline absorbance was controlled to be stable and equal to one unit of absorbance, and 10 μL of supernatant was added to initiate the catalyzed reaction. Specific activity was expressed as units per mg protein^{-1} using a molar extinction coefficient of $40 \text{ M}^{-1} \text{ cm}^{-1}$, defining one unit as the quantity of enzyme transforming one micromole of substrate at 25 mM (equivalent to one unit of absorbance) in one minute [3]. Supernatants were assayed by triplicate.

2.5. GSH content determination

Fifty LG embryos from each replicate were rinsed with cold amphibian Ringer's solution and mechanically separated from the jelly coat. The embryos were homogenized, mixed with 10% trichloroacetic acid 1:1 (v/v) and centrifuged at $10,000 \times g$ for 10 min at 4 °C. GSH content was immediately measured as acid-soluble thiols by triplicate in 0.1 mL of the supernatant, using 1 mL of 1.5 mM DTNB in 0.25 M sodium phosphate buffer, pH 8.0 [3,4]. The mixture was

incubated for 10 min and the absorbance at 412 nm was measured. Acid soluble thiols were quantified using a calibration curve with pure GSH as a standard.

2.6. Nuclear protein expression analysis

2.6.1. Nuclear protein extracts

Nuclear proteins were extracted from groups of 200 embryos at LG stage after exposure to 0, 2, 8 and 14 mg/L of CPF, using homogenate buffer (H) containing 10 mM Hepes buffer (pH 7.4), 1.5 mM MgCl$_2$, 10 mM KCl, 1 mg/mL leupeptin, 1 mg/mL aprotinin and 0.5 mM dithiothreitol. Dejellied embryos were homogenized, and a 1000 × g centrifugation during 10 min was carried out. The pellet was washed 3 times in buffer H and resuspended in extraction buffer (E) (20 mM Hepes buffer (pH 7.4), 25 % glycerol, 0.42 M NaCl, 1.5 mM MgCl$_2$, 0.2 mM EDTA, 1 mg/mL leupeptin, 1 mg/mL aprotinin, 0.5 mM PMSF (phenylmethylsulfonyl fluoride) and 0.5 mM DTT (dithiothreitol). The samples were transferred to a new tube where they were kept on ice for 30 min. Finally, the homogenates were shaked for 30 min and centrifuged at 16,000 × g for 20 min and the supernatant containing the nuclear protein extract was kept for the next step.

2.6.2. Nuclear protein analysis

One-hundred μg of nuclear proteins were separated by a 10% SDS-PAGE and transferred to a nitrocellulose membrane (Sigma Aldrich) for 60 min at 300 mA. Membranes were stained by Ponceau S to check protein loading, washed, and blocked with 5% non-fat powdered milk in TBST buffer (5% Tween 20 in Tris-buffered saline pH 7.6) for one hour. Polyclonal antibody (c-Fos) was diluted 1:250 in TBST buffer and incubated with the membrane at RT for 90 min. Secondary antibody anti-mouse, rabbit and rat IgG Biotin conjugate (1:5000) was mixed with Avidin Alkaline Phosphatase conjugate in TBST buffer for 30 minutes prior to incubation with the membrane for 90 min at room temperature (RT). The membranes were then washed two times for 10 min and incubated with 5 mL AP buffer containing 33 μl NBT and 16.5 μl BCIP in the darkness until color development. For Mek and Erk1/2 analysis, blocked membranes were incubated with the respective polyclonal antibody (1:200) in TBST buffer at RT for 60 min. The membranes were washed three times for 5 min with TBST and incubated with secondary antibody anti-Rabbit igG (whole molecule)-Peroxidase diluted (1:5000) in TBST buffer for 60 min at RT. After two washes for 10 min with TBST, a final 5 min-wash was performed with TBS. ECL solution was prepared as two solutions mixed prior to membrane incubation (solution A: Tris-HCl 100 mM (pH 8.6), 50 μl Luminol 250 mM prepared in DMSO (dimethyl sulfoxide) and 22 μl p-coumaric acid 90 mM prepared in DMSO; and solution B: Tris-HCl 100 mM (pH 8.6), 3 μl H$_2$O$_2$ (30% W/V).

2.7. Protein determination

Protein content was determined according to Lowry et al. [17] using bovine serum albumin as standard.

2.8. Data analysis

Effective concentration-50 (EC$_{50}$) was calculated using PriProbit version 1.63 designed by Masayuki Sakuma [18]. The observed effects on control animals were less than 10% so Abbot's correction was not necessary. Lowest-Observed-Effective-Concentration (LOEC) and No-Observed-Effective-Concentration (NOEC) were assessed from experimental data by Student's t-test [19].

Mean ± SEM of enzyme activities were calculated from two independent experiments with triplicate treatments, using the average measurements. Statistical differences between treatments were assessed by ANOVA and Fisher's lowest significant differences (LSD) post hoc test.

Western blot protein levels were analyzed using ImageJ software [20]. The results were expressed in relative units with respect to controls, considering protein loading in each lane by Ponceau S staining as no commercial antibodies could be found to cross-react with R. arenarum constitutive proteins.

3. Results

3.1. EC$_{50}$ determination and morphological malformation analysis

We assessed embryo arrest as the endpoint to determine CPF toxicity in R. arenarum embryos at LG. The EC$_{50}$ estimated by Probit analysis was 21.3 mg/L (15.5–27.5 mg/L, 0.95 confidence limits). The lowest concentration causing significant arrest (LOEC) was 16 mg/L (p = 0.028), which was similar to the EC$_{10}$ estimated by Probit regression (14.8 mg/L). The NOEC was estimated in 11.0 mg/L CPF using EC$_1$, while the experimental concentration of 8 mg/L caused not significant differences in embryo arrest with respect to control (p = 0.15).

Embryos exposed to 2 mg/L CPF showed no morphological alterations when compared to control embryos. As the concentration increased, an abnormal gastrulation was distinguished. Some embryos exposed to 4 mg/L CPF showed an incomplete blastopore dorsal lip, and at 16 mg/L the malformations were observed among the entire cohort, becoming impossible to identify the cells composing the yolk plug (Fig. 1). At 32 mg/L CPF, all the embryos showed incomplete gastrulation and were unable to continue their development.

3.2. Effects on ChE specific activity and antioxidant defenses

Exposure to increasing concentrations of CPF up to 14 mg/L, the highest concentration tested, did not significantly affect ChE activity in embryos at LG stage (Fig. 2). Cholinesterase activity in control LG embryos was about 0.21 mIU/mg protein, which was found to be very low compared to ChE levels in other stages.

Glutathione content was significantly increased in embryos exposed to CPF with respect to control (Fig. 3A). When LG embryos were exposed to 2–8 mg/L, GSH content increased almost 300% (p < 0.001). The highest CPF concentration tested of 14 mg/L produced a moderate increase in GSH content with respect to control (55%), significantly lower than 2 and 8 mg/L CPF. In turn, GST activity was diminished in LG embryos exposed to CPF (Fig. 3B). A 35% decreased GST activity was observed in gastrula exposed to 2 mg/L CPF with respect to control (p = 0.005), similar to 14 mg/L exposure (32% of inhibition; p = 0.008). Catalase activity was unaffected by the lowest CPF concentration (Fig. 3C), but 8 mg/L CPF yielded a significant increase in the activity (168%; p < 0.001) with respect to control. In embryos exposed to 14 mg/L, catalase activity diminished with respect to 8 mg/L-exposed embryos but was above the levels measured in control embryos (79%; p = 0.002).

3.3. C-Fos and its regulatory Mek-Erk pathway levels

We analyzed the effect of the tested concentrations of CPF on the expression of c-Fos protein. In control embryos, a single band of 68 KDa was observed, but in embryos exposed to 2 and 8 mg/L CPF an additional 64 KDa-band was detected (Fig. 4A). The 68 KDa-band was increased in 2 mg/L-exposed embryos about 50% with respect to control. The levels of c-Fos protein in embryos exposed to 14 mg/L CPF showed no differences with respect to control. In contrast, Erk showed no substantial variations in response to any of CPF treatments (data not shown). Western blot analysis of Mek showed increments of 6X, 8.5X and 7.6X with respect to control levels
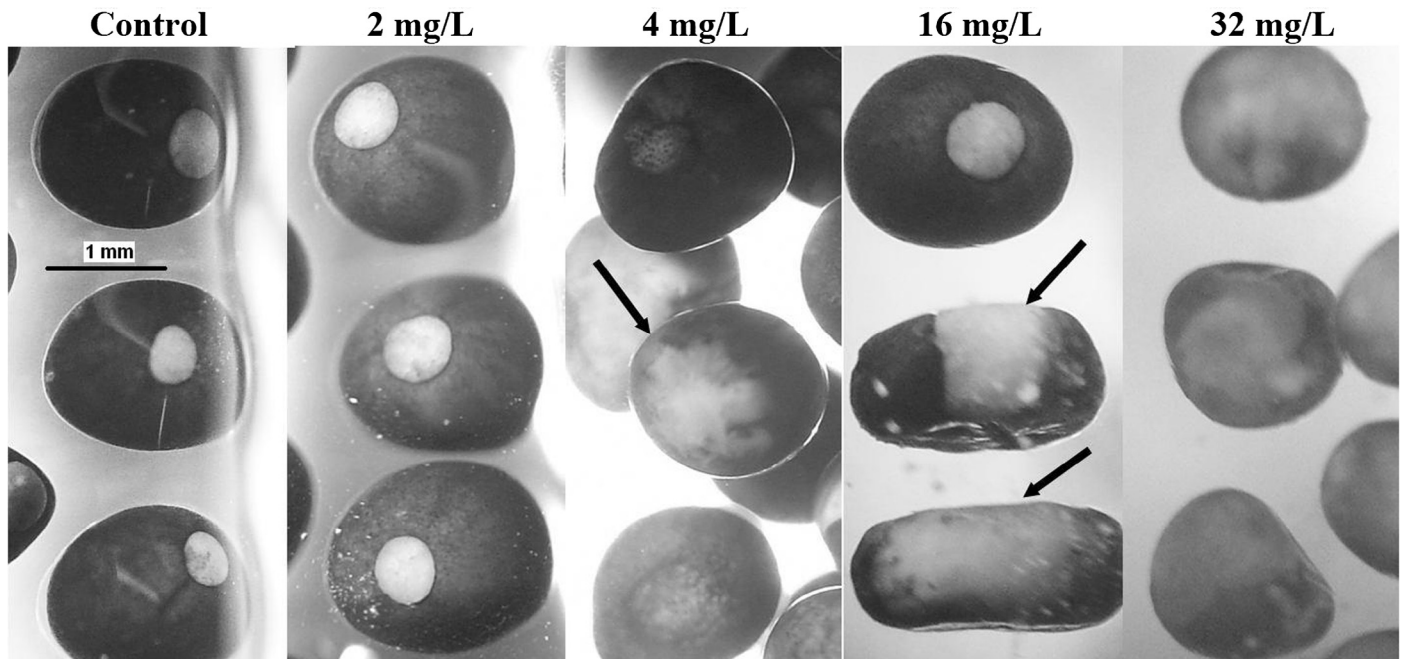
Fig. 1. Morphological alterations in *R. arenarum* embryos at late gastrula (LG) exposed to CPF. Arrows indicate malformed yolk plug in embryos exposed to 4 and 16 mg/L CPF.

in nuclear extratcs from embryos exposed to 2, 8 and 14 mg/L CPF respectively (Fig. 4B).

4. Discussion

Our results indicate that CPF may affect AP-1 transcription factor, regulating the expression of c-Fos mediated by Mek in exposed gastrula. This effect could be detected earlier and at lower concentrations than those eliciting the responses of other traditional biomarkers. While the EC_{50} for embryo arrest in LG stage was 21.3 mg/L CPF, malformations were detected in a significant percentage of gastrula exposed to 4 mg/L. Indeed, biochemical biomarkers related to oxidative stress effects and antioxidant responses were succesfully detected in gastrula exposed to 2 mg/L, where c-Fos and Mek also responded as molecular biomarkers related to underlying mechanisms to those effects. In fact, the specific biomarker for OP exposure, AChE, was hardly detected in the gastrula and did not respond to
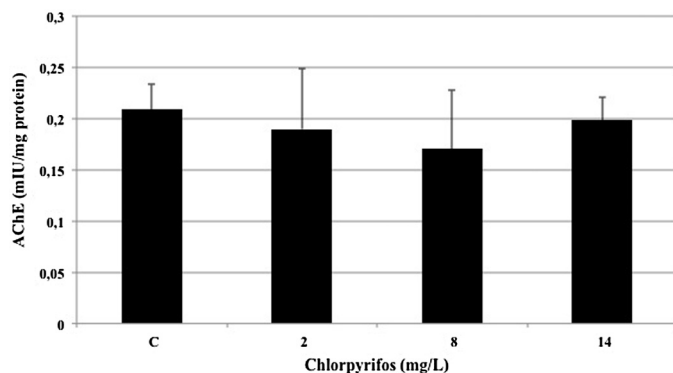

Fig. 2. ChE activity in *R. arenarum* embryos at late gastrula (LG) stage exposed to CPF. Mean data and SEM are shown from two independent experiments with three measurements by sample. No significant differences between treatments were found ($p = 0.05$).

CPF exposure. It is also noticed that the exposure to higher concentrations close to CPF EC_{50} for arrest in gastrula stage negatively affects the responses of some biochemical biomarkers that become similar to controls.

The analysis of malformations caused by CPF in embryos at LG stage showed as the main alteration an undefined edge of the yolk plug because of the abnormal cell distribution. The incomplete blastopore dorsal lip may result from an altered internalization of the endoderm because of ectoderm epiboly and involution of cells from the mesoderm, migrating toward the interior of the blastopore and beneath the surface. These malformations observed in some embryo at exposures to low CPF concentrations would progressively come out in other embryo with no signs of teratogenesis, increasing the number of failures in development [7]. It has been reported that *X. laevis* larvae exposed to CPF and malathion show developmental alterations [21,22]. Thus, CPF should be considered as teratogenic to aquatic wildlife.

Cholinesterase activity increases drastically along *R. arenarum* development, from 0.2 mIU/mg protein in gastrula (present work) to about 15 mIU/mg protein in complete operculum stage at the end of the embryonic phase [13], and even higher (70 mIU/mg protein) in larval stages [6]. In early stages of development such as LG, AChE function is different from cholinergic transmission ending, and is instead related to neurological development, neuronal plasticity and cell migration [23]. The fact that AChE remained unaltered toward CPF exposure firstly suggests that at LG stage there may be present a resilient isoform of AChE [24], or that it is unavailable to OP attack. Secondly, it highlights that another mechanism different from the classical cholinergic inhibition is acting to cause mortality at early embryonic development. It has been proven that CPF itself, rather than its active metabolite, inhibits DNA synthesis leading to a decrease in the number of neural cells among other effects [2,25,26].

Late gastrula *R. arenarum* embryos showed oxidative stress and antioxidant response to CPF exposure at low (1/10 EC_{50} arrest) concentrations. Catalase is one of the enzymes involved in the main antioxidant response in early amphibian embryos [3,27]. An increment of catalase activity is reported in response to environmental
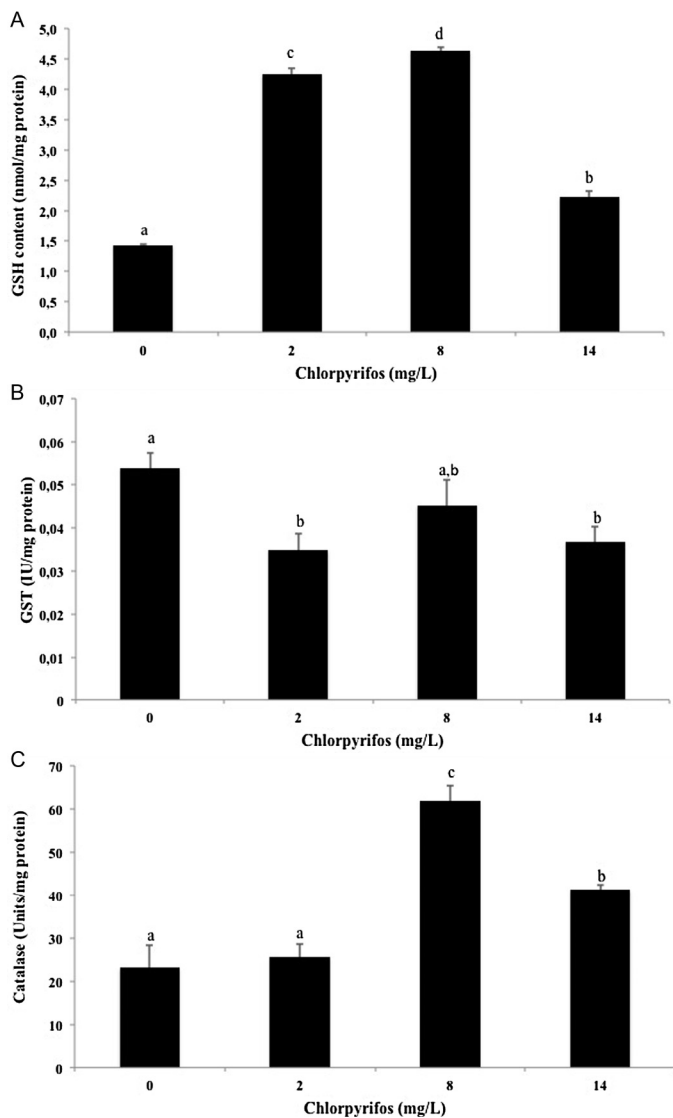
and p53 in the early phases of cell growth when most cells are in active division [30].

Molecular analysis of c-Fos protein as a putative early biomarker was performed since it participates as one of the members of AP-1 transcription factor, which regulates gene expression in cellular proliferation. Increased expression and phosphorylation of c-Fos contributes to activation of AP-1, which may be triggered by oxidants [31]. The exposure of gastrula to low (2–8 mg/L) CPF concentrations resulted in an increase of a 68-KDa band and the appearance of a 64-KDa band, both corresponding to c-Fos protein. It is possible that CPF may be regulating not only c-Fos expression but also its activation through phosphorylation, with the upper and the lower band being the phosphorylated and unphosphorylated c-Fos proteins respectively. The regulatory pathway leading to c-Fos phosphorylation showed a very well defined increase in the nuclear upstream-kinase Mek. However, nuclear Erk did not show a significant response to CPF exposure, and we were not able to determine the phosphorylated form of Erk in *R. arenarum* nuclear protein extract using primary antibodies arising against standard species. It has been established that both Mek and Erk are capable of translocating to the nucleus during many cellular processes to induce transcription via c-Fos activation [32]. Although Erk1/2 has been generally reported to be the activating kinase of c-Fos, other MAPKs such as p38 have also been involved in c-Fos induction by some signaling pathways [33]. Interstingly, the upregulation of c-Fos and Jun through MAPK pathway by DNA damage has been involved in apoptotic response to oxidative stress [34]. There may be then a direct link between oxidative stress caused by CPF in LG embryos, c-Fos induction, and the subsequent arrest observed later in the development. This fact was well established by Cheng et al. [35], who showed that the increase in ROS was involved as a signal in c-Fos expression by the Ras pathway, a fact that was abolished by catalase and GSH. In fact, ROS production leading to Erk 1/2 activation through MEK1-mediated phosphorylation was established as the chain of events leading to the inhibition of proliferation in breast cancer cell lines exposed to CPF [36]. When toad gastrula are exposed to higher CPF concentrations approaching the EC_{50} for arrest, the increase in the oxidative stress may lead to a failure in

Fig. 3. Antioxidant responses in *R. arenarum* embryos at late gastrula (LG) stage exposed to CPF. Mean data and SEM are shown from two independent experiments with three measurements by sample. Significant differences assessed by ANOVA-LSD test are indicated by different letters. **A:** GSH content; a-d: $p < 0.001$. **B:** GST activity; a,b: $p < 0.01$. **C:** Catalase activity; a–c: $p < 0.002$.

challenge causing oxidative stress [28]. We observed that catalase increase was accompanied by an increment in the endogenous content of GSH in embryos exposed to CPF. Both catalase expression and GSH synthesis through γ-glutamylcysteine synthetase expression are upregulated by oxidative stress by the transcription factor Nrf-2 through its binding to the antioxidant response element ARE [29]. Thus, CPF may be increasing both responses through ROS generation. Higher CPF concentrations (14 mg/L) limited the increase of GSH content and catalase activity. As GSH neutralizes free radicals directly acting as an antioxidant agent or through GSH-peroxidase activity, its levels may be effectively reduced by an increasing ROS production due to higher CPF concentrations in the same way as for catalase activity [6]. Catalase enzyme possesses an heme group in its catalytic site that is highly sensitive to oxyradicals. Oxidative stress may be also associated with the decrease in GST activity in gastrula exposed to CPF, as observed in other situations [6]. The rise in GSH content in treated embryos could be related to the need of keeping a reduced cellular environment for the correct activity of several transcription factors such as NF-κB AP-1 complex
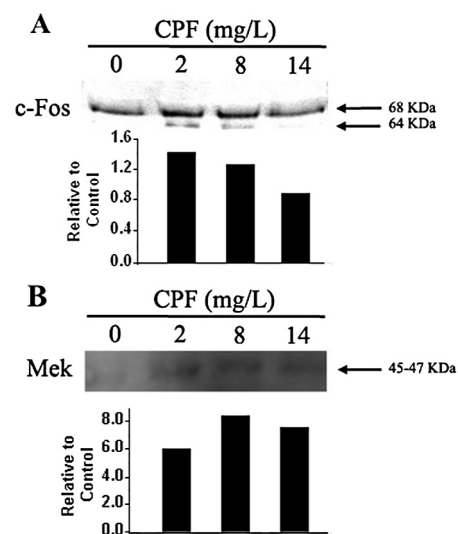

Fig. 4. c-Fos and Mek nuclear protein levels in *R. arenarum* embryos at late gastrula (LG) stage exposed to CPF. Nuclear protein extracts were separated by SDS-PAGE and analyzed by Western blot. Data are from one experiment representative of two. **A:** c-Fos: 64 and 68 KDa bands. **B:** Mek: 45–47 KDa band. Protein levels were analyzed using ImageJ software and expressed in relative units with respect to controls (68 KDa-band for c-Fos). Protein loading in each lane was checked by Ponceau S staining.

molecular responses mediating cellular defenses and trigger apoptotic/ necrotic processes.

In conclusion, *R. arenarum* late gastrula exposed to low CPF concentrations show an early response to oxidative stress that may be linked to Mek pathway activation and c-Fos regulation and to developmental alterations preceding embryo arrest. These biochemical and molecular events may be very early biomarkers of OP exposure, instead of the very low and unresponsive ChE activity observed in toad gastrula.

Acknowledgments

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