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Diclofenac-enriched artificial sediment induces oxidative stress in *Hyalella azteca*

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ABSTRACT

Diclofenac is a nonsteroidal anti-inflammatory drug widely used in Mexico where it is sold over the counter. It enters water bodies through municipal and industrial discharges, posing a risk to water systems and aquatic organisms. Diclofenac-enriched artificial sediment was used to evaluate the toxicity of this pharmaceutical on the sentinel species *Hyalella azteca*, using oxidative stress biomarkers in order to determine if the set of tests used in this study is a suitable early damage biomarker. The median lethal concentration (72-h LC_{50}) was determined and oxidative stress was evaluated using lipid peroxidation, protein carbonyl content to evaluate oxidized protein content, and the activity of superoxide dismutase, catalase, and glutathione peroxidase. All biomarkers were significantly altered. Diclofenac induces oxidative stress in *H. azteca* and the set of tests used (lipid peroxidation, protein carbonyl content, antioxidant enzyme activities) constitutes an adequate early damage biomarker for evaluating the toxicity of this pharmaceutical group in aquatic species.

1. Introduction

Diverse studies have shown that pharmaceutical drug concentrations have increased markedly in water bodies throughout the world (Snyder et al., 2005; Montforts et al., 2007), becoming a major source of pollution. These compounds have therefore been identified as emerging contaminants in aquatic ecosystems (Kim et al., 2007; Zuccato et al., 2006). The main pharmaceuticals detected include nonsteroidal anti-inflammatories (diclofenac, ibuprofen, acetylsalicylic acid, naproxen, ketorolac, paracetamol), stimulants (methylxanthines such as caffeine) and carbamazepine, atorvastatin, fluoxetine and 17α-ethynylestradiol (Khetan and Collins, 2007; Bormann et al., 2006; Zuccato et al., 2006; Gagné et al., 2005; Ashton et al., 2004).

In Mexico, one of the highest selling and most frequently used medicinal groups is nonsteroidal anti-inflammatories (NSAIDs), in particular diclofenac (Gómez-Oliván et al., 2009). NSAIDs come in a large variety of pharmaceutical forms and are used irrationally since they can be purchased over the counter.

The aryl acetic derivate diclofenac has analgesic, antipyretic and anti-inflammatory properties. It is widely used to relieve pain in acute and chronic inflammatory conditions, rheumatic syndromes, degenerative disease, acute lumbar pain, tendinitis, bursitis, sciatica, gout, dental surgery, dysmenorrhea and migraine (Mycek et al., 2004). Its main mechanism of action is competitive inhibition of the enzyme cyclooxygenase (COX) which synthesizes prostaglandins from arachidonic acid. It also blocks the action of the neuromodulator glutamate which amplifies reflex response to peripheral pain sensitization and stimulates the release of endogenous opioids and serotonin (Foegh and Ramwell, 2005).

Diverse studies report that trace concentrations of NSAIDs in the environment elicit toxic effects in photosynthetic organisms such as *Synechocystis* sp. and *Lemna minor* (Zuccato et al., 2006) and impair reproduction in *Daphnia magna* and *D. longispina* (Marques et al., 2004). In particular, exposure to diclofenac at the concentrations usual in surface waters has been associated with adverse effects in the brown trout *Salmo trutta*, including kidney and gill impairment and alterations in immunological parameters (Khetan and Collins, 2007).

The biomarkers used to characterize toxic response must be carefully selected. They are defined as a change in any biologic, physical or chemical response associated with toxic effects or exposure to environmental chemicals (van der Oost et al., 2003). In our study, oxidative stress was selected as a biomarker of toxicity.

Oxidative stress is produced by disruption of the balance between reactive oxygen species (ROS) and the antioxidant sys-

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tems of the body. ROS, such as superoxide radical anion (O_2^-), the hydroxyl radical ($OH^•$) and hydrogen peroxide (H_2O_2), are produced in cells as a result of metabolic processes (Vlahogianni et al., 2007). Such ROS can induce cellular damage by promoting lipid peroxidation (LPO) of cell membranes, and have recently been implicated in the regulation of many cellular events, such as transcription factor activation, gene expression and differentiation, and cellular proliferation (Park et al., 2007). Antioxidant systems such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) are therefore required by the cell in order to offset the effects of ROS (Sinha et al., 2007). Thus, prooxidant/antioxidant balance and ROS scavenging are essential in order to maintain cellular homeostasis (Valavanidis et al., 2006). Other studies have shown that exposure to environmental contaminants stimulates ROS formation in cells (Sinha et al., 2007), increasing LPO and protein carbonyl content (PCC) and altering antioxidant enzyme activities (Parvez and Raisuddin, 2005; Vlahogianni et al., 2007).

The occasional amphipod *Hyalella azteca* offers many advantages as a sediment and water quality bioindicator, including easy reproduction and maintenance under laboratory conditions and high sensitivity to diverse xenobiotics.

The aim of this study was to evaluate the toxicity induced on *H. azteca* by diclofenac-enriched artificial sediment using oxidative stress biomarkers, in order to determine if this set of tests constitutes an adequate early damage biomarker.

2. Materials and methods

2.1. Specimen procurement, culture and maintenance

H. azteca was collected from its natural habitat in Lake San Miguel de Almaya, municipality of Capuluac (State of Mexico) and transported to the laboratory in plastic bags with constant aeration.

For culturing, specimens were maintained in reconstituted water ($NaHCO_3$ = 174 mg L^{-1}, $MgSO_4$ = 120 mg L^{-1}, KCl = 8 mg L^{-1} and $CaSO_4 \cdot 2H_2O$ = 120 mg L^{-1}) pH 7.5–8.5 at room temperature with constant oxygen (6.4–6.6 mg L^{-1}, O_2) and a 16 h:8 h light:dark photoperiod, and were fed ground lettuce *ad libitum* (Galar-Martínez et al., 2006).

Specimens used in the toxicity assays were third-generation neonates obtained by sexual reproduction from a four-month culture.

2.2. Artificial sediment preparation

The artificial sediment was 70% sand (0.2 mm), 20% kaolinite (<0.002 mm) and 10% organic matter (0.2 mm). The organic matter source was lamb compost inactivated by dry heat at 55–60 °C for 3 days. The sediment was sterilized with three 15-min autoclave cycles at 121 °C and 15-lbs pressure, separated by 1-h intervals (Martínez-Tabche et al., 2000).

2.3. Determination of LC_{50} and oxidative stress

Test systems consisted in 150-mL polyethylene containers holding a 3:1 ratio of reconstituted water and artificial sediment, and supplied with constant oxygen and a 16 h:8 h light:dark photoperiod at room temperature. Intoxication systems were static and no food was provided to specimens during exposure periods.

The median lethal concentration (LC_{50}) of diclofenac in *H. azteca* was determined in five test systems enriched with different concentrations of diclofenac (0.42, 0.67, 1.0, 1.7 and 2.7 mg kg^{-1} sediment) and a diclofenac-free control system, placing 10 specimens in each. The number of dead (immobile) specimens was counted after 72 h. Five replicates of the assay were performed. The 72-h LC_{50} of diclofenac and its 95% confidence limits ($p < 0.05$) were estimated by Probit analysis (EPA Probit Analysis Program v1.5).

For the sublethal toxicity assay, test systems were enriched with 46.7 μg diclofenac kg^{-1} sediment (equal to 1/10 of the LC_{50}) and 150 mg wet weight of *H. azteca* was added (entire organisms were used). The exposure times used were 0, 24, 48 and 72 h after which, test specimens were removed and suspended in 1 mL of Tris buffer solution pH 7. The mixture was maintained in an ice bath throughout the procedure and homogenized. The supernatant was centrifuged at 12,500 rpm and −4 °C for 15 min. The following biomarkers were evaluated: LPO; the activity of the antioxidant enzymes SOD, CAT and GPX; and PCC to determine oxidized protein content. All tests were made on the supernatant except for LPO determination in which the cellular pellet was used.

2.4. Determination of lipid peroxidation

LPO was determined by the Büege and Aust (1978) method. To 500 μL supernatant was added Tris–HCl buffer pH 7.4 to attain a volume of 1 mL. This was incubated at 37 °C for 30 min; 2 mL of TCA–TBA reagent (0.375% thiobarbituric acid in 15% trichloroacetic acid) was added and the sample was shaken in a vortex. It was then heated in boiling water bath for 45 min, allowed to cool and the precipitate removed by centrifugation at 3000 rpm for 10 min. Absorbance was determined at 535 nm using a reaction blank. Results were calculated as mM malondialdehyde mg $protein^{-1}$ using the molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}.

2.5. Determination of SOD activity

SOD activity was determined according to the Misra and Fridovich (1972) method. To 20 μL supernatant in a 1-cm cuvette were added 150 μL of a carbonate buffer solution (50 mM sodium carbonate and 0.1 mM EDTA) pH 10.2 and 100 μL adrenaline (30 mM). Absorbance was read at 480 nm, at 30 s and 5 min. SOD activity was determined by interpolating the data on a type curve. Results were calculated as IU SOD mg $protein^{-1}$.

2.6. Determination of CAT activity

CAT activity was determined according to Radi et al. (1991). To 20 μL supernatant were added 1 mL of an isolation buffer solution (0.3 M saccharose, 1 mM EDTA, 5 mM HEPES and 5 mM KH_2PO_4) and 0.2 mL of hydrogen peroxide (20 mM). Absorbance was read at 240 nm, at 0 and 60 s. Results were obtained by substituting the absorbance value of each time in the formula: catalase concentration = (A60 − A0)/MEC, where the MEC of H_2O_2 equals 0.043 mM^{-1} cm^{-1}. Results were calculated as mM H_2O_2 mg $protein^{-1}$.

2.7. Determination of GPX activity

GPX activity was determined using the Paglia and Valentine (1967) method. To 100 μL supernatant were added 900 μL of buffer reagent solution (5 M K_2HPO_4, 5 M KH_2PO_4, 3.5 mM reduced glutathione, 1 mM sodium azide, 2 U glutathione reductase and 0.12 mM NADPH, pH 7.0; Sigma) and 200 μL of H_2O_2 (20 mM). Absorbance was read at 340 nm at 0 and 60 s. Activity was estimated using the molar extinction coefficient of NADPH (6.2 mM^{-1} cm^{-1}). Results were calculated as mM NADPH mg $protein^{-1}$.

2.8. Determination of protein carbonyl content

PCC was determined by the method of Levine et al. (1994). Soluble proteins were obtained by centrifugation of samples at 10,500 rpm for 30 min. To 100 μL of this supernatant was added 150 μL of 10 mM dinitrophenylhydrazine in 2 M HCl (Sigma) prior to incubation at room temperature for 1 h in the dark. Subsequently, 500 μL of 20% trichloroacetic acid was added and the sample was allowed to rest for 15 min at 4 °C. Next, this was centrifuged at 16,000 rpm for 5 min. The bud was rinsed three times in 1:1 ethanol:ethyl acetate (Baker), dissolved in 150 μL of 6 M guanidine (Sigma) pH 2.3, and incubated at 37 °C for 30 min. Absorbance was read at 366 nm and results were expressed as nmols of reactive carbonyls (C=O) formed mg^{-1} protein based on their molar extinction coefficient of 21,000 M^{-1} cm^{-1}.

2.9. Determination of total protein content

To 25 μL of supernatant was added 75 μL deionized water and 2.5 mL Bradford's reagent (0.05 g Coommassie Blue dye, 25 mL of 96% ethanol and 50 mL H_3PO_4, in 500 mL deionized water). The test tubes were shaken and allowed to rest for 5 min prior to evaluation of absorptivity at 595 nm and interpolation on a bovine albumin curve (Bradford, 1976).

2.10. Statistical analysis

Results were subjected to a one-way analysis of variance (ANOVA) and differences between means were compared using the Tukey–Kramer multiple comparisons test, with p set at <0.05.

3. Results

3.1. LC_{50} determination

The LC_{50} of diclofenac in the acute toxicity assay was 0.467 mg kg^{-1} at 72 h, with a 95% confidence interval of (0.344–0.57). The X^2 linear adjustment test was not significant at $p < 0.05$.