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DNA damage and oxidative stress induced by acetylsalicylic acid in *Daphnia magna*

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ABSTRACT

Acetylsalicylic acid is a nonsteroidal anti-inflammatory widely used due to its low cost and high effectiveness. This compound has been found in water bodies worldwide and is toxic to aquatic organisms; nevertheless its capacity to induce oxidative stress in bioindicators like *Daphnia magna* remains unknown. This study aimed to evaluate toxicity in *D. magna* induced by acetylsalicylic acid in water, using oxidative stress and DNA damage biomarkers. An acute toxicity test was conducted in order to determine the median lethal concentration (48-h LC_{50}) and the concentrations to be used in the subsequent subacute toxicity test in which the following biomarkers were evaluated: lipid peroxidation, oxidized protein content, activity of the antioxidant enzymes superoxide dismutase, catalase, and glutathione peroxidase, and level of DNA damage. Lipid peroxidation level and oxidized protein content were significantly increased ($p < 0.05$), and antioxidant enzymes significantly altered with respect to controls; while the DNA damage were significantly increased ($p < 0.05$) too. In conclusion, acetylsalicylic acid induces oxidative stress and DNA damage in *D. magna*.

1. Introduction

Thirty million people worldwide use nonsteroidal anti-inflammatory drugs (NSAIDs) daily (Morera et al., 2007). The most common members of this group of pharmaceuticals in terms of consumption and biological action include naproxen, paracetamol, diclofenac, ibuprofen and acetylsalicylic acid (ASA), among others (Katzung, 2007).

Due to its high effectiveness and low cost, ASA has remained for over 90 years as one of the most widely used pharmaceuticals (Katzung, 2007), and as the other members of the NSAIDs, its mechanism of action involves inhibition of prostaglandin synthesis due to the irreversible blocking of the enzyme cyclooxygenase (COX), which is responsible for catalyzing the conversion of arachidonic acid to endoperoxides.

Human and veterinary pharmaceutical agents, including NSAIDs, are regarded as emerging contaminants and are found in water bodies worldwide, in which they enter through domestic and industrial wastewater discharges (Cleuvers, 2004; Kent et al., 2006). NSAIDs, such as ASA, have been detected all over the world in significant quantities in domestic effluents (1.51 μg/L), superficial waters (0.01–0.5 μg/L) (Stumpf et al., 1996; Parolini et al., 2009) and water from treatment plants (>1 μg/L) (Ternes, 1998). The most active metabolite of ASA was detected in the public water supply system at concentrations >4.1 μg/L (Ternes et al., 2001), and in Spain ASA has been quantified at concentrations of 13 μg/L (Farré et al., 2001; Heberer, 2002) and even 59.6 μg/L in wastewater (Metcalfe et al., 2003).

Diverse studies point out that trace concentrations of ASA may induce toxicity in organisms such as daphnids and algae (Cleuvers, 2004; Khetan and Collins, 2007) as well as *Vibrio fischeri* (Brun et al., 2006). Marques et al. (2004a,b) demonstrated that chronic exposure to ASA and its metabolites at concentrations of 1.8 mg/L affects reproduction in cladocerans like *Daphnia magna* and *Daphnia longispina*, causing abortions and abnormal neonates. The mechanism of its toxic action has not been elucidated, however other NSAIDs have shown to induce oxidative stress in aquatic species, as is the case of acetaminophen on *Hyalella azteca* (Gómez-Oliván et al., 2012), ibuprofen, acetaminophen and diclofenac on common carp (*Cyprinus carpio*) (Islas-Flores et al., 2013; Nava-Álvarez et al., 2014), and diclofenac, ibuprofen and naproxen on *D. magna* (Gómez-Oliván et al., 2013).

Oxidative stress is produced by disruption of the balance of reactive oxygen species (ROS) and the antioxidant systems in the organism. ROS are formed as a result of metabolic processes carried out by the cells, but may be increased by many pollutants such as metals and hydrocarbons, among others (Vlahogianni et al., 2007). The most important

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antioxidant enzymes are: superoxide dismutase (SOD, converts O_2^- to H_2O_2), catalase (CAT, reduces H_2O_2 to water), and glutathione peroxidase (GPX, detoxifies H_2O_2) and organic hydroxyperoxides formed, for example, by lipid peroxidation (LPX) (Barata et al., 2005). Defects in the antioxidant defense that detoxify excess ROS may lead to significant oxidative damage including deactivation of enzymes, protein degradation, DNA damage and LPX (Halliwell and Gutteridge, 1999).

In aquatic toxicology, species of the genus *Daphnia* are commonly used (Santojanni et al., 2003), since they present many advantages like its wide distribution in freshwater bodies, its short life cycle, and the fact that it is relatively easy to grow in the laboratory and is sensitive to a wide variety of aquatic pollutants (Rand, 1995).

The aim of this study was to evaluate toxicity induced on *D. magna* by ASA in water, using oxidative stress and DNA damage biomarkers.

2. Material and methods

2.1. Specimen procurement, culture and maintenance

D. magna was obtained from Mexican Institute of Oil (IMP) and cultured in the laboratory of environmental toxicology of the Autonomous University of Mexico State (UAEMex), for several generations.

For the culturing of specimens, daphnids were maintained in ASTM hard water medium under a light:dark photoperiod of 16:8 h at 20 ± 2 °C. The hard water medium had a total hardness of 200–250 mg $CaCO_3$/L, a pH ranging from 8.0 to 8.4 with constant aeration (U.S.EPA, 2002), and daphnids were fed with 5 mL of concentrated algae *Scenedesmus* sp. (3×10^6 cells/mL). Toxicity assays (acute and sublethal) were performed with 24 ± 3 h neonates.

2.2. Acute toxicity assay

Test systems consisted in 1500-mL plastic containers and 1000 mL of ASTM hard water medium. The median lethal concentration (LC_{50}) of ASA in *D. magna* was determined in five test systems spiked with different concentrations of ASA (77.5, 82.5, 88.5, 94 and 101.5 mg/L) and an ASA-free control system, placing 10 specimens in each. Dead (immobile) specimens were counted after 48 h. The 48-h LC_{50} of ASA and its 95% confidence limits ($p < 0.05$) were estimated by Probit analysis (EPA Probit Analysis Program v.1.5). A static nonrenewal model with the following characteristics was used: 16:8 h light:dark cycles, temperature of 20 ± 2 °C and constant aeration. No food was provided to specimens during the exposure period. The study was performed in triplicate for each test concentration and control, and different vessels were used for each replicate.

2.3. Sublethal toxicity assay

Sublethal toxicity of ASA was determined using biomarkers of oxidative stress (SOD, CAT and GPX activity, LPX, and protein carbonyl content (PCC) and biomarkers of genotoxicity (comet assay and detection of oxidized bases by modified comet assay).

Test systems consisted in 1500-mL plastic containers and 1000 mL of ASTM hard water medium. The test concentrations used were 0 (control group) and 8.8 mg/L of ASA, equal to the lowest observed adverse effect level (LOAEL) obtained from the previous acute assay. After the pharmaceutical was added, the system was mechanically shaken for 10 min to homogenize it and 500 mg of *D. magna* (wet weight) was introduced and exposed for 48 h. A static nonrenewal model with the following characteristics was used: 16:8 h light:dark cycles, temperature of 20 ± 2 °C and constant aeration. No food was provided to specimens during the exposure period. The study was performed in triplicate for each test concentration and its corresponding control and different vessels were used for each replicate.

To evaluate the biomarkers of oxidative stress, after the exposure period ended specimens were weighed and homogenized with 1 mL phosphate buffered saline (PBS), pH 7.4. The homogenate was divided into two parts: one was used to determine LPX and the other was centrifuged at 16,000 *g* for 15 min at 4 °C. The supernatant was used to determine PCC as well as activity of the antioxidant enzymes SOD, CAT and GPX. Total protein content was used to express the results of all the biomarkers assayed.

To evaluate genotoxicity, 150 organisms per treatment were exposed to 10 μM/H_2O_2 (positive control), 0 (control) and 8.8 mg/L ASA under the same conditions than the oxidative stress test. After 24 and 48 h of exposure, organisms were removed from the intoxication systems and were disgregated in a mortar with 500 μL of PBS at 4 °C, and after mildly homogenized for up to 1 min. Cellular viability (95 ± 0.7416%) was confirmed using 0.4% trypan blue. The study was also performed in triplicate for each test concentration and controls, and different vessels were used for each replicate.

2.4. Determination of lipid peroxidation degree

LPX was determined using the Büege and Aust (1978) method. 500 μL of supernatant was added Tris–HCl buffer solution pH 7.4 (Sigma-Aldrich, St. Louis) 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 (Fluka-Sigma-Aldrich, Toluca, Mexico) in 15% trichloroacetic acid (Sigma-Aldrich, St. Louis)] was added and the sample was shaken in a vortex. It was then heated in a boiling water bath for 45 min and allowed to cool and the precipitate removed by centrifuging at 1000 *g* for 10 min. Absorbance was determined at 535 nm using a reaction blank. Results were expressed as mM of malondialdehyde/mg protein using the molar extinction coefficient of 1.56 × 105 M/cm.

2.5. Determination of SOD activity

Superoxide dismutase (SOD; EC 1.15.1.1) activity was determined according to the Misra and Fridovich (1972) method. 20 μL of supernatant in a 1-cm cuvette was 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 expressed as IU SOD mg protein g wet tissue^{-1}.

2.6. Determination of CAT activity

Catalase (CAT; EC 1.11.1.6) activity was determined according to Radi et al. (1991). 20 μL of supernatant was 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 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 reading in the formula: catalase concentration = (A60 − A0) / MEC, where the MEC of H_2O_2 equals 0.043 mM^{-1} cm^{-1}. Results were expressed as mM H_2O_2 mg protein g wet tissue^{-1}.

2.7. Determination of GPX activity

Glutathione peroxidase (GPX; EC 1.11.1.9) activity was determined by the Paglia and Valentine (1967) method. 100 μL of supernatant was added 900 μL of buffer reagent solution [5 M K_2HPO_4 (Sigma-Aldrich, St. Louis, MO, USA), 5 M KH_2PO_4 (Vetec-Sigma-Aldrich, St. Louis, MO, USA), 3.5 mM reduced glutathione (Sigma-Aldrich, St. Louis), 1 mM sodium azide (Sigma-Aldrich,), 2 U glutathione reductase (Sigma-Aldrich) and 0.12 mM NADPH pH 7.0 (Sigma-Aldrich)] and 200 μL of H_2O_2 (20 mM); all reagents were obtained from Sigma-Aldrich. Absorbance was read at 340 nm, at 0 and 60 s. Activity was estimated using the molar extinction coefficient of NADPH (6.2 mM/cm). Results were expressed as mM NADPH/mg protein.