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Toxicological evaluation of ametryn effects in Wistar rats

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ABSTRACT

São Paulo state, Brazil, is one of the main areas of sugar cane planting in the world. Extensive use of ametryn, a triazine herbicide, in sugar cane agriculture and the properties of this herbicide suggest it could be present in the environment as a potential contaminant of soil, surface water, groundwater, and river sediment. In order to clarify the mechanism through which ametryn could be toxic, an *in vivo* study with Wistar rats was conducted using hematological, biochemical, molecular, morphological and genotoxic approaches. For this purpose, two sub-lethal ametryn concentrations (15 mg and 30 mg/kg/day) were administered to 42 rats divided into three groups ($n = 12$) by gavage during 56 days, whereupon blood, liver and bone marrow were collected. The results showed ametryn genotoxic activity by *in vivo* micronuclei testing. This event probably occurred as consequence of oxidative stress induction demonstrated by *GSTM1* transcript levels increase (indicating complexation between ametryn and/or metabolites with GSH) and by SOD activity decrease. Also, *Mn-SOD* transcripts were increased, probably avoiding mtDNA damage caused by EROS. These mechanisms displayed hepatic stellate cell (HSCs) activation because two major biomarkers were regulated, connexin and cadherin. *N-cad* transcripts were increased on both exposed groups while *E-cad* decreased in the T1 group, indicating epithelial-to-mesenchymal transition. In addition, *Cx43* transcripts were decreased suggesting an increase in collagen content. Volumetric proportion of sinusoids was significantly decreased in T1 group and no significant alteration in hepatocyte volume was observed, indicating an increase in the space of Disse, due to fibrosis. Hepatocyte nuclei showed significant decrease in diameter and volume. Few hematological alterations were found. We emphasize the importance of other approaches, such as cell death and proliferation assays, so that ametryn toxicity can better be understood.

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1. Introduction

Recently, there has been a rapid decline in biodiversity in many ecosystems and this fact has been associated to environmental stressors such as pesticides in high concentrations, suspensions, and organic pollution, which are a threat to worldwide biota (Liess et al., 2013). Our research group has demonstrated testicular toxicity in Wistar rats exposed to sub-lethal doses of ametryn (Dantas et al., 2015), highlighting oxidative stress induction and decrease of Leydig cell and sperm number, corroborating this association.

Ametryn is a triazinic herbicide. They are considered one of the most important classes of chemical pollutants due to their toxicity and high resistance (Wang et al., 2011). They have been extensively used as selective herbicides for the control of broad leaf and grassy weeds in many agricultural crops over the past years. Triazines are relatively stable, and can be identified in drinking water, food, and fish (Stara et al., 2012). Residues of ametryn have been also detected in fodder and cow milk, and minimal concentrations have

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also been found in root crops such as cassava and yams (Asongalem and Akintonwa, 1997).

Triazines are known to induce oxidative stress, cellular and DNA damage and cell death (Severi-Aguiar et al., 2013). These contaminants may stimulate reactive oxygen species and alterations in antioxidant systems (Stara et al., 2012). Ametryn demonstrates low acute and chronic toxicity by the oral route, and has been classified at the toxicity III level (moderately toxic) for oral ingestion. In male rats, ametryn has a lethal dose (DL_{50}) of 508 mg/kg (Takayasu et al., 2010). Rabbits and dogs showed an increase in liver weight, and degenerative and inflammatory effects in that organ with doses of 70 mg/kg/day and 60 mg/kg/day, respectively, of this herbicide (EPA, 2010).

In vertebrates, liver is one of the target organs of triazine herbicides, such as atrazine, which is associated with deleterious effects on rat hepatocytes. In these cells, oxidative, genotoxic and morphological damage was observed, causing alterations at the gap junctions and affecting hepatic homeostasis (Campos-Pereira et al., 2012). Therefore, this study aimed at the evaluation of the toxicological potential of this herbicide using hematological, morphological, biochemical, molecular and genotoxic biomarker assays, looking for hepatic effects after chronic exposure to realistic ametryn concentrations.

2. Material and methods

2.1. Experimental delineation

The study was approved by the Ethics Committee of Centro Universitário Hermínio Ometto, UNIARARAS (protocol 072/2011), and was conducted in accordance to the ethical guidelines of the Brazilian Committee of Animal Experimentation (COBEA). Animals were maintained throughout the experiment at the Center of Animal Experimentation, Centro Universitário Hermínio Ometto (UNIARARAS), on a 12-h light/dark cycle at a temperature of 25 °C and air humidity of 60%. They received standard rat chow and potable water *ad libitum*. Thirty-six adult male Wistar rats (90 days old) were divided into three groups with 12 animals in each group, distributed in two different cages (A and B) with 6 rats in each. The control group (Co) ($n = 12$) received only filtered water and the two experimental groups T1 and T2 ($n = 12$ for each group) were exposed to 15 mg or 30 mg/kg/day (EPA, 2005), respectively, of the herbicide ametryn (HERBIPAK 500BR, Milenia Agrociências S. A., Londrina, PR, Brazil). Considering a daily intake of 0.2 μg/L of ametryn in water (Jacomini et al., 2011), and other forms of ametryn ingestion, such as by milk and/or root crops (Asongalem and Akintonwa, 1997) we inferred that the doses presented above may be environmentally relevant (Dantas et al., 2015). The animals were weighed, and received by gavage, an aqueous solution of ametryn (T1 and T2) or water only (Co) for a chronic period of 56 days. For the micronucleus test, a positive control group (PG) ($n = 6$) received by gavage water and, one day before the euthanasia, a single dose (20 mg/kg) of methyl methane sulfonate (MMS) (Kuriyama et al., 2005).

2.2. Euthanasia and collection of material for analysis

Animals were anesthetized by intramuscular administration of xylazine (20 mg/ml; Rompun®, Bayer S.A., São Paulo, SP, Brazil) and ketamine (50 mg/ml; Ketalar®, Parke-Davis & Co., Wellington, New Zealand). Next, a median laparotomy was performed and the chest cavity was opened to expose the heart. Blood samples were obtained by cardiac puncture using a syringe for the determination of hematological parameters. Animals from cages A were fixed by perfusion. After a brief saline wash to clear the blood vessels, they were perfused with glutaraldehyde 4% and paraformaldehyde 4% in 0.1 M sodium caccodylate buffer (pH 7.2) for 25–30 min, according Sprando (1990). The liver was dissected out and fixed overnight in the same solution, then prepared for embedding in hydroxyethyl methacrylate (Historesin, Leica), using a routine technique. For morphological and morphometrical evaluation, liver fragments were sectioned at 3 μm thickness and stained with hematoxilin/eosin (HE). Animals from cage B, that were not perfused, and after anaesthesia, their livers were dissected out and weighed. A left lobe fragment was frozen at −80 °C for biochemical analysis and another fragment was immersed in TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) for genic expression analysis.

2.3. Determination of hematological parameters

To estimate hematological parameters, 0.08 ml blood was mixed with 0.02 ml Ethylene Diamine Tetracetic Acid-EDTA (33.33 mg/ml) and fed to the auto analyzer (ANALYSER 6604,388, TKS COULTER, Hialeah, Flórida, USA). Parameters measured were as follows: Total RBC (red blood cells), hemoglobin content (Hb), hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), leucocyte (Leu) and platelet count (Pl).

2.4. Biometry, morphometry and stereology

Liver was weighed and the hepatosomatic index (HSI) was expressed as a percentage of total body weight in relation to the liver weight (HSI = liver weight/total body weight) × 100. Representative areas of hepatic tissue were photographed with a Leica DM2000 microscope and subjected to morphometric and stereological analyses with an image analysis system: Pro-Plus software version 4.5 (Media Cybernetics). Stereological analysis of the liver was made using 15 random sections per animal. Analysis was performed with a 494 point grid to determine the proportion of the liver components (hepatocytes, sinusoids, central vein) in the experimental groups.

Volume, expressed in ml, of each component described above was determined as the product of the hepatic volume and volumetric proportions (Mori and Christensen, 1980). Proportion between hepatocyte nucleus and cytoplasm was assessed using a grid mask with 494 points placed over images at 1000× magnification. Three thousand points over nuclei and cytoplasm of hepatocytes were counted per animal. Nuclear and cellular diameter of hepatocytes was obtained assessing 30 nuclei/animal over images at 1000× magnification. Nuclear volume was calculated using the $4/3\pi r3$ formula, where r was the mean nucleus radius. Individual volume of each hepatocyte was obtained from the nucleus volume and the proportion between nucleus and cytoplasm (Mori and Christensen, 1980).

2.5. Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was isolated from approximately 100 mg rat livers with the TRIzol® reagent (Invitrogen, Carlsbad, CA, USA), including the digestion of contaminating DNA with DNAse I, amplification grade (Invitrogen), following the manufactures's instructions. RNA purity and concentration were determined spectrophotometrically. cDNA was synthesized from 2 μg RNA in the presence of dithiothreitol, dNTP, random primers, RNAseOUT, and SuperScript™ II Reverse Transcriptase (Invitrogen) in a final volume of 20 μl. Semi quantitative RT-PCR was used to amplify Glutathione S-transferase mu 1 (*GSTM1*), Manganese superoxide dismutase (*Mn-SOD*), Connexin 43 (*Cx43*), N-cadherin (*N-cad*) and E-cadherin (*E-cad*) mRNA, and to compare their expression between a metryn-exposed and no exposed groups.

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