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Effects of benzo[a]pyrene on the blood and liver of *Physalaemus cuvieri* and *Leptodactylus fuscus* (Anura: Leptodactylidae)

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ABSTRACT

Benzo[a]pyrene (BaP) is a bio-accumulative toxic compound found in the atmosphere, water, and soil that may affect the life cycle of amphibians. In this study, a few contamination biomarkers, such as hepatic melanomacrophages (MMs), mast cells, erythrocyte micronuclei (MN) and white blood cells were used to determine how BaP acts in these cells in the anurans *Physalaemus cuvieri* and *Leptodactylus fuscus*. Animals of both species were divided into three treatment groups: 1 day, 7 days and 13 days, subcutaneously injected 2 mg/kg BaP diluted in mineral oil and control group with only mineral oil. After 7 days, BaP caused the frequency of MN to increase in both species while reducing melanin area. The micronucleus frequency increased due to the genotoxicity of BaP, while the decreasing melanin area may be related to the inhibition of tyrosinase activity, an enzyme responsible for regulating melanogenesis, decreasing the synthesis of melanin. The mast cell density increased in all groups and in both species as a response to the inflammatory action of BaP. These cells respond to nonspecific inflammatory effects leading, therefore, to this response in all treatments. The percentage of leukocytes remained unchanged probably due to great intraspecific variability. Additionally, the leukocyte profiles of both species were characterized and the differences were attributed to extrinsic factors. In short, BaP can affect the integrity of several organs and tissues, and cell functions leading to the conclusion that this compound is hepatotoxic, genotoxic and immunotoxic for anurans.

1. Introduction

In recent years, the number of ecotoxicological studies on amphibians has grown rapidly (Burlibaşa and Gavrilă, 2011), justified by a few peculiar characteristics of this taxon. These animals are very sensitive to pollutants, due to characteristics such as skin permeability, water-dependent reproduction, exposed embryogenesis, and a life-cycle that is dependent on both aquatic and terrestrial environments, thus maximizing their exposure to contaminants (Conrad, 2010; Burlibaşa and Gavrilă, 2011; Blaustein et al., 2011; Pérez-Iglesias et al., 2016; De Oliveira et al., 2017).

Contaminants such as chemical pollutants are distributed in the organisms through blood flow (WHO, 1995) and exposure can, therefore, induce nuclear abnormalities such as the formation of micronuclei, which consists of chromosomal fragments or whole chromosomes in anaphase (Fortin et al., 2015). Micronuclei were detected in mature erythrocytes of different fish species (Pastore et al., 2014) and amphibians when exposed to BaP or pesticides, for example (Grinfeld et al., 1986; Candioti et al., 2010; Pérez-Iglesias et al., 2014). Hematological parameters can also be affected, due to the exposure to contaminants (Winkaler et al., 2008). These changes of the physiological status provide information on the level of damage, indicating the healthy status of anurans and other animals (Sayed, 2015). In this context, differential leukocyte count also provides information on the immunological status (Newman et al., 1997).

In addition to micronuclei formation and quantitative changes of leukocytes, melanomacrophages and mast cells are also responsive to the action of xenobiotics (Ma et al., 2011; Franco-

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Belussi et al., 2013, 2016; Möller et al., 2014; Passantino et al., 2014; Çakıcı, 2015; Regnault et al., 2014, 2016). For this reason, these cells are used, respectively, as contamination biomarkers and inflammatory response indicators in several tissues (Paetow et al., 2012; Franco-Belussi et al., 2013; Santos et al., 2014).

Melanomacrophages are macrophages that produce and store pigments such as melanin, lipofuscin, and hemosiderin (Agius and Roberts, 2003; Ribeiro et al., 2011). Melanin, the major pigment of this phagocytic cell absorbs and neutralizes free radicals, cations and other potentially toxic agents derived from the degradation of phagocytized cellular material (Zuasti et al., 1989). These cells are found in hematopoietic organs such as liver and spleen (Agius, 1980; Cesarini, 1996) of ectothermic animals (fish, amphibians, and reptiles) (Wolke, 1992; Fournie et al., 2001; Loumbourdis and Vogiatzis, 2002; Fishelson, 2006) while having detoxification, bactericidal and innate immunity as main functions (Agius and Roberts, 2003; Passantino et al., 2014; Franco-Belussi et al., 2013). Mast cells specialize in secreting mediators of lipids, leukotrienes, proteases, and histamine, which contribute to the inflammatory process (Shakoory et al., 2004) while playing an important role in inflammatory response mechanisms associated with a wide range of stress conditions, such as exposure to xenobiotics (Lauriano et al., 2012).

Polycyclic Aromatic Hydrocarbons (PAHs) are highly studied toxic compounds due to its toxicity at low and moderate concentrations, and its persistence in the environment (Brandt et al., 2002). Benzo[a]pyrene (BaP) is considered a high-risk contaminant due to genotoxicity (Mouchet et al., 2005) and ability to induce micronucleus formation in erythrocytes (Fortin et al., 2015), hepatotoxicity (Latif et al., 2010; Regnault et al., 2014, 2016; Pastore et al., 2014), immunotoxicity (Phalen et al., 2014) and also change the patterns of the leukocyte cells (Sorensen, 1991; Sayed, 2015). Because it results from the incomplete combustion of organic matter, it can be present naturally in fires (Zakaria et al., 2002; Ou et al., 2004; Luo et al., 2008; Jiao et al., 2009) or result from anthropogenic action, such as the use of oil products and fossil fuel combustion (Zakaria et al., 2002; Kim et al., 2008; Morillo et al., 2008; Stogiannidis and Laane, 2015).

BaP is adsorbed by particles present in water, suspended in the air (WHO, 1998; Srogi, 2007), in sediments and in the soil (Stark et al., 2003; Morillo et al., 2008; Harris et al., 2011). Because it is practically everywhere, anurans may be exposed to it via inhalation, feeding and through the skin (Collins et al., 1991; Kanaly and Harayama, 2000). In addition, this compound may bioaccumulate in anurans (Brandt et al., 2002), becoming quite harmful since after its absorption, BaP is biotransformed in the liver by the cytochrome P450 1A1 (CYP1A1) (Caruso and Alaburda, 2008; Wakx et al., 2016). Throughout its metabolism, toxic byproducts are formed, especially the 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetra (a) pyrene (BPDE) (Madureira et al., 2014).

Although toxic compounds are produced during BaP metabolism and disrupt homeostasis (Chovatiya and Medzhitov, 2014), anurans have defense cells such as melanomacrophages, mast cells, and leukocytes, which are capable of attenuating toxicity, due to their functions. The consequence of the protective response of these cells is the homeostasis restoration (Chovatiya and Medzhitov, 2014), enabling the survival and adaptation of the animals in anthropic environments (Blaustein et al., 2011). In general, the effects of pollutants on the internal morphology of adult anurans are poorly known (Fanali et al., 2017). After undergoing biotransformation in the liver, the metabolites of BaP reach target organs through circulation, causing toxicity (Van Veld et al., 1988; Buhler and Williams, 1988; Costa et al., 2011; Fanali et al., 2017). Therefore, the purpose of the study was to determine the effects of BaP on hepatic melanomacrophages, hepatic mast cells, leukocyte composition and erythrocytes integrity of the of *P. cuvieri* and *L. fuscus*.

During its reproductive period, *P. cuvieri* performs mating vocalization in newly flooded environments, usually inside animal footprints, when deep and filled with water (Uetanabaro et al., 2008), while *L. fuscus* vocalizes on the ground, on the banks of temporary ponds or depressions subject to flooding (Uetanabaro et al., 2008). Both species coexist, have similar living habits, and are phylogenetically close and abundant in the study region. Because these two species share the same habitat and expose themselves to similar environmental conditions, they could be used as sentinel species to evaluate the cause-effect relationships of chemical contaminants in the environment.

2. Material and methods

2.1. Animal sampling

Seventy-two adult males, 36 *P. cuvieri* and 36 *L. fuscus,* were used in the experiment. This project was approved by the Ethics Committee on Animal Use, protocol 094 and RAN/IBAMA/MMA 18573-1. The animals were collected in northwestern São Paulo, Brazil, from December to February, period that includes the species reproductive season. The animals were acclimated for one week, in $28 \times 21 \times 15$ cm boxes containing 2 cm of soil, which was kept moist. Room temperature was kept at 27 °C, with natural photoperiod. During the experimental period, all animals were fed with *Drosophila* shortly after the injections to avoid further stress.

2.2. Experiments with benzo[a]pyrene

The studied parameters were evaluated at 1, 7 and 13 days after all animals were subcutaneously injected 2 mg/kg benzo[a]pyrene (B1760; purchase from Sigma-Aldrich), diluted in 0.02 ml of Nujol mineral oil, while the control consisted of mineral oil only. The solution was injected every 48 h on the animal back, near the hind limb. The solution concentration was adapted and based on the work of Padrós et al. (2003). The animals ($N = 6$, per group) were exposed for 1, 7 and 13 days and each group had its respective control with the same experimental period. This experimental model was replicated for each species.

After the experiment, the hind limb was anesthetized with xylocaine (with the excess removed with cotton to avoid smear interference) and blood was drawn from the femoral vein using a heparinized needle and a syringe. Smear was performed immediately after collection, due to the small blood sample and rapid coagulation.

The animals were then euthanized with benzocaine solution (1 g/L) and had their liver sampled, which was weighed in an analytical balance (0.05 g precision) and analyzed as the following procedures.

2.3. Micronucleus analysis

After drying, slides were fixed in methanol at 4 °C for 20 min, stained with Giemsa 7.5% for 15 min and observed under a light microscope (Leica DM4000 B). A total of 1000 erythrocytes were counted for each animal.

2.4. Leukocyte analysis

For differential leukocyte count, the blood smear was stained with Panoptic (Hematocor-Biológica®) to identify leukocytes variety and establish their relative proportions. For each animal, 100 leukocytes were counted under a light microscope (Leica DM4000