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UV-B affects the immune system and promotes nuclear abnormalities in pigmented and non-pigmented bullfrog tadpoles

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ABSTRACT

Ultra-Violet (UV) radiation is a stressor of the immune system and causes DNA damage. Leukocytes can change in response to environmental changes in anurans, making them an important biomarker of stressful situations. The initial barrier against UV in ectothermic animals is melanin-containing cells in skin and in their internal organs. Here, we tested the effects of UV exposure on immune cells and DNA integrity in pigmented and non-pigmented tadpoles of *Lithobates catesbeianus*. We used an inflammation model with lipopolysaccharide (LPS) of *Escherichia coli* to test synergic effects of UV and LPS. We tested the following hypotheses: 1) DNA damage caused by UV will be more pronounced in non-pigmented than in pigmented animals; 2) LPS increases leukocytes in both pigmented and non-pigmented animals by systemic inflammation; 3) The combined LPS and UV exposure will decrease the number of leukocytes. We found that the frequency of immune cells differed between pigmented and non-pigmented tadpoles. UV exposure increased mast cells and DNA damage in erythrocytes in both pigmented and non-pigmented tadpoles, while leukocytes decreased after UV exposure. Non-pigmented tadpoles experienced DNA damage and a lower lymphocyte count earlier than pigmented tadpoles. UV altered immune cells likely as a consequence of local and systemic inflammation. These alterations were less severe in pigmented than in non-pigmented animals. UV and LPS increased internal melanin in pigmented tadpoles, which were correlated with DNA damage and leukocytes. Here, we described for the first time the effects of UV and LPS in immune cells of pigmented and non-pigmented tadpoles. In addition, we demonstrated that internal melanin in tadpoles help in these defenses, since leukocyte responses were faster in non-pigmented animals, supporting the hypothesis that melanin is involved in the initial innate immune response.

1. Introduction

Ultra-Violet (UV) radiation causes behavioral and physiological changes in amphibians, contributing to their worldwide decline [1]. For example, UV radiation promotes DNA damage [1,2] by producing pyrimidine dimers that block transcription of gene and lead to mutation or cell death [3]. Another effect of UV in amphibians is disruption of the immune system [4]. Previous studies have found that exposing tadpoles in early developmental stages to ultra-violet-B (UV-B) radiation decreased their fitness as a result of the impact on the immune functions [5]. Thus, UV exposure in different life stages affects the ability of amphibians to cope with subsequent infections [5]. In addition, the combined effects of UV-B and pathogens increase the mortality of amphibian embryos more than pathogens alone [6], since UV radiation promotes systemic immunosuppression, which makes amphibians more sensitive to subsequent viral, fungal, and bacterial infections [7]. However, little is known about the systemic effects of UV. For example, how they interact with other environmental factors and defenses of amphibians against UV exposure [7]?

Hematological alterations usually reflect changes in the physiology of the organism. For example, leukocytes respond effectively to stressful situations. Therefore, these cells allow us to measure the level of damage an animal underwent [8]. Several studies have reported that the number of leukocytes change in response to different environmental variables [9–12]. Immune cells in tissues also respond to environmental change. For example, the density of liver mast cells in *Physalaemus nattereri* increases after 3 h of UV exposure [2]. Mast cells produce mediators to inflammatory process and activate neutrophils [13,14] and these cells are used to indicators of inflammation in tissues [15]. In this study, mast cells were used to measure inflammation in tissues of tadpoles.

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Amphibians are susceptible to several pathogens, due to their complex life cycle. Their skin is also permeable to harmful microorganisms. For example, *Escherichia coli* is commonly found in environments where anurans live. The pathogenicity of this bacterium is associated with LipoPolySaccharides (LPS) in its cell wall. Specifically, the LPS activates the immune response in hosts, by stimulating mononuclear phagocytes to synthesize cytokines [16], such as the Tumor Necrosis Factor α (TNF-α) and several Interleukins (IL-1, IL-6, and IL-10; [17]). The LPS of *E. coli* has been commonly used in experimental models to analyse the systemic inflammatory process of rodents [18] and fish [16]. Here, we used the LPS by test synergic effects of UV and LPS.

Ectothermic animals have melanin on the surface of internal organs [19]. In the skin, melanin is the first barrier against UV [20]. Melanin also protects tissues against DNA damage by absorbing UV radiation and transforming it into heat [21]. Melanin has also an immune role in internal organs, since hydrogen peroxidases and their quinone precursors act as bactericides [22]. This is especially important for ectothermic animals, because their enzymatic reaction rates, such as enzyme-mediated repair of DNA damage induced by UV-B, decrease at low temperatures [22,23]. In addition, previous studies found that amphibian embryos died when exposed to both UV and pathogens at the same time [6]. Therefore, it is expected that the inflammation promoted by LPS would be stronger in animals exposed previously to UV. Conversely, melanin protects internal organs against UV [2]. As a result, pigmented animals are less affected due to the role of the melanin in mediating immune responses.

Here, we conducted three experiments to test if UV radiation affects immune cells and causes DNA damage on pigmented and non-pigmented tadpoles of *Lithobates catesbeianus*. Specifically, we tested the hypotheses that: 1) DNA damage caused by UV will be more pronounced in non-pigmented than in pigmented animals, since melanin can protect tissue against UV effects; 2) LPS increases leukocytes in both pigmented and non-pigmented animals by systemic inflammation; 3) The combined effect of LPS and UV exposure will decrease the number of leukocytes, since UV can disrupt the immune system.

2. Methodology

2.1. Animal Model

We used pigmented and non-pigmented, i.e. albine, tadpoles of the American Bullfrog (*Lithobates catesbeianus*; Anura: Ranidae) between stages 38 and 40 [24], supplied by Ranaville Agro Indústria Ltda, São Roque, São Paulo, Brazil. This species is bred in large scale frog farms, making it easy to obtain a homogeneous and large set of specimens, both pigmented and non-pigmented, raised under controlled conditions. Tadpoles were kept in aquaria with water equivalent to 1 L per individual, at room temperature (27.0 ± 0.5 °C), and 12:12 light: dark photoperiod. Animals were fed daily for 7 days before experiments. Animal handling followed the NIH Guide for Care and Use of Laboratory Animals and procedures were approved by the Ethics and Animal Experimentation Committee of the Sao Paulo State University (CEUA-IBILCE/UNESP 096/2014).

2.2. Experiment 1: Effects of UV on Immune Cells and DNA Damage Assay

To test the UV effects on leukocyte profile and erythrocyte abnormalities, we designed a 5 × 2 fully-crossed factorial experiment, in which we varied the *time of exposure* to UV (control group, 6, 12, 18, and 24 h) and the *incidence of pigmentation* of tadpoles (pigmented and non-pigmented). Animals exposed for longer time to UV did not survive in a pilot study (data not shown). Each experimental group had six tadpoles (replicates). The control group consisted in animals kept under the same conditions without UV exposure. UV radiation was implemented using Philips TL 20 W/12 RS lamps, whose emission are 60% UVB and 40% UVA, < 3% gamma radiation, and irradiation of 2.4 Wm^{-2} of UVB (after [25,26]). The doses were approximately 10% of the natural UVA and UVB levels, which are average levels of summer months of southeastern Brazil [25]. Animals were kept in an incubator (EletroLab model 121 FC, with Philips TLT 20 W/75RS fluorescent lamp) with controlled temperature (27 ± 0.5 °C) and photoperiod (12:12 h light: dark). After exposure, animals were euthanized with a lethal dose of benzocaine diluted in water (5 g/L), and blood and liver samples were collected. Blood was removed from the *vena caudalis dorsalis* of the tadpole, with syringes and heparinized needles.

2.3. Experiment 2: Effect of LPS on Leukocytes and Hepatic Mast Cells

To test the LPS effects on leukocyte profile and erythrocyte abnormalities, we designed a 3 × 2 fully-crossed factorial experiment, in which we varied the *time of exposure* to LPS (control group, 12 and 24 h) and the *incidence of pigmentation* of tadpoles (pigmented and non-pigmented). Each experimental group had six tadpoles (replicates). Tadpoles were inoculated intraperitoneally with a single dose of 3 mg/kg LPS of *E. coli*, Serotye 0127:B8 (Sigma, St. Louis, MO; after [16]) diluted in a sterile physiological solution with osmolality adjusted for amphibians (60% of mammalians). The control group consisted of pigmented and non-pigmented animals administered with a sterile physiological solution. These exposure times were chosen because during the inflammatory response, mast cells degranulate after 24 h [15], suggesting that the LPS are detectable during this time frame. After the end of the experiment we collected blood and liver samples.

2.4. Experiment 3: Combined Effects of UV and LPS

To test the combined effects of UV and LPS on leukocyte profile and erythrocyte abnormalities, we designed a 2 × 2 fully-crossed factorial experiment, in which we varied the *time of exposure* to LPS and UV (12 and 24 h) and the *incidence of pigmentation* of tadpoles (pigmented and non-pigmented). Each experimental group had six tadpoles (replicates). Animals received an intraperitoneal single dose of LPS and exposed to UV following the same procedures described above.

2.5. Histological Processing

For hepatic mast cell analysis, the liver was fixed in Karnovsky fixative solution (0.1 M Sørensen phosphate buffer, pH 7.2 phosphate buffer containing 5% paraformaldehyde, and 2.5% glutaraldehyde) for 24 h at 4 °C. Subsequently, samples were washed in water, dehydrated in an alcoholic series, and embedded in historesin (Leica-historesin embedding kit). Sections of 2 μm obtained in a microtome (RM 2265, Leica, Switzerland) were stained with toluidine blue and borax for mast cells detection. Sections were observed under a light microscope (Leica DM4000 B) with an image capture system (Leica DFC 280). Ten histological sections were made per animal to estimate mast cell density (mm^2).

2.6. Leukocytes Profile and Nuclear Abnormalities of Erythorcytes

Peripheral blood smears were fixed in methanol at 4 °C for 20 min and then stained with a 7.5% Giemsa solution for 15 min. For leukocyte count, we recorded the relative proportions of each cell type per 100 leukocytes per animal under a light microscope (Leica DM4000 B). Then, we calculated the total circulating leukocytes to obtain the total amount of each cell type.

We assessed nuclear abnormalities in 1000 cells per animal under a light microscope (Leica DM4000 B), with 1000× magnification. Nuclear abnormalities in erythrocytes recorded were micronuclei, buds, binucleate erythrocytes, and anucleated cells [2]. Results were expressed as the frequency of nuclear abnormalities per 1000 cells.