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The effects of predicted environmentally relevant concentrations of ZnO nanoparticles on the behavior of *Gallus gallus domesticus* (Phasianidae) chicks☆

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ABSTRACT

The toxicity of ZnO nanoparticles (NPs) has been the subject of several investigations; however, concentrations much higher than the ones potentially found in the environment are often tested. In addition, groups of animals such as birds have not been used as model in studies in this field, fact that creates an important ecotoxicological gap in them. The aim of the present study is to investigate the effects of the exposure to environmentally relevant concentrations of ZnO nanoparticles on the anti-predatory behavior of chicks (*Gallus gallus domesticus*). The test animals were daily exposed to an environmentally relevant concentration of ZnO nanoparticles (0.245 mg kg^{-1}) and to a toxic concentration of it (245.26 mg kg^{-1}) through intraperitoneal injection for two days. We set a control group for comparison purposes. According to our results, ZnO nanoparticles did not affect the locomotor activity of, and did not cause anxiolytic or anxiogenic effect on, birds in the open field test. However, based on the lowest cluster score recorded during the social aggregation test, chicks exposed to ZnO nanoparticles failed to recognize the grunt of a hawk (*Rupornis magnirostris*) as predatory threat. Only birds in the control group recognized the test snake (*Pantherophis guttatus*) as potential predator. The higher Zn concentration in the brains of animals exposed to ZnO nanoparticles evidenced the capacity of these nanomaterials to cross the blood–brain barrier, even at low concentrations. This blood-brain barrier crossing could have affected the structures or neuronal mechanisms that modulate the defensive response of birds. Assumingly, even the minimal exposure to low concentrations of ZnO nanoparticles can affect birds. Our outcomes corroborate previous studies about the biological risks of water surface contamination by metal-based nanomaterials.

1. Introduction

Zinc oxide (ZnO) is one of the most used and studied nanoparticles (NPs) in the world (Baun et al., 2008; Espitia et al., 2012; McCall, 2011). This component is found in commercial products such as toothpastes, cosmetics, sunscreens, textile material, wall paints and construction materials (Xia et al., 2008; Zvyagin et al., 2008; Smijs & Pavel, 2011; Vanderiel & Jong, 2012). Several studies have already investigated the toxicological potential of this NP at different biological organization levels (Pati et al., 2016; Sheida et al., 2017; Almansour et al., 2017; Wang et al., 2017; Hou et al., 2018). Based on a number of multi-media and multi-compartment modeling studies on surface water, and despite the known causal link between the exposure to ZnO nanoparticles and the damages caused by them (cytotoxicity, genotoxicity, neurotoxicity, reproductive toxicity and immunotoxicity), little is known about their effects at predicted environmental concentrations (Boxall et al., 2007; Gottschalk et al., 2009; Sun et al., 2014; Dumont et al., 2015). These studies provided information to ecotoxicologists about possible ZnO nanoparticle concentration ranges that can be used to design acute and chronic toxicity tests and to evaluate toxicity data.

The evaluation to measure the toxicity of ZnO nanoparticles is

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often based on quantitative toxicity values (EC50, LC50, MIC) [see review by Bondarenko et al. (2013)] that may not correspond to concentrations found in natural environments. Many studies in this field have used non-vertebrate experimental models such as bacteria, crustaceans, algae, nematodes, yeasts and protozoa as subjects (Bondarenko et al., 2013). Therefore, there is a gap in the knowledge about the effects of exposing vertebrates to predictive and environmentally relevant concentrations of ZnO nanoparticles. There is only one study about the toxicity of ZnO nanoparticles in a vertebrate model based on realistic environmental conditions: low ZnO concentrations potentially found in surface water. According to Souza et al. (2018a), mice exposed to ZnO nanoparticles at dose 5.625×10^{-5} mg kg^{-1}, for five days, presented behavioral changes. This dose corresponded to the amount of ZnO nanoparticles animals can intake on a daily basis (Souza et al., 2018a).

Our aim is to understand whether similar effects would be observed in other groups of vertebrates subjected to chronic, or eventual, contact with ZnO nanoparticles. Groups of birds remain unexplored by the nanotoxicology field due to several factors including their worldwide distribution, migratory abilities, different foraging habits, conspicuous nature and sensitivity to environmental pollutants. However, the exposure of bird groups to contaminants has been better documented than the exposure of other terrestrial vertebrate groups (Jaspers, 2015), but most studies involving birds focus on traditional toxicological tests and pay little attention to non-lethal effects in the short-term. There is no report on the toxicity of these nanomaterials in animals exposed to them, either at doses/concentrations based on acute toxicity tests (EC50, LC50, MIC) or at predicted environmental concentrations. Consequently, the importance of assessing the potential risks associated with the exposure of birds to ZnO nanoparticles is undeniable, since these animals can get in touch with such NPs in different real conditions.

Evaluating the effect of pollutants, including ZnO nanoparticles, on the typical behavior of birds is essential, since small biological changes may impair the fitness of these individuals and the dynamics of the population. The natural behavior of these animals is featured by their defensive anti-predatory response, whose primary function is to reduce threats faced by them or their vulnerability (Yang et al., 2004). Predation is the most important cause of reproductive failure in most bird species (Ricklefs, 1969; Montgomerie & Weatherhead, 1988; Maziarz et al., 2018), although birds adopt several anti-predator behaviors (Caro, 2005) to broaden their chances to achieve successful breeding. The anti-predatory behavior, besides being an innate animal response, is particularly important because disturbances in mechanisms used to recognize potential predators can lead to increased predation risks (Yang et al., 2004). Anti-predatory defensive behavior changes and sensitive biomarkers are important to assess xenobiotic effects on the biology of many species (Mendes et al., 2017; Souza et al., 2018b; Cardoso et al., 2018).

We aimed at investigating whether the short exposure to ZnO nanoparticles, even at predicted environmental concentrations, could cause behavioral changes in *Gallus gallus domesticus* (Phasianidae) chicks, as well as at inferring the ecological risks of such NPs. We also sought to correlate bird exposure to possible changes in birds' total body mass and relative brain and liver masses, which are commonly used as systemic toxicity biomarkers. Our study emerges as an incremental step to a series of studies about the toxicity of ZnO nanoparticles and opens new windows for further investigations related to the nanotoxicology of birds.

2. Materials and methods

2.1. Nanoparticles

Nanocrystals without any surface modification (uncoated NPs) were the ZnO nanoparticles (purity > 99.99% - Sigma Aldrich, Saint Louis, MO, USA; CAS number 544906) used in the present study. Their chemical characterization was previously performed by our research group (Souza et al., 2018a). The stock solution of ZnO nanoparticles (18 mg L^{-1}) was prepared in distilled water and dispersed for 10 min in sonicator to prevent aggregation. Transmission electron microscopy (TEM) and x-ray diffraction (XDR) after 30min sonication were the instruments of choice to feature the ZnO solution. We used the Alpha-T spectrometer (Perkin Elmer Lambda 1050) to record the Fourier transform–infrared (FTIR) spectra of the samples. Based on the TEM analysis, the crystalline and polygonal particles (Fig. 1A–F) had individual diameter 68.96 ± 33.71 nm (Fig. 2A). Fig. 2B shows the XRD patterns of the ZnO nanoparticles. Peaks at $2\theta = 31.67°$, $34.31°$, $36.14°$, $47.40°$, $56.52°$, $62.73°$, $66.28°$, $67.91°$, $69.03°$ and $72.48°$ were attributed to (100), (002), (101), (102), (110), (103), (200), (112), (201) and (004) ZnO nanoparticles, respectively. We did not identify impurity peaks and it suggested the high quality of ZnO nanoparticles. Fig. 2C shows the FTIR spectra of the samples.

2.2. Animals and experimental design

The species *Gallus gallus domesticus* is used as experimental model to test environmental toxicology worldwide (Carvalho et al., 2008; Gul et al., 2017). We used 45 male autosexed commercial hybrid chicks belonging to variety "Embrapa-021", which derives from the White Cornish x White Plymouth Rock crossing, in our study. The chicks were provided by a commercial incubator at their seventh day of life. We divided the models in groups with 15 animals. Each group was placed in cages (70 cm length x 50 cm width x 25 cm height) stored in a test room at controlled temperature ($24° \pm 2$ °C) under 250-W infrared bulbs, and subjected to artificial 12 h light/dark cycles. Food and water were provided *ad libitum*. Wire-mesh lids prevented the birds from jumping out of the cage. The present study was approved by the Institutional Care and Use Committee of the university the research was conducted in. We followed the care and test conformity standards for birds according to local, state and national regulations. Animals were subjected to the least suffering, external stress, pain and discomfort possible. We did not exceed the number of animals necessary to produce trustworthy scientific data.

The chicks acclimated to laboratory conditions for 48 h before the experiment. We distributed the birds presenting counterbalanced body biomass in the following experimental groups: (*i*) control (not exposed to ZnO nanoparticles – n = 15); (*ii*) exposed to ZnO nanoparticles at predictive environmentally relevant concentrations (EC1x = 245.26 μ kg^{-1} = 0.245 mg kg^{-1} – n = 15); and (*iii*) exposed to high concentrations of ZnO nanoparticles (EC1000x = 245,260.00 μ kg^{-1} = 245.26 mg kg^{-1} – n = 15). We administered a solution of ZnO nanoparticles diluted in distilled water in the test models through intraperitoneal injection for two days. Each animal was daily weighed to accurately adjust the drug dose.

We adopted the 2-day exposure period because wild birds just drink water from contaminated sources occasionally. Other water sources used by these animals may not necessarily be contaminated; therefore, our experimental design sought to approximate the simulated conditions to the most realistic scenario possible. We intravenously administered the test solution to assure the delivery of equal doses. Birds can drink different water amounts; therefore,