Published in final edited form as:

Environ Sci Nano. 2014 February 1; 2014(1): 27–36. doi:10.1039/C3EN00001J.

Alterations of intestinal serotonin following nanoparticle exposure in embryonic zebrafish

Rıfat Emrah Özel^a, Kenneth N. Wallace^b, and Silvana Andreescu^a

Kenneth N. Wallace: kwallace@clarkson.edu; Silvana Andreescu: eandrees@clarkson.edu

^aDepartment of Chemistry and Biomolecular Science, Clarkson University, 8 Clarkson Ave. Potsdam, NY, 13699-5810, USA

^bDepartment of Biology, Clarkson University, 8 Clarkson Ave. Potsdam, NY, 13699-5805, USA

Abstract

The increased use of engineered nanoparticles (NPs) in manufacturing and consumer products raises concerns about the potential environmental and health implications on the ecosystem and living organisms. Organs initially and more heavily affected by environmental NPs exposure in whole organisms are the skin and digestive system. We investigate the toxic effect of two types of NPs, nickel (Ni) and copper oxide (CuO), on the physiology of the intestine of a living aquatic system, zebrafish embryos. Embryos were exposed to a range of Ni and CuO NP concentrations at different stages of embryonic development. We use changes in the physiological serotonin (5HT) concentrations, determined electrochemically with carbon fiber microelectrodes inserted in the live embryo, to assess this organ dysfunction due to NP exposure. We find that exposure to both Ni and CuO NPs induces changes in the physiological 5HT concentration that varies with the type, exposure period and concentration of NPs, as well as with the developmental stage during which the embryo is exposed. These data suggest that exposure to NPs might alter development and physiological processes in living organisms and provide evidence of the effect of NPs on the physiology of the intestine.

Introduction

The rapid development of nanotechnology has offered opportunities for large scale production of a variety of nanosized materials and engineered nanoparticles (NPs) with unique properties and functions, and their routine implementation into commercial products^{1, 2}. In addition to the many beneficial aspects of nanotechnology, the new, unusual and often unpredictable physicochemical properties of NPs can also cause adverse health and environmental effects, altering normal physiological processes in living systems^{3–6}. Therefore, understanding how NPs interact with the environment and biological species is of critical importance⁷. NPs have been found to alter skeletal development⁸, tissue formation⁹, induce reactive oxygen species (ROS)¹⁰, cause DNA damage¹¹, inflammation⁹, oxidative stress¹², decreased cell proliferation¹³ and increase mortality in high concentrations⁸. The toxic effect of NPs from the same origin can change from one cell line to the other⁸ while the toxicological and environmental effects of some NPs remain controversial^{14, 15}.

This journal is © The Royal Society of Chemistry [year]

Correspondence to: Silvana Andreescu, eandrees@clarkson.edu.

[†]Electronic Supplementary Information (ESI) available: TEM images, particle size distribution and zeta-potential values of NPs used. Additional zebrafish micrographs of unhatched disintegrated or dead embryos after high concentration CuO NPs treatment and viability data.

Organs initially and more heavily affected by environmental NPs exposure in whole organisms are the skin and digestive system. While the skin will be exposed to NPs on a regular basis, exposure will often be transient and this organ forms a strong barrier to the exterior environment. The digestive system, on the other hand, needs to function as a barrier but also needs to absorb nutrients making it more susceptible to NPs toxicity. Additionally, NPs taken in with or without food will remain within the lumen until excretion or might become trapped for longer periods of time within the numerous intestinal folds. Concentration and retention of NPs within the digestive system can then result in amplified and longer exposure times. As a result, initial dysfunction may be more frequently observed in this organ system. Herein, we investigate the effect of nickel (Ni) and copper oxide (CuO) NPs on the physiology of the intestine of a living aquatic system, zebrafish embryos. These NPs are widely used in a variety of commercial applications and therefore can be released in the environment where they might induce toxicity to the ecosystem and affect development of living organisms. Ni NPs are being used as catalysts, ink materials and ceramic additives^{16–18}. The toxicity of soluble and insoluble Ni (*i.e.* NiSO₄, NiCl₂·6H₂O, NiO) on aquatic organisms has been demonstrated through diminished locomotor activity, hatching rate, respiratory problem and carcinogenic effect^{19–21}. Although there are several observational toxicology studies describing the qualitative effects of Ni NPs^{8, 22, 23}, their impact in living organisms remains obscure^{1, 24–26}. CuO NPs are used in catalysis and added to other materials to increase mechanical properties^{27, 28}. Studies have shown that exposure to CuO NPs causes DNA damage, oxidative stress, increases cell death^{24, 25} and inhibited growth of organisms^{1, 26}. Studies to assess intestinal effects of these NPs in an intact living organism have not been reported.

Accumulation of NPs within the intestine can cause developmental defects, which may alter the normal intestinal enteroendocrine secretory response including the serotonin (5HT) producing enterochromaffin cells (EC).²⁹ Alterations of EC cells by NPs internalization might change 5HT secretion, which plays a role in intestinal motility. Thus alteration of 5HT levels may alter movement of contents through the digestive tract and absorption of nutrients. Our goal in this study is to assess physiological changes of 5HT within the intestine following NPs exposure. We use carbon fiber microelectrodes (CFME) and differential pulse voltammetry (DPV), which we have previously developed to detect real time physiological concentrations of 5HT in the live zebrafish intestine^{30, 31}. CFMEs are commonly used in neuroscience research to analyze the physiological concentrations of neurotransmitters³⁰. The small electrode size provides real-time *in vivo* measurement capabilities with high spatial resolution allowing for detection of 5HT concentrations within different regions of the intestine. Here, we find that intestinal 5HT concentrations change with both type and concentration of NP exposure. Results show that sensitivity to NP exposure is dependent on the exposure period and the developmental stage during which the embryo is exposed. Once the digestive system is open to the exterior, allowing NPs to be internalized, interaction of NPs with EC cells results in increased 5HT concentration. With functional ECs at the end of embryogenesis, NP exposure changes 5HT concentration but the response appears to be dependent on NPs composition.

Materials and methods

Reagents

Ni (99.9%, 20 nm, slightly passivated by oxygen) and CuO (99%, 40 nm) NPs without surfactants, stabilizers and organic coatings were purchased from SkySpring Nanomaterials Inc. (Houston, TX). 2-propanol (99.5% ACS grade), potassium phosphate (monobasic) and chitosan (from shrimp shells) were from Sigma-Aldrich. Sodium phosphate (dibasic) and nickel chloride were from Fisher Scientific. Serotonin hydrochloride (99%) was from Acros

Organics, acetic acid (glacial) was from J.T. Baker and methanol (100%) was from LabChem Inc. Copper (Cu^{2+}) and Nickel (Ni^{2+}) standard solutions (AAS grade) with nitrate (NO_3^-) counter-ions were purchased from Alfa Aesar.

NPs dispersion in E3 (egg water)

E3 solution (pH 6.9 – 7.2) contains 5 mM NaCl, 0.17 mM KCl, 0.33 mM MgSO_4 , and 0.33 mM CaCl_2 in distilled, deionized water. A stock NPs dispersion of 100 ppm was prepared by sonication for each Ni and CuO NPs. Then, these stock dispersions were diluted down to specific concentrations needed (1, 5, 10, 20, 50, 100 ppm (mg NPs powder/liter E3)) with E3. The mixtures were sonicated for 10 min before exposure. The concentration of copper and nickel ions were measured by amperometry with an Epsilon BAS system coupled to an automated FIA injection after ultrafiltration of the NP dispersions at the end of four-day period. Carrier and supporting electrolyte was E3 with a flow rate of 6 ml/min.

Fish stock and exposure of zebrafish embryos to NPs

Adult AB wild type (WT) zebrafish embryos were maintained at 28.5°C on a 14h light/10h dark cycle in an Aquatic Habitats recirculating system. At 24 hour post-fertilization (hpf), chorions of the embryos were manually removed. Embryos were divided into 10 per well in 6-well plates and exposed to 3 ml of NP dispersions with specific concentrations, prepared in E3 solution. At 5 day post-fertilization (dpf), embryos were assessed for NP effects on physiological serotonin concentrations. Malformation and mortality studies were carried out in the same manner. Embryos were monitored every 24 hours for five days. For hatching inhibition tests, healthy embryos were collected at 4 – 5 hpf. The number of hatched embryos was counted every 24 hour for three consecutive days. Food exposure was done with food from Zeigler (Larval AP100), with a particle size less than 100 microns. To obtain statistically representative data, five replicate sets of experiments were carried for each type of particle and concentration, using 10 embryos.

Immunohistochemistry

Zebrafish embryos were fixed in formaldehyde (4%) for 2 hours at room temperature. Fixed embryos were washed with PBST and methanol, and kept for 1 h at -20°C . Embryos were then digested with Proteinase K (0.5 mg/ml) in PBST for 20 min. at room temperature and incubated at 4°C overnight with primary antibody (rabbit anti-serotonin antibody, 1:500 dilution, Sigma). After incubation, embryos were washed with PBST for 45 min and then incubated with secondary anti-rabbit antibody (1:500 dilution in 5% Goat serum, Molecular Probes-Invitrogen) for 2 h. Embryos were washed in PBST for 45 min. Before fluorescence imaging, intestines of embryos were dissected and kept in Vectashield (Vector Lab.). Intestines were analyzed on a Nikon Eclipse TE2000 inverted compound microscope. Images were displayed using Hamamatsu Orca camera in IP lab software.

Embryo digestion

Embryos were treated with NPs dispersion of 100 ppm for four days. At the end of the fifth day, embryos were collected and washed thoroughly with PBS three times and then sonicated overnight after tricaine treatment. Samples from both Ni and CuO NPs exposed embryo homogenate were acidified with pure nitric acid and digested on a hot plate until all nitric acid evaporated. Metal concentrations were measured by amperometry at the characteristic reduction potentials of each ion: at -0.45 V for Ni^{2+} and -0.05 V for Cu^{2+} (vs. Ag/AgCl).

Physicochemical characterization of NPs

Particle size distribution and zeta potential (ζ -potential) were measured at 25°C with a Brookhaven Zeta Plus analyzer with particles suspended in distilled water, egg water and PBS (pH = 7.4). ζ -potential measurements were based on the electrophoretic mobility of the particles and calculated with built-in software. A JEOL JSM-2010 instrument was used for transmission electron microscopy (TEM). Aliquots of NPs prepared for TEM were placed directly on a copper TEM grid and dried under vacuum.

Fabrication and characterization of microelectrodes

Microelectrodes were fabricated from carbon fibers from WPI as described³¹. Prior use, electrodes were immersed into isopropanol for 10 min, air-dried, and electrochemically treated in 0.1 M PBS (pH 7.0) by repetitive oxidation in the potential range from -0.4 to 1.4 V at 500V/s and dip coated with 1% chitosan³². The electrochemical parameters such as scan increment, pulse amplitude, width and period were respectively 4.0 mV, 50 mV, 50 ms and 200 ms. Each microelectrode was tested prior to use, as described³¹.

In vivo electrochemical measurements

DPV was utilized as detection technique for all *in vivo* measurements in a potential range of 0 to 0.8 V. Measurements were carried out with the microsensor inserted into the middle intestine with a micromanipulator, after fixing embryos on agarose gel. All measurements were conducted on live embryos. Background voltammograms were recorded in E3 followed by the insertion in intestine. All voltammograms are shown after background subtraction. Each CFME was utilized for only one *in vivo* experiment.

Statistical Analysis

We have used Past software for the statistical analyses of the data. NP exposed group of embryos were compared to the control group by using one-way ANOVA, with Bonferroni correction to evaluate the significance levels. Statistically significant differences between groups were defined as $p < 0.05$ and < 0.001 which are indicated with one and two asterisks, respectively, in the graphs.

Results

Physicochemical characterization of NPs

TEM analysis of fresh and aged NPs reveals a size range of 30 to 50 nm in dry state with some aggregations (Supporting Information (SI) Figure S1) with agglomeration becoming prominent when introduced to aqueous media, especially to PBS (pH 7.4; physiological pH) and E3 (Table SI and SII). Therefore, zebrafish encounters these aggregates as the embryos spend much of their time on the bottom of the container in which they are grown. Particle size distributions and ζ -potentials in PBS and E3 show each of the NPs have similar sizes, surface charge and aggregation profile. The specific surface areas of NPs were 40–60 and 50 m²/g with densities of 0.2 and 0.7 g/cm³ for Ni and CuO, respectively. Thermogravimetric analysis (TGA) of particles showed negligible weight loss of ~0.6% for CuO and ~ 3.1 % for Ni NPs respectively (Figure S2). Additionally Fourier Transform Infrared Spectroscopy (FTIR) shows no organic traces on the particles. These results suggest that the NPs are free of surfactants or surface coatings. Particles were dispersed in E3 medium without other treatment.

Effect of NP exposure on hatching rate and embryonic viability

Hatching from the chorion is a developmentally timed event; embryos remain in the chorion on average up to 72 hpf³³. As the embryo develops, enzymes are produced by the hatching

gland which digests the inner layer of the chorion allowing the embryo to escape as a result of increasing movements^{34, 35}. Hatching rates have been used as an indicator of overall health of the developing embryo and an indicator of NPs toxicity for aquatic species³⁶. We exposed embryos to a range of increasing Ni and CuO NPs concentrations beginning at 1 ppm up to 100 ppm. Generally, increasing concentrations decreased hatching rates for both NPs.

Both exposure conditions show visible accumulation of NPs on the chorion, with higher accumulation for Ni, indicating enhanced adsorption of these particles on the chorion (Figure S3). No significant changes were observed for up to 10 ppm Ni NPs. However, 50 and 100 ppm reduce the hatching rate to 30 % (Figure S3A). For CuO NPs, hatching rate decreased dramatically to ~70 % even at 1 ppm. At 50 to 100 ppm, more than 98% of the embryos fail to hatch (Figure S3B). The effects of NPs on hatching rate may be due to dissolved ions as many NPs are not able to pass through the pores of the chorion^{36, 37}. Previous studies have demonstrated that ions can pass through the chorion and be concentrated in the perivitelline fluid, increasing hatching interference, which can be reversed with the metal chelator DTPA³⁶. This suggests that these NP exposures may also concentrate ions in the perivitelline fluid and therefore decrease the rate of hatching with higher NPs exposure. Additionally, high mortality rate among embryos was observed at these concentrations (Figure S4).

We further studied the effect of these NPs on the viability of the embryos during embryogenesis after removal of the protective chorion layer. Dose and time-dependent exposures displayed a more lethal impact of the Ni as compared to the CuO NPs (Figure S5). This can be due to the enhanced accumulation of Ni NPs on embryos as can be seen in Figure S5C. The magnetic properties of Ni NPs can increase aggregation and coverage of the embryos by particles further affecting organ development.

Effect of NP composition and concentration

Within the intestine, 5HT is produced and secreted by ECs. Mammalian ECs have been shown to respond to touch, stretch and nutrient stimulation with 5HT release playing a critical role in motility and secretion often becoming altered in diseased states^{38–40}. Internalization of the NPs within the intestine could result in activation of the mechanoreceptors and alteration of the EC response, and thus the 5HT level. We therefore, use 5HT concentration to assess the physiological state of the intestine after exposure to NPs. 5HT concentrations in embryos were monitored using CFMEs inserted in the mid-intestine and quantified using DPV (Figure 1 right panel). Embryos were exposed to NP dispersions in E3 after manual dechoriation at 24 hpf at concentrations ranging from 1 to 100 ppm and incubated until the end of embryogenesis at 5 dpf. Intestinal 5HT concentration in control unexposed zebrafish embryos is $30.8(\pm 3.4)$ nM. Exposures of embryos to up to 10 ppm Ni NPs did not show significant changes of the physiological 5HT level. However, 5HT decreases significantly to $5.1(\pm 3.8)$, $8.8(\pm 0.4)$ and $3.9(\pm 3.8)$ nM for higher concentrations exposure of 20, 50 and 100 ppm Ni NP (Figure 2A). On the other hand, embryos exposed to CuO NPs (1 – 100 ppm) show a general increase in the intestinal 5HT at levels greater than or equivalent to those of the unexposed embryos. The highest 5-HT concentration of $55.1(\pm 3.8)$ nM was obtained after 5 ppm CuO NP exposure (Figure 2B). No significant change was observed for 100 ppm CuO, which we attribute to higher agglomeration of the particles at this concentration.

For comparison and validation purposes, changes in the physiological 5HT levels were also assessed with anti-5HT immunohistochemistry. Fluorescence images do not provide quantitative assessment of 5HT concentrations (Figure 1A and B). Similar trends were obtained with both DPV and immunohistochemistry (Figure 2 left panel). However, the

fluorescence images displayed slight changes which cannot be used for *in vivo* 5HT assessment. These results suggest that CFMEs can be utilized as a sensitive and powerful tools for *in vivo* evaluation of 5HT after NPs exposure.

Changes of 5HT concentration at different phases of intestinal development

Zebrafish intestinal development first begins with a period of growth (proliferation phase) followed by a differentiation phase⁴¹. Direct NPs exposure to intestinal epithelial cells does not occur during the first two days of embryogenesis. During that period, the digestive system primarily grows in size through mitosis of the epithelial layer⁴¹. The differentiation phase involves the opening of the anterior intestine to the external environment on the third day followed by opening of the posterior intestine on the fourth day⁴² with development of contractions between the fourth and fifth days⁴³. Once the intestine is open to the external environment, NPs can be internalized within the lumen potentially affecting differentiating epithelial cells by direct exposure resulting in more dramatic alterations in intestinal physiology. Previously, we have found accumulation of NPs primarily within the pharynx and the anterior intestine⁸. As epithelial cells are not fully differentiated and barrier function may not be fully developed on the third and fourth dpf, epithelial cells may be more sensitive to NPs during this period. NPs are then likely to act on different tissues affecting digestive 5HT concentration differently depending upon which phase embryos are exposed.

To evaluate 5HT changes at different developmental stages, we separated NPs exposure into either growth or differentiation phase. For each NPs exposure we choose a concentration of 50 ppm which altered 5HT concentrations over the entire course of embryogenesis as seen in Figure 2. For the growth phase, embryos were exposed to Ni or CuO NPs beginning at 24 hpf to 60 hpf followed by washout into E3 and detection of 5HT concentration at 5 dpf. 5HT concentration demonstrates a significant increase (from 30.8 (± 3.4) nM in the control to 37.2 (± 3.8) nM in for Ni and 48.8 (± 5.4) nM) with CuO NP exposure (Figure 3). We next exposed embryos to NPs during the differentiation phase beginning at 3 dpf and continuing to 5 dpf. During this phase, NPs can enter the anterior digestive system at 3 dpf, and in addition the posterior at 4 dpf. This may allow accumulation and concentration of NPs adjacent to epithelial cells. Embryos exposed to Ni NPs demonstrate more dramatic increases in 5HT concentration within the intestine during this period when compared to exposure during the proliferation phase. CuO NP exposure shows increased levels of 5HT, which are slightly above those observed in the growth phase exposure (Figure 3A). This suggests that once the digestive system opens to the exterior environment, NP exposure will have greater physiological and developmental affects likely due to internalization and concentration of NPs directly interacting with the epithelial layer, as well as NP dissolution. However, the observed effects appear to be composition dependent.

NP exposure to embryos with a functional digestive system

NP exposure during the course of embryogenesis is likely to result in altered ECs that produce different steady state concentrations of 5HT due to altered development. In contrast, at the end of embryogenesis, the digestive system is functional and ECs should respond in a similar way to adult cells. Exposure during this period should then reveal how the intestine responds to toxic NPs. We exposed 5 dpf embryos to 50 ppm Ni and CuO NPs for a five hour period and recorded alterations in 5HT concentration. Exposure during this period almost doubles the 5HT concentration for both the Ni and CuO NP exposures with calculated concentrations of 56.2(± 2.3) and 63.8(± 9.8) nM respectively (Figure 4A). While NP exposure may stimulate mechanoreceptors within the intestine, they are less likely to stimulate taste receptors. We therefore compared alterations in 5HT concentration resulting from NP exposure to changes due to feeding. After incubation with food for the same 5 hour period, embryos consistently demonstrate a near tripling in the intestinal 5HT concentration

(80.67(±2.2) nM) (Figure 4A). Lower 5HT concentration due to NPs as compared to food may then be due to stimulation of only mechanical response in the former, with mechanical coupled with nutrient stimulation in the latter. This demonstrates that the digestive system may also have alternate mechanisms to discern toxicity of ingested NPs to increase 5HT release, thus increasing motility and intestinal secretion in order to expell NPs.

NPs internalization and dissolution

To confirm NPs accumulation within the embryos and evaluate the amount of internalized particles we acid-digested embryos exposed to 100 ppm Ni and CuO NPs. Concentrations of 4.67(±0.87) and 0.66(±0.04) ppm of Ni and Cu were found per 150 digested embryos, which demonstrate presence of these particles within the embryos, with higher accumulations being observed for Ni as compared to CuO NPs. Consequently, calculated NPs taken up per embryo were 31 and 4.4 ng for Ni and CuO NPs, respectively. Additionally, intestinal cross sections show NPs localized within the intestinal lumen (Figure 4B and C). Retention of these NPs within the intestine can directly stimulate ECs that could cause a sustainable release of 5HT and trigger serotonin syndrome or serotonin toxicity³⁹, or they could interact with 5HT altering reuptake and release from ECs. The toxicity of Ni and CuO NPs can be due to the release of ions⁴⁴ especially in the growth phase when the intestine is not open which would increase NPs residence time. Concentration of soluble Ni and Cu ions in E3 was quantified by using inductively coupled plasma mass spectrometry (ICP-MS)⁸ and amperometry at the reductive potentials of Cu²⁺ (−0.05 V) and Ni²⁺ (−0.45V), which allows trace metal detection with high sensitivity⁴⁵. 100 ppm Ni and CuO NPs dispersions were incubated in E3 for 4 days at 28.5°C. The concentration of Cu²⁺ ions in the suspension after centrifugation and filtration was estimated to be 0.92 (± 0.02) ppm within 4 days exposure. For Ni, we found a higher dissolution rates by both ICP-MS⁸ and electrochemistry of 4.49 (±0.87) ppm within 4 days exposure, indicating that embryos are exposed to higher amounts of cations which might be responsible for the observed 5HT changes over the course of embryogenesis. Taken into consideration the dissolution percentage of the NPs, ions concentrations taken up by embryo were estimated as 1.55 and 0.04 ng for Ni²⁺ and Cu²⁺, respectively. These results indicate that embryos were exposed to low concentrations of soluble ions produced by dissolution of NPs in the egg water. To evaluate the effect of Ni²⁺ and Cu²⁺ on intestinal 5HT levels, embryos were exposed to 5 ppm Ni²⁺ and 1 ppm Cu²⁺ solution at 24 hpf and incubated until the end of embryogenesis at 5 dpf. Intestinal 5HT concentration decreased to 25.84 (±1.55) nM (Figure 2A inset). This result suggests that Ni²⁺ contributes to the observed intestinal 5HT decrease, yet their effect is less than that of the parent NPs. Similarly, 5HT levels decreased to 23.72 (±2.4) nM upon exposure to copper ions. This finding suggests that Cu²⁺ has differential effects on 5HT concentrations compared to CuO NPs.

Discussions

Zebrafish is a commonly used aquatic model for early-life stage toxicity evaluation of many different environmental contaminants^{46, 47}. Zebrafish organs and tissues are homologous to more complex mammalian species at the anatomical, physiological and molecular level. Conventional approaches to evaluate nanotoxicity involve assessment of the LD₅₀ as well as the ability to cause developmental defects⁸, which typically occurs at high dose NPs exposure. The presence of low concentrations of NPs however, may represent a more common environmentally relevant situation since low-levels of NPs are more likely to be released from a variety of products or manufacturing sources. Low concentrations of NPs encountered in the environment would not frequently result in observable malformations or lethality. NP exposure might instead induce a variety of more chronic problems such as reduction in viability, inflammation, or altered organ function. One of the primary sites of

NPs accumulation in zebrafish embryos is the digestive tract. Here we have utilized the embryonic zebrafish intestine to assess physiological changes of the intestinal 5HT concentration following NP exposure. We used changes in the physiological 5HT concentrations to assess this organ dysfunction due to NP exposure. In our previous work, we monitored 5HT within the intestine of live 5dpf zebrafish embryos by electrochemistry using inserted CFMEs. The physiological 5HT level was found to be $30.8(\pm 3.4)$ nM³¹. In this work, we utilize this technique to monitor changes of 5HT in zebrafish embryos exposed to NPs. Here we demonstrate increases in 5HT up to $55.1(\pm 3.8)$ nM after 5 ppm CuO NP exposure and decreases as low as $3.9(\pm 3.8)$ nM for 100 ppm Ni NP. We have previously demonstrated that changes in 5HT concentration of this magnitude can significantly modify intestinal motility with changes in distance, velocity, and frequency of contractions⁴⁹ suggesting that the alterations in 5HT concentration that we observe here, create physiological changes within the digestive system including nutrient movement and absorption.

We expose embryos to two types of same size NPs, Ni and CuO, of various concentrations and time periods. In the aquatic environment, NPs can undergo agglomeration, sedimentation and dissolution⁵⁰. Species living in environments containing NPs can internalize small amounts of these NPs along with natural nutrients^{51, 52}. As a result, the gut becomes one of main targets of NPs exposure. The digestive system is constantly exposed to the external environment through ingested food and it is likely to accumulate environmental sources of NPs due to extensive folding and intermittent release of ingested materials. Intestinal cross-section of exposed embryos in our study identified ingested Ni and CuO NPs in the intestinal lumen after 5 hour exposure. NPs in contact with the intestinal epithelium trigger physiological response and alter the normal functioning of this organ. We have shown previously that high NP concentration exposure induces thinning of the epithelial barrier⁸ which can disrupt overall function of the intestine. In this study we identified significant changes in the 5HT concentration within the intestine in embryos exposed to NPs and showed that these changes are concentration, time, and composition dependent.

Exposure to Ni NPs at 20 ppm and higher concentrations throughout the entire course of embryogenesis results in striking decreases in intestinal 5HT down to 16.5% or lower concentrations compared to WT. With increasing concentrations of Ni NPs we have previously observed reduced intestinal epithelial differentiation and overall thinning of the intestinal wall⁸. Concentrations of 20 ppm and higher of Ni NP may also begin to affect differentiation of epithelial EC cells and affect their ability to produce WT levels of 5HT. Additionally, interaction between 5HT and metal ions has been reported⁵³. In this study, release of Ni(II) ions from NPs could form metal-ligand complexes with 5-HT, e.g. Ni-serotonin, further sequestering 5HT. Ni(II) ions released from NPs may also further reduce 5HT concentrations by reducing 5HT production and increasing cellular uptake. Previous reports find that Ni(II) ions suppress expression of tryptophan hydroxylase while enhance expression of the monoamine transporter⁵⁴ reducing the overall 5HT concentration. This could partially explain the decrease in 5HT concentration ($25.84(\pm 1.55)$ nM) that we observe after exposure to 5 ppm Ni²⁺.

In contrast, exposure to CuO NPs throughout embryogenesis consistently increased intestinal 5HT. In this case, embryos internalized fewer CuO NPs as determined by digestion experiments, and thus are exposed to lower doses of particles. In contrast to NP exposure, we observed decreased concentration of 5HT with addition of 1 ppm Cu(II) ions. Reduction of 5HT concentration may be due in part to the previously reported ability of Cu(II) ions to induce oxidation of 5HT to methidequinone derivative⁵⁵. The difference between CuO NPs and Cu(II) ions on 5HT concentration suggests that the affect of increased 5HT with CuO NP exposure occurs by additional mechanisms than ion release.

Differences between Ni and CuO NPs exposure during the course of embryogenesis on 5HT concentration suggest different developmental and signalling pathways are affected and demonstrate the complexity of NP induced toxicity during embryogenesis.

While Ni and CuO exposure over the entire course of embryogenesis demonstrates variable 5HT release, NP exposure during specific phases of embryogenesis, suggests a general increase in 5HT concentration. We find that exposure during the initial phase of embryogenesis when the intestine grows and is not open to the exterior increases 5HT for both Ni and CuO NPs. This can be a consequence of the released ions and suggests that both Ni and CuO NPs affect intestinal development even when the sole exposure route is the outer surface of the embryo. In the differentiation phase however, after the intestine is open to the exterior, 5HT concentration is higher than during exposure in the growth phase for both NPs. This suggests that once NPs gain access to the intestinal lumen, the organ has increased sensitivity over a wider range of NPs compositions and concentrations. The response may be due to toxic effects initiated by different compositions and released ions. This can include activation of ECs by mechanical stimulation in the presence of the NPs. Variations in 5HT may also be due to the quantity of different types of NPs that enter the intestine. Increased 5HT may also indicate inflammatory response during which 5HT concentration is often altered. CuO NPs in particular have been shown to trigger oxidative stress and induce inflammation in many aquatic species^{56–58}.

When embryos are exposed to NPs for a five hour period at the end of embryogenesis and the effect was compared to that of a functional intestine containing ingested food, the 5HT level followed the same trend for both the Ni and CuO NP exposures. Ingestion of food provides the normal 5HT response with a near tripling in concentration. Increases in 5HT are likely due to a combination of proteins, fats, and sugars in the intestinal lumen activating taste receptors on a host of enteroendocrine cells. NPs on the other hand are not likely to activate similar cascades of receptors and we therefore expect to observe a different response with short-term exposure. While Ni and CuO NPs also demonstrate a dramatic increase in 5HT concentration after five hour exposure, these concentrations are less than the level observed with food, indicating that different responses are being activated by NPs within the intestine.

Conclusions

This study is the first to demonstrate physiological alterations of 5HT within embryonic zebrafish intestine as a result of NPs exposure. We observed time and dose-dependent decreases in the hatching rate and viability of embryos after Ni and CuO NPs. Monitoring the alterations of physiologically important markers can help better understand how NPs might affect the physiology of aquatic species exposed to environments that contain NPs. These findings provide evidence that environmental exposure to NPs might affect the physiology of developing organs in characteristic ways, and indicate the need for more fundamental investigations to assess interaction of NPs (and their soluble ions) with chemical messengers like 5HT and better understand toxic effects of NPs. This information could be used to predict long term physiological effects of NP exposure in living organisms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NSF-1336493 to SA and KW, NSF-0954919 to SA and by NIH 1R15DK089474-01 to KW. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the

author(s) and do not necessarily reflect the views of the National Science Foundation or the National Institute of Health. The authors acknowledge a seed grant from the Institute for a Sustainable Environment at Clarkson. We would like to acknowledge R. Alkasir for the help with the NPs dissolution experiments and Dr. D. Andreescu for the helpful discussions.

References

1. Baek Y-W, An Y-J. Science of The Total Environment. 2011; 409:1603–1608. [PubMed: 21310463]
2. Roco MC. Environmental Science & Technology. 2005; 39:106A–112A.
3. Nel A, Xia T, Mädler L, Li N. Science. 2006; 311:622–627. [PubMed: 16456071]
4. Lewinski N, Colvin V, Drezek R. Small. 2008; 4:26–49. [PubMed: 18165959]
5. Oberdörster G, Oberdörster E, Oberdörster J. Environ Health Perspect. 2005; 113
6. Maurer-Jones MA, Gunsolus IL, Murphy CJ, Haynes CL. Analytical chemistry. 2013
7. Marquis BJ, Love SA, Braun KL, Haynes CL. Analyst. 2009; 134:425–439. [PubMed: 19238274]
8. Ispas C, Andreescu D, Patel A, Goia DV, Andreescu S, Wallace KN. Environmental Science & Technology. 2009; 43:6349–6356. [PubMed: 19746736]
9. Xiong D, Fang T, Yu L, Sima X, Zhu W. Science of The Total Environment. 2011; 409:1444–1452. [PubMed: 21296382]
10. Schanen BC, Karakoti AS, Seal S, Drake Iii DR, Warren WL, Self WT. ACS Nano. 2009; 3:2523–2532. [PubMed: 19769402]
11. AshaRani PV, Low Kah Mun G, Hande MP, Valiyaveetil S. ACS Nano. 2008; 3:279–290. [PubMed: 19236062]
12. Ahamed M, AlSalhi MS, Siddiqui MKJ. Clinica Chimica Acta. 2010; 411:1841–1848.
13. Pan Z, Lee W, Slutsky L, Clark RAF, Pernodet N, Rafailovich MH. Small. 2009; 5:511–520. [PubMed: 19197964]
14. Das M, Patil S, Bhargava N, Kang J-F, Riedel LM, Seal S, Hickman JJ. Biomaterials. 2007; 28:1918–1925. [PubMed: 17222903]
15. Zhang H, He X, Zhang Z, Zhang P, Li Y, Ma Y, Kuang Y, Zhao Y, Chai Z. Environmental Science & Technology. 2011; 45:3725–3730. [PubMed: 21428445]
16. Ozay O, Aktas N, Inger E, Sahiner N. International Journal of Hydrogen Energy. 2011; 36:1998–2006.
17. Eluri R, Paul B. Journal of Manufacturing Processes. 2012; 14:471–477.
18. Seifollahi Bazarjani M, Kleebe H-J, Müller MM, Fasel C, Baghaie Yazdi M, Gurlo A, Riedel R. Chemistry of Materials. 2011; 23:4112–4123.
19. Pane EF, Richards JG, Wood CM. Aquatic Toxicology. 2003; 63:65–82. [PubMed: 12615421]
20. Hoang TC, Tomasso JR, Klaire SJ. Environmental Toxicology and Chemistry. 2004; 23:86–92. [PubMed: 14768871]
21. Kienle C, Köhler HR, Filser J, Gerhardt A. Environmental Pollution. 2008; 152:612–620. [PubMed: 17720287]
22. Cuevas A, Liberda E, Chen LC. Toxicology Letters. 2010; 196:S284–S285.
23. Yin H, Too HP, Chow GM. Biomaterials. 2005; 26:5818–5826. [PubMed: 15949547]
24. Karlsson HL, Cronholm P, Gustafsson J, Möller L. Chemical Research in Toxicology. 2008; 21:1726–1732. [PubMed: 18710264]
25. Midander K, Cronholm P, Karlsson HL, Elihn K, Möller L, Leygraf C, Wallinder IO. Small. 2009; 5:389–399. [PubMed: 19148889]
26. Kasemets K, Ivask A, Dubourguier H-C, Kahru A. Toxicology in Vitro. 2009; 23:1116–1122. [PubMed: 19486936]
27. Dziembaj R, Molenda M, Chmielarz L, Zaitz MM, Piwowarska Z, Rafalska-Łasocha A. Catalysis Today. 2011; 169:112–117.
28. Sunkara S, Vendra VK, Kim JH, Druffel T, Sunkara MK. Catalysis Today. 2013; 199:27–35.
29. Ozel RE, Hayat A, Wallace KN, Andreescu S. RSC Advances. 2013

30. Njagi J, Ball M, Best M, Wallace KN, Andreescu S. *Analytical chemistry*. 2010; 82:1822–1830. [PubMed: 20148518]
31. Özel RE, Wallace KN, Andreescu S. *Analytica Chimica Acta*. 2011; 695:89–95. [PubMed: 21601035]
32. Njagi J, Ispas C, Andreescu S. *Analytical chemistry*. 2008; 80:7266–7274. [PubMed: 18720950]
33. Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. *Developmental Dynamics*. 1995; 203:253–310. [PubMed: 8589427]
34. Kawaguchi M, Yasumasu S, Shimizu A, Sano K, Iuchi I, Nishida M. *FEBS J*. 2010; 277:4973–4987. [PubMed: 21070609]
35. Inohaya K, Yasumasu S, Yasumasu I, Iuchi I, Yamagami K. *Dev Growth Differ*. 1999; 41:557–566. [PubMed: 10545028]
36. Lin SJ, Zhao Y, Xia T, Meng H, Ji ZX, Liu R, George S, Xiong SJ, Wang X, Zhang HY, Pokhrel S, Madler L, Damoiseaux R, Lin S, Nel AE. *Acs Nano*. 2011; 5:7284–7295. [PubMed: 21851096]
37. Xia T, Zhao Y, Sager T, George S, Pokhrel S, Li N, Schoenfeld D, Meng H, Lin S, Wang X, Wang M, Ji Z, Zink JJ, Mädler L, Castranova V, Lin S, Nel AE. *ACS Nano*. 2011; 5:1223–1235. [PubMed: 21250651]
38. Raybould HE. *Autonomic neuroscience : basic & clinical*. 2010; 153:41–46. [PubMed: 19674941]
39. Mohammad-Zadeh LF, Moses L, Gwaltney-Brant SM. *Journal of Veterinary Pharmacology and Therapeutics*. 2008; 31:187–199. [PubMed: 18471139]
40. Spiller RC. *Current opinion in pharmacology*. 2011; 11:68–74. [PubMed: 21398180]
41. Wallace KN, Akhter S, Smith EM, Lorent K, Pack M. *Mechanisms of Development*. 2005; 122:157–173. [PubMed: 15652704]
42. Wallace KN, Pack M. *Developmental biology*. 2003; 255:12–29. [PubMed: 12618131]
43. A. S. Holmberg T, Fritsche R, Pelster B, Holmgren S. *Journal of Fish Biology*. 2003; 62:318–331.
44. Zhang H, Ji Z, Xia T, Meng H, Low-Kam C, Liu R, Pokhrel S, Lin S, Wang X, Liao Y-P, Wang M, Li L, Rallo R, Damoiseaux R, Telesca D, Mädler L, Cohen Y, Zink JJ, Nel AE. *ACS Nano*. 2012; 6:4349–4368. [PubMed: 22502734]
45. Wang J, Lu JM, Hocevar SB, Farias PAM, Ogorevc B. *Analytical chemistry*. 2000; 72:3218–3222. [PubMed: 10939390]
46. de Esch C, Sliker R, Wolterbeek A, Woutersen R, de Groot D. *Neurotoxicology and Teratology*. 2012; 34:545–553. [PubMed: 22971930]
47. Rinkwitz S, Mourrain P, Becker TS. *Progress in Neurobiology*. 2011; 93:231–243. [PubMed: 21130139]
48. Hansen MB, Witte AB. *Acta Physiologica*. 2008; 193:311–323. [PubMed: 18462271]
49. Roach G, Wallace RH, Cameron A, Ozel RE, Hongay CF, Baral R, Andreescu S, Wallace KN. *Developmental biology*. 2013; 376:171–186. [PubMed: 23353550]
50. Klaine SJ, Alvarez PJJ, Batley GE, Fernandes TF, Handy RD, Lyon DY, Mahendra S, McLaughlin MJ, Lead JR. *Environmental Toxicology and Chemistry*. 2008; 27:1825–1851. [PubMed: 19086204]
51. Zhu X, Wang J, Zhang X, Chang Y, Chen Y. *Chemosphere*. 2010; 79:928–933. [PubMed: 20371096]
52. Unrine JM, Shoults-Wilson WA, Zhurbich O, Bertsch PM, Tsyusko OV. *Environmental Science & Technology*. 2012; 46:9753–9760. [PubMed: 22897478]
53. Limson J, Nyokong T, Daya S. *Journal of pineal research*. 1998; 24:15–21. [PubMed: 9468114]
54. Slotkin TA, Seidler FJ. *Toxicol Appl Pharm*. 2008; 233:211–219.
55. Hadi N, Malik A, Azam S, Khan NU, Iqbal J. *Toxicology in Vitro*. 2002; 16:669–674. [PubMed: 12423649]
56. Blinova I, Ivask A, Heinlaan M, Mortimer M, Kahru A. *Environmental Pollution*. 2010; 158:41–47. [PubMed: 19800155]
57. Heinlaan M, Ivask A, Blinova I, Dubourguier H-C, Kahru A. *Chemosphere*. 2008; 71:1308–1316. [PubMed: 18194809]

58. Gomes T, Pinheiro JP, Cancio I, Pereira CG, Cardoso C, Bebianno MJ. Environmental Science & Technology. 2011; 45:9356–9362. [PubMed: 21950553]

Highlight

Initial evidence of the adverse effect of nanoparticles on the physiology of intestine is demonstrated with comprehensive quantitative analyses.

Fig. 1. Assessment of 5-HT levels in zebrafish embryos after NP exposure using anti-5HT immunohistochemistry (fluorescence images are on the left) and differential pulse voltammograms (graphs on the right) for unexposed wild type control embryos (A) and embryos subjected to 50 ppm Ni NPs (B) and CuO NPs (C). Exposure of Ni NPs causes slight decrease in fluorescence intensity in the intestine, thus a decrease in 5-HT concentration (B). In contrast, CuO NPs enhances production of 5-HT levels within the intestine leading to a visible increase in fluorescence intensity (C). All images are in same scale. Microelectrodes are inserted in the mid-intestine. The supporting electrolyte was E3 medium.

Fig. 2. Changes of *in vivo* intestinal 5HT concentrations after exposure to Ni NPs (Inset: alteration of intestinal 5HT after 5 ppm Ni(II) exposure) (A) and CuO NPs (Inset: alteration of intestinal 5HT after 1 ppm Cu(II) exposure) (B) dispersions at concentration ranging from 1 to 100 ppm from 24 hpf to 5 dpf. Microelectrodes are inserted in the mid-intestine. The error bars represent standard deviation for $n = 3-5$ replicate measurements at each concentration. The supporting electrolyte was E3 medium. One and two asterisks indicate $p < 0.05$ and < 0.001 , respectively.

Fig. 3.

Time dependent variation of 5HT after NPs exposure. 5 dpf embryos were exposed to 50 ppm Ni and CuO NPs for two days either during the proliferation phase when the digestive system is not open to the exterior or the differentiation phase when the digestive system opens to the exterior (A). Schematic illustration of the NPs exposure procedure. NP exposure was begun at indicated day (either 1 dpf or 3 dpf) and washed out after two days. Average voltammetric responses obtained from CFMEs of intestinal 5HT concentration of 5 dpf embryos (B). The error bars represent standard deviation for $n = 3 - 5$ replicate measurements at each time point. The supporting electrolyte was E3 medium. One and two asterisks represent $p < 0.05$ and < 0.001 , respectively.

Fig. 4. 5 dpf embryos were exposed to 50 ppm Ni and CuO NPs for a 5 hour period. A separate group was provided food for the same period. After the exposure, average DPV responses obtained from chitosan modified microelectrodes used for the *in vivo* assessment of intestinal 5HT concentration (A). Embryos exposed to NPs during the 5 hour period were cross-sectioned to determine accumulation within the intestinal lumen. NPs are present in embryos exposed to Ni (B) and CuO (C) NPs (arrows in each image). The error bars represent standard deviation for $n = 3$ replicate measurements for each condition. The supporting electrolyte was E3 medium. Two asterisks indicate $p < 0.001$.