Gender-specific Biological Responses in Juvenile Rats Orally Exposed to Three Engineered Nanomaterials

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ABSTRACT

Engineered nanomaterials (ENMs) are widely used in medicine, food, and agriculture, as well as general household applications, and exposure to them is ubiquitous. Children represent a vulnerable population because perturbations in cell growth and signaling can disrupt temporally-sequenced developmental processes leading to long-term functional deficits. Little is known about the uptake, distribution, and biological responses of ENMs and their toxicity in developing animals.

In the study presented here, three nanoparticles (NPs) provided by The National Institute of Environmental Health Sciences Consortium for Nanotechnology Health Implications Research were tested: P25 TiO_2, 30 nm Al_2O_3, and 50 nm ZnO. Three litters (five male and five female) of juvenile Sprague Dawley rats were daily administered 10 mg/kg NPs or vehicle control (water) by oral gavage between postnatal day (PND) 17-20. Basic neurobehavioral (acoustic startle response, locomotor activity, and rotarod) and cardiac (ECGenie) assessment were performed 4 hours post administration of the final dose. Animals were sacrificed on PND 21, and selected tissues were collected, weighted, and processed for histopathology or biochemical analysis. Neurotransmitter concentrations in brain tissues were quantified by HPLC with electrochemical detection.

No change was observed for body weight (bw) or brain-to-bw ratio for male and female pups. Liver-to-bw ratio were significantly increased for male pups receiving TiO_2 P25 (0.0417±0.0028) and Al_2O_3 NP and (0.0409±0.0021) for female pups administered TiO_2 P25 (0.0420±0.0040) compared to control (male: 0.0389±0.0025; female: 0.0395±0.0021). No neurobehavioral effects were found. Heart rate was significantly decreased for female pups administered TiO_2 P25 (441±43.3 beats per minute [bpm]) compared to vehicle control (511±46.0 bpm). No significant changes were observed for neurotransmitter levels in brain tissue.

Gender-specific responses were observed in juvenile rats orally administered TiO_2 P25 and Al_2O_3 NP. These data suggest that developing animals present a valuable population to oral NP exposure, and more knowledge is needed for potential long-term metabolic and cardiovascular perturbations.

Introduction

Little is known about the uptake, distribution, and biological responses of NPs and their toxicity in developing animals. Moreover, the developing intestinal tract is more permeable than in the adult suggesting that oral NP exposure in developing animals could be higher than that observed in adult animals. Thus, evaluating the effects of NP exposure in developing animals is essential for understanding the potential risks posed by NP use. The biological response to NPs was studied in female and male neonatal and juvenile rats, by a comprehensive assessment of the NP tissue distribution, functional neurobehavioral and cardiovascular endpoints, and untargeted and targeted metabolomics analyses.

Methods

Nanoparticles (NPs). Three NPs provided by The NIEHS Consortium for Nanotechnology Health Implications Research (NHIR) were tested: 30 nm TiO_2 P25, 30 nm Al_2O_3, and 50 nm ZnO.

Formulation, Characterization and Stability Tests. Dosing solutions were formulated as described in the 'Discrete Sonication' protocol published by [1]. Dosing solutions were prepared fresh every day.

Animals. Female Sprague Dawley rats with their standardized litter of 5 male and 5 female pups, were obtained from Charles River Laboratories in Raleigh, NC. Dams were housed with their litter. The animal rooms were maintained at 72 ± 3 °F, 30-70% RH and a 12:12 light cycle. Pups were dosed by oral gavage with 10 mg/kg NP daily between PND 17-20. The pups were euthanized by live decapitation. The liver and brain were collected and weighed. Duodenum, jejunum, ileum, colon, liver, kidney, spleen, and lymph node were examined by hematoxylin and eosin histologic staining under light microscopy and were within normal limits. The intestinal sections were prepared using the improved Swiss roll technique, following the published protocol [2].

Locomotor Activity Testing. The motor activity test was done using the San Diego Instruments Photobeam Activity System (PAS). The photobeam test is designed to assess the locomotor activity using the collection and recording of beam breaks over time as the animal moves.

Acoustic Startle Response. The Startle Response System Test (San Diego Instruments, SR-Lab) was performed where the session started after 300 s acclimation period where pups were exposed to background noise of 69dB, followed by the acoustic startle as a single pulse of 120 dB.

Rotarod. The Rotarod test was performed using a Stoelting Rotarod, model 52790, using 1¼ inch diameter drums. The test is completed when all pups fall off the rod, or when 150 s has expired and the rod stops moving, whichever happened first.

ECGenie. Cardiac repolarization (electrocardiograms [EGC]) was measured by ECGenie (Mouse Specific, Inc.) using the software LabChart8. ECGenie detects cardiac electrical activity through the animals' paws.

Enhanced Darkfield and Hyperspectral Imaging of Tissues. H&E stained tissue sections from dosed and control animals were images using a near-inferred darkfield microscope with a hyperspectral imaging unit (CytoViva Inc., Auburn).

Neurotransmitter Analysis of Brain Tissue. The level of six neurotransmitters were quantitated in brain tissues from male and female pups using ultra high pressure liquid chromatography (UPLC) coupled with electrochemical detection (ECD). Samples were analyzed on a Luna Omega 1.6 µm Polar C18, 2.1X150 mm column (Phenomenex, Torrance, CA) coupled to a LPG-3400RS pump, WPS-3000TBRS autosampler, and a 5600A CoulArray electrochemical detector (Thermo Scientific, Waltham, MA).

Statistical Analysis. Unpaired t-test, P<0.05 (GraphPad Prism 7.04).

Results

ENM Dose Formulation and Characterization

Table 1. Characterization of NP dosing solutions (2 mg/mL) was done by DLS and NTA at 0 and 4 hours after preparation. The criteria adopted for a stable dosing solution was a change in hydrodynamic diameter less than 30% over 4 hours.

Nanoparticles	Time (h)	Dynamic Light Scattering (DLS)		Nanoparticle Tracker (NTA)
		Hydrodynamic Diameter (nm)	Polydispersity index (PdI)	Mean diameter (nm)
TiO_2 P25	0	476.5 ± 23	0.534	114 ± 76
	4	503.7 ± 32	0.561	123 ± 54
Al_2O_3	0	148.3 ± 1.3	0.124	191 ± 96
	4	151.6 ± 2.1	0.131	213 ± 72
ZnO	0	406.0 ± 16	0.324	110 ± 70
	4	410.5 ± 9.8	0.197	128 ± 55

Neurobehavioral Testing

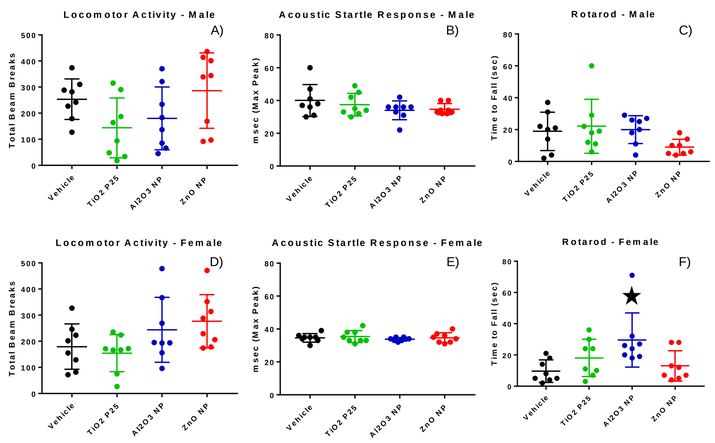


Figure 1. Basic neurobehavioral assessment of A-C) male and D-F) female pups administered TiO_2 P25 NP, Al_2O_3 NP, and ZnO NP were performed by measuring A+D) locomotor activity, B+E) acoustic startle response, and C+F) rotarod. Black star = significant difference (P<0.05) from vehicle control.

Heart Rate

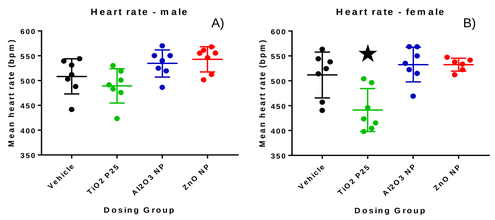


Figure 2. Electrocardiograms for unrestrained and awake A) male and B) female pups were performed non-invasively by ECGenie over a period of 10 min. The mean heart rate (bpm). Black star = significant difference (P<0.05) from vehicle control.

Body Weight and Organ-to-Body Weight for Male and Female Juvenile Rats

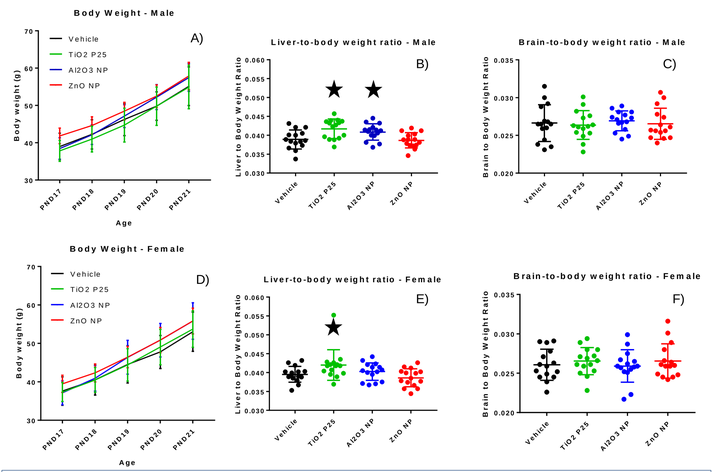


Figure 3. Body weight for A) male and D) female pups dosed with TiO_2 P25, Al_2O_3, ZnO, and vehicle control between PND 17-20. Pups were sacrificed on PND 21, and the liver-to-body weight ratio for B) male and E) female and brain-to-body weight ratio for C) male and F) female pups were calculated. In male pups TiO_2 P25 and Al_2O_3 caused a significant increase in B) liver-to-body weight ratio as indicated by a star. TiO_2 P25 also caused a significant increase in E) liver-to-body weight ratio in female pups. Black star = P<0.05.

Results

Enhanced Darkfield Microscopy and Hyperspectral Imaging of ENMs in Tissues.

Figure 4. Enhanced Darkfield Microscopy (EDM) and Hyperspectral Imaging (HSI) of the intestinal tract of male and female juvenile pups administered vehicle control or Al_2O_3 NP. Analysis of TiO_2 P25 and ZnO NP are ongoing. Histopathology analysis showed no histological changes in the intestinal tissues.

Images from three male and three female pups, 24 h after administration of the last oral dose, were collected by EDM, followed by HSI, using a 60x oil objective. Spectral Libraries (SL) were built for each tissue, and filtered against at least five images of control tissue to remove any spectral not specific to NP. The filtered SL was used to map (Spectral Angle Mapping) the location of spectra unique to NPs in each image. The mapped areas are highlighted in the HSI images, and indicated by circles or arrows in **Figures 4 and 5**. Number of individual or aggregates of NPs were counted using ImageJ.

Duodenum: Al_2O_3 NP was found in lumen, ducts, and crypts. In the tissue Al_2O_3 NP was most frequently located in the lamina propria and muscularis mucosa, but also found in villi enterocytes and muscularis externa.

Jejunum: Al_2O_3 NP was found in lumen, ducts, and crypts. As for duodenum, Al_2O_3 NP was most frequently located in the lamina propria and muscularis mucosa. NPs were not found in enterocytes.

Ileum: Al_2O_3 NP was found in lumen, ducts, and crypts. In the tissue NPs were located in lamina propria, muscularis mucosa, and Peyer's Patches.

Colon: Al_2O_3 NP was found in lumen, ducts, and crypts. In the tissue Al_2O_3 NP was most frequently located in the lamina propria and muscularis mucosa, and less in muscularis externa.

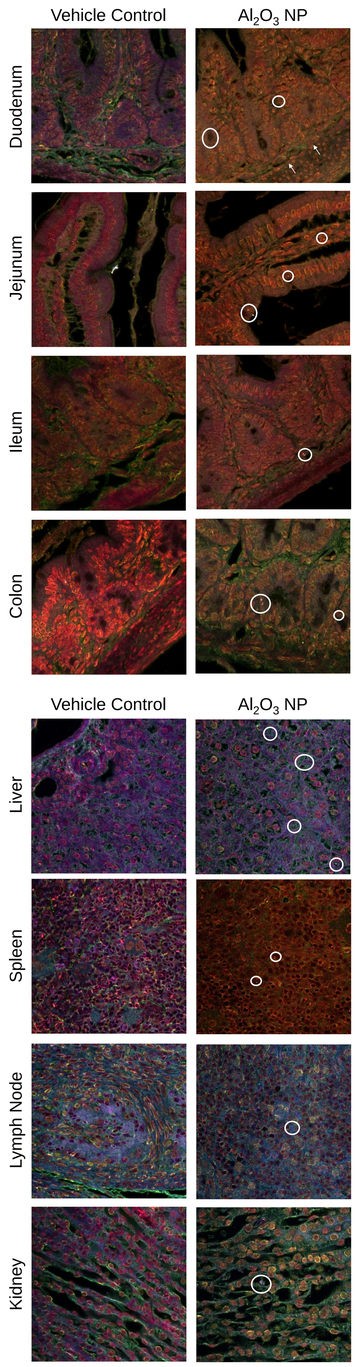


Figure 5. EDM and HSI of liver, spleen, mesenterial lymph node, and kidney tissues of male and female juvenile pups administered vehicle control or Al2O3 NP. Analysis of TiO2 P25, and ZnO NP are ongoing.

A mild to occasionally moderate lymphocytic portal hepatitis focused on bile ductules is present in both vehicle controls and Al2O3 NP exposed pups.

Liver: The hepatic architecture of Sprague Dawley rats at PND 21 is very similar to that of adults, and is fully matured by PND 28.

- Al2O3 NP was found in hepatocytes in most images, but also frequently in Kupffer cells. Occasionally NP was also found in small blood vessels.

Spleen: The splenic follicles and germinal centers are not present at PND 21, and the periarteriolar lymphoid sheaths are immature.

- Al2O3 NP was found throughout the spleen.

Lymph Node: Mesenteric lymph node reaches adult morphology at PND 21, while mandibular and axillary lymph nodes are still undergoing maturation.

- Al2O3 NP was found throughout the lymph node.

Kidney: The kidney has three major parts; cortex, medulla, and papilla, which matures at different rates, but are all mature at PND 30 [3]. At PND 21 nephrogenesis is considered complete. Although morphological mature at PND 21, functional maturation is ongoing.

- Al2O3 NP was found in different areas of the kidneys.

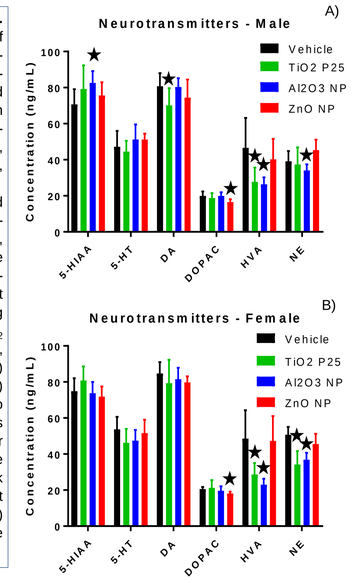
Table 2. ImageJ analysis of HSI images after SAM. The number of NP per image was determined and the average ± standard deviation reported for each tissue. The pixel size for each Al_2O_3 NP area was also reported. This pixel area does not translate into NP size or provide information regarding single versus aggregated NPs. It describes the size of the area where NP(s) are found by SAM.

Tissue	NP Found in # Image		Average NP Count per Image		Average Pixel Area	
Gender	Male	Female	Male	Female	Male	Female
Duodenum	14/15	15/15	7.87 ± 6.51	58.1 ± 104	21.9 ± 65.7	12.6 ± 20.0
Jejunum	13/15	12/15	12.7 ± 18.4	9.08 ± 10.8	8.91 ± 10.8	10.4 ± 28.7
Ileum	12/15	10/10	7.41 ± 10.2	20.1 ± 22.2	9.44 ± 11.2	10.37 ± 15.9
Colon	15/15	14/15	7.18 ± 10.7	21.6 ± 40.5	10.6 ± 27.3	6.16 ± 8.46
Liver	13/13	15/15	30.4 ± 17.6	29.1 ± 33.3	10.8 ± 19.6	7.20 ± 16.5
Spleen	10/10	14/15	3.20 ± 1.83	5.64 ± 4.19	8.56 ± 6.36	7.61 ± 8.80
Kidney	15/15	12/15	4.29 ± 2.58	8.27 ± 14.8	17.5 ± 47.2	3.99 ± 5.21
Lymph Node	12/15	14/15	3.21 ± 2.73	3.08 ± 2.56	8.22 ± 8.96	10.3 ± 12.5

Results

Neurotransmitter Concentrations in Brain Tissue

Figure 6. Concentration of six neurotransmitters: 5-Hydroxy-indoleacetic acid (5-HIAA), serotonin or 5-hydroxy-tryptamine (5-HT), dopamine (DA), 3,4-Dihydroxy-phenylacetic acid (DOPAC), homo-vanillic acid (HVA), and norepinephrine (NE) were determined in the right brain half following exposure to TiO_2 P25 NP, Al_2O_3 NP, and ZnO NP in A) male and B) female pups. No significant changes in neurotransmitter concentration were observed. Black star = significant difference (P<0.05) from vehicle control.



Conclusions

- Gender-specific differences in rotarod, heart rate, liver-to-bw ratio, and neurotransmitter levels in brain were observed for juvenile pups administered Al_2O_3 NP, TiO_2 P25 and ZnO NP by gavage between PND 17-20 and sacrificed on PND 21.
- Neurobehavioral tests (Startle Response, Locomotor Activity, and Rotarod) were not impacted by NP exposure, with the exception of female pups dosed with Al_2O_3 NP. Here exposure seemed to increase the female pups' performance in the test.
- Heart rate was decreased for female pups dosed with TiO_2 P25.
- NP exposure did not impact pup body weight. However, the liver-to-bw ratio was significantly increased for male pups following Al_2O_3 NP and TiO_2 P25 administration, and following TiO_2 P25 for female pups. Brain-to-bw ratio was not significantly impacted by exposure to the three NPs.
- HSI located Al_2O_3 NP in most of the eight collected tissues. Despite the high standard deviation for the number of mapped areas per image, the findings suggest that there was a higher number of NP in the female duodenum, ileum, and colon compared to male pups. HSI investigations with the other NPs are ongoing.
- For neurotransmitter concentrations in the right brain TiO_2 P25 caused significant increase for 5-HIAA in male pups, and significant decrease in DA and HVA, while for female pups TiO_2 P25 significantly decreased HVA and NE. Al_2O_3 NP administration significantly increased 5-HIAA in male pups, and HVA and NE were significantly decreased in both genders. ZnO NP led to a significant decrease in DOPAC.

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