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Titanium dioxide nanoparticles enhance mortality of fish exposed to bacterial pathogens

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ABSTRACT

Nano-TiO_2 is immunotoxic to fish and reduces the bactericidal function of fish neutrophils. Here, fathead minnows (*Pimephales promelas*) were exposed to low and high environmentally relevant concentration of nano-TiO_2 (2 ng g^{-1} and 10 μg g^{-1} body weight, respectively), and were challenged with common fish bacterial pathogens, *Aeromonas hydrophila* or *Edwardsiella ictaluri*. Pre-exposure to nano-TiO_2 significantly increased fish mortality during bacterial challenge. Nano-TiO_2 concentrated in the kidney and spleen. Phagocytosis assay demonstrated that nano-TiO_2 has the ability to diminish neutrophil phagocytosis of *A. hydrophila*. Fish injected with TiO_2 nanoparticles displayed significant histopathology when compared to control fish. The interplay between nanoparticle exposure, immune system, histopathology, and infectious disease pathogenesis in any animal model has not been described before. By modulating fish immune responses and interfering with resistance to bacterial pathogens, manufactured nano-TiO_2 has the potential to affect fish survival in a disease outbreak.

1. Introduction

From 1916 to 2011, an estimated 165,050,000 metric tonnes of titanium dioxide (TiO_2) pigment (nano and bulk combined) were produced worldwide (Jovanović, 2015). Nano-TiO_2 is used as a constituent in personal, household, and food products. As an ingredient in food products nano-TiO_2 has an estimated human consumption of 1 mg kg^{-1} body weight per day (Weir et al., 2012). Nano-TiO_2 is also considered as an additive of drinking water in water treatment plants in a protocol for the removal of arsenic from water (EPA., 2010). The most frequent predicted concentration of nano-TiO_2 in surface water is 21 ng L^{-1} (Gottschalk et al., 2009), while the highest potential concentration is 16 μg L^{-1} (Mueller and Nowack, 2008). The nano-TiO_2 concentration of waste water effluent is documented in the μg L^{-1} range (Gottschalk et al., 2009; Kiser et al., 2009; Westerhoff et al., 2011). However, in urban runoff this concentration can be as high as 0.6 mg L^{-1} (Kaegi et al., 2008), and in raw sewage up to 3 mg L^{-1} of nano-TiO_2 has been detected (Kiser et al., 2009; Westerhoff et al., 2011). Nano-TiO_2 can be absorbed by the gills, skin, and intestine of fish, although the highest potential uptake is through diet (Handy et al., 2008). The experiments with perfused intestines of fish demonstrated TiO_2 uptake across the intestine both for the nano-TiO_2 and its bulk counterpart with average particle aggregates diameter of up to 1124 ± 331 nm (Al-Jubory and Handy, 2013). Although nano-TiO_2 is classified as a non-bioaccumulative substance in the fish embryos with the bioconcentration factor (BCF) < 100 (López-Serrano Oliver et al., in press), it is still present in the juvenile and adult fish body upon exposure (Ates et al., 2013; Fouqueray et al., 2013) with BCF of 181 (Zhu et al., 2010). Another study with adult fish determined BCF in the range 600–700, indicating possible increase of risk (Zhang et al., 2006). Since nano-TiO_2 can be transferred via the trophic food chain to fish (Fouqueray et al., 2013; Zhu et al., 2010), and although observed biomagnification factor is < 1, this suggests that

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the fish can internalize nano-TiO_2 on a daily basis through diet leading to chronic exposure (Zhu et al., 2010).

Nano-TiO_2 has a strong bactericidal effect and can kill fish pathogens *in vitro* (Cheng et al., 2008, 2009). Therefore, addition of nano-TiO_2 to the water of fish farms has been recommended in order to prevent or mitigate bacterial disease outbreaks (Cheng et al., 2008). However, methods that are successfully used for bacterial killing *in vitro* are frequently not efficient when applied to *in vivo* bacterial killing, due to the differences in the intracellular environment and the specific antibacterial function of phagocytic cells (Segal, 2005). It was recently demonstrated that nano-TiO_2 acts as a strong immunomodulator of fish neutrophil function (Jovanović et al., 2011). Cell-mediated immunity and the phagocytic cells are the primary targets of nano-TiO_2 immunotoxicity in aquatic animals. Immunotoxicity is manifested through lysosomal destabilization, frustrated phagocytosis, and change in function of the phagocytic cells (Jovanović and Palić, 2012).

Aeromonas hydrophila is a Gram-negative motile rod and one of the most important bacterial pathogens of aquatic animals in temperate waters (Angka, 1990; Esteve et al., 1993). *A. hydrophila* infection causes a systemic disease resulting in dermal ulceration, tail or fin rot, ocular ulceration, and erythrodermatitis, which leads to the descriptive disease appellations of "hemorrhagic septicemia", "red sore disease", "red rot disease", and "scale protrusion disease", among others (Cipriano, 2001). In the acute form of disease, rapid septicemia is the most common cause of mortality (Cipriano, 2001). Pathogenic mechanisms include the production of a cytotoxic enterotoxin, a type 3 secretion system, hemolysins, and an exotoxin (Grim et al., 2013), along with cytotoxic and haemolytic activities of the bacterial extracellular polysaccharides (Rodríguez et al., 2008), which collectively have lethal effects on renal tubular epithelium, precipitating acute renal failure. It is important to note that *A. hydrophila* is a member of the normal intestinal flora of healthy fish (Trust et al., 1979). The presence of the bacteria itself in fish does not indicate the disease *per se* and stress is often considered to be a contributing factor in disease outbreaks caused by *A. hydrophila* (Cipriano, 2001).

Edwardsiella ictaluri is a Gram-negative rod from Enterobacteriaceae family. It is the causative agent of Enteric Septicemia Disease that affects a variety of fish species (Baxa et al., 1990). Clinical signs, apart from signs of generalized systemic bacterial infection, include the presence of an open ulcer on the frontal bone of the skull between the eyes, and intradermal petechial hemorrhage of the jaws (Miyazaki and Plumb, 1985). The infection is initiated by transport of bacteria from the environment through the olfactory sac to the brain, with subsequent systemic dissemination of bacteria, causing generalized septicemic infection (Miyazaki and Plumb, 1985). During the infection, *E. ictaluri* may overcome phagocytic activities of neutrophils and other granulocytic cells, and multiplies intracellularly in foci of inflammation (Miyazaki and Kaige, 1985). Therefore, previously observed suppression of fish neutrophil function caused by nano-TiO_2 (Jovanović et al., 2011) has the potential to favor non-bactericidal phagocytosis of *E. ictaluri*.

Nano-TiO_2 is immunotoxic to fish and changes the function of fish neutrophils *in vivo*. After exposure of fathead minnows to 10 μg/g body weight of nano-TiO_2 for 48 h, respiratory burst, degranulation of primary granules, and neutrophil extracellular trap (NET) release were significantly reduced (Jovanović et al., 2011). The potential of nano-TiO_2 to interfere with resistance to infectious disease as a consequence of the ability to modulate immune responses has not been studied, and there are no available reports addressing possible outcomes of nanoparticle pre-exposure followed by bacterial challenge. The aim of this study was to determine if the outcome of bacterial challenge would be more severe in fish that are exposed to environmentally relevant concentrations of nano-TiO_2, as compared with bacterial-challenged fish without prior exposure to nano-TiO_2. Our hypothesis was that fish exposed to nano-TiO_2 would have higher morbidity and mortality than non-exposed fish after challenge with *A. hydrophila* and *E. ictaluri*.

2. Materials and methods

2.1. Animal care

Fathead minnows (*Pimephales promelas*) with average weight 2.5 ± 0.5 g were maintained in the Iowa State University, College of Veterinary Medicine, Ames, Iowa, USA. Fish were housed in a water recirculation system supplied with dechlorinated tap water at 20 °C in 120 L tanks, and fed twice daily with live brine shrimp larvae and dried flake food. Fish were cared for in accordance with approved Iowa State University animal care guidelines.

2.2. Bacterial culture

A. hydrophila (fish pathogen group, outbreak strain, USDA), and *E. ictaluri* (fluorescent transformed strain 93–146 pAKgfp1 (Karsi and Lawrence, 2007)) were plated on trypticase soy agar (TSA) with 5% of sheep blood plates and incubated at 37 °C overnight (*A. hydrophila*) or at 27 °C for two days (*E. ictaluri*). Morphologically distinct colonies were selected and placed in trypticase soy broth in a sterile tube. Cultures of *A. hydrophila* or *E. ictaluri* were incubated at 37 °C or 27 °C, respectively, to achieve logarithmic growth. The optical density of the broth culture was measured spectrophotometrically at 450 nm. Using a previously determined growth curve, colony forming unit (CFU) was determined based on optical density. After diluting the cultures with Hank's Balanced Salt Solution without Ca, Mg and Phenol Red (HBSS) to obtain the desired CFU, they were used immediately for intraperitoneal (i.p.) injections. To confirm the actual CFU used for bacterial challenge, the diluted cultures were plated on TSA sheep blood plates and enumerated.

2.3. Nanoparticle characterization

Nano-TiO_2 (anatase, nanopowder, < 25 nm, 99.7% purity; Sigma–Aldrich Corp, St. Louis, MO, USA) was used in all experiments. Nano-TiO_2 was suspended in sterile HBSS, pH = 7.3. The suspensions of nanoparticles were used as non-filtered or were filtered through a 220 nm general purpose filter. The non-filtered nano-TiO_2 suspension contained particles with average aggregate diameter of 585 nm, average zeta potential of −16.4 mV, and conductivity of 16 mS cm^{-1}. Polydispersity index (PDI) was 0.21. After filtration, the aggregate size had an average diameter of 86 nm, zeta potential of −8.87 mV, and conductivity of 15.4 mS cm^{-1} as determined by dynamic light scattering (DLS) technique with Malvern Zetasizer Nano ZS-90 instrument (Malvern Instruments Ltd, Malvern, Worcestershire, UK). Since the fish were later injected with the 24 h aged suspension (concentration had to be analytically verified first) DLS measurements were also performed on a 24 h aged suspension. Prior to measurements suspension was sonicated for 10 min in a benchtop portable sonicator. The detailed characterization of the nano-TiO_2 is provided in the Supplementary Information.

2.4. Nano-TiO_2 accumulation in fish tissues

To determine the accumulation of nano-TiO_2 in fish organs, fish were injected i.p. with 10 μg g^{-1} body weight with non-filtered nano-TiO_2 suspension in HBSS. Negative control was injected