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Vascular toxicity of silver nanoparticles to developing zebrafish (*Danio rerio*)

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Abstract

Nanoparticles (NPs, 1–100 nm) can enter the environment and result in exposure to humans and other organisms leading to potential adverse health effects. The aim of the present study is to evaluate the effects of early life exposure to polyvinylpyrrolidone-coated silver nanoparticles (PVP-AgNPs, 50 nm), particularly with respect to vascular toxicity on zebrafish embryos and larvae (*Danio rerio*). Previously published data has suggested that PVP-AgNP exposure can inhibit the expression of genes within the vascular endothelial growth factor (VEGF) signaling pathway, leading to delayed and abnormal vascular development. Here we show that early acute exposure (0–12 hours post-fertilization, hpf) of embryos to PVP-AgNPs at 1 mg/L or higher results in a transient, dose-dependent induction in VEGF-related gene expression that returns to baseline levels at hatching (72 hpf). Hatching results in normoxia, negating the effects of AgNPs on vascular development. Interestingly, increased gene transcription was not followed by the production of associated proteins within the VEGF pathway, which we attribute to NP-induced stress in the endoplasmic reticulum (ER). The impaired translation may be responsible for the observed delays in vascular development at later stages, and for smaller larvae size at hatching. Silver ion (Ag⁺) concentrations were < 0.001 mg/L at all times, with no significant effects on the VEGF pathway. We propose that PVP-AgNPs temporarily delay embryonic vascular development by interfering with oxygen diffusion into the egg, leading to hypoxic conditions and ER stress.

1. Introduction

The vascular system is one of the first organs to develop in embryonic vertebrates. Vascular development occurs in two phases: vasculogenesis, *de novo* formation of vessels, and angiogenesis, which involves the sprouting of new vessels from pre-existing ones (Shirininifard et al., 2013). The vascular endothelial growth factor (VEGF) signaling pathway regulates vasculogenesis and angiogenesis via endothelial cell proliferation, promoting cell

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7. Declaration of interest statement

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migration and inhibition of apoptosis (Herbert and Stainier, 2011). In zebrafish (*Danio rerio*), VEGFa is the main driver of vascular development; *vegfa* is transferred maternally and expressed at a very early stage (1–4 cell stage), declining to very low levels at 8.5 hours post fertilization (hpf) or 80% epiboly (Liang et al., 2001, Covassin et al., 2009). Expression of embryonic *vegfa* begins at 12 hpf reaching peak levels at 24–30 hpf (Liang et al., 2001). Loss of endothelium-specific receptor tyrosine kinase, VEGFR-2, and VEGF function inhibit vascular development (Gore et al., 2012). In contrast, overexpression of *vegfa* results in ectopic vasculature development as well as pericardial edema in later stages of zebrafish embryos (Liang et al., 2001). Similar results have been reported in mice with overexpression of VEGF resulting in severe abnormalities in heart development and embryonic lethality (Miquerol et al., 2000).

Hypoxia is a major regulator of blood vessel formation, and stimulates angiogenesis in order to increase oxygen delivery to hypoxic regions. Hypoxia increases expression of several genes within the VEGF signaling pathway (Liu et al., 1995, Stone et al., 1995, Neufeld et al., 1999). A very sensitive molecular marker of hypoxia is the hypoxia-inducible factor 1 (HIF1), which triggers the VEGF signaling pathway in vascular endothelial cells (Liu et al., 1995).

Zebrafish is a popular vertebrate model for investigating vascular toxicity, as its gene pattern of vasculogenesis and angiogenesis is similar to humans. Embryonic zebrafish develop *ex utero* and are also transparent, allowing for the visualization of vascular morphological changes in real time (Serbedzija et al., 1999, Stainier et al., 1993, Quaife et al., 2012, Delov et al., 2014, Liu and Stainier, 2012). For instance, vascular development in zebrafish larvae can be tracked in real time by visualizing the whole circulatory system using transgenic lines with fluorescent markers (Raldúa and Piña, 2014). In addition, zebrafish embryos are not completely dependent on a functional cardiovascular system during early embryo development, allowing for the study of severe cardiovascular defects (Bakkers, 2011).

Silver nanoparticles (AgNPs) are commonly used as bactericides in many commercial products. Nearly 400 commercial products are known to contain AgNPs (NanotechProject, 2014), culminating in the global production of approximately 320 tons of AgNPs per year (Nowack et al., 2011). A handful of *in vitro* studies have reported that AgNPs are vasculotoxic. In endothelial cells, AgNPs (40–50 nm: 5 mg/L) inhibit vascular development via the Src kinase and PI3K/Akt pathways, both downstream of the VEGF signaling pathway (Sheikpranbabu et al., 2009, Gurunathan et al., 2009, Kalishwaralal et al., 2009). Other inorganic nanoparticles (copper oxide: 100 mg/L; 15- and 40-nm gold particles: 25 mg/L) can also inhibit vasculogenesis via the VEGF signaling pathway (Chang, 2015, Pan et al., 2014). In human umbilical vein endothelial cells, gold NPs inhibit cell migration and tube formation via Akt phosphorylation downstream of VEGFR2/PI3K affecting cell-surface ultrastructure and cytoskeleton formation (Pan et al., 2014). Finally, both micron-sized particles (PM_{2.5}) and ultrafine particles (carbon black, <100 nm) alter the development of endothelial cells. In hepatic vessels, they cause platelet accumulation, which has been associated with prothrombotic changes and elevated risk of myocardial infarction (Khandoga et al., 2004, Peters et al., 2001).

Evidence for the vascular toxicity of nanoparticles on whole organisms is mostly anecdotal, with the majority of published studies not designed to specifically test for these effects. Zebrafish embryos exposed to AgNPs (5–35 nm: >50 mg/L) responded with decreased blood flow, pericardial edema and cardiac arrhythmia (Asharani et al., 2008). These are, however, non-specific responses that can be induced by a myriad of causes. In a follow-up study, the same AgNPs were shown to cause abnormal cardiac development and circulatory defects (Asharani et al., 2011). Further, increased mortality and deformity rates were partly attributed to depressed heart rates resulting in diminished blood flow to the brain and the spinal cord (Massarsky et al., 2013, Asharani et al., 2008).

The objective of the present study is to evaluate the vascular toxicity of polyvinylpyrrolidone-coated silver nanoparticles (PVP-AgNPs) on embryonic zebrafish, following exposure during vascular (2–96 hpf) and heart (48–96 hpf) development. Based on previously published data, we reasoned that exposure of embryonic zebrafish to PVP-AgNPs would inhibit the VEGF signaling pathway, leading to delayed and abnormal vascular development. This is the first study to systematically investigate the mechanisms of vascular toxicity by PVP-AgNPs *in vivo*.

2. Materials and Methods

2.1 Characterization of PVP-AgNPs

PVP-AgNPs (50 nm) were purchased from Nanocomposix Inc. (San Diego, CA, USA). The original concentration of PVP-AgNPs was 4,730 mg/L and was stored at 4°C under dark conditions. PVP-AgNP solutions were diluted 10,000 times in embryo media (Replenish®, Seachem Laboratories Inc., Madison, GA, USA: 13–14% calcium, 1% magnesium, 0.12% potassium, and 0.6–0.7% sodium) and particle-free water separately before analysis. Particle size distributions were obtained using nanoparticle tracking Analysis (NTA) (Nanosight LM-10, Malvern Instruments, Worcestershire, UK). Zeta potentials of particles in fish embryo media (12 µL Replenish in 100 mL test solution) were measured using a Malvern Zetasizer Nano ZS (Malvern, UK; $\lambda=633$ nm) at 25 °C (Table 1) (EPA, 2002). Samples were allowed to equilibrate for 30 s inside the instrument prior to analysis; measurements were based on the mean of 12–16 runs performed at rates of 30–70 kcps.

2.2. Toxicity tests

Transgenic adult zebrafish (TG(*flil1a:EGFP*)) expressing enhanced green fluorescent protein (EGFP) under control of the *flil1* promotor were obtained from ZFIN (Eugene, OR, USA). Transgenic and AB wild-type (WT) zebrafish were maintained separately at the Aquatic Ecology Laboratory (Purdue University) at a temperature of $28 \pm 1^\circ\text{C}$ and a photoperiod of 14L:10D. Fish were fed twice daily with a combination of hatched *Artemia* nauplii in the morning and commercial food (Tetramin) in the afternoon. Genders were housed separately until the day before breeding, then placed in tanks at a 2:1 male:female ratio. Fish were left undisturbed overnight and fertilized embryos were collected 1 h after the light was turned on the next morning. Embryos within 1 hpf were randomly placed in plastic petri dishes containing 25 mL exposure solutions. Half of the exposure solution was renewed daily. Each test consisted of three replicates and each experiment was repeated at least three times from

embryos collected from different mating events. Ultrapure water (Millipore; $R > 18 \text{ M}\Omega\cdot\text{cm}$) was used for diluting PVP-AgNPs into a series of concentrations (0.1, 1.0, and 10 mg/L) as exposure solutions. Every 100 mL of exposure solution contained 12 μL embryo medium. Mortality was recorded at 24, 48, 72 and 96 h.

2.3 Silver ion release from PVP-AgNPs

Ag^+ release from PVP-AgNPs was quantified from exposure solutions at 0, 24, 48, 72 and 96 h using an ion-selective electrode (ISE, Model 9616BNWP, Thermo Fisher Scientific, USA). The system was calibrated with a series of silver nitrate (AgNO_3) concentrations (1–20,000 $\mu\text{g/L}$). Concentrations were quantified immediately prior to exposure tests.

2.4 Effects on the cardiovascular system

2.4.1. Vascular development and size of larvae at hatching—Development of intersegmental vessels (ISV) in zebrafish follows a well-defined pattern with pairs of ISVs sprouting from the dorsal aorta (DA) at regular spatial and temporal intervals following an anterior to posterior sequence (Childs et al., 2002, Isogai et al., 2003). The common cardinal vein (CCV) grows across the yolk of zebrafish embryos and is extensively remodeled and regresses as the heart migrates dorsally within the pericardium.

ISV length, interval between ISVs, angles between ISVs and the DA, and regression of CCV were recorded from TG(*fli1a:EGFP*) embryos using an Olympus BX-51 optical microscope fitted with a DP71 camera (Olympus America, Jupiter, FL, USA). Measurements of eggs were collected after enzymatic dechoriation using pronase (2 mg/mL in embryo media for 1 min) and after anesthesia with tricane methanesulfonate (MS222, 0.2 g/kg). Measurements were taken from digital images from at least 15 embryos per dose and developmental stages (24, 48, 72 and 96 hpf) from the first 5 ISVs located anterior to the yolk sac extension (see insert in Fig. 1, red rectangle indicates the position of ISVs). Additional endpoints, which could be quantified in transparent WT embryos, included measurement of standard larvae length using a Nikon SMZ 1500 (Nikon USA, Melville, NY, USA) and NIS-Elements D 3.2 software, and quantification of heartbeat rates using an Olympus CX-41 with a DP70 camera.

2.4.2. Oxygen consumption—Oxygen intake by WT zebrafish larvae was quantified using a multi-frequency phase fluorometer (MFPF) (Tau Theta LLC, USA). Oxygen sensors were prepared by mixing hydrophobic platinum(II)-5,10,15,20-tetrakis-(2,3,4,5,6-pentafluorophenyl)-porphyrin (PtTFPP) (Frontier Scientific, Logan, UT) and TiO_2 microparticles (Sigma-Aldrich, St. Louis, MO, USA) in a polystyrene matrix. These were deposited onto the bottoms of 1.5-mL clear glass vials using 200- μL micropipette tips, which were cured in the dark overnight (Stensberg et al., 2014). 48-hpf larvae were added into each vial with test solution, which were immediately sealed with Teflon-lined caps. Changes in oxygen levels were recorded every 6 h until 96 hpf using Tau theta software (Tau Theta LLC). Negative controls with PVP-AgNPs dispersed in “fish tank” water without larvae were tested in parallel. Each treatment had a minimum 8 biological replicates.

2.4.3 Gene expression—Gene expression within the VEGF pathway (*vegfr*, *vegfr*, *pi3k*, *pkc*, *plc*, *bcl2*) was quantified at several time points (2, 4, 6, 12, 24 and 72 hpf) using quantitative RT-PCR (qRT-PCR). Expression of *hif1* in the 10 mg/L PVP-AgNP group and controls was quantified at 12, 24, 48, and 72 hpf. For more information on gene functions and primers used, see Table S1 (Supporting Information).

A minimum of three biological replicates consisting of groups of six-pooled zebrafish embryos/larvae was used per condition and were ran in triplicate. Total RNA was extracted using TriSure (Bioline) following re-suspension of RNA in water treated with diethyl pyrocarbonate. Total RNA was quantified using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). DNase I (Thermo Scientific, Lithuania) was added at 37 °C for 30 min to remove genomic DNA contamination, and cDNA was synthesized using high-capacity reverse-transcription kits (Applied Biosystems, USA) following the manufacturer's instruction. Elongation factor 1 alpha 1 (*ef1a*) was used as the housekeeping gene, as it is stably expressed throughout zebrafish development and is unaffected by AgNP exposure (data not shown). Primers were purchased from Integrated DNA Technologies (Coralville, IA, USA) and gene expression quantified using a Bio-Rad CFX96 system (Bio-Rad Laboratories, Philadelphia, PA, USA). Each reaction (20 µL total volume) contained 10 µL of 2X iQTM SYBR Green Supermix (Bio-Rad Laboratories 170-8880), 10 µM gene specific primers (forward and reverse), 60 ng of cDNA template, and nuclease-free water. All quantitative PCR reactions were performed in duplicate. Gene expression levels were determined by the comparative C_q method (Livak and Schmittgen, 2001).

2.4.4 Protein expression—Western blotting was used to characterize the effect of PVP-AgNPs on VEGF protein expression at 12 and 24 hpf. Twenty embryos were placed in a tube and homogenized using a lysis buffer (50 mM Tris, pH 7.8, 150 mM NaCl, 1% Nonidet P-40). Total protein concentration was determined using bicinchoninic acid (BCA Assay Kit, Thermo Scientific, Rockford, IL, USA). Samples were resolved using a 12% SDS–polyacrylamide gel, then transferred to polyvinylidene difluoride (PVDF) membranes for Western blot analysis using an Odyssey infrared imager (Li-Cor, Lincoln, NE, USA). Primary antibodies used include polyclonal anti-VEGF (Anaspec, Fremont, CA, USA) and polyclonal anti β-actin (GenScript, Piscataway, NJ, USA), which showed stability at different developmental stages of zebrafish, including PVP-AgNP treated groups. Secondary antibodies were labeled with IR-DYE 800 and IR-DYE 700 (Li-Cor). Intensity of the VEGF immunoreactivity was normalized to the level of β-actin in the sample.

2.5 Data analysis

All statistical analyses were conducted using SPSS 22.0. Unless otherwise noted, results are expressed as means ± SE (standard error) of three or more biological replicates. Data was first assessed for normality. One-way analysis of variance (ANOVA) followed by post-hoc Tukey's multiple comparison tests and LSD tests (for Western blot protein expression test) were used to compare means across treatments.

3. Results

3.1 Characterization and stability of PVP-AgNPs

The hydrodynamic diameter of PVP-AgNPs in freshly prepared fish embryo medium was determined by NTA to be 51.1 ± 0.04 nm. This is comparable to the size of PVP-AgNPs dispersed in pure water (53.3 ± 0.03 nm; Fig. S1). All zeta potential values were negative, ranging from -7.52 to -13.10 mV (Table 1). For Ag^+ concentrations, an ISE standard curve was developed for the range between 1 and 20,000 $\mu\text{g/L}$ and applied toward the analysis of AgNP solutions in fish media, which were monitored for up to 96 h. In all cases, Ag^+ concentrations were below detection levels (1 $\mu\text{g/L}$; data not shown).

3.2 Vascular developmental morphology

Early ISV sprouting and sprout extension development occurs between 20–25 hpf. Disruption of somite shape or on the VEGF signaling pathway can perturb the orientation of ISV sprouting relative to the DA. As expected, effects on vascular development were highly dependent on the embryonic stage. In controls, the length and interval of ISV sprouting more than doubled between 24 and 72 hpf, and remained high at 96 hpf (Figs. 1A, 1C). In contrast, the angle between ISV and DA decreased by roughly 20% during development between 24 to 96 hpf (Fig. 2B). Compared with the 24-hpf control group, embryos exposed to 1.0 and 10 mg/L PVP-AgNPs experienced a delay in vasculogenesis, made evident by a decrease in ISVs length and a complete lack of ISV development (Figs. 1B–D, 2A). This delay was also evident in the 0.1 mg/L group at 48 hpf. No differences in ISV length were observed at 72 hpf between treated and control specimens, however the highest dose (10 mg/L) caused a decrease in interval distance between ISVs (Fig. 2C). The reduction in sprout-to-DA angle occurred at a delayed rate in PVP-AgNP-treated embryos, as no changes were observed between 48 and 96 hpf (Fig. 2B). CCV is the only vessel passing over the yolk for the first 60 h of zebrafish development, providing all venous blood and return to the heart during the first week (Bello et al., 2004). We observed a clear delay in regression of the CCV in the PVP-AgNP treated group. In control specimens and at the lowest PVP-AgNP dose, the CCV was remodeled into a tube by 72 hpf (Fig. 3), however, regression had not yet occurred in larvae treated with 1.0 or 10 mg/L PVP-AgNP. But, by 96 hpf, there was no longer any difference in the degree of CCV regression between groups (data not shown).

3.3 Functional tests

PVP-AgNP effects on heart rate (48 and 72 hpf), oxygen consumption (48 to 96 hpf), and standard body length (96 hpf) were quantified in order to examine whole-organism responses. PVP-AgNPs induced dose-dependent bradycardia in larvae at 48 and 72 hpf exposure, starting at 1 mg/L PVP-AgNP (Fig. 4). Further, a significant decrease in oxygen consumption was observed in all concentrations starting at 84 hpf (Fig. 5A). From 91 to 96 hpf, groups treated with 1.0 and 10 mg/L PVP-AgNP responded with a significant decrease in oxygen consumption compared with controls (Fig. 5B). Oxygen was also measured over time in control vials filled with fish embryo media without larvae, with no apparent changes (data not shown). This confirmed that oxygen consumption was driven by larvae rather than microbial sources (e.g., bacteria). Finally, a significant reduction in total standard length was

observed for larvae exposed to PVP-AgNPs at 1.0 and 10 mg/L, and high mortality was caused at 10 mg/L group at 96 hpf (Figs. 6 and S2).

3.4 Molecular mechanisms: gene and protein expression

The VEGF signaling pathway plays a critical role on cardiovascular development and is up-regulated under hypoxia. HIF1 is a transcription factor highly inducible by cellular hypoxic conditions. To investigate the molecular mechanisms of PVP-AgNPs' effect on vascular development in embryos, relative expression of genes within the VEGF pathway (*vegfr*, *vegfr*, *pi3k*, *pkc*, *plc* and *bcl2*) was quantified at six time points (2, 4, 6, 12, 24 and 72 hpf) (Figs. 7 and S3A). Except for *bcl2*, the expression of all other genes changed in a dose- and time-dependent manner. The highest concentration of PVP-AgNPs (10 mg/L) significantly induced *vegfr*, *vegfr*, *plc*, *pkc*, and *pi3k* from early life stage embryos (4 and 6 hpf). A peak in expression of *pi3k* was observed at 4 hpf, whereas expression of the other genes peaked at 12 hpf and decreased thereafter. By 24 hpf, gene expression in specimens treated with PVP-AgNP was much reduced although still higher than the controls, and was completely back to background levels around the time of hatching (72 hpf). Expression of the same six genes was also measured in embryos exposed to silver ion (AgNO_3 , 0.01, 0.1 and 1.0 mg/L) at 2, 4, 6, 12 and 24 hpf (Fig. S3B); with the exception of *bcl2*, gene expression did not change significantly during this period. Expression of *hif1* was also quantified at 4 time points (12, 24, 48 and 72 hpf) in controls and groups treated with 10 mg/L PVP-AgNP (Fig. 8). PVP-AgNPs treated group, *Hif1* peaked in expression at 12 hpf and decreased gradually thereafter until reaching baseline levels at 72 hpf. To determine whether changes in gene expression could be induced by other nanoparticles, we performed a control study with 50-nm PVP-AuNPs at 10 mg/L. However, its impact on mRNA expression of VEGF-related genes was modest at best, relative to that observed for PVP-AgNPs (Fig. S3C). Finally, to determine if the above changes in mRNA were translated to the protein level, changes in the expression of VEGF after PVP-AgNP exposure was performed in 12 and 24 hpf zebrafish embryos (Fig. 9). However, VEGF expression did not differ across treatment groups.

4. Discussion

We evaluated the vascular toxicity of 50-nm PVP-AgNPs on zebrafish embryos and larvae for up to 96 hpf. NTA and zeta potential values indicated the AgNPs were stable in solutions over this period. Very importantly, our results indicate that the vasculotoxic effects are induced by the AgNPs themselves, as the Ag^+ concentrations were consistently $< 1 \mu\text{g/L}$. We show that an acute (0–12 hpf) exposure of embryos to PVP-AgNPs results in a reversible dose-dependent induction in VEGF-related gene expression, which reverts to baseline levels just around hatching. Increased gene transcription, however, was not followed by the translation and production of VEGF. Correlating with the lack of VEGF expression, vascular development of PVP-AgNPs treated embryos was delayed rather than enhanced. The threshold for delayed vascular development and growth by PVP-AgNPs was determined to be 1 mg/L.

We propose that zebrafish eggs exposed to PVP-AgNPs at 10 mg/L experience a physical interference that limits the diffusion of oxygen, leading to embryonic hypoxia and

subsequent alterations in gene transcription (Fig. 10A). In this scenario, the overexpression of genes within the VEGF pathway in the early life stages of embryos may be induced from the lack of oxygen. This argument is supported by the high levels of expression of *hif1* at 12 hpf, with reversion to baseline levels by 24–72 hpf (Fig. 8). Exposure to PVP-AgNPs, however, did not result in increased VEGF production, possibly because of NP aggregation on the endoplasmic reticulum (see below) (Fig. 10B). Hatching results in normoxia, effectively removing the hypoxia-driven effects of NPs on gene expression.

Effects on cardiovascular development after exposure to PVP-AgNPs were consistent with previous studies with zebrafish embryos reporting bradycardia and decreased peripheral circulation (Asharani et al., 2008, Massarsky et al., 2013). Abnormal or delayed development of ISVs and dorsal migration of the CCV caused by PVP-AgNPs could explain smaller growth rates of larvae at hatching.

NPs are known to aggregate on the chorion surface of fish eggs (Laban et al., 2010), causing the depletion of oxygen exchange and hypoxia (Zhu et al., 2012). In Japanese medaka (*Oryzias latipes*), 35-nm AgNPs (62.5–1,000 µg/L) have been reported to disrupt the network of fine filaments from eggs, resulting in increased anaerobic respiration by embryos (Wu and Zhou, 2012). Individual AgNPs (up to 46 nm) are known to be transported into embryos through chorion pore canals (CPCs) (Lee et al., 2007); AgNPs can also aggregate on the chorion exterior of zebrafish embryos within the first 24 hours (Osborne et al., 2013). These earlier observations support our hypothesis that the early induction (12 hpf) of mRNA expression of *hif1* and other genes within the VEGF signaling pathway is caused by the physical blockage of oxygen diffusion by AgNPs.

The absence of VEGF production, despite a three to five-fold upregulation of *vegfa*, is in accord with recent findings indicating the inhibition of protein translation by NP aggregation on the endoplasmic reticulum (ER). Internalization of NPs into the ER has been reported for citrate-AgNPs and PVP-AgNPs (75–100 nm), as well as zinc oxide NPs (42 nm) and hydroxyapatite NPs (60 nm) (Zucker et al., 2013, Chen et al., 2014, Han et al., 2014). The accumulation of NPs can induce ER stress, a self-protection mechanism against ER damage or dysfunction (Garg et al., 2012, Lin et al., 2008). ER stress has been studied in human Chang liver cells and Chinese hamster lung fibroblasts exposed to AgNPs <100 nm, and is thought to be caused by the phosphorylation of RNA-dependent protein kinase-like ER kinase (PERK) and its downstream eukaryotic initiation factor 2α, phosphorylation of inositol-requiring protein 1 (IRE1) (Zhang et al., 2012). In zebrafish embryos and hepatocytes, 120-nm AgNPs at loadings as low as 0.1 mg/L induced ER stress as measured by increased transcription of the biomarkers *bip* and *synv* (Christen et al., 2013). The induction of ER stress in zebrafish embryos by PVP-AgNPs may explain why the induced expression of VEGF-related genes is not accompanied by protein translation.

5. Conclusions

PVP-AgNPs causes dose-dependent hypoxia in zebrafish embryos starting at loadings of 1 mg/L, resulting in delayed vascular development and decreased larvae growth. PVP-AgNPs also caused bradycardia and impaired the tolerance of larvae to hypoxia. The adverse effect

on vascular development appears to be specific to AgNPs, as a parallel study with PVP-AuNPs did not produce a significant response. This is the first *in vivo* study to focus specifically on the vascular toxicity of PVP-AgNPs; the consequences of lower larvae size at hatching on long-term viability remain unknown, and may require additional studies for a more comprehensive correlation with NP-induced changes in vascular development. We note that 1 mg/L of AgNP is a relatively high concentration that is unlikely to be environmentally relevant (Gottschalk et al., 2009, Gottschalk et al., 2013). Lastly, the apparent disconnect between AgNP-induced mRNA expression and protein translation may be attributable to the simultaneous induction of ER stress by the same NPs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

A. Representative trunk region of transgenic (TG) (*fli1a*) zebrafish embryo from a 24-hour post fertilization (hpf) control. Intersegmental vessels (ISVs) sprout from the dorsal aorta (DA) at regular spatial and temporal intervals from anterior to posterior (right to left). Boxed area contains five ISVs used to measure these parameters; arrow “a” points to length of ISV (dashed line); arrow “b” points to angle between ISV and DA; arrow “c” points to interval spacing between ISVs. **B.** Embryos treated with 0.1 mg/L PVP-AgNPs exhibit disordered ISVs; **C, D.** Embryos treated with 1.0 and 10 mg/L PVP-AgNPs exhibit atrophied ISVs, relative to **A**.

Figure 2.

Changes in intersegmental vessel (ISV) morphology across development in control and PVP-AgNP treated embryos. **A.** ISV length increased with development. A reduction of ISV sprout growth was observed at 24 hpf in embryos exposed to 1 and 10 mg/L ($n = 20$), and at 48 hpf in all treated embryos ($n = 10$). **B.** Angle between ISVs and DA decreased with development. Angle was significantly larger at 72 hpf ($n = 10$) and 96 hpf ($n = 5$) in embryos exposed to 10 mg/L PVP-AgNP, relative to control. **C.** Interval distance between ISVs increased with development stage. At 72 hpf and 96 hpf, embryos treated with 10 mg/L PVP-AgNP had a smaller interval distance relative to control. * $p < 0.05$; # $p < 0.1$. Lines over tops of bars denote significant differences between different stages, $p < 0.05$.

Figure 3. Effects of PVP-AgNPs on common cardinal vein (CCV) regression; boxed area indicates region of interest. The CCV (white arrow) in the control specimen is a tube of vascular endothelial cells along the anterior margin of the yolk at 72 hpf; however, no regression is observed in embryos treated with 10 mg/L PVP-AgNP. Bar = 200 µm.

Figure 4. Heartbeats (mean $\pm$ SE) of zebrafish embryos at 48 and 72 hpf. Significant reduction in heartbeat rate was observed at 48 hpf for embryos treated with 10 mg/L PVP-AgNP; a decrease in heartbeat rate was also observed at 72 hpf for embryos treated with 1 mg/L PVP-AgNP (* $p < 0.05$). C = control.

Figure 5. Body length (mean $\pm$ SE) of zebrafish larvae after exposure to PVP-AgNPs for 96 h; larvae exposed to 1 and 10 mg/L AgNPs were smaller (* $p < 0.05$). C = control.

Figure 6.

Changes in oxygen level (mean $\pm$ SE) after exposing embryos to PVP-AgNPs, up to 96 hpf.

A. Continuous decrease in oxygen level was observed for zebrafish embryos at all AgNP doses, but with significantly less consumption versus control at 84 hpf (a–c: 0.1, 1.0 and 10 mg/L groups, $p < 0.05$); at 96 hpf, only the two highest AgNP doses (1.0 and 10 mg/L) differed from control (b,c: $p < 0.05$). **B.** Expansion of boxed area.

Figure 7. Relative increases in mRNA expression (mean $\pm$ SE) for *vegf*, *vegfr*, *plc*, *pkc*, *pi3k*, and *bcl2*, at different time points of exposure to PVP-AgNPs (* $p < 0.05$). C = control.

Figure 8.

Relative increase of mRNA expression for hypoxia-induced factor (hif), induced by 10 mg/L PVP-AgNPs at 12, 24, 48, and 72 hpf (* $p < 0.05$).

Figure 9. VEGF protein expression by whole zebrafish embryos, as a function of PVP-AgNP exposure (in mg/L). **A.** Representative Western blots of VEGF and β -actin produced by embryos at 12 and 24 hpf, showing negligible changes in VEGF production. **B.** Changes in VEGF protein expression (mean $\pm$ SE) at 12 and 24 hpf; again, no significant differences were observed between groups ($p > 0.05$). C = control.

Figure 10.

Mechanistic model accounting for the observed effects of PVP-AgNPs on the cardiovascular development in zebrafish embryos. **A.** PVP-AgNPs coat the surface of eggs, inducing hypoxia and subsequent expression of genes in the VEGF signaling pathway, with peak expression at 12 hpf and reduction to background levels by 72 hpf. Induction of these genes, however, does not lead to increased protein production. At 48 hpf, delayed vascular development and bradycardia become evident; at 85 hpf, oxygen consumption is significantly decreased. Upon hatching (96 hpf), larvae exposed to PVP-AgNPs are released into normoxia but are also smaller relative to control specimens. **B.** Proposed molecular mechanism of cardiovascular toxicity by PVP-AgNPs. At the embryonic stage, the chorion is covered by PVP-AgNPs, blocking oxygen intake. Hypoxia-induced expression of VEGF pathway genes increases rapidly; at the same time, intracellular PVP-AgNPs enter the endoplasmic reticulum (ER) and block protein synthesis. The latter effect not only blocks hypoxia-induced angiogenesis, but can also increase the mortality of zebrafish embryos at a later development stage.

Table 1

Zeta potential values in fish embryo medium.

PVP-AgNP Test Solution	0.1 mg/L	1 mg/L	10 mg/L
Temperature (°C)	25		
Mean Value (mV)	−7.52	−9.27	−13.10
Standard deviation (mV)	7.20	7.80	15.6