Published in final edited form as:

J Appl Toxicol. 2017 May ; 37(5): 530–544. doi:10.1002/jat.3387.

Disposition of Intravenously or Orally Administered Silver Nanoparticles in Pregnant Rats and the Effect on the Biochemical Profile in Urine

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Abstract

Here is presented a comprehensive investigation of the distribution of polyvinylpyrrolidone (PVP)-stabilized AgNP (20 or 110 nm) in pregnant rats after a single injection or oral gavage dose. The biological impacts of AgNP exposure were evaluated by metabolomic analysis, and measurement of biomarkers of cardiovascular injury, oxidative stress, and inflammation. The investigation provided a basic understanding of the distribution, internal dose, persistence, metabolomics, and elimination of AgNP following exposure in pregnant rats.

Few investigations have been conducted on the disposition and fate of silver nanoparticles (AgNP) in pregnancy. The distribution of a single dose of polyvinylpyrrolidone (PVP)-stabilized AgNP was investigated in pregnant rats. Two sizes of AgNP, 20 and 110 nm, and silver acetate (AgAc) were used to investigate the role of AgNP diameter and particle dissolution in tissue distribution, internal dose, and persistence. Dams were administered AgNP or AgAc intravenously (i.v.) (1 mg/kg) or by gavage (p.o.) (10 mg/kg), or vehicle alone, on gestation day 18 and euthanized at 24 or 48 h post-exposure. The silver concentration in tissues was measured using inductively-coupled plasma mass spectrometry. The distribution of silver in dams was influenced by route of administration and AgNP size. The highest concentration of silver ($\mu\text{g Ag/g tissue}$) at 48 h was found in spleen for i.v. administered AgNP, and in lungs for AgAc. At 48 h following p.o. administration of AgNP, the highest concentration was measured in cecum and large intestine, and for AgAc in placenta. Silver was detected in placenta and fetuses for all groups. Markers of cardiovascular injury, oxidative stress marker, cytokines, and chemokines were not significantly elevated in exposed dams compared to vehicle-dosed control. NMR metabolomics analysis of urine indicated that AgNP and AgAc exposure impact the carbohydrate, and amino acid metabolism. This study demonstrates that silver crosses the placenta and is transferred to the fetus regardless of the form of silver.

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Keywords

Silver nanoparticles; *in vivo* distribution; pregnant rats; maternal-fetal transfer; metabolomics analysis; biomarkers; oral gavage; intravenous injection; cytokines; oxidative stress

INTRODUCTION

The broad-spectrum bactericidal properties of silver nanoparticles (AgNP) (Berger, et al. 1976, Hamilton-Miller, et al. 1993, Zhao and Stevens Jr 1998) have led to their widespread use in industries, including textile, personal hygiene, medical applications, and other consumer products. The integration of silver nanomaterials to such a broad selection of products increases the risk of human exposure, both through direct exposure and environmental exposure. This practice makes it essential to understand the potential risks and effects that AgNP exposure poses on human health. In addition, maternal exposure during pregnancy may have effects on the developing fetus, and it is therefore important to understand the distribution, internal dose, persistence, and potential maternal-fetal transfer of nanosilver during pregnancy in order to perform meaningful health risk assessments.

Nanosized particles have been demonstrated to cross the human placenta *ex vivo* (Menjoge, et al. 2011, Myllynen, et al. 2008, Wick, et al. 2010). The gestational/lactational translocation of particles has been demonstrated in rodents, including titanium dioxide (TiO₂) (Shimizu, et al. 2009), diesel exhaust particles (Hemmingsen, et al. 2009, Hougaard, et al. 2008, Ramdhan, et al. 2009), and fullerene C₆₀ (Snyder, et al. 2015, Sumner, et al. 2010a, Tsuchiya, et al. 1996). A few studies have investigated the transfer of AgNP across the placenta in rats (Melnik, et al. 2013) and mice (Austin, et al. 2015, Austin, et al. 2012, Wang, et al. 2013). Following intravenous (i.v.) administration of AgNP in pregnant mice, silver was detected in extra-embryonic tissues (visceral yolk sac, placenta, and endometrium), and embryos (Austin, et al. 2015, Austin, et al. 2012). Silver was also found the placenta and fetal tissues of mice following intraperitoneal administration of AgNP, and while the highest dosing concentration resulted in the highest recovered dose in the placenta, the lowest dose resulted in the highest detected silver concentrations in the embryo and fetal liver (Wang, et al. 2013). Transfer of silver through placenta or breast milk to fetuses and pups in rats, respectively, have been demonstrated following gavage (p.o.) administration of AgNP (Melnik, et al. 2013).

The mechanisms and consequences of prenatal exposure to AgNP in fetuses and offspring are not yet well understood. No teratogenicity on skeletal system was observed in the offspring of rats exposed prenatally to AgNP, but the placenta from exposed dams were smaller in weight, volume and width (Mahabady 2012). Silver has been shown to cross the blood brain barrier as well as the cerebrospinal fluid barrier to enter tissues of the central nervous system in animals (Lansdown 2007). Following oral AgNP administration in pregnant rats, silver was found to reach the offspring's brain (Lale Ataei and Ebrahimzadeh-bideskan 2014, Lee, et al. 2012, Wu, et al. 2015). After prenatal exposure to AgNP, silver was detected in hippocampus tissues of offspring, as was cell apoptosis (Lale Ataei and Ebrahimzadeh-bideskan 2014). That prenatal exposure to AgNP can induce apoptosis in the

brains of newborn rats, was confirmed by the findings of elevated expression of the apoptosis mediator, procaspase-3 (Ganjuri, et al. 2015). Also, the growth-associated protein-43 (GAP-43) a neuron-specific phosphoprotein that plays a major role in initial development and remodeling of neural connections, was found in significant lower levels in the hippocampus of rat offspring following prenatal exposure to AgNP compared to controls (Wu, et al. 2015). Wu and colleagues also found that offspring at postnatal day 35 performed less well in the Morris Water Maze, suggesting impaired spatial cognition in rat offspring following prenatal exposure (Wu, et al. 2015). Postnatal neurobehavioral disorders as a resulting from prenatal exposure to AgNP has also been reported in mice (Ghaderi, et al. 2015).

The metabolomics analysis of responses to chemical exposure, including ENMs, have been studied, and utilized as a discovery tool, to determine the metabolites that are affected by exposure (Bu, et al. 2010, Buesen, et al. 2014, Hadrup, et al. 2012, Li, et al. 2013, Li, et al. 2014a, Tang, et al. 2010, Zhang, et al. 2015). Investigations of the biochemical profile in urine from rats exposed p.o. to AgNP demonstrated an increase in the level of two metabolites, uric acid and its degradation product, allantoin, whereas AgAc only increased allantoin levels in female rats (Hadrup, et al. 2012). However, to our knowledge, no studies have investigated the metabolic impact of ENMs during pregnancy using metabolomics analysis.

Silver has been shown to cross the placenta in both i.v. dosed mice (Austin, et al. 2015, Austin, et al. 2012, Wang, et al. 2013) and p.o. dosed rats (Melnik, et al. 2013), but a comprehensive examination of the role of NP size and route of delivery in distribution, internal dose, and persistence has not been presented previously. To study maternal-fetal transfer, embryology, and reproductive toxicology, the rat is a commonly used and well-characterized model (de Rijk, et al. 2002). Herein we report the results of a comprehensive investigation of the distribution and maternal-fetal transfer of AgNP in pregnant rats after administration of a single i.v. or p.o. dose. This study was conducted as part of a National Institute of Environmental Health Sciences (NIEHS) Centers for Nanotechnology Health Implications Research (NCNHIR) Consortium investigation of the role of size and surface coating on the effects of nanosilver. To investigate the role of nanoparticle size in tissue distribution and biological outcome, two AgNP sizes were chosen, 20 and 110 nm. This paper describes the investigation of PVP coated particles, and the results of the investigation of citrate coated particles will be reported in a separate paper. The two sizes were selected to encompass the range of sizes of commercially available nanosilver. The i.v. route was chosen to examine tissue distribution and elimination of silver in pregnant rats while avoiding absorption from the intestinal tract, and first pass elimination in the liver, and the oral route was chosen to investigate ingestion of nanosilver. The levels of markers for cardiovascular injury, oxidative stress, and inflammation were measured to study the responses in dams of AgNP exposure. Metabolomics analysis of urine was performed to investigate the biochemical changes caused by AgNP exposure. The results presented here are basic to an understanding of the distribution, internal dose, persistence, and biological impacts of AgNP in pregnant rats.

METHODS

Characterization of Silver Nanoparticle (AgNP)

In this study PVP-stabilized 20 or 110 nm AgNP, 1 mg Ag/mL in sterile water (Nanotechnology Characterization Lab [NCL] IDs NIEHS-2 and NIEHS-4, respectively), manufactured by nanoComposix, Inc. and supplied to the Nanotechnology Characterization Laboratory (NCL) were used (NCL 2011). The size and coating were selected by the NIEHS NCNHIR Consortium. The extensive characterization by NCL of the selected AgNP were done on behalf of NIEHS, including the determination of silver concentration in the provided solution. NCL assayed each preparation in duplicate for the following: Sterility and endotoxin contamination; hydrodynamic size/size distribution by dynamic light scattering (DLS); size/size distribution by transmission electron microscopy (TEM); hydrodynamic diameter (Z-average); surface charge by Zeta potential analysis and silver concentration by inductively coupled plasma mass spectrometry (ICP-MS) and the results are summarized in Table 1. Samples of NIEHS-2 and NIEHS-4 were obtained from NCL and stored refrigerated until time of use.

Housing and Dosing Administration of Pregnant Rats

Timed pregnant Sprague Dawley rats, approximately 10 weeks old were obtained from Charles River Laboratories in Raleigh, NC. Dams were singly housed in standard polycarbonate caging and were fed Purina 5002 rodent chow and Durham (NC) municipal tap water, ad libitum. A temperature of 72 ± 3 °F, relative humidity of 30-70%, and a 12:12 light cycle were maintained in the animal rooms. Dams were acclimated for 5-7 days prior to dosing.

All doses were administered on gestational day (GD) 18. Dosing solution of AgNP were prepared by using AgNP suspensions as supplied and mixed well prior to administration. Silver acetate (AgAc) (Sigma-Aldrich, St. Louis, MO, USA) was PVP-stabilized in 100 µg PVP/mL of water at a concentration of 1.55 mg/mL (1 mg Ag/mL). Rats were weighed before dosing, and the dose administered was determined by the syringe difference method. The charged syringe was weighed prior to dosing and the empty syringe was weighed after dosing. The exact dose administered was determined as the difference between the weight measurements. The AgNP and AgAc doses administered were calculated based on the weight of the dose administered and the concentration of the dosing solution. Dosing information was collected and calculated in a Debra™ data collection and reporting system (LabLogic Systems Ltd., Sheffield, England). Intravenous doses (i.v.) were administered via a lateral tail vein at a concentration of 1 mg Ag/kg body weight, and oral doses were administered by oral gavage (p.o.) at a concentration of 10 mg Ag/kg body weight. Each dosing group had 6 dams. Control dams received vehicle doses of 100 µg PVP/mL in water at 1 mL/kg i.v. or 10 mL/kg p.o. The dose for i.v. administration was selected to be well under the lethal dose for i.v. exposure in mice of >200 mg Ag/kg (Xue, et al. 2012). The dose of 10 mg/kg selected for p.o. is equivalent to 2000 times the reference dose for daily oral exposure to silver by EPA (EPA 1991), but not toxic in mice at repeated daily doses (Bergin, et al. 2015). Dams were placed in glass metabolism cages that allow for separate collection of urine and feces on dry ice. Urine and feces were collected at 24 h intervals,

prior to dosing (0h), 24 and 48h post administration. Three dams per group were euthanized at 24 and 48 h post exposure and tissues: blood, brain, heart, kidney, liver, spleen, lung, pancreas, stomach and small intestine (upper GI tract), large intestine and cecum (lower GI tract), skin, bone (femur), carcass, fetus, and placenta were collected, weighed, and stored frozen (-20°C). A portion of each blood sample was processed to plasma for biomarker analysis and the remainder was used for silver analysis. Blood volume in rats was estimated to be 7.4% of body weight based on the literature, muscles to be 40.43%, bones 7.3%, and skin 19.03% (Brown, et al. 1997).

Measurement of Silver Content in Tissues

Quantitative analysis of silver in tissues and excreta was conducted by ICP-MS, adapted from methods described previously (Poitras, et al. 2015). In short, an aliquot representing about half of the organ was processed per sample except for GI tissues where the whole organ was processed with contents. For each set of digestions, method blanks were prepared and analyzed to monitor background silver content. Sample preparation was conducted in a room equipped with yellow lights to minimize the potential for photo-oxidation of silver. The silver concentration for all samples was analyzed using an X-Series I ICP-MS (Thermo Electron Corporation, Bremen, Germany) equipped with a concentric glass nebulizer and Peltier-cooled glass spray chamber. Quality control samples were processed with the study samples to monitor method performance, including pre-digestion spiked method blanks. The quantification limit was 0.5 ng Ag/mL digested solution, with a linear range from 0.5 to 100 ng/mL. Quantitation limits depended on the size of feces, urine, or tissue sample used for the digestion, and were approximately 50 ng/g for feces, 5 ng/g for urine, and 12.5 ng/g for liver (Poitras, et al. 2015).

Measurements of Cardiovascular Injury Markers and Inflammation

Levels of markers for cardiovascular injury and inflammation were measured in plasma samples collected from dams following exposure to AgAc, AgNP or vehicle control. To evaluate the cardiovascular injury concentrations of plasminogen activator inhibitor (PAI)-1, von Willebrand factor (vWF), soluble intercellular adhesion molecule 1 (ICAM-1), and soluble e-Selectin were measured quantitatively with a Milliplex MAP Cardiovascular Immunoassay Panel (RCVD1-89k, EMD Millipore, Billerica, MA, USA). Inflammatory markers counted eight chemokines and cytokines: Eotaxin, interleukins; IL-1 β , IL-4, IL-5, IL-6, monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor alpha (TNF- α), and vascular endothelial growth factor (VEGF), which were quantified utilizing Milliplex MAP Cytokine/Chemokine and Cardiovascular Immunoassay panels (RECYTMAG-65K, EMD Millipore, Billerica, MA, USA). Assays were run according to the manufacturer's protocol, and analyzed using a Luminex 200 (Luminex, Austin, TX, USA). The results were reported using Luminex xPONENT® software version 3.1.

Detection of 8-Hydroxy-2'-deoxyguanosine (8-OH-dG) in Urine

Levels of 8-OH-dG in urine were measured using an ELISA assay, following the instructions of the (New 8-OH-dG-Check, Jaica, Japan; Supplier: Genox, MD, USA). Following centrifuged (10 min $\times$ 5,000 g), 30 μL urine was diluted with 120 μL PBS. For the assay a 50 μL aliquot of the diluted samples were used. Statistical analyses were performed using a

Wilcoxon matched pairs test using GraphPad Prism 5.0 (San Diego, CA) and significant differences were $p < 0.05$.

NMR Metabolomic Analysis of Urine

Metabolomic analysis was done on urine samples collected from dams at 48 h post exposure to vehicle, AgAc, 20 nm or 110 nm AgNP. The sample preparation for NMR metabolomics analysis has been described previously (Snyder, et al. 2015, Sumner, et al. 2015). In short, to 540 μL of urine, 60 μL of Chenomx ISTD solution (Chenomx, Edmonton, Alberta, Canada) and two internal standards [4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) and imidazole] were added. The internal standards were used for line shape analysis and chemical shift referencing, pH determination, and metabolite identification using the NMR library in Chenomx NMR Suite 8.1 Professional software (Chenomx, Edmonton, Alberta, Canada). A 500 μL aliquot of sample was transferred into a 5 mm NMR tubes. A Bruker Avance III 700 MHz NMR spectrometer (located at the David H. Murdock Research Institute at Kannapolis, NC, USA) using the first increment of a NOESY sequence (Beckonert, et al. 2007), with a 100 ms mixing time, a 2 s relaxation delay, a spectral width of 16 ppm, 32k data points, and 32 transients were used to acquire ^1H NMR spectra. All ^1H NMR spectra were collected at 25° C, zero filled, and Fourier transformed after exponential multiplication with a line broadening factor of 0.5. The water resonance was suppressed using resonance irradiation during the relaxation delay and mixing time. Phase and baseline of the spectra were manually corrected for each spectrum, and spectra were referenced to DSS (δ 0 ppm). Assessment of the quality of each NMR spectrum was done for the level of noise and alignment of identified markers.

Analysis of NMR spectra was conducted as previously described (Church, et al. 2014, Pathmasiri, et al. 2012, Sumner, et al. 2009, Sumner, et al. 2010a, Sumner, et al. 2015, Sumner, et al. 2010b). Briefly, NMR data were processed by automated integration (increments of 0.04 ppm) over the spectral window (binning), excluding the region of water suppression, and DSS signal, and intensities each of the bins was normalized to the total integral of the bins of each spectrum. Normalized binned data (bin region and integral value) were transported for data reduction and visualization using SIMCA-P+ 13.0 (Umetrics, Umeå, Sweden). SIMCA-P+ 13.0 for the binned data were used to generate principal component analysis (PCA) and orthogonal partial least squares projection to latent structures discriminant analysis (OPLS-DA). Data were preprocessed by mean centering and Pareto scaling prior to multivariate data analysis. Loadings plots and variable importance for projection (VIP) plots were investigated to determine the bins that best define the separation of exposure groups and those bins were matched to metabolites using Chenomx NMR Suite 8.1 Professional software. The library matched marker metabolites that best differentiated the study groups were used for pathway mapping using MetaCore module in GeneGo (Thompson Reuters, Philadelphia, PA), and descriptive statistics were calculated using SAS 9.4.

RESULTS

Pregnant rats were exposed to three forms of silver: AgAc, 20 nm AgNP, or 110 nm AgNP in this comprehensive distribution study. Dams either received silver i.v. (1 mg/kg) or by p.o. (10 mg/kg). To compare with the distribution of silver ions, additional group of pregnant rats received AgAc at equivalent doses of silver (1 mg/kg i.v., 10 mg/kg p.o.). Following administration of silver and vehicle no adverse clinical signs were noted. To evaluate the distribution, internal dose, and persistence of silver, the total silver in tissues and excreta was determined by ICP-MS.

Distribution of Silver in Pregnant Rats after i.v. Administration

Tissue Concentration ($\mu\text{g Ag/g tissue}$)—The concentration ($\mu\text{g Ag/g tissue}$) of silver in tissues at 24 or 48 h post administration is presented in Table 2. The results demonstrated that the form of silver played an important role in tissue distribution. In dams receiving 20 or 110 nm AgNP, the highest concentration of silver was found in spleen at 48 h post exposure (1.1 $\mu\text{g Ag/g}$ for 20 nm AgNP, and 3.6 $\mu\text{g Ag/g}$ for 110 nm AgNP). In contrast, for AgAc dosed dams, the highest concentration of silver at 48 h was found in lungs (1.3 $\mu\text{g Ag/g}$). At 48 h post administration, the second highest concentration of silver in AgAc and 20 nm AgNP exposed dams was detected in placenta (0.8 $\mu\text{g Ag/g}$ and 0.9 $\mu\text{g Ag/g}$, respectively), while for dams administered 110 nm AgNP, the second highest concentrations were found in liver (2.7 $\mu\text{g Ag/g}$), followed by the placenta (1.1 $\mu\text{g Ag/g}$). Silver concentration in liver was dependent on AgNP size, such that the concentrations of silver were an order of magnitude higher for 110 nm AgNP dosed dams (2.7 $\mu\text{g Ag/g}$) than for 20 nm AgNP (0.2 $\mu\text{g Ag/g}$). Silver was measured in fetuses for all three forms of silver and increased from 24 to 48 h post administration. The results show that silver crosses the placenta regardless of its form, and that the concentration is similar in the placenta between 24 and 48 h. However, the concentrations of silver in placenta were higher than the concentrations measured in blood, and also higher than the concentration measured in fetuses. Levels at least 3-fold above background were also detected in blood, liver, spleen, lungs, heart, kidney, bone, stomach and small intestine, cecum and large intestine, and pancreas for all three forms of nanosilver. Levels at least 3-fold above background were also detected in muscles and brain for AgAc and 20 nm AgNP, but not for 110 nm AgNP. Silver was not detected in adipose tissue at 48 h in any of the groups.

Recovery of Administered Dose—Dams dosed with 20 nm AgNP had the highest percentage of recovered silver in skin at 48 h (4%), whereas the highest percentage in dams administered 110 nm AgNP was found in liver at 48 h (12%) (Table 3). The percentage of silver recovered in skin at 48 h for dams that received AgAc and 110 nm AgNP were similar (5%). The levels of silver in the liver for 20 AgNP exposed dams were 1% at 48 h, 12-fold lower compared to 110 nm AgNP. For AgAc, the percentage of silver in the liver was 2% at 48 h. The percentage of silver recovered in placenta at 48 h was; 1.6-2.1% for all three groups, and 0.8-1.0% in fetuses. The percentage of dose detected in fetus and placenta were similar over the 24-48 h time interval for all groups, with the exception of fetuses of 110 nm AgNP dosed dams where the concentration increased. The percent recovery of administered dose at 48 h post i.v. and p.o. administration is presented in Table 4. The percentage of dose

recovered in the tissues following i.v. administration was greater than an order of magnitude compared to recovery in tissues following p.o. administration.

Distribution of Silver in Pregnant Dams after Gavage Administration

Tissue Concentration ($\mu\text{g Ag/g tissue}$)—The highest tissue concentrations of silver 24 h after administration of both sizes of AgNP were found in the lower GI tract (3–4 $\mu\text{g Ag/g}$), which decreased (0.9 $\mu\text{g Ag/g}$) at 48 h (Table 5). The concentration of silver in the lower GI tract of dams administered AgAc was 16 $\mu\text{g Ag/g}$ at 24 h and decreased to 0.4 $\mu\text{g Ag/g}$ at 48 h, a 40-fold decrease compared to the approximately 3–4 fold decrease observed for both AgNPs. Besides the GI tract tissues, placental tissue was found to contain the highest silver concentration (0.9 $\mu\text{g Ag/g}$ at 48 h for AgAc and 0.4 $\mu\text{g Ag/g}$ for AgNPs). The concentration of silver at 48 h in fetuses of AgAc dosed dams was 2-fold higher than that of 110 nm AgNP dosed dams and 3-fold higher compared to 20 nm AgNP exposure. On average, the concentrations of silver found in placenta and fetuses were similar at 24 and 48 h for all three forms of silver. Fetus concentrations were generally an order of magnitude lower than the placental concentration, which was also observed for fetuses of i.v. exposed dams. The concentrations of silver in placenta were higher than in blood for all groups. Silver concentrations in fetuses were lower than those detected in the dams' blood at 48 h following AgAc and 20 nm AgNP exposure. With 110 nm AgNP, silver concentrations measured in blood of dams dosed were below the detection limit, however the silver concentration detected in their fetuses were similar to AgAc and 20 nm AgNP exposure groups.

Recovery of Administered Dose—The highest recovered percentage of dose in AgNP exposed dams was found in GI tract tissues, with higher recovery in the lower GI than in the upper at both 24 and 48 h (Table 6). The percentage recovered in GI tract decreased dramatically between 24 and 48 h, probably due to passage of the dose through the GI tract and elimination in feces. The second highest percentage for AgNP exposed dams was found in placenta (<0.2%), while for AgAc dosed dams placenta had the highest percentage (0.9%). The recovered administered dose in fetuses was <0.1%. Interestingly, the percentage of nanosilver was approximately 10-fold higher in the placenta compared to the liver, and several orders of magnitude higher than the kidneys.

Markers of Cardiovascular Injury, Inflammation, and Oxidative Stress

Markers of cardiovascular injury—Pregnant rats exposed i.v. to nanosilver were compared to time-matched vehicle groups at 24 and 48 h post exposure revealed that 110 nm AgNP caused a lower PAI-1 expression level (Table 7). For p.o. exposed dams, levels of PAI-1 were lower at 48 h for AgAc and 110 nm AgNP compared to vehicle. While the standard deviations were large, vWF levels in dams dosed i.v. with 110 nm AgNP exhibited a lower level of expression compared to vehicle exposed dams at 48 h, and AgAc showed an overall increase in vWF levels. For p.o. administered 110 nm AgNP, vWF was non-detectable at 24 h and lower than vehicle at 48 h. On average, the vWF and PAI-1 responses were lower for nanosilver administered p.o. compared to i.v.

Levels of inflammatory markers—Marker of inflammation in plasma were determined in pregnant rats receiving i.v. AgAc, 20 nm AgNP, or vehicle control at 24 and 48 h after administration, by evaluating the level of eight cytokines and chemokines (Table 8). While the standard deviations of cytokine and chemokine levels were high, the levels decreased from 24 to 48 h, and only eotaxin remained detectable at 48 h post administration in dams dosed with silver, but eotaxin, IL-4, and IL-6 increased from 24 to 48 h in dams receiving vehicle control.

Oxidative stress—Comparison between the three timepoints; predose (0 h), 24 and 48 h post dose, for the exposure groups vehicle control, AgAc, 20 nm AgNP, and 110 nm AgNP administered either i.v. or p.o. showed that 110 nm AgNP caused a significant elevation in 8-oxo-2'-deoxyguanosine (8-OH-dG) at 24 h compared to pre-exposure levels (Figure 1). There was an overall trend of increasing levels of 8-OH-dG from pre-exposure levels (0 h) to 48 h post exposure for all groups, including vehicle control. On average, the 8-OH-dG concentration were similar between i.v. and p.o. exposed dams.

NMR Metabolomics Analysis

Metabolomics analysis of urine collected at 48 h post dosing from pregnant rats administered AgAc, 20 or 110 nm AgNP, or vehicle control either i.v. or p.o. (Figure 2). Library matched metabolites that separate AgAc, 20 nm or 110 nm AgNP i.v. exposed dams from vehicle control dams, are listed in Table 9. Metabolites that distinguish AgAc, 20 nm or 110 nm AgNP p.o. exposed dams from dams receiving vehicle are listed in Table 10. It was found for both route of administration and forms of silver that there are common as well as unique metabolites that separate silver exposure groups from vehicle-dosed control.

In pregnant rats administered either form of silver (AgAc, 20 nm or 110 nm AgNP) i.v., the metabolites 1-methylnicotinamide, 3-hydroxyphenylacetate, citrate, glutamine, lactose, N-acetylglutamine, proline, and trigonelline differentiated the exposure groups from the vehicle control (Table 9). For dams receiving silver p.o., the metabolites isoleucine, lactose, and N-acetylglutamine separated all three groups from vehicle control (Table 10). In common for all silver exposed groups were that lactose and N-acetylglutamine separated them from vehicle control dosed dams (Tables 9 and 10).

The metabolites 1-methylnicotinamide, acetate, isoleucine, lactose, taurine, and trigonelline were differentiating metabolites in urine of AgAc dosed dams, regardless of route of administration (Tables 9 and 10). For 20 nm AgNP exposed dams a total of 15 metabolites were altered regardless of administration route compared to vehicle control (2-oxoglutarate, 3-hydroxyphenylacetate, acetoacetate, citrate, creatinine, dimethylsulfone, glucose, isoleucine, lactate, lactose, N-acetylaminoacids, N-acetylglutamine, N-acetylglycine, proline, and sucrose). Following 110 nm AgNP exposure the metabolites 1-methylnicotinamide, 3-hydroxyphenylacetate, allantoin, dimethylsulfone, lactose, N-acetylglutamine, proline, and trigonelline were found to separate both i.v. and p.o. dosed dams from vehicle control.

The most significant perturbed pathways as a result of i.v. exposure to AgAc and 20 nm, or 110 nm AgNP when compared to vehicle were: amino acid metabolism (involving the metabolites betaine, glycolic acid, L-glutamine, and L-proline), amino acid metabolism

(involving the metabolites 2-oxoglutaric acid, glycolic acid, L-glutamine, L-methionine, and L-proline), and tricarboxylic acid (TCA) metabolism and transport (2-oxoglutaric acid, citric acid, and succinic acid), respectively (Table 11). When AgAc, 20 nm, or 110 nm AgNP were administered p.o. the most perturbed pathway for each exposure group compared to vehicle control were: carbohydrate metabolism, sucrose pathway (D-sucrose, D-glucose, and lactose), TCA metabolism and transport (2-oxoglutaric acid, citric acids, and succinic acid), and alanine, glycine, and cysteine metabolism and transport (glycolic acid and L-alanine), respectively (Table 11).

DISCUSSION

The increasing number of investigations reporting maternal-fetal transfer of engineered nanomaterials (ENMs) and resulting negative impact on fetuses are concerning. Important aspects of prenatal exposure to ENMs are to understand maternal-fetal transfer, internal dose of ENMs in both maternal and fetal tissues, as well as resulting pre- and postnatal toxicological outcome. Few studies have reported the disposition, persistence, and fate of AgNP in pregnancy and potential maternal-fetal transfer, and understanding these parameters in biological systems is the basis for toxicology and risk assessment.

Here, a comprehensive investigation of silver distribution in pregnant rats was conducted following a single i.v. or p.o. dose of PVP-stabilized AgAc, 20 nm AgNP, 110 nm AgNP or vehicle control to provide data for modeling of risks associated with exposure to nanosilver during pregnancy. Following i.v. administration of 20 nm AgNP, spleen was the tissue at 48 h found to contain the highest concentration of silver, followed by placenta>lungs>cecum and large intestine ~ liver ~ blood ~ kidney ~ skin. After i.v. administration of 110 nm AgNP, the highest silver concentration in tissue at 48 h was also found in spleen, followed by the liver>placenta>cecum and large intestine>blood ~ kidneys ~ lung. At 48 h following i.v. administration of AgAc, the highest silver concentration was found in the lungs followed by placenta>spleen ~ liver ~ cecum and large intestine. The silver concentration in spleen of dams receiving 20 nm AgNP i.v. was found to be 2-fold lower than in 110 nm AgNP dosed dams, while the silver concentration in the liver was an order of magnitude lower. It has been suggested that NP smaller than the pores of liver fenestrae (~100 nm) can be taken up by the liver, whereas larger particles are absorbed with greater affinity in the spleen (Dziendzikowska, et al. 2012, Liu, et al. 1992).

Excretion of silver through the feces via the liver following i.v. exposure could either happen as free ions, as in the case of AgAc, or possibly as NP. Hepato-biliary excretion has been demonstrated to be dependent on NP size, so that smaller NP are excreted faster than larger NP (Cho, et al. 2009, Cho, et al. 2010, Hirn, et al. 2011). Here we found that the recovered percentage of silver in feces were similar for 20 nm and 110 nm AgNP dosed dams at 48 h (10% and 6%, respectively), while the recovered percentage in feces for AgAc dosed dams were several fold higher (39%). Excretion of silver through urine was minimal following both i.v. and p.o. administration, and the concentration of silver in the kidneys was low for all three groups (0.2-0.5 µg/g tissue). For quantum dots the cut-off size for renal excretion has been reported to be approximately 5.5 nm (Choi, et al. 2007). This suggests that silver ions are not effectively excreted in urine. For p.o. administered AgNP, the highest

concentrations of both sizes of AgNP were found in the cecum and large intestine, with both demonstrating a 3- to 4-fold decrease in concentration between 24 and 48 h post exposure. This observation corresponds with the high amount of silver recovered in feces. For dams receiving either size of AgNPs, the second highest concentration of silver at 48 h was found in placenta, followed by kidneys. For dams receiving AgAc p.o., the highest concentration of silver at 48 h was found in placenta, followed by cecum and large intestine > liver > kidneys. Low levels of silver were detected in the blood for all three animal groups at both 24 and 48 h post administration, and little of the administered dose was found in the analyzed tissues.

In this study, silver was detected in both placenta and fetus for all animal groups, illustrating that silver enters and crosses the placenta regardless of the route of administration and the form of silver. Interestingly, while the concentration of silver in the placenta was approximately 6-fold higher following i.v. administration compared to p.o. at 48 h post dosing, the placenta was the tissue with the second highest concentration of silver following p.o. administration. The concentrations of silver were approximately a magnitude higher in the placenta compared to the fetus, demonstrating that the transfer of silver to the fetus was substantially impeded by the placenta. The maternal-fetal transfer of ENMs and translocation across the placenta is not well understood, as discussed in recent reviews (Buerki-Thurnherr, et al. 2012, Pietroiusti, et al. 2013, Rollerova, et al. 2015, Stapleton and Nurkiewicz 2014). The diameter of the uterine and placental blood vessels change during normal placental development (Osol and Mandala 2009, Osol and Moore 2014). The diameter of the uterine artery measured in non-pregnant women was 1.4 mm, while at 21 week of pregnancy the diameter was 2.8 mm, increasing to 2.9 mm at week 30, and 3.4 mm at week 36 (Palmer, et al. 1992). In pregnant rats at GD 20, uterine arteries reaching male or female fetuses have a diameter of 0.72 or 0.73 mm, respectively, while the diameter of radial artery is 0.63-0.65 mm and the diameter of spiral arteries are 0.15-0.16 mm (Gopalakrishnan, et al. 2016). During pregnancy, the blood flow to the uterus increases from 0.1 mL/min in the non-pregnant rat, to 9 mL/min on GD 20 (Ahokas, et al. 1983, Buelke-Sam, et al. 1982), representing a 90-fold increase in blood flow, with approximately 6% of the cardiac output distributed to the placentas on GD 20. Here we administered silver at GD 18 and determined the concentration of silver in tissues on GD 19 and GD 20. The increased blood flow to the placenta (0.7 ml/min/g at GD 18 and 0.4 ml/min/g at GD 20) during the later stages of pregnancy (Ahokas, et al. 1983) ensures that a substantial portion of i.v. administered materials will distribute to the placenta. Liver receives a substantial portion of the cardiac output (blood flow is 0.1 ml/min/g at GD18 and 0.07 ml/min/g at GD 20) as well as the spleen (blood flow is 1.3 ml/min/g at GD 18 and 1.1 ml/min/g at GD 20). Interestingly, the concentration of silver in the placenta was similar to the liver for dams i.v. dosed with AgAc, while for 20 nm AgNP dosed dams it was 2-3 fold higher than in liver. In contrast, in dams receiving 110 nm AgNP, silver concentrations in placenta were approximately half that in liver. These results indicate that nanoparticle size plays a critical role in retention of material in the placenta compared with other organs. For p.o. administered materials, the concentrations of silver measured in the placenta were even more striking compared to the other tissues, since they were the highest measured at 48 h after the lower GI, and several times higher than those found in liver and spleen. It is important to remember that the placenta is not just a filter protecting the developing fetus from maternal

blood substances, but also plays an important role in for example fetal brain development (Bonnin and Levitt 2012, Hsiao and Patterson 2012, Zeltser and Leibel 2011). The internal concentration of silver in the placenta might point to the mechanisms behind the reported impact on fetal brain and neurological development following prenatal AgNP exposure (Ganjuri, et al. 2015, Ghaderi, et al. 2015, Lale Ataei and Ebrahimzadeh-bideskan 2014, Wu, et al. 2015).

Circulating biomarkers like selected pro- and anti-inflammatory cytokines and chemokines were analyzed in the plasma of pregnant rats to understand possible correlations with nanosilver exposure. Cytokine levels were consistent in nanosilver exposed dams with previous reports of limited toxicity of ENMs such as AuNP (Rattanapinyopituk, et al. 2014, Yang, et al. 2012) and [$^{14}\text{C}(\text{U})$] C_{60} (Snyder, et al. 2015). Here it was found for all forms of nanosilver, that the highest cytokine levels were at 24 h post exposure. Intratracheal (i.t.) installation of citrate-capped 20 nm AgNP in male Sprague-Dawley rats revealed elevations of serum cytokines: G-CSF, MIP-1 α , IL-1 β , IL-2, IL-6, IL-13, IL-10, IL-18, IL-17, TNF α , and RANTES at 1 day post exposure, but decreased, with only IL-2, IL-13 and TNF α remaining elevated at day 7 (Holland, et al. 2015). In this study, markers of cardiovascular injury, PAI-1, and vWF were evaluated. PAI-1 was lower in dams receiving 110 nm AgNP i.v. compared to vehicle, AgAc, and 20 nm AgNP exposed dams, but not for p.o. administration. For 110 nm AgNP administered p.o., a lower level of vWF resulted compared to the other groups. It is possible that i.v. or p.o. exposure to nanosilver does not evoke the same cardiovascular response as i.t. exposure, or that the cardiovascular injury marker assay employed here was not as sensitive to detect possible cardiovascular responses.

Broad-spectrum NMR metabolomics analysis of urine from dams receiving nanosilver either i.v. or p.o. and collected at 48 h was conducted as a discovery tool to determine metabolites that were most important to differentiate between two sizes of AgNP and route of administration during pregnancy. Published studies of metabolomics analysis of urine obtained from female Wistar Hannover Galas rats p.o. administered PVP-stabilized 14 nm AgNP, by high performance liquid chromatography – quadrupole time-of-flight mass spectrometry, exhibited metabolic differences compared to control specimens, as an increase in levels of uric acid and allantoin utilization (Hadrup, et al. 2012). In this investigation, uric acid itself was not found to be important for differentiating AgNP p.o. exposure from vehicle controls, but the degradation product of uric acid, allantoin, was found to separate p.o. administered AgAc and 110 nm AgNP (Table 10), as well as for dams dosed i.v. with 110 nm AgNP from vehicle control dams (Table 9). NMR-based metabolomics analysis has also been used to understand the exposure in rats or mice of other NP, such as TiO $_2$ (Bu, et al. 2010), manganese oxide (MnO) (Li, et al. 2014b), MnO embedded iron oxide NP (Li, et al. 2013), aluminum oxide (Al $_2\text{O}_3$) (Zhang, et al. 2015), silica dioxide (SiO $_2$) (Buesen, et al. 2014), zirconium dioxide (ZrO $_2$) (Buesen, et al. 2014), and barium sulfate (BaSO $_4$) (Buesen, et al. 2014). Combining results from metabolomics analysis, serum chemistry, hematology, and histopathology in male and non-pregnant female rats have indicated that a disturbance in energy and amino acid metabolism might be attributed to slight liver and heart injury caused by TiO $_2$ (Bu, et al. 2010). MnO NP exposure in male rats was found to impact metabolites involved in lipid, energy, amino acid, and other nutrient metabolism (Li, et al. 2013). The metabolomics analysis of urine from exposed pregnant rats (when compared to vehicle

control) demonstrated that a single AgNP dose impacted the glycosphingolipid metabolism or TCA cycle when administered i.v. and for AgNP delivered p.o., the TCA cycle and amino acid metabolism and transport are most perturbed (Table 11). It is interesting that the literature reported metabolomics analysis of the effects of NP exposure in male or non-pregnant female rodents reported changes associated with lipid, energy, amino acid, and protein metabolism pathways, and the results presented here also find perturbation in dams of energy, and amino acid metabolic pathways as a result of AgNP exposure. Collectively these data suggest that exposure to ENMs can impact metabolism, and further research is needed to determine the possible health implication of ENMs exposure in all physiological states.

It is essential to have a comprehensive knowledge of the internal dose of each ENM in tissues in order to understand its pathology and the role of the material's physiochemical properties. Several mechanisms behind adverse outcomes following exposure are possible, including direct particle exposure in tissues, or indirect effects mediated by the autonomic nervous system, systemic immunological effects (Utell, et al. 2002), endocrine disruption (Iavicoli, et al. 2013, Larson, et al. 2014), metabolic imbalance, and signaling pathways (Hussain, et al. 2014, Nel, et al. 2006). Regardless of the mechanisms behind the adverse outcome of ENM exposure, a basic knowledge of distribution and disposition is fundamental in toxicology. Distribution, disposition, and elimination also reveal if a ENMs are persistent. Persistence (biodurability) of ENMs in biological systems is considered one of the main contributors to particle and fiber toxicity (Utembe, et al. 2015), since the longer the ENM persists, the higher the risk there is for an adverse outcome. The most classic example of the serious consequences of persistence in toxicology might be asbestos, which is not metabolized and has an estimated clearance half-time measured in years to decades (Churg and Wright 1994). Also, in the case of persistent ENMs, the release of constituents, such as ions, from the material, may be a major cause of adverse effects. The dissolution:translocation relationship of ENMs is believed to be a central part of nanotoxicology (Borm, et al. 2006). The toxicity of ENMs is expected to result from material properties and biological fate, with persistent ENMs, and ENMs that rapidly dissolve to release toxic constituents representing areas of concern. Our working hypothesis is that AgNPs are rapidly removed from circulation after dosing, enter into the liver and spleen, and are then slowly released back into the circulation or eliminated in feces. This contrasts with studies with other ENMs such as C₆₀, which has very limited clearance, with less than 2% of the dose per 24 hours (Snyder, et al. 2015, Sumner, et al. 2010a, Sumner, et al. 2015).

This report has demonstrated that the distribution of silver in pregnant rats depends on both route of administration and form of nanosilver, and that maternal-fetal transfer of silver across the placenta occurs regardless of both parameters. Our findings also illustrate that the carbohydrate and amino acid pathways were perturbed by silver. This study demonstrates the importance of understanding the relationships between the distribution, disposition, internal dose, and persistence of ENMs during pregnancy and the health aspects of human exposure.

Acknowledgements

This study was supported by National Institute of Environmental Health Sciences (U19 ES019525), and National Institute of Diabetes and Digestive and Kidney Diseases (1U24DK097193-01). The authors are grateful to Andrew Novokhatny, Zachery Acuff, Susan McRitchie, and Dr. Kelly Mercier for providing analytical and statistical assistance with the metabolomics data analysis and to Rose Ewald for critical comments and discussion.

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Figure 1.
8-OH-dG (mean $\pm$ standard deviation) in urine collected from pregnant rats following administration of a single a) i.v. or b) p.o. dose of vehicle, AgAc, 20 nm and 110 AgNP (n=3).

Figure 2. Metabolomics analysis of urine from pregnant rats. The effect of form of nanosilver shown in OPLS-DA plots of A) i.v. and B) p.o. administration at 48 h post dosing. Vehicle samples are shown in yellow, AgAc in red, 20 nm AgNP green and 110 nm AgNP blue.

Table 1

Methods for analysis of AgNP stock standard solutions and characteristics.

Characterization Assay	Method ID	20 nm AgNP (NIEHS-2)	110 nm AgNP (NIEHS-4)
Endotoxin Quantification – Kinetic Turbidity (EU/mL)	STE-1.2	1.1	<0.5
Hydrodynamic Size/Size Distribution by DLS (Z-Avg [nm])	PCC-1	26	112.3
Size by TEM (nm)	PCC-7	20.5 ^a	111.3 ^b
Surface Charge by Zeta Potential (mV)	PCC-2	–37.1	–25.9
Silver concentration by ICP-MS (mg/g)	PCC-8	1.09	1.10

^aTwo populations observed; <9% were smaller than 10 nm, the remainder averaged 20 nm

^bTwo populations observed; <13% were smaller than 60 nm, the remainder averaged 111 nm

Table 2

Concentration of silver ($\mu\text{g Ag/g}$) in tissues following i.v. administration (1 mg/kg) of AgAc, 20 nm AgNP or 110 nm AgNP to pregnant rats.

Tissue	AgAc		20 nm AgNP		110 nm AgNP	
	24 h	48 h	24 h	48 h	24 h	48 h
Liver	1.03 $\pm$ 0.399	0.511 $\pm$ 0.534	0.312 $\pm$ 0.044	0.228 $\pm$ 0.130	2.28 $\pm$ 1.69	2.71 $\pm$ 1.63
Blood	0.663 $\pm$ 0.195	0.180 $\pm$ 0.033	0.375 $\pm$ 0.0485	0.214 $\pm$ 0.0916	0.594 $\pm$ 0.0566	0.348 $\pm$ 0.0720
Spleen	2.95 $\pm$ 0.927	0.524 $\pm$ 0.493	1.42 $\pm$ 0.499	1.14 $\pm$ 0.636	6.79 $\pm$ 2.16	3.60 $\pm$ 0.467
Lungs	1.38 $\pm$ 0.958	1.31 $\pm$ 0.906	0.519 $\pm$ 0.103	0.397 $\pm$ 0.0950	0.436 $\pm$ 0.0839	0.305 $\pm$ 0.0831
Heart	0.162 $\pm$ 0.0306	0.0584 $\pm$ 0.000479	0.127 $\pm$ 0.0168	0.0863 ^a	0.119 $\pm$ 0.0204	0.104 $\pm$ 0.0144
Kidney	0.405 $\pm$ 0.0944	0.233 $\pm$ 0.0364	0.431 $\pm$ 0.0520	0.222 $\pm$ 0.0843	0.472 $\pm$ 0.0968	0.373 $\pm$ 0.161
Brain	0.0358 $\pm$ 0.000661	0.0233 $\pm$ 0.00150	0.0297 ^a	0.0424 ^a	<LOQ ^b	<LOQ
Skin	0.224 $\pm$ 0.0488	0.265 $\pm$ 0.141	0.196 $\pm$ 0.147	0.194 $\pm$ 0.0735	0.188 $\pm$ 0.107	0.248 $\pm$ 0.189
Muscle	0.0833 $\pm$ 0.0616	0.0556 $\pm$ 0.0210	0.0284 $\pm$ 0.00378	0.0146 $\pm$ 0.00525	<LOQ	<LOQ
Adipose	0.0453 $\pm$ 0.0191	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Bone	0.311 $\pm$ 0.0455	0.119 $\pm$ 0.0370	0.243 $\pm$ 0.0780	0.144 $\pm$ 0.0789	0.120 $\pm$ 0.0386	0.136 $\pm$ 0.0299
Stomach and Small Intestine	0.377 $\pm$ 0.215	0.145 $\pm$ 0.0958	0.319 $\pm$ 0.0378	0.167 $\pm$ 0.148	0.214 $\pm$ 0.165	0.136 $\pm$ 0.0376
Cecum and Large Intestine	0.414 $\pm$ 0.118	0.443 $\pm$ 0.175	0.976 $\pm$ 0.431	0.370 $\pm$ 0.222	0.554 $\pm$ 0.150	0.459 $\pm$ 0.153
Pancreas	0.196 $\pm$ 0.0561	0.170 $\pm$ 0.116	0.321 $\pm$ 0.204	0.138 $\pm$ 0.0553	0.0732 $\pm$ 0.0214	0.0831 $\pm$ 0.0485
Placenta	0.881 $\pm$ 0.337	0.756 $\pm$ 0.184	0.696 $\pm$ 0.0682	0.926 $\pm$ 0.436	0.719 $\pm$ 0.0364	1.15 $\pm$ 0.239
Fetus	0.0603 $\pm$ 0.0106	0.0759 $\pm$ 0.019	0.0445 $\pm$ 0.00763	0.0549 $\pm$ 0.0174	0.0316 $\pm$ 0.000507	0.0492 $\pm$ 0.00674

All data are reported as Mean $\pm$ SD (n= 3)

^a Only one rat had levels above quantitation limit.

^b <LOQ = below limit of quantitation = quantitation limit; 0.0125 $\mu\text{g/g}$ for tissues.

Percent recovered of administered dose of silver in tissues following i.v. administration (1 mg/kg) of AgAc, 20 nm AgNP or 110 nm AgNP to pregnant rats

Table 3

Tissue	AgAc		20 nm AgNP		110 nm AgNP	
	24 h	48 h	24 h	48 h	24 h	48 h
Liver	4.33±1.63	2.08±2.02	1.44±0.203	1.02±0.635	8.69±6.04	12.1±7.11
Blood	4.88±1.39	1.33±0.257	2.80±0.346	1.57±0.664	4.41±0.418	2.55±0.516
Spleen	0.529±0.124	0.114±0.118	0.274±0.0631	0.193±0.110	1.44±0.587	0.780±0.0878
Lungs	0.534±0.402	0.496±0.325	0.204±0.0454	0.138±0.0379	0.157±0.0247	0.0974±0.0139
Heart	0.0504±0.005	0.0181±0.00348	0.0404±0.00715	0.0234 ^a	0.0332±0.00344	0.0317±0.000950
Kidney	0.248±0.0583	0.125±0.0233	0.265±0.0367	0.125±0.0454	0.274±0.0505	0.221±0.0734
Brain	0.0213±0.00126	0.0215±0.000908	0.0169 ^a	0.0240 ^a	<LOQ ^b	<LOQ
Skin	4.34±0.995	5.49±2.95	3.89±3.00	3.80±1.47	3.75±2.11	5.23±4.08
Muscle	3.42±2.53	2.44±0.901	1.19±0.158	0.605±0.137	<LOQ	<LOQ
Adipose	0.324±0.143	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Bone	2.21±0.302	0.902±0.273	1.77±0.609	0.995±0.486	0.881±0.275	1.05±0.214
Stomach and Small Intestine	1.11±0.567	0.530±0.197	1.36±0.442	0.593±0.419	0.681±0.533	0.627±0.250
Cecum and Large Intestine	1.33±0.0429	1.49±0.599	3.63±1.58	1.15±0.563	0.167±0.0650	0.145±0.387
Pancreas	0.141±0.0523	0.109±0.0832	0.161±0.114	0.103±0.0475	0.0843±0.047	0.0797±0.037
Placenta	1.41±0.304	1.60±0.470	1.28±0.274	2.10±1.10	1.26±0.181	1.93±0.483
Fetus	0.529±0.0521	1.01±0.214	0.379±0.108	0.835±0.253	0.263±0.0333	0.751±0.0456

All data are reported as Mean ± SD (n= 3)

^aOnly one rat had levels above quantitation limit.

^b<LOQ = below limit of quantitation = quantitation limit; 0.0125 µg/g for tissues.

Table 4

Total percent recovery of administered dose 48 h following i.v. (1 mg/kg) or p.o. (10 mg/kg) of AgAc, 20 nm AgNP or 110 nm AgNP to pregnant rats.

Sample	AgAc		20 nm AgNP		110 nm AgNP	
	i.v.	p.o.	i.v.	p.o.	i.v.	p.o.
Tissues	17.7±6.90	0.601±0.198	12.9±4.01	0.53±0.29	26.95±4.97	0.430±0.159
Urine	0.0722±0.0244	<LOQ ^b	0.0297±0.0044	0.0268 ^a	<LOQ	<LOQ
Feces	38.9±11.2 ^c	88.4±20.9	9.68±5.94	27.2±8.97	5.97±2.62	92.7±6.76
Overall	56.7±5.96	89.0±20.7	22.6±9.30	27.7±8.73	32.92±6.96	93.1±6.66

All data are reported as Mean ± SD (n= 3)

^aOnly one rat had levels above the reportable limit

^b<LOQ = below limit of quantitation = quantitation limit; 0.050 µg/g for feces, 0.005 µg/g for urine, and 0.0125 µg/g for tissues.

^cData for feces from 0-48 h

Table 5

Concentration of silver ($\mu\text{g Ag/g}$) in tissues following p.o. administration (10 mg/kg) of AgAc, 20 nm AgNP or 110 nm AgNP to pregnant rats.

Tissue	AgAc		20 nm AgNP			110 nm AgNP		
	24 h	48 h	24 h	48 h	24 h	24 h	48 h	48 h
Liver	0.187 $\pm$ 0.106	0.324 $\pm$ 0.380	0.0592 $\pm$ 0.0293	0.0380 $\pm$ 0.0126	0.0205 $\pm$ 0.0150	0.0170 $\pm$ 0.00377		
Blood	<LOQ ^b	0.117 $\pm$ 0.0168	0.124 $\pm$ 0.0120	0.0646 $\pm$ 0.00519	<LOQ	<LOQ		
Spleen	0.0802 $\pm$ 0.0648	0.104 $\pm$ 0.0702	0.220 $\pm$ 0.0329	<LOQ	0.172 ^a	<LOQ		
Lungs	0.137 $\pm$ 0.0239	0.177 ^a	0.965 ^a	<LOQ	<LOQ	<LOQ		
Heart	0.0604 ^a	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ		
Kidney	0.461 $\pm$ 0.506	0.147 $\pm$ 0.0413	0.187 $\pm$ 0.0545	0.0868 $\pm$ 0.0136	0.104 $\pm$ 0.0387	0.0795 $\pm$ 0.0179		
Brain	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ		
Stomach and Small Intestine	1.55 $\pm$ 2.51	0.0726 $\pm$ 0.0286	0.0657 $\pm$ 0.0324	0.0307 $\pm$ 0.0141	0.584 $\pm$ 0.468	0.0193 $\pm$ 0.00657		
Cecum and Large Intestine	16.0 $\pm$ 25.6	0.400 $\pm$ 0.0248	3.76 $\pm$ 2.24	0.884 $\pm$ 0.750	3.02 $\pm$ 1.04	0.901 $\pm$ 0.449		
Placenta	0.751 $\pm$ 0.0249	0.919 $\pm$ 0.271	0.518 $\pm$ 0.0302	0.354 $\pm$ 0.307	0.201 $\pm$ 0.125	0.351 $\pm$ 0.106		
Fetus	0.0537 $\pm$ 0.000831	0.0663 $\pm$ 0.0165	0.0266 $\pm$ 0.00239	0.0207 $\pm$ 0.0180	0.0211 $\pm$ 0.00183	0.0353 $\pm$ 0.00848		

All data are reported as Mean $\pm$ SD (n= 3)

^a Only one rat had concentrations > quantitation limit.

^b <LOQ = below limit of quantitation = quantitation limit; 0.0125 $\mu\text{g/g}$ for tissues.

Table 6

Percent recovered of administered dose of silver following p.o. administration (10 mg/kg) of AgAc, 20 nm AgNP or 110 nm AgNP to pregnant rats.

Tissue	AgAc		20 nm AgNP		110 nm AgNP	
	24 h	48 h	24 h	48 h	24 h	48 h
Liver	0.0836±0.0628	0.134±0.150	0.0287±0.0143	0.0168±0.00584	0.00967±0.00739	0.00753±0.00139
Blood	<LOQ ^b	0.0866±0.0117	0.0993±0.0127	0.0525±0.00494	<LOQ	<LOQ
Spleen	0.00165±0.00155	0.00233±0.00116	0.00540±0.00181	<LOQ	0.00402 ^a	<LOQ
Lungs	0.00403±0.000107	0.00628 ^a	0.00316 ^a	<LOQ	<LOQ	<LOQ
Heart	0.00229 ^a	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Kidney	0.0286±0.0307	0.00785±0.00210	0.0123±0.00380	0.00535±0.000782	0.00631±0.00260	0.00444±0.00107
Brain	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Stomach and Small Intestine	0.524±0.841	0.0270±0.007	0.276±0.175	0.144±0.048	0.239±0.214	0.0079±0.004
Cecum and Large Intestine	7.06±11.48	0.139±0.084	15.0±12.28	2.37±1.95	0.975±0.130	0.314±0.151
Placenta	0.112±0.00432	0.161±0.0216	0.0908±0.00605	0.116±0.00232	0.0372±0.0258	0.0638±0.0104
Fetus	0.0531±0.000121	0.0863±0.0244	0.0230±0.00103	0.0486±0.00456	0.0205±0.00326	0.0511±0.00691

All data are reported as Mean ± SD (n= 3)

^a Only one rat had concentrations > quantitation limit.

^b <LOQ = below limit of quantitation = quantitation limit; 0.0125 µg/g for tissues.

Table 7

Markers of cardiovascular injury from serum of pregnant rats at 24 or 48 h following i.v. or p.o. administration of AgAc, 20 or 110 nm AgNP.

Sample	Exposure duration	Route	PAI-1 (mean $\pm$ std.)	vWF (mean $\pm$ std.)
Vehicle	24 h	i.v.	1,314 $\pm$ 156	222 $\pm$ 49.6
	48 h		1,632 $\pm$ 1073	272 $\pm$ 236
AgAc	24 h		1,328 $\pm$ 437	258 $\pm$ 135
	48 h		970 $\pm$ 266	502 $\pm$ 167
20 nm AgNP	24 h		1,723 $\pm$ 482	291 $\pm$ 77.3
	48 h		759 ^b	322 ^b
110 nm AgNP	24 h		62.1 $\pm$ 87.9	126 $\pm$ 58.2
	48 h		406 $\pm$ 72.9	88.1 $\pm$ 77.1
Vehicle	24 h	p.o.	315 $\pm$ 68.4	71.5 $\pm$ 101
	48 h		406 $\pm$ 73	88.1 $\pm$ 77.1
AgAc	24 h		398 $\pm$ 53.3	119 $\pm$ 29.6
	48 h		151 $\pm$ 95.6	26.5 $\pm$ 41.7
110 nm AgNP	24 h		307 $\pm$ 56.2	ND ^a
	48 h		265 $\pm$ 116	6.73 $\pm$ 11.7

All data are reported as Mean $\pm$ SEM ng/ml plasma of duplicates from n = 3.

^aND = not detected

^bn=1 (for vehicle – Error for all readings on rats 2 and 3, for 20 nm AgNP 48 h – only one sample analyzed.)

Table 8

Cytokine/Chemokine expression for pregnant rats exposed i.v. to AgAc, 20 nm AgNP and vehicle after 24 or 48 h. All data are reported as mean $\pm$ SEM pg/ml of plasma of duplicates.

Cytokine	Vehicle		AgAc		20 nm AgNP	
	24 h	48 h	24 h	48 h	24 h	48 h
Eotaxin	146 ^b	336 $\pm$ 475	ND	ND	ND	55.3 ^b
IL-1 β	ND ^a	ND	ND	ND	ND	ND
IL-4	263 ^b	779 $\pm$ 1,072	44.5 $\pm$ 77.2	ND	ND	ND
IL-5	ND	ND	ND	ND	ND	ND
IL-6	489 ^b	3,561 $\pm$ 5,036	91.5 $\pm$ 158	4.66 $\pm$ 8.07	30.3 $\pm$ 27.2	ND
MCP-1	78.1 ^b	57.7 $\pm$ 81.6	57.4 $\pm$ 52.1	42.2 $\pm$ 36.5	62.8 $\pm$ 42.7	ND
TNF- α	ND	ND	ND	ND	ND	ND
VEGF	ND	ND	ND	ND	ND	ND

All data are reported as Mean $\pm$ SD pg/ml of plasma of duplicates (n= 3)

^aND = not detected

^bn=1 (for vehicle – Error for all readings on rats 2 and 3, for 20 nm AgNP 48 h – only one sample analyzed.)

Table 9

Library matched metabolites that are deemed to be important for differentiating pregnant rats administered a single i.v. (1 mg/kg) dose of 110 nm AgNP from vehicle at 48 h. Metabolites often fall in multiple bins, which is noted by the parenthesis.

Library Matched Metabolite	AgAc vs Vehicle		20 nm AgNP vs Vehicle		110 nm AgNP vs Vehicle	
	VIP ^a Range	Fold Change range ^b	VIP Range	Fold Change range	VIP Range	Fold Change range
1-Methylnicotinamide	1.4	(-1.7)				
1-Methylnicotinamide Lactose Trigonelline	1.3	(-1.1)	1.6	(-1.2)	1.1	(-1.2)
2-Oxoglutarate (1-2 bins)			3.1	1.2	3.7 – 3.9	(-2.3) – (-2.1)
3-Hydroxyphenylacetate (1-2 bins)	1.1	(-1.9)	1.1 – 1.2	(-2.8) – (-2.7)		
3-Hydroxyphenylacetate N-acetylglutamine	1.7	(-1.3)	2.1	(-1.7)	1.0	(-1.3)
Acetate	3.8	1.4			4.0	2.9
Acetoacetate			1.0	1.1	2.3	1.7
Acetoin 2-Hydroxyisobutyrate			1.0	(-1.2)		
Allantoin					3.5	(-1.9)
Choline			1.0	(-1.1)		
Citrate (1-2 bins)	1.4	(-1.3)	4.9	1.3	1.1 – 4.4	(-1.5) – 1.5
Creatinine			2.2	(-1.1)	2.1	(-1.3)
Dimethylamine					3.1	(-2.3)
Dimethylsulfone			1.4	(-1.2)		
Formate	1.2	(-1.7)				
Glucose					1.3	(-1.4)
Glucose Lactose			1.5	(-1.3)	1.0	(-1.3)
Glucose Sucrose			1.3	(-1.1)		
Glutamine	3.4	(-1.6)	3.1	(-1.8)	1.9	(-1.7)
Hippurate (3 bins)			2.6 – 3.8	1.6		
Hippurate Glycolate	3.0	1.1	3.7	1.2		
Homocystine	2.2	(-1.3)	2.7	(-1.8)		
Isoleucine			1.1	(-1.1)		
Lactate			1.8	(-1.2)	1.8	1.5
Leucine Isoleucine Fatty acids	1.4	1.1				
Methionine					1.3	1.7
N-Acetylglutamine			1.6	(-1.2)		
N-Acetylglutamine Glutamine Methionine			1.1	(-1.1)	1.1	(-1.2)
N-Acetylglycine N-Acetylaminocids			2.2	(-1.1)		
Proline (1-2 bins)	1.3	(-1.1)	1.3	-1.1	1.2	(-1.3)
Succinate					4.1	3.6
Sucrose			1.1	(-1.1)		
Taurine Betaine TMAO	3.1	(-1.1)			2.6	(-1.4)
Trans-Aconitate	1.4	(-1.2)				
Unknowns (5 - 13 bins)	1.1 – 1.6	(-1.8) – (-1.1)	1.0 – 1.4	(-2.2) – 1.3	1.1 – 2.4	(-8.3) – 2.0

^aVIP = variable importance for projection

^bA negative fold change means that the vehicle median was higher than the AgNP median, while a positive fold change means that the AgNP median was higher than the vehicle median.

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Table 10

Library matched metabolites that are deemed to be important for separating pregnant rats administered a single p.o. (10 mg/kg) dose of 20 nm AgNP from vehicle at 48 h. Metabolites often fall in multiple bins, which is noted by the parenthesis.

Library Matched Metabolite	AgAc vs Vehicle		20 nm AgNP vs Vehicle		110 nm AgNP vs Vehicle	
	VIP ^a Range	Fold Change range ^b	VIP Range	Fold Change range	VIP Range	Fold Change range
1-Methylnicotinamide Lactose Trigonelline	1.2	(−1.2)			1.5	(−1.1)
2-Oxoglutarate (2 bins)			3.1 – 3.3	1.5 – 1.6		
3-Hydroxyphenylacetate					1.0	(−1.5)
3-Hydroxyphenylacetate N-Acetylglutamine			1.5	(−1.6)	1.3	(−1.2)
Acetate	5.5	2.3	1.3	(−1.3)		
Acetoacetate trans-Aconitate			2.3	(−1.2)		
Alanine	1.1	1.1			1.1	1.0
Allantoin	3.3	(−1.7)			1.3	(−1.6)
Benzorate (2-3 bins)	1.7 – 2.5	2.9 – 11.5			1.2 – 1.8	1.3 – 2.7
Citrate (2 bins)			4.9 – 5.6	(−1.4)		
Dimethylsulfone			1.0	1.1	1.7	1.1
Glucose	1.6	(−1.4)				
Glucose Lactose			1.1	1.2		
Glucose Sucrose	2.7	1.8	1.4	1.2		
Glucose Taurine	2.2	(−1.5)				
Hippurate (3 bins)					2.9 – 4.4	1.6
Hippurate Glycolate					4.4	1.2
Homocystine					1.7	(−1.2)
Isoleucine	1.0	1.1				
Lactate (2 bins)	1.8 – 3.5	1.2 – 2.0	1.1	1.1		
Lactose Glucose	2.3	1.9				
Leucine Isoleucine Fatty acids			1.7	1.3	2.0	1.2
N,N-Dimethylglycine	2.1	1.4				
N-Acetylglutamine	1.5	(−1.1)	1.5	(−1.2)	1.8	(−1.1)
N-Acetylglycine N-Acetylaminoc acids			1.2	(−1.0)		
Proline			1.0	(−1.1)	1.3	1.1
Succinate	4.9	2.0	2.8	(−1.6)		
Sucrose			1.5	1.3		
Sucrose Creatinine			1.6	1.2		
Trans-Aconitate			1.2	(−1.4)		
Valine/Leucine			1.4	1.4		
Unknowns (3-8 bins)	1.0 – 2.8	(−1.6) – 1.5	1.1 – 3.4	(−1.1) – 1.2	1.4 – 2.1	(−1.2) – 1.2

^aVIP = variable importance for projection

^bA negative fold change means that the vehicle median was higher than the AgNP median, while a positive fold change means that the AgNP median was higher than the vehicle median.

Table 11

List of the most perturbed pathways in the library matched metabolomics analysis and the metabolites that distinguish the AgAc, 20 nm or 110 nm AgNP and the vehicle control 48 h post i.v. or p.o. exposure.

Treatment	Route	Most perturbed pathway	p-Value	Pathway metabolites that differ from silver and vehicle
AgAc vs Vehicle	i.v.	Ala,Ser,Cys,Met,His,Pro,Gly,Glu, and Gln metabolism and transport	9.0×10^{-5}	<ul style="list-style-type: none"> • Betaine • Glycolic acid • L-Glutamine • L-Proline
	p.o.	Carbohydrate metabolism: Sucrose metabolism and transport	5.4×10^{-4}	<ul style="list-style-type: none"> • D-Sucrose • D-Glucose • Lactose
20 nm AgNP vs Vehicle	i.v.	Ala,Ser,Cys,Met,His,Pro,Gly,Glu, and Gln metabolism and transport	3.6×10^{-6}	<ul style="list-style-type: none"> • 2-Oxoglutaric acid • Glycolic acid • L-Glutamine • L-Methionine • L-Proline
	p.o.	TCA metabolism and transport	2.1×10^{-9}	<ul style="list-style-type: none"> • 2-Oxoglutaric acid • Citric acid • Succinic acid
110 nm AgNP vs Vehicle	i.v.	TCA metabolism and transport	2.1×10^{-9}	<ul style="list-style-type: none"> • 2-Oxoglutaric acid • Citric acid • Succinic acid
	p.o.	Alanine, Glycine, and Cysteine metabolism and transport	3.6×10^{-6}	<ul style="list-style-type: none"> • Glycolic acid • L-Alanine • L-Leucine

Ala (Alanine), Ser (Serine), Cys (Cystine), Met (Methionine), His (Histidine), Pro (Proline), Gly (Glycine), Glu (Glutamic acid), Gln (Glutamine)