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Interference of silver nanoparticles with essential metal homeostasis in a novel enterohepatic fish *in vitro* system

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Silver nanoparticle toxicity has been extensively studied in several vertebrate cells. Its interaction with cellular essential metals homeostasis, however, has largely been overlooked. In this study, we used a novel *in vitro* model of the fish enterohepatic system to investigate the effect of citrate coated AgNP (cit-AgNP) and AgNO₃ on the homeostasis of copper, iron and zinc. The intestine and the liver are key tissues for whole body absorption and processing of metals. The enterohepatic system is based on a co-culture of intestinal cells (rainbow trout gut, RTgutGC) grown on permeable supports and hepatic cells (rainbow trout liver, RTL-W1) grown in the sub-located well. We have investigated early responses to sub-toxic and toxic doses of cit-AgNP and AgNO₃. Viability assays indicated that lysosomes were a target of cit-AgNP. Moreover, in comparison to AgNO₃, cit-AgNPs elicit a similar but attenuated metal stress response (induction of MT mRNA and ATP7A protein trafficking). Metal quantification revealed that, while intestinal cells accumulated similar amounts of silver following non-toxic exposure to equivalent amounts of either AgNO₃ or cit-AgNP, cells exposed to AgNO₃ excreted significantly more Ag to the basolateral chamber resulting in higher Ag accumulation in RTL-W1 cells. In addition, application of toxic doses of AgNO₃ resulted in a reduction of intracellular zinc and iron. Silver nanoparticles were detected by STEM/EDX in RTgutGC after 3 hours of exposure but not after 24 hours suggesting rapid intracellular dissolution. Thus, Ag is a potent disruptor of essential metals homeostasis and cit-AgNP, which tend to be more difficult to excrete by the cell, can prolong this effect.

Keywords: Metal homeostasis, *in vitro*, STEM/EDX, ATP7A trafficking, Metallothionein

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Environmental Significance Statement: Our knowledge of the uptake and processing of silver nanoparticles (AgNP) at the fish intestine is limited due to lack of an appropriate model to study interactions at the cell/medium interface. The intestinal uptake is important especially considering the emerging use of AgNP in food supplements, food packaging and as a disease control agent in aquaculture. Here we show that silver and AgNP accumulate in intestinal cells, inducing a rapid molecular response, which results in silver ions detoxification processes but also in a reduction in intracellular essential trace elements such as zinc and iron. This effect should be considered when evaluating potential long-term effects of silver and AgNP exposures.

1. Introduction

Silver is much less toxic to humans than it is to bacteria. This difference in sensitivity is at the root of its widespread use in several consumer products. In addition, the antibacterial properties of silver in nanoparticle form is currently used in several products. These include medical applications, food supplements and food packaging, which implies the possibility of dietary uptake¹. The use of AgNP have also been reported in aquaculture as a means to preventing diseases^{2,3}. Uptake of AgNP can also occur via accidental exposure in polluted waters and fish were shown to accumulate significant amounts of silver in long-term AgNP mesocosm exposures⁴. However, uptake of silver and AgNP at the fish intestine is currently poorly understood due lack of an appropriate model to study interactions at the cell/medium interface⁵. The implications of silver exposure and the extent to which silver and AgNP are toxic to humans and the environment is still under debate⁶⁻⁸. For example, although it is evident that toxicity of AgNP relates to its capacity to release Ag ions which, in turn, results in oxidative stress⁹⁻¹³, its role in affecting the homeostasis of essential trace metals has only recently been considered.^{14,15} – Disruption of essential metal homeostasis might be an important mechanism of toxicity of such particles.

Non-essential metals, such as silver, enter cells by hijacking systems evolved to uptake essential metals¹⁶. Specifically, the uptake and intracellular metabolism of silver is inextricably linked to that of copper in virtually all species from bacteria to humans⁶. Molecular mechanisms shared by silver and copper include uptake via copper transporter 1 (CTR1)^{17,18}, excretion via Cu-ATPases^{19–21}, and blood transport via the same plasma proteins²². Most importantly, silver can substitute copper in cupro-enzymes thereby inhibiting their activity (e.g. Ceruloplasmin^{23–25} and the Cu/Zn-SOD^{26,27}). In mammals, silver has also been shown to disrupt the homeostasis of other essential trace elements such as zinc¹³, iron²⁸ and selenium¹¹. In fish, silver has been shown to be particularly toxic by interfering with chloride and sodium uptake^{29,30}. The latter effect has also been shown specifically for AgNP in rainbow trout (*Oncorhynchus mykiss*) *in vivo*³¹ and *in vitro*³².

While uptake and homeostasis of essential and non-essential metals such as copper and silver are tightly regulated by copper transporters^{9,35,36}, metal nanoparticles seem to bypass such mechanisms and enter the cell via endocytosis^{5,32,35,36}. Therefore, AgNP uptake might be more difficult to control by the cell, resulting in the entry of a “big cargo” of zero-valent AgNP into cellular vesicles. Once inside the cell, AgNP seem to be directed to lysosomes whose internal milieu is characterized by an acidic pH that likely induces dissolution of silver from the NP surface^{5,32}. It has been repeatedly shown that factors affecting ions dissolving from NPs, such as silver (Ag) ions in the case of AgNP or cobalt in the case of tungsten-carbide cobalt-NPs, are the cause of toxicity but cannot fully explain the observed biological effects^{6,37}. Silver nanoparticle dissolution and speciation of the dissolved metal in the exposure media is an important determinant of toxicity for aquatic species where the NP is not taken up, or the internalization is minimal, such as algae³⁸ and fish embryo^{39,40}. However, the nature of the NPs interaction at the gill or intestinal epithelia of fish results in internalization via endocytosis, which minimizes the influence of extracellular AgNP chemical modifications and dissolved metal speciation. Indeed, modulation of chloride or cysteine concentrations in the exposure media, affected the toxicity of ionic silver (AgNO₃) but not the toxicity of AgNP in intestinal fish

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Environmental Science: Nano Accepted Manuscript

cells⁵. Therefore, the behavior of AgNP in the extracellular environment cannot fully predict its bioavailability and ensuing toxicity.

Determination of the exposure scenarios that enhance or reduce NPs uptake, bioavailability, and toxicity is often difficult. This is due to the lack of appropriate biological models to study such phenomena. To allow investigations at the NP/cell interface we have used a model of the fish intestine derived from rainbow trout, the RTgutGC cell line^{41,42}. RTgutGC is a unique intestinal fish cell line, which, when grown on permeable supports (transwells), forms a cell monolayer dividing the system into an upper (apical) and a lower (basolateral) compartment, thereby mimicking the intestinal lumen and the portal blood, respectively (Figure 1 and^{42,43}). RTgutGC cells grown on transwells develop a polarized epithelium characterized by the expression of tight junction (ZO-1) and Na/K-ATPase proteins at the apical and basolateral membrane respectively⁴². Most importantly, RTgutGC cells tolerate exposures to well-defined synthetic simplified media that mimic different possible environmental scenarios (e.g. pH, salinity, presence of proteins, etc.) occurring at the intestinal lumen, thus allowing the study of silver and AgNP interactions with the media components and, once internalized, with the intracellular environment (Figure 1 and^{5,42}).

This paper's focus is on the effect of silver in its ionic- (AgNO_3) and nano- (citrate-coated silver nanoparticles; cit-AgNP) form on the homeostasis of essential trace metals – copper, zinc and iron using RTgutGC cells grown on permeable supports in combination with fish hepatic cells (rainbow trout liver, RTL-W1⁴⁴) grown in the sub-located well. This multi-cellular system represents a novel model of the fish enterohepatic system which allows the evaluation of the effect of silver compounds in the intestinal cells and on liver cells following intestinal processing. Moreover, we show the specific interference by silver on the homeostasis of copper, through the monitoring of the Cu-ATPase protein trafficking in a mammalian intestinal model, the colorectal adenocarcinoma cells (Caco2).

2. Experimental

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2.1 Nanoparticles characterization

Citrate coated silver nanoparticles (cit-AgNPs) were purchased as aqueous suspension with a concentration of total silver of 1 g/L (9.27 mM, pH 6.46, nominal size: 19 nm, NanoSys GmbH, Switzerland). Extensive characterization of the same cit-AgNP in the same exposure solutions used in this study is described elsewhere^{5,45}. Cit-AgNP dispersed in nanopore water (16-18 MΩcm⁻¹; Barnstead Nanopure Skan AG, Switzerland) show a hydrodynamic diameter, measured by Dynamic Light Scattering, that is stable at ~30 nm and the ζ-potential is -35 mV. In the exposure media L-15/ex and L-15/FBS (see below), cit-AgNP form agglomerates of ~540 and ~230 nm after 3 hours and of ~1100 and ~250 nm in size after 24 hours and possess ζ-potentials of -18 and -9 mV, respectively⁵. In the exposure media DMEM, cit-AgNP form agglomerates of ~355 nm after 3 hours and possess ζ-potentials of -20 mV. A scanning transmission electron microscope (STEM, Hitachi HD-2700) was used to perform the Energy dispersive X-ray spectroscopy (EDX) analyses of cit-AgNP dispersed in nanopure water (Figure S1).

2.2 Cell lines and culture media

Routine cell culture: The RTgutGC cells were established by Kristin Schirmer, in the laboratory of Niels C. Bols (University of Waterloo). The RTL-W1 were provided by N.C. Bols. RTgutGC and RTL-W1 cells were routinely cultured as described in ⁴¹ and ⁴⁴, respectively. Briefly, cells were grown in 75 cm² flasks (TPP, Trasadingen, Switzerland) in Leibovitz's L-15 medium without phenol red (Invitrogen, Basel, Switzerland) supplemented with 5% Foetal Bovine Serum (FBS, Gold, PAA Laboratories GmbH, Pasching, Austria) and 1 % penicillin/streptomycin (Sigma-Aldrich, Buchs SG, Switzerland; 10000 U/ml penicillin, 10 mg/ml streptomycin). We will refer to this media as L-15/FBS. Cells were maintained at 19°C in normal atmosphere and split into three flasks once confluent, which was every 2 to 3 weeks. Confluent flasks were washed twice with Versene

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(Invitrogen/Gibco, Basel, Switzerland) and cells were detached using trypsin (0.25% in phosphate-buffer saline, PBS, Biowest, Nuaille, France). Human intestinal cells, Caco-2, were purchased from ATCC (ATCC® HTB-37™) and grown in D-MEM medium with phenol red (Gibco/Invitrogen, Basel, Switzerland) supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were maintained at 37°C and 5% CO₂. Sub-culturing was performed as for RTgutGC and RTL-W1 but every 3 days.

Cell culture on permeable membranes: RTgutGC and Caco-2 cells were seeded onto commercially available transparent tissue culture inserts for multiwell plates (pore size = 0.4 µm; polyethylene terephthalate [PET] from Greiner bio-one, Frickenhausen, Germany). Insert size was chosen depending on the application: inserts for 24-well plates (0.33 cm² surface area) were used for cell viability, confocal microscopy and electron microscopy; inserts for 12-well plates (1.13 cm² surface area) for quantitative RT-PCR (qPCR) analyses; and inserts for 6-well plates (4.25 cm² surface area) for silver and cit-AgNP quantification. Previous to cell seeding, to enhance cell attachment, inserts were incubated at room temperature for 3 hrs (hrs) with fibronectin (50 µg/mL; Roche Applied Science, Basel, Switzerland) in PBS (Bioswissstec AG, Schaffhausen, Switzerland), then washed once with PBS⁴². To initiate cultures on inserts, RTgutGC and Caco-2 cells, dislodged by trypsin, were re-suspended in respective growth medium and added at a density of 62500 cell/cm². The insert well (or top compartment) is referred to as the “apical chamber” whereas the sub-located well (or bottom compartment) as “basolateral chamber” (Figure 1). Apical chamber was filled with 0.3, 1 and 3.5 mL of growth medium (L-15/FBS), for 24-, 12- and 6-well inserts, respectively. These were then placed in the corresponding culture wells (referred to as basolateral compartment), which were subsequently filled with, respectively, 1, 1.7 and 4 mL of L-15/FBS. The media was changed weekly for RTgutGC and every 48 hrs for Caco-2 cells. It was determined that confluent and polarized epithelia would form in 3 weeks for RTgutGC⁴² and 2 weeks for Caco-2 cells⁴⁶, after which the epithelia are defined as developed.

Co-culture RTgutGC/RTL-W1: Co-cultures were obtained by seeding 150000 RTL-W1 cells/cm² in the bottom of the basolateral chamber (e.g. 12-well plates; Greiner Bio-ONE, Frickenhausen, Germany). Previous to exposure, RTL-W1 were incubated for 48 hrs in L-15/FBS. Then monolayers were washed twice with PBS and the exposure medium L-15/ex was applied⁴⁷. Subsequently, developed RTgutGC epithelia grown on inserts were washed likewise and mounted on top (Figure 1).

TEER Measurement: The development of a confluent intestinal epithelium was monitored daily through 'blank'-corrected measurements of transepithelial electrical resistance (TEER) using an epithelial tissue voltohmmeter (EVOMX; World Precision Instruments) fitted with chopstick electrodes (STX-2). As blank controls, empty (no cell) inserts, treated identically to the inserts containing cells, were used. A TEER of ~30 or ~250 $\Omega \times \text{cm}^2$ was used as a criterion for the presence of a sufficiently tight epithelium for RTgutGC^{42,43} or Caco2 cells⁴⁶, respectively.

2.3 Exposures of cells to silver

Experimental solutions were prepared freshly in the respective cell exposure media. Silver nitrate (Sigma-Aldrich, Buchs SG, Switzerland) stock solution was prepared at a concentration of 10 mM in nanopure water and diluted right before each experiment in the appropriate cell exposure medium. Silver nitrate (AgNO_3) and cit-AgNP stock solutions were prepared to contain identical nominal concentrations of total silver. Exposure media were L-15/FBS or L-15/ex. L-15/ex has an identical composition in salts, glucose and pyruvate to L-15 but lacks amino acids and vitamins⁴⁷. To initiate exposure, the developed RTgutGC cell epithelium was first washed twice with either L-15/FBS or L-15/ex depending on the planned exposure conditions. Subsequently, for the viability assay 0.3 mL per transwell of cit-AgNP or AgNO_3 suspensions were applied at concentrations ranging from 0.1 to 500 mM in the respective media. RTL-W1 were exposed only indirectly i.e. in the basolateral chamber (see Figure 1). For the qPCR and metal analysis 1 and 3.5 mL of cit-AgNP or AgNO_3 suspensions were added to the 12 and 6 well transwell in the respective media. Cells were exposed to

a non-toxic concentration of 1 μM of cit-AgNP or AgNO_3 in L-15/ex and L-15/FBS or at toxic (EC20) concentrations of 3.4 μM AgNO_3 and 10 μM cit-AgNP in L-15/ex as determined by the viability assay (see below). The exposure of cell analyzed by electron or confocal microscopy in indicated below. Cells were incubated in the dark at 19 $^{\circ}\text{C}$ for 3 or 24 hrs depending on the assay to be performed (see below).

2.4 Cell viability assays

Assessment of cell viability was accomplished by means of the fluorescent cell viability indicator dyes, alamar Blue (AB, Invitrogen, Basel, Switzerland), CFDA-AM (Invitrogen, Basel, Switzerland) and Neutral Red (NR, Sigma-Aldrich, Buchs SG, Switzerland), as previously described⁴⁷. Responses of AB and CFDA-AM were comparable and therefore only AB data are shown. Alamar blue is a cellular marker of cellular metabolic activity and Neutral Red of lysosome membrane integrity⁴⁸. Cell viability was assessed on RTgutGC cells cultured in inserts for 24 well plates (0.33 cm^2) and RTL-W1 seeded in the bottom of the basolateral chamber. At the time of assessment (3 or 24 hrs), the exposure medium was aspirated from both the apical and basolateral compartment and cells washed once with PBS. Then, 100 and 400 μL of AB, at 5% (v/v) in PBS, was applied to the apical and basolateral chamber, respectively. Cells were incubated in the dark at 19 $^{\circ}\text{C}$ for 30 min and then fluorescence recorded with a Tecan Infinite M200 multiwell plate reader (Maennedorf, Switzerland) at an excitation/emission wavelength of 530/595. To account for the small area of the insert and the fact that the position of inserts may shift slightly in the wells, readings were adjusted so as to measure 21 points, of which an estimated nine central measurements covered the insert surface area. These were averaged for analysis assuming that they best reflect the fluorescence in the centre of the inserts, which was supported by a very small variation among these nine values⁴². Afterward, the AB working solution was discarded and 400 μL of NR solution, containing 1.5% v/v NR in PBS, was added and incubated for 120 min. Then, cells were fixed with 400 μL fixative (0.5% v/v

formaldehyde and 1% w/v CaCl_2). Finally, NR was extracted from the lysosomes using 400 μL of an extraction solution (1% v/v acetic acid and 50% v/v ethanol) and gently shaken on a horizontal shaker (TiMix 2, Johanna Otto GmbH, Hechingen, Germany) for 10 min. NR fluorescence was measured at excitation/emission wavelengths of 530/645nm using the same plate reader.

2.5 Metal analyses

Metal quantification was assessed on RTgutGC cells cultured on inserts (4.25 cm^2) and in RTL-W1 cells cultured underneath in 6 well plates. After exposure, the basolateral and apical media were aspirated carefully, taking care to avoid carry-over from possible cit-AgNP sedimented on the cell monolayer, and transferred to an Eppendorf tube for silver quantification. Next, to ensure thorough removal of loosely bound silver, cells were washed twice with a solution of 0.5 mM Cysteine in PBS. Cells were then lysed by applying 0.5 mL of 50 mM NaOH to the well and incubated at room temperature for 2 hrs⁴⁹. An aliquot (100 μL) of the cells' lysate was used for protein quantification using the modified Lowry assay (Thermo Scientific, Waltham, USA) with bovine serum albumin as standard. For metal determination, media and cell lysates were desiccated using a concentrator (Concentrator Plus, eppendorf, Hamburg, Germany) and digested over-night by adding 0.8 mL of 65% HNO_3 . Acidified solutions were then re-suspended and transferred to a Teflon tube containing 0.2 mL of 30% H_2O_2 and digested further in a high-performance microwave digestion unit (MLS-1200 MEGA, Switzerland) at a maximum temperature of 195°C for 20 min. The digest was then diluted 10 times with nanopure water and measured. Metal concentration was measured in media and cells by ICP-MS (Element 2 High Resolution Sector Field ICP-MS, Thermo, Finnigan, Germany). The reliability of the measurement was determined using specific water references (M105A, IFA System, Tull, Austria). Metal concentration in cell lysates is reported as ng metal per mg of protein to take into account cell growth. Silver accumulation and transport across the intestinal epithelium is reported as absolute amount (i.e. ng Ag in the medium of the apical or basolateral compartment, and in the cell monolayer).

2.6 RNA extraction, cDNA synthesis and quantitative PCR (qPCR)

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Messenger RNA quantification was assessed in RTgutGC cells cultured on inserts (1.13 cm²) and in RTL-W1 cells cultured underneath in 12 well plates. RNA extraction, DNase treatment, cDNA synthesis and measurement of mRNA levels were performed as described previously⁵⁰. Briefly, after exposure, total RNA was extracted by adding 600 µL of TRIzol® Reagent (Invitrogen, Basel, Switzerland) directly onto the RTgutGC cell monolayer grown in inserts or onto the RTL-W1 cells. The quantity and quality of isolated RNA was determined by spectrophotometry with an ND-1000 Nanodrop (Labtech Int., East Sussex, UK) and by electrophoresis using 0.5 µg of total RNA in a 1% agarose gel. At each time point, total RNA was extracted from an untreated and a treated sample. Messenger RNA levels are reported as fold change of the treated from the untreated sample. Normalization was based on the geometric mean expression of two reference genes (β-actin and elongation factor 1α) determined using geNorm software⁵¹. Primer sequences are reported in Table S1.

2.7 Electron microscopy and Energy-dispersive X-ray spectroscopy

In order to prepare samples for electron microscopy, RTgutGC cells were cultured on inserts as indicated above. Developed RTgutGC epithelia were exposed to 50 µM cit-AgNP dispersed in L-15/ex for 3 hrs and to 10 µM cit-AgNP dispersed in L-15/ex and L-15/FBS for 24 hrs. All these exposures were not cytotoxic (data not shown). Previous to fixing, cell monolayers were washed as described in the metal analysis section. The PET membrane containing the cells was carefully excised from the insert. The samples were fixed in phosphate-buffered 2.5 % glutaraldehyde, postfixed in 1% osmium tetroxide and block stained with 1% uranyl acetate, then dehydrated in a graded ethanol series and finally embedded in Epon-resin (EMbed 812, EMS). After polymerization at 60°C, ultrathin sections of about 100 nm thickness were cut on an ultra-microtome (Reichert Ultracut C) perpendicular to the cell monolayer. Due to different material properties of resin and

membrane, some folding occurred (see Figures 5 and 6). For scanning electron microscopy (SEM) the ultrathin sections were transferred onto silicon chips. No post-staining was applied. Micrographs were taken on a FEI Magellan 400 at 2kV by backscatter electron detection. Large area scans were performed by stitching individual image tiles (FEI Maps software). A scanning transmission electron microscope (STEM, FEI Tecnai F30ST) was used to acquire electron micrographs and the energy dispersive X-ray (EDX) spectroscopy measurements.

2.8 ATP7A trafficking

Evaluation of the copper efflux pump (ATP7A) function, via monitoring of its trafficking behaviour, was evaluated via imaging and co-localization of ATP7A with actin, or with the Trans Golgi Network (TGN). The human ATP7A antibody was kindly gifted by Dr Adam Southon (Melbourne University). The TGN marker, the antibody Golgin97 and the actin stain, rhodamine phalloidin, were purchased from Invitrogen, Basel, Switzerland. This assay was only possible in Caco2 cells because of antibody specificity. The trafficking assay in CaCo2 cells was performed as previously described⁵² including appropriate controls such as excess Cu (100 μ M CuSO₄) and Cu starvation via pre-exposure with the Cu chelator, bathocuproine disulphate (BCS). Developed Caco2 epithelia were exposed for 18 hrs in low serum media (DMEM, Invitrogen, Basel, Switzerland, supplemented with 5% FBS) containing 200 μ M of BCS prior to treatment with 200 μ M BCS, 100 μ M CuSO₄, 10 μ M AgNO₃ or 80 μ M cit-AgNP dissolved or dispersed in DMEM for 3 hrs. Toxicity in CaCo2 cells was evaluated as described for RTgutGC. All doses of exposure were not cytotoxic (data not shown). Following exposures, cells were prepared for confocal microscopy. Two inserts were washed twice with PBS and fixed by incubation in a solution of 3.7 % paraformaldehyde (Sigma-Aldrich, Buchs SG, Switzerland) in PBS for 15 min at room temperature. Permeabilization was achieved by incubating the cells in a solution of 0.2% (v/v) Triton (Sigma-Aldrich, Buchs SG, Switzerland) in PBS for 30 minutes (min) at 4°C. The “image-it” solution (Molecular Probes, Invitrogen, Basel, Switzerland) was used for blocking nonspecific signals following manufacturer instructions. Primary

antibodies were diluted (ATP7A 1:400; Golgin97 1:200) in 1% goat sera and 0.05 % Triton in PBS. Alexa Fluor® 488-anti-rabbit antibody and Alexa Fluor® 635-anti-mouse antibody were used at a 1:200 dilution as secondary antibodies for ATP7A and Golgin97, respectively. Primary antibody incubation was performed overnight at 4°C, while incubation with secondary antibody and the f-actin staining (Rhodamine-phalloidin, Invitrogen, Basel, Switzerland), applied at 2 Unit/mL in PBS, was done for one hour at room temperature. Nuclear counter staining was achieved by incubating cells with 300 nM DAPI (Invitrogen, Basel, Switzerland) for 5 min at room temperature. Finally, the PET membrane holding the cells was excised and mounted on a microscope slide (Thermo Scientific, Zug, Switzerland) covered with ProLong Antifading solution (Invitrogen, Basel, Switzerland). Imaging was performed using a Leica SP5 (Wetzlar, Germany) upright confocal laser-scanning microscope, equipped with the acquisition software LAS AF 2.6.v. The 63X OIL (NA 1.4) objective was used. Images at each wavelength (405 nm for DAPI, 488 nm for Alexa488 conjugated secondary antibody, 543 nm for Rhodamine-phalloidin and 633 nm for the Alexa635 conjugated secondary antibody) were acquired sequentially. Signals from five Fields Of View (FOVs; 5337±350 µm²) were analyzed by the co-localization module of the LAS AF 2.6v software. The ATP7A trafficking experiment was performed three times.

2.9 Statistical analyses

Statistical analysis was performed using GraphPad Prism Version 5 (GraphPad Software, USA). Fluorescent units obtained in the cell viability assays were converted to percentage viability of control cells. Concentrations leading to 50% reduction in viability (EC₅₀) were determined by the nonlinear regression sigmoidal dose-response curve fitting module using the Hill slope equation. EC values are presented as mean and 95% confidence intervals. Difference between curves was assessed by F-test ($p < 0.05$). Statistical analysis for multiple groups of data was performed by analysis of variance (ANOVA) followed by Tukey's *post hoc* test. When comparing to a control group,

Dunnett's test was applied after the ANOVA. Values of $p < 0.05$ were considered statistically significant.

3. Results and Discussion

3.1 Citrate coated AgNP characterization in exposure medium

Characterization of the cit-AgNP (NanoSys GmbH, Switzerland) dispersed in the exposure media L-15/ex and L-15/FBS has been previously reported^{5,45}. Briefly, cit-AgNP agglomerate rapidly in L-15/ex reaching 1064 nm in size and a ξ -potential of about -18 mV in 24 hrs. On the other hand, in L-15/FBS, cit-AgNP agglomerate moderately to about 250 nm and show a ξ -potential of -9 mV. In DMEM without FBS after 3 hours, cit-AgNP agglomerate to about 360 nm and ξ -potential of -20 mV which is similar to their size and charge in L-15/ex. This data suggests that in the absence of FBS, the nanoparticle agglomeration can be mainly explained by the media ionic strength^{37,53}. In addition, elemental analysis by STEM/EDX verified the elemental composition of cit-AgNP (Figure S1).

3.2 Toxicity of silver compounds depends on media composition

The cit-AgNP toxicity was initially studied by measuring the viability of polarized RTgutGC cells after 24 hrs of exposure to either AgNO₃ or cit-AgNP at various equivalent silver concentrations and in two exposure media, in L-15/ex and L-15/FBS. Viability of RTL-W1 cultured under RTgutGC was also evaluated. For the sake of simplicity, we chose to express all our data, AgNO₃ and cit-AgNP exposures, in equivalents of total silver, in μ M, (Figure 2).

While silver nitrate is more toxic than cit-AgNP in L15/ex (5 and 22-fold when considering lysosome integrity or metabolic activity, respectively), an opposite scenario is shown when exposing cells in L-15/FBS (Table S1, Figure 2). This effect can be attributed to the scavenging properties of

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amino acids and proteins, present in L-15/FBS but not in L-15/ex, which have been shown to form thiolate complexes with silver ions⁵. Therefore, the exposure medium has an important role in mediating the toxicity of dissolved silver, but less so in mediating the toxicity of cit-AgNP. Silver NPs can enter the cell via endocytosis and release silver ions into intracellular vesicles such as lysosomes^{5,32,36,45,54} thus, reducing the scavenging effect of amino acids and proteins. In support of this hypothesis, lysosome membrane integrity measured via the Neutral Red assay is significantly more sensitive than metabolic activity in RTgutGC exposed to cit-AgNP (Figure 2 and³⁹). RTL-W1 exposed to cit-AgNP seeded in the basolateral chamber (under the polarized RTgutGC) were little impacted with a statistically significant reduction in viability of about 20 and 30% in metabolic activity only in cells exposed to 100 and 500 μM of cit-AgNP, respectively. Moreover, the response of RTL-W1 was similar to RTgutGC exposed to AgNO_3 , where the metabolic activity endpoint was more sensitive than lysosome integrity suggesting that ionic silver was the major cause of cytotoxicity.

According to the cell viability data, 1 μM of AgNO_3 or cit-AgNP was non-toxic for RTgutGC cells in both exposure media. A reduction of about 20% (EC20) in lysosome integrity was observed at 3.4 μM of AgNO_3 or 10 μM of cit-AgNP in L-15/ex. These exposure concentrations were selected to evaluate silver uptake and transcriptional responses in RTgutGC.

3.3 Uptake of silver nitrate and cit-AgNP

Uptake of silver in RTgutGC is independent of the silver form (i.e. ionic or nano) but is dependent on the exposure media type (i.e. L-15/FBS or L-15/ex) (Figure 3 and Table 1). RTgutGC exposed to 1 μM of silver equivalent in ionic- (AgNO_3) or nano- (cit-AgNP) form accumulated comparable levels of intracellular silver. However, exposures in L-15/ex resulted in a higher accumulation of silver (~ 3-fold, for both AgNO_3 or cit-AgNP) when compared to exposures in L-15/FBS (Figure 3,

Table 1). This effect is attributable to the stabilizing properties of FBS that resulted in less cit-AgNP precipitation and by cysteine complexation with Ag ions that might reduce uptake⁵.

Previous studies have shown that AgNP are able to enter epithelial cells and accumulate into cellular vesicle-like structures including lysosomes^{32,55–58}. In this study cit-AgNP entered and accumulated in RTgutGC cells in a dose dependent manner as indicated by ICP-MS analysis (Figure 3A).

However, nanoparticulate silver was detectable by STEM/EDX in a vesicle-like structure only in cells exposed for 3 hrs. In cells exposed for 24 hrs, nanoparticulate silver was not detectable by EDX (Figure 4; Figure S2). Therefore, our results support previous studies showing that AgNP can exert toxicity using the “Trojan horse” mechanism and dissolve rapidly intracellularly^{35,58,59}. Using an innovative approach that combined microscopy and X-ray absorption spectroscopy, Veronesi and co-authors⁵⁸, have shown that in hepatocytes following 24 hrs exposure to cit-AgNP, around 50% of silver was in nano-form (Ag⁰) and the remaining dissolved silver was complexed mainly to glutathione.

In addition, AgNO₃ appears to be transported across the RTgutGC epithelium into the basolateral chamber and absorbed by RTL-W1 cells more efficiently than cit-AgNP (Figure 3).

3.4 Intracellular effects of silver nitrate and cit-AgNP on metal homeostasis

RTgutGC cells exposed to toxic concentrations (EC20) of silver nitrate showed a marked reduction in the intracellular essential trace elements, zinc and iron, and possibly also copper (though not statistically significant; Table 1). This reduction in intracellular essential trace metals could be due to the activation of the Metal regulatory Transcription Factor 1 (MTF1) by intracellular silver^{60,61}, which has been shown to induce several essential metal excretory proteins, including the zinc transporter 1 (ZnT1), the iron-regulated transporter 1 (IREG1 or ferroportin-1) and the copper ATPase (ATP7A)⁶². Induction of ZnT1 was also measured in this study in RTgutGC cells, along with Metallothionein b (MTb), another target gene of MTF1, supporting the hypothesis that excess

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intracellular silver activates MTF1 (Figure 5). Alteration of copper homeostasis was moreover shown at the post-translational level via the monitoring of ATP7A trafficking in Caco2 cells (Figure 6). ATP7A is a pivotal protein in the regulation of cellular Cu homeostasis responsible for normal delivery of Cu to cupro-enzymes, occurring at the TGN, and excretion of Cu in conditions of excess Cu, occurring at the cell periphery. ATP7A was also shown to traffic to the cell periphery in response to intracellular silver excess⁶³. ATP7A cellular location is therefore indicative of intracellular Cu and Ag availability^{64,65}. It is therefore understandable why the trafficking of ATP7A is so important for its function and for the regulation of intracellular copper homeostasis. Indeed, in Menkes disease, defective ATP7A proteins lose their trafficking properties⁶⁴. In this study, the ratio of ATP7A co-localizing with the TGN or with actin as marker of cell periphery was lower in cells exposed to CuSO₄, used as a positive control²¹, and AgNO₃ (Figure 6). This indicates a more peripheral localization of ATP7A as expected in conditions of intracellular copper or silver excess^{21,65}. ATP7A also co-localized toward the periphery in cells exposed to cit-AgNP, although less than in cells exposed to AgNO₃. The ratios of co-localization of ATP7A with actin divided by the ratio of ATP7A with TGN were 3.0 and 1.8 for cit-AgNP and AgNO₃, respectively. A similar response, i.e. induction of trafficking of copper ATPases, was shown in human hepatocytes (HepG2) exposed to copper oxide NP⁶⁶, indicating that copper or silver NPs can induce the trafficking of these proteins in response to excess intracellular metal concentrations. Such response, induction of copper excretion via ATP7A trafficking, could explain the reduced serum copper and ceruloplasmin in human ingesting colloidal silver⁶⁷.

Measurement of MTb mRNA levels were an excellent marker of intracellular silver bio-reactivity (i.e. induction of a biological response; Figure 5). RTgutGC exposed to 1 µM silver equivalent (AgNO₃ or cit-AgNP) accumulated similar amounts of silver (Table 1). However, AgNO₃ exposed cells expressed about twice as much MTb mRNA levels than cells exposed to cit-AgNP (Figure 5) suggesting that cit-AgNP might be accumulated in a less bio-reactive form, i.e. metallic Ag⁰, and

remain in a non-bio-reactive form until dissolution occurs. The induction of MTb by cit-AgNP most likely occurs following dissolution into lysosomes which in turn results in lysosome damage⁵ and leakage of silver into the cytoplasm. Extracellular dissolution of cit-AgNP, estimated at 2% in L-15/ex⁴⁵, likely has a negligible effect on RTgutGC. Exposure to 200 nM of AgNO₃, an equivalent amount of silver dissolved from 10 µM of cit-AgNP, did not induce MTb expression (Figure 5). MTb was also induced 6.3-fold in RTL-W1 under RTgutGC exposed to 3.4 µM AgNO₃ in L-15/ex (Figure S4). This is in agreement with the higher transport of Ag to the basolateral compartment in these inserts (Figure 3). Excluding MTb, all other genes measured were not changed in their mRNA abundance compared to controls in RTL-W1 (not shown). The mRNA level of other genes involved in metal detoxification and homeostasis processes were measured, and in addition to MTb and ZnT1 also glutathione reductase (GR) was induced in cells exposed to 3.4 µM of AgNO₃ in L-15/ex and 1 µM of AgNO₃ and cit-AgNP in L-15/FBS, indicating an oxidative response. Messenger RNA of transporter proteins Divalent Metal Transporter 1, ATP7A and Na/K-ATPase (NAK) were not affected at the mRNA level in RTgutGC (Figure 5). In summary, cit-AgNP accumulated in RTgutGC cells, disrupting lysosome membrane integrity and increasing MTb mRNA levels, but did not induce a significant disruption of essential metal homeostasis as both intracellular essential metals and ATP7A trafficking were not significantly different from controls.

4. Conclusions

We reveal that silver is a potent disruptor of essential metal homeostasis. RTgutGC cells exposed to toxic concentrations of silver nitrate displayed a reduction in intracellular zinc, iron and copper. We hypothesized that this reduction might be due to the induction of MTF1 target genes including the zinc exporter ZnT1 and the copper exporter, ATP7A. Indeed, an increase of ZnT1 mRNA levels was shown in RTgutGC cells exposed to silver nitrate. Furthermore, we show that ATP7A function is

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Environmental Science: Nano Accepted Manuscript

induced, at the posttranslational level, by silver nitrate and by cit-AgNP, although the latter to a smaller extent. RTgutGC accumulated similar amounts of silver following exposure to equivalent amounts of total silver in cit-AgNP or AgNO₃ form. However, the latter was a more potent inducer of Metallothionein, and of the oxidative stress marker glutathione reductase as well as ZnT1 mRNA levels and of ATP7A protein trafficking. All together, these transcriptional responses in RTgutGC cells and ATP7A trafficking responses in Caco2 cells indicate that, in these intestinal cells, nanoparticulate silver exert a similar but attenuated response to ionic silver (AgNO₃). Metallic silver (Ag⁰) present in cit-AgNP might therefore not be bio-reactive until it is dissolved and leaks out of lysosomes. Furthermore, our viability and electron microscopy data show that cit-AgNP targets lysosomes.

The enterohepatic fish cell *in vitro* system allowed to elucidate of the effect of AgNO₃ and cit-AgNP in liver cells following the intestinal cells uptake and processing. Overall, the intestinal cell protected the liver cells from acute toxicity but did not prevent the transport of silver ions into the basolateral chamber. We believe that this novel *in vitro* system will be a useful tool for fish nutrition and toxicology.

Disturbance of essential metal homeostasis has been linked to several human diseases including neurodegenerative disease^{68–70} and metabolic diseases^{71,72}. Although such diseases are unknown in fish, disturbances of essential metal homeostasis have been shown to affect fish development and reproduction^{73–75} which could adversely affect fish populations. The disturbance of essential metal homeostasis by silver and AgNP should therefore also be considered in situations of low-concentration, chronic exposures for the protection of both human and environmental health.

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TABLES

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DOI: 10.1039/C9EN00310JTable 1: Intracellular metal content expressed as ng metal per mg of protein¹.

Exposure in L-15/ex				
RTgutGC	Ag	Fe	Zn	Cu
Control	< 0.01	55.0 ± 1.24	177 ± 12.3	4.00 ± 0.58
AgNO ₃ 1 µM	19.0 ± 3.50 ^b	50.0 ± 9.08	156 ± 11.7	3.70 ± 0.65
AgNO ₃ 3.4 µM	54.0 ± 11.4 ^b	34.1 ± 0.41*	120 ± 24.6*	2.86 ± 1.02
cit-AgNP 1 µM	21.3 ± 6.37 ^b	51.5 ± 8.30	174 ± 52.0	3.60 ± 0.77
cit-AgNP 10 µM	313 ± 104 ^a	49.6 ± 3.69	169 ± 19.8	3.64 ± 1.16
RTL-W1				
Control	< 0.01	107 ± 40.4	190 ± 40.6	5.52 ± 0.49
AgNO ₃ 1 µM	6.67 ± 5.79 ^b	91.7 ± 50.9	165 ± 63.7	7.45 ± 4.29
AgNO ₃ 3.4 µM	32.3 ± 16.7 ^a	106 ± 54.8	218 ± 36.7	5.96 ± 2.26
cit-AgNP 1 µM	1.69 ± 0.61 ^b	75.3 ± 16.2	137 ± 13.7	5.68 ± 1.31
cit-AgNP 10 µM	6.74 ± 3.69 ^b	79.0 ± 39.7	173 ± 14.3	4.50 ± 0.81
Exposure in L-15/FBS				
RTgutGC	Ag	Fe	Zn	Cu
Control	< 0.01	45.0 ± 1.4	77.7 ± 47.3	3.1 ± 2.2
AgNO ₃ 1 µM	6.45 ± 1.65	36.1 ± 19.3	64.2 ± 31.4	3.4 ± 2.4
cit-AgNP 1 µM	7.37 ± 7.32	34.4 ± 25.4	63.4 ± 33.1	3.8 ± 2.8

¹ Metal concentration in cell lysates is reported as ng metal per mg of protein determined from the same samples to take into account differences in cell numbers. Values represent mean ± SD from three or four independent experiments, N=3 or 4. Statistical comparisons are within each metal. For Ag, values bearing different lettering are significantly different (P< 0.05, 1-way ANOVA, Tukey test). For other metals, asterisk indicate difference from respective silver-free control (P< 0.05, 1-way ANOVA, Dunnet test).

FIGURES

Figure 1: Schematic representation of the experimental system. Shown in the apical chamber are the factors influencing Ag bioavailability for AgNO_3 and cit-AgNP (Derived from^{5,45}).

Figure 2: Toxicity of AgNO_3 and cit-AgNP as function of total silver in exposure media (L-15/FBS and L-15/ex). The endpoints measured, metabolic activity (Alamar blue) and lysosome integrity (Neutral Red), were recorded after 24 hrs of exposure. Values shown are averages and 95% confidence intervals (dashed lines) of three independent experiments (N=3). RTL-W1 cells, sitting in the basolateral chamber, were exposed to cit-AgNP in L15/ex RTgutGC cells seeded on permeable membranes in the apical chamber. EC50s (shown in Table S1) were determined by the non-linear regression sigmoidal dose-response curve fitting module using the Hill slope equation. Asterisk indicate difference between curves assessed by F-test (GraphPad Prism Ver. 5.0, San Diego, CA).

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Figure 3: Silver determination by ICP-MS in (A) RTgutGC/RTL-W1 cell culture system (see Figure 1) exposed to AgNO₃ or cit-AgNP in L-15/ex for 24 hrs. Values represent mean $\pm$ SD from three or four independent experiments, N=3 or 4 Bars bearing different lettering are significantly different ($P < 0.05$, 1 way ANOVA, Tukey test) and (B) RTgutGC cells system (without RTL-W1) exposed to AgNO₃ or cit-AgNP in L-15/FBS for 24 hrs. Asterisk indicate statistical differences within groups (t-test, $p < 0.05$).

Figure 4: STEM images and EDX spectra of RTgutGC cells exposed to cit-AgNPs. (A) STEM image of RTgutGC cells exposed for 3 hrs to 50 μ M cit-AgNP in L-15/ex, the corresponding EDX spectra is shown in panel (C). Elemental Ag is shown in area 1, in a vesicle near the plasma membrane. Area 2 shows the spectra of the background. (B) STEM image of RTgutGC exposed for 24 hrs to 10 μ M cit-AgNP in L-15/ex, the corresponding EDX spectra is shown in panel (D). Elemental Ag was not detectable in any of the electron dense areas (1-3) at this later time point. Area 4 shows the spectra of the background. In panel B, a lysosome is enclosing area 2, arrows indicate desmosomes. Scale bar is 300 nm in both images.

Figure 5: Normalised mRNA levels of Metallothionein b (MTb), Zinc Transporter 1 (ZnT1), Copper transporting ATPase (ATP7A or Menkes protein), Glutathione Reductase (GR), Divalent Metal Transporter 1 (DMT1), Glucose-6-Phosphate Dehydrogenase (G6pd) and Sodium-Potassium ATPase (NaK) measured in RTgutGC cells exposed for 24 hrs to: (A) toxic doses (~20% reduction

in viability) of AgNO₃ and cit-AgNP. 200 nM AgNO₃ is 2% of 10 µM which is the measured dissolved fraction of silver from of cit-AgNP in L-15/ex (Yue et al., 2014); (B) non-toxic concentrations of AgNO₃ and cit-AgNP in L-15/ex; (C) non-toxic concentrations of AgNO₃ and cit-AgNP in L-15/FBS, note the y-axis scale was adjusted. All values are expressed as a ratio of the expression in those cells exposed to control media (see Figure S3). Values represent mean normalised fold change ± SD, N=3. Statistical difference from respective control is indicated by an asterisk (1-way ANOVA, followed by Dunnett's test p< 0.05).

Figure 6. Metal dependent intracellular ATP7A protein localization. Caco2 cells were grown in the presence of 200 μ M BCS for 18 hrs followed by the indicated treatment for 3 hrs and processed for immunostaining. Co-localization of ATP7A (in green) with cellular markers of cell periphery (in red, f-actin) and trans golgi network (TGN, in purple, Golgin97) is shown in panels A-E. ATP7A co-localization in: A, control (DMEM +10% FBS); B, Cu deficient (200 μ M BCS); C, Cu excess (100 μ M CuSO_4); D, Ag excess (10 μ M AgNO_3); E, AgNP excess (80 μ M cit-AgNP). Overlapping signal intensities (co-localization) were analysed and quantitated by using a laser scanning confocal microscope Leica SP5 and software 9LAS AF 2v and are shown in panel F. G, schematic representation of ATP7A co-localization under low Cu and high Cu and Ag intracellular concentrations. Values represent mean $\pm$ S.D. from three independent experiments, N=3. Treatment effect on ATP7A co-localization with the TGN or actin (i.e. cell periphery) compared to control is indicated by an asterisk (*, $P < 0.05$, 1-way ANOVA, Dunnett's test). Boxed numbers are ratios of ATP7A co-localization with actin divided by co-localization with TGN. Insets are enlarged X2. Scale bars = 25 μ m.