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# Acute hazard assessment of silver nanoparticles following intratracheal instillation, oral and intravenous injection exposures

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## ABSTRACT

With ever-increasing production and use of nanoparticles (NPs), there is a necessity to evaluate the probability of consequential adverse effects in individuals exposed to these particles. It is now understood that a proportion of NPs can translocate from primary sites of exposure to a range of secondary organs, with the liver, kidneys and spleen being some of the most important. In this study, we carried out a comprehensive toxicological profiling (inflammation, changes in serum biochemistry, oxidative stress, acute phase response and histopathology) of Ag NP induced adverse effects in the three organs of interest following acute exposure of the materials at identical doses *via* intravenous (IV), intratracheal (IT) instillation and oral administration. The data clearly demonstrated that bioaccumulation and toxicity of the particles were most significant following the IV route of exposure, followed by IT. However, oral exposure to the NPs did not result in any changes that could be interpreted as toxicity in any of the organs of interest within the confines of this investigation. The finding of this study clearly indicates the importance of the route of exposure in secondary organ hazard assessment for NPs. Finally, we identify Connexin 32 (Cx32) as a novel biomarker of NP-mediated hepatic damage which is quantifiable both (*in vitro*) and *in vivo* following exposure of physiologically relevant doses.

## ARTICLE HISTORY

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## KEYWORDS

Liver; kidneys; spleen; Ag NPs; different routes of exposure; inflammation; oxidative stress; acute-phase response; pathology; Connexin 32


## Introduction

The ever-increasing utilization of engineered nanoparticles (NPs) in various products and applications has led to considerable interest in the field of nanotechnology (Kermanizadeh, Jacobsen et al. 2020 ; Radwan et al. 2021). Unfortunately, the same unique nano-specific chemical and physical characteristics which make NPs desirable could also contribute to toxicity in exposed individuals. With the inevitable rise of public and occupational exposure from increasing production and use of NPs, there is an urgent need to assess the potential health consequences of being exposed to these NPs (Jacobsen et al. 2017; Karakus, Bilgi, and Winkler 2021; Kermanizadeh et al. 2019). The assessment of risk to human health in a 'nano' context is based upon the physicochemical properties of the particle in question, dose-response relationship of adverse effects

and the exposure scenarios. The exposure scenario is essential as it influences the exposure concentrations and the route of exposure; the latter being critical in governing the translocation, distribution, accumulation in a variety of target organs.

The skin, lungs and the gastrointestinal tract (GIT) are in constant contact with the external environment and are the primary exposure sites for NPs (Huang and Tang 2021). It is now understood that a proportion of NPs can translocate from these primary sites to a range of secondary organs with the liver, kidneys and spleen being some of the most important in terms of the accumulation of relatively large quantities of NPs (Balasubramanian et al. 2010; Kermanizadeh et al. 2015; Lee et al. 2013; Lipka et al. 2010). Importantly, with the advances over the last decade in the field of nanomedicine, there is now potential for intravenous (IV) and

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 Supplemental data for this article can be accessed [here](#).

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direct injection of NPs into the bloodstream (Kermanizadeh, Jacobsen, et al. 2020). The presence of NPs in the blood will result in the particles reaching a number of extra-pulmonary organs quickly and in very large concentrations (Balasubramanian et al. 2010; Kermanizadeh et al. 2015). This has been exemplified by the consensus that for particulates in the blood, the liver is key and the forefront to the xenobiotic challenge.

Silver (Ag) NPs are used in various applications which include but are not limited to additives to textiles and plastics, in water filters and disinfectants, as health supplements, health care devices as well as routinely being utilized in food preservation and packaging fundamentally due their inherent anti-microbial properties (Ahamed, Al Salhi, and Siddiqui 2010; Radwan et al. 2021). Therefore, there is very likely scenario for these particles to be ingested by consumers and reach the GIT or be inhaled during the manufacturing process (Geiser and Kreyling 2010; Kermanizadeh et al. 2015). It is believed that once in the submucosal tissue, certain particulates are able to enter the lymphatic system and the bloodstream. As touched upon nanomedicines can be deliberately introduced into the body via injection hence direct entry of NPs into the circulatory system (Kermanizadeh, Jacobsen, et al. 2020; Lagopati et al. 2021). The presence of these NPs in the blood will result in direct distribution to a wide range of target organs, including the liver, kidneys and spleen (Geraets et al. 2014; Hadrup and Lam 2014; Hadrup, Sharma, et al. 2020). Crucially, it is widely demonstrated that the liver has significance with regards to NPs accumulating in the organ following IV exposure compared to other organs (Kermanizadeh et al. 2015; Lipka et al. 2010). Alongside the kidneys, the liver might be responsible for the clearance of NPs from the blood (Geiser and Kreyling 2010; Semmler-Behnke et al. 2008).

In this study, we investigated the biokinetics and acute hazard potential of Ag NPs following three different routes of exposure (namely intratracheal instillation (IT), oral gavage and IV). The focus of the study was placed on three extrapulmonary organs identified for accumulating large quantities of materials. The Ag NPs was selected as a highly soluble particle. Here, we assessed a wide and comprehensive array of biomarkers/end-points of damage

which included organ-specific inflammation, changes in serum biochemistry (biomarkers of general toxicity and functional and metabolic activity), anti-oxidant depletion, acute-phase response and histopathology of the liver, kidneys and the spleen. Finally, we identify a novel biomarker of NP-induced hepatic injury (connexin 32) which might prove to be useful as a means of assessing liver damage which is quantifiable both *in vitro* and *in vivo* following exposure of physiologically relevant doses. This would allow for more meaningful *in vitro* and *in vivo* data comparisons and bridges the gap between data generated from *in vitro* and animal models and for better linking of the two different testing strategies.

## Materials and methods

### Nanoparticles and AgNO<sub>3</sub>

The Ag NPs was sourced from Fraunhofer IME – Germany (NM300-K), sub-sampled under Good Laboratory Practice conditions and preserved under argon in the dark until use. AgNO<sub>3</sub> was purchased from Sigma, UK.

### Characterization of the Ag NPs

The Ag NPs used within this study were extensively characterized using a combination of analytical techniques to identify their primary physical and chemical properties important for understanding their toxicological potential. These primary physico-chemical properties have been described in detail previously (Kermanizadeh, Pojana, et al. 2013) and reproduced below. Furthermore, the primary size as measured via TEM and the hydrodynamic size distribution of the particles in PBS (exposure medium) was determined at a concentration of 10 µg/ml by Dynamic Light Scattering (DLS) using a Malvern Zetasizer nano series – Nano ZS (USA) (Table 2).

### Animals and treatments

This study used eight-week-old female C57BL/6N mice (19.5–21 g), which were obtained from The University of Edinburgh Animal Unit (UK) and from Taconic (Denmark). The mice were housed in polypropylene cages with bedding (sawdust) and enrichment at controlled environmental conditions.

**Table 1.** The outline of the experimental treatment groups – 60 female mice were randomly divided at random into 12 exposure groups.

	24-hr Exposure			
	Control (PBS)	Ag NP low dose (25 µg per animal)	Ag NP high dose (100 µg per animal)	AgNO <sub>3</sub> (80 µg per animal)
IT	5	5	5	5
Oral gavage	5	5	5	5
IV	5	5	5	5

**Table 2.** The main primary physical and chemical properties of investigated NPs reproduced from Keramanzadeh, Pojana, et al. (2013).

NP code	NP type	Primary size (nm)	Surface area [m <sup>2</sup> /g] (BET)	Coating	Size in PBS (nm) <sup>‡</sup>	Zeta potential (mV)
NM300-K	Ag	15	–	4% Polyoxyethylene glycerol trioleate and 4% Tween 20	44.6 ± 8.1	–10.2 –9.88 –10.6

The Ag NPs were also characterized in PBS within 30 mins of preparation.  
<sup>‡</sup>Size by DLS in biological media measured within 30 min of sonication.

The mice were allowed to acclimatize and randomly divided into 12 groups of five animals. The experimental groups are summarized in Table 1. The *in vivo* procedures followed the guidelines for care and handling of laboratory animals according to the EC Directive 86/609/EEC, the Danish law and UK home office were approved by the local ethical committee for animal research and by the Danish Animal Experiment Inspectorate (under the Danish Ministry of Justice, permission 2015-15-0201-00465) and by both Heriot-Watt University and the University of Edinburgh.

In this experiment, the animals were exposed to the particle or controls either via the IT, oral gavage or IV (via the lateral tail vein) routes. The IT instillation was conducted as previously described (Kyjovska et al. 2015). The Ag NPs were dispersed in sterile PBS (two doses of either 25 or 100 µg per animal) in a volume of 100 µl for the IT and IV exposures and 500 µl for the oral route. These experiments incorporated negative controls (PBS vehicle only and an Ag ion control (80 µg of AgNO<sub>3</sub> per animal)). The NP doses utilized within this investigation were selected based on previous acute dosing studies including those conducted as part of FP7 funded EU project ENPRA, keeping in mind the overall aim and the selection of dosage which would allow for distinction of adverse effects following different routes of exposure. The *in vivo* IV dose, 100 µg NPs per animal, corresponds to approximately 0.5 µg/10<sup>6</sup> hepatocytes based on previous literature regarding liver cell numbers and

particle retention in the liver following IV exposure (Gaiser et al. 2013). The high IT doses reflect pulmonary deposition in mice equivalent to 1–10 working days of 8 hr at the Danish occupational exposure limit of 3.5 mg/m<sup>3</sup> for materials such as carbon black (Poulsen, Saber, Mortensen, et al. 2015). The mice were kept under 3.5% isoflurane anesthesia during the IV and IT process. The animals were conscious within minutes after these treatments and suffered no ill effects. The mice were monitored over the entire 24-hr exposure period. Following the exposure, the mice were sacrificed by exsanguination while anesthetized (200 µl of ZRF cocktail composed of Zoletil 250 mg/ml, Rompun 20 mg/ml and Fentanyl 50 µg/ml) after collection of intracardial blood. The liver was removed, the caudate lobe (glutathione measurements) and the left lobe (biokinetics/inflammation/biomarker analysis) were snap frozen in liquid nitrogen and stored at –80 °C, while the right medial lobe was fixed in a 4% formaldehyde solution for histological and biokinetics analysis. Additionally, the spleen and kidneys were also removed and either snap-frozen or fixed in 4% formaldehyde.

#### **Detection of Ag concentration in mouse liver, spleen and kidneys**

The Ag content in the tissues was determined in the vehicle control and 100 µg/mouse dose groups by High Resolution Inductively Coupled Plasma Mass Spectrometry (HR-ICP-MS) (ELEMENT XR,

Thermo Fisher Scientific, UK). In short, ~80 mg of fixed tissue was transferred into digestion tubes. A volume of 2 ml of 60% nitric acid was added for the complete digestion of the samples. The digests were kept at 120 °C overnight. At this juncture, the samples were diluted prior to analysis by HR-ICP-MS. The Ag content was measured as  $^{107}\text{Ag}$ ; while  $^{109}\text{Ag}$  was used for the control. The low-resolution mode was selected for maximal sensitivity and there was no interference for the isotopes. In our trials, external calibration with internal standard correction by rhodium as  $^{103}\text{Rh}$  was carried out. For quantification, the tissues from three random animals were selected from each group.

#### ***Analysis of serum biomarkers relating to toxicity***

The collected intracardial blood samples were centrifuged and serum frozen at  $-80\text{ }^{\circ}\text{C}$  for the analysis of biomarkers and the acute-phase response. The serum biochemistry measurements included lactate dehydrogenase (LDH), aspartate aminotransferase (AST), alanine aminotransferase (ALT), cholesterol, triglycerides and albumin. Prior to measurements, the samples were thawed, centrifuged for 10 min at 2000 *g* at room temperature with the analysis performed on a Cobas 8000 modular analyzer (Roche, USA).

#### ***Acute-phase response***

The acute-phase protein serum amyloid A3 (SAA3) was measured in the prepared serum (described above) utilizing a commercially available mouse SAA3 ELISA kit (Abcam, UK - ab157723) according to the manufacturer's instructions.

#### ***Tissue homogenization***

The liver samples were thawed on ice and homogenized in a homogenization buffer (RIPA lysis buffer supplemented with a complete protease inhibitor mixture and a protein phosphatase inhibitor) (Abcam, UK). For tissue homogenization, pre-filled bead mill tubes (Thermo Fisher, UK) and an evolution homogenizer was used (Percellys, France). The samples were thoroughly mixed and stored on ice for 10 min before centrifugation at 10000 *g* for 10 min. The supernatants were transferred to a fresh

tube and centrifuged for a further 10 min. The protein concentrations were measured utilizing a Coomassie Plus Bradford assay reagent (Thermo Scientific, UK) and the supernatants stored at  $-80\text{ }^{\circ}\text{C}$  until use.

#### ***Liver cytokine levels***

The levels of Interleukin 10 (IL10), IL6, Monocyte Chemoattractant Protein-1 (MCP-1) and keratinocyte-derived chemokine (KC) and Tumor Necrosis Factor- $\alpha$  (TNF- $\alpha$ ) was determined in the cell supernatant of homogenized liver tissue using R&D Systems magnetic Luminex® Performance Assay multiplex kits (bead-based immunoassay; Bio-technie, USA) according to the manufacturers instruction. The proteins were detected via a Bio-Rad Bio-Plex MAGPIX multiplex reader. The technology is based on analyte-specific antibodies pre-coated onto magnetic microplates embedded with fluorophores at set ratios for each unique microparticle region being recognized by the MAGPIX reader.

#### ***Western blotting***

The changes in the levels of the two proteins of interest (CD36 and connexin 32) was quantified by Western blotting. In short, 40  $\mu\text{g}$  of denatured protein from each sample was added to a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel (Thermo Fisher, UK). The electrophoresis products were transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, USA), blocked with blotting-grade blocker (Abcam, UK) and 0.1% Tween 20 at 1 hr at room temperature. Subsequently, the membranes were incubated with primary anti-CD36 and anti-connexin 32 antibodies (1:1000) (Abcam, UK) over night and anti-IgG peroxidase-conjugated secondary antibody (1:5000) (Abcam, UK). In these experiments, GADPH was used as an internal control (Abcam, UK). The proteins were detected using the Clarity™ ECL Western substrate kit (Bio-Rad, USA) and visualized via a Fusion FX7 imager (Witec AG, Switzerland). Finally, immunoblotting signals were quantitated using Image Studio 4.0 (LI-COR Biotechnology, USA).



### Glutathione depletion

The mouse liver and kidney samples were weighed, thawed on ice and homogenized in 2 ml of lysis buffer. The homogenized samples were incubated on ice for 10 min before being centrifuged at 10000g for 5 min to generate lysates. Glutathione was quantified in the lysate by reaction of sulfhydryl groups with the fluorescent substrate *o*-phthalaldehyde (Sigma, UK) using a fluorimeter with an excitation wavelength of 350 nm and emission wavelength of 420 nm. The protocol was modified to include measurements of total glutathione by reducing oxidized glutathione dimers (GSSG) by addition of 7  $\mu$ l of 10 mM sodium dithionite the samples and incubation at room temperature for 1 hr.

### Histological analysis of liver, kidneys, and spleen

The tissue samples were trimmed, dehydrated and embedded in paraffin wax using a Shandon duplex tissue processor (SCE 0540). After embedding, the tissue was sectioned at a thickness of 5  $\mu$ m using a BROMMA 2218 HistoRange Microtome (LKB). The cut sections were attached to glass microscope slides and stained using hematoxylin and eosin (H&E) before examination using a Carl Zeiss Axiovert inverted microscope (Germany). Three random animals (five slides per animal) were chosen from each group for histological analysis.

### Statistical analysis

The data are expressed as mean  $\pm$  standard error of the mean (SEM). For statistical analysis, the detection of significant differences was calculated using a two-way full factorial ANOVA with *post hoc* multiple comparisons (Tukey) and a defined significance level of  $p < 0.05$ . All statistical analysis was carried out utilizing SPSS 26.

## Results

### Particle characterization

The main physicochemical characteristics for the Ag NPs used within this study has been reproduced from previously published work. Additionally and importantly, the Ag NPs were also characterized in the exposure medium. The hydrodynamic size distribution and zeta potential of the materials in PBS is presented in (Table 2). Of note, no endotoxin contamination ( $\leq 0.25$  EU/ml) was detected for the Ag NPs. The TEM images of the Ag NPs is provided as Supplementary information (Supplementary Figure 1).

### Bodyweight changes in the alcohol fed and/or Ag NP-exposed animals

There was no significant change in the body weight of Ag NP or AgNO<sub>3</sub> exposed animals (data not shown). In addition, none of the animals showed any visible signs of discomfort during or subsequent to any of the treatments.

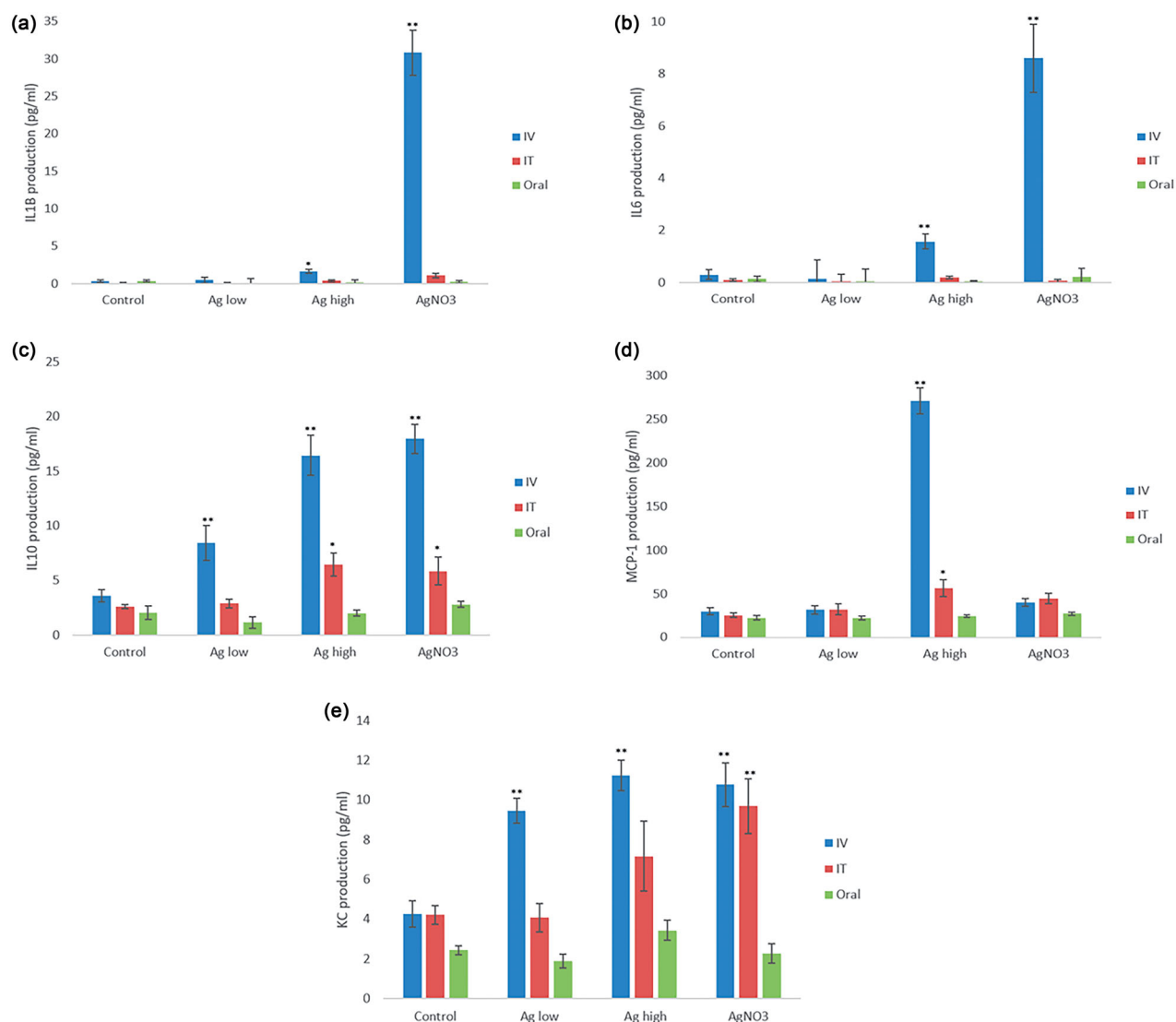
### Ag biokinetics and organ accumulation following different routes of exposure

To investigate the tissue distribution and disposition of Ag NPs following exposure of the animals *via* the different routes of administration, the concentrations of silver in liver, kidneys and spleen were determined by HR-ICP-MS with the data presented in Table 3. Silver was below the limit of detection in the organs of mice in the control group. As expected, after a single intravenous dose of the NPs the highest concentrations of Ag was detected in the liver and spleen. In comparison, IT administration resulted in smaller quantities of Ag in all three target organs. Interestingly, very little Ag was detected in any of extra-pulmonary organs following oral exposure to the NPs.

**Table 3.** Silver content in the liver, kidneys and spleen determined by HR-ICP-MS following exposure to 100  $\mu$ g of NP per animal via three different routes of exposure ( $n = 3$ ).

	Liver ( $\mu$ g/g wet tissue)	Kidneys ( $\mu$ g/g wet tissue)	Spleen ( $\mu$ g/g wet tissue)
PBS	<0.01	<0.01	<0.01
IV	36.4 $\pm$ 11.7	2.3 $\pm$ 1.7	37.5 $\pm$ 13.6
IT	1.9 $\pm$ 0.9	0.4 $\pm$ 0.3	0.2 $\pm$ 0.1
Oral	<0.01	0.08 <sup>#</sup>	0.09 $\pm$ 0.07

<sup>#</sup>Ag only detected in one animal.



**Figure 1.** Cytokine production (a) IL1 $\beta$ , (b) IL6, (c) IL10, (d) MCP-1 and (e) KC from liver of mice following IV, IT or oral exposure of 25 or 100  $\mu$ g of Ag NPs for 24 hr. The values represent mean  $\pm$  SEM ( $n = 5$ ) with significance indicated by \* $p < 0.05$  and \*\* $p < 0.005$ .

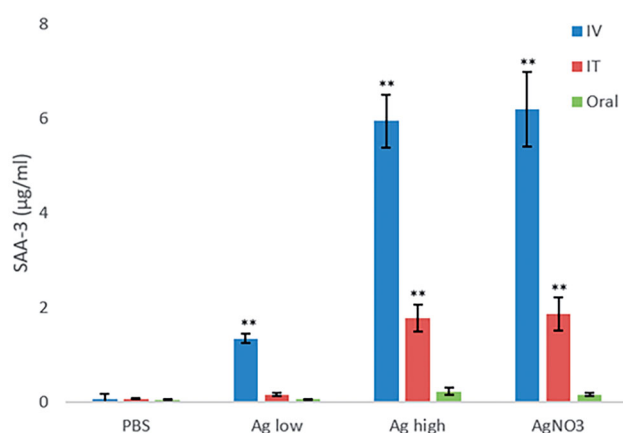
### Hepatic inflammation

The analysis of liver-specific inflammation demonstrated significant alterations in the levels of Interleukin 10 (IL10), Monocyte Chemoattractant Protein-1 (MCP-1) and keratinocyte-derived chemokine (KC) in tissues from mice treated with Ag NPs in a dose-dependent manner (Figure 1). For these cytokines, the data demonstrated that exposure via IV route resulted in the most significant alterations as compared with the PBS treated control animals. This being said, smaller yet significant increases in levels of IL10 and MCP-1 were noted following IT exposure to the Ag NPs at the highest dose (Figure 1(c,d)). Moreover, a significant increase in the levels of IL6

and IL1 $\beta$  were noted following exposure to the NPs via the IV route (Figure 1(a,b)). Next, the data showed no changes in any of the cytokines investigated following oral exposure to the Ag NPs. Interestingly, one of the most notable changes in the overall hepatic cytokine response was an increase in the anti-inflammatory IL10 level following exposure to Ag NPs, suggesting an overall anti-inflammatory and immune-tolerant milieu of the healthy liver.

### Acute phase response

The acute-phase response is a vital systemic response to disturbances to local and/or systemic



**Figure 2.** SAA3 levels measured in the serum of control and NP-exposed mice at 25 and 100 µg for 24 hr via IT, IV, or oral routes. The values depict mean  $\pm$  SEM ( $n = 5$ ) with significance indicated by \* $p < 0.05$  and \*\* $p < 0.005$ .

homeostasis caused by a variety of factors including infection, injury, trauma or immunological disorders. The most significant proportion of acute-phase response proteins are manufactured and secreted by the liver in response to cytokines IL1 and IL6. Here, SAA3 levels were measured as an indicator of such a response following the NP challenge. The data showed that the exposure to Ag NPs resulted in a dose-dependent increase in SAA3 levels in the appropriate animals, most notable for the IV route of exposure but also following IT exposure albeit at lower levels. There was no evidence of an SAA3 response following oral exposure at the specific doses and time-points measured (Figure 2).

### Glutathione depletion

As a measure of oxidative stress, the levels of reduced and total glutathione content were measured in the liver and kidney homogenates (Figure 3). The data showed a clear decrease in both reduced GSH and total GSH in the livers of Ag NP-exposed animals at 24 hr at the highest dose of 100 µg per animal following IV exposure. Additionally, a small yet significant decrease in reduced GSH levels were noted following IT exposure. Finally, as one of the few positive observations relating to the oral route of exposure within the whole study, a small yet significant decrease in total GSH in the liver was noted following exposure to the highest dose of the NPs (Figure 3(a)).

Similarly, in the kidneys, the IV route of exposure resulted in the most evident decrease in the

antioxidant levels in the organs (Figure 3(b)). Moreover, the IT exposure of Ag NPs at 100 µg per animal resulted in a small decrease in total GSH in the kidneys. The oral exposure to the Ag NPs at these doses and time point did not alter kidney antioxidants.

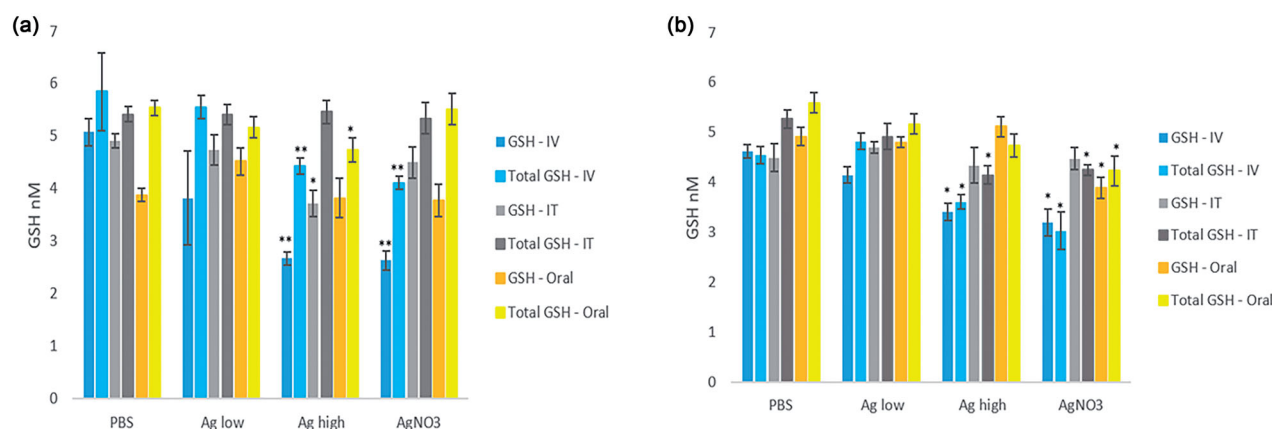
### Blood biochemistry

The analysis of blood biomarkers showed some statistically significant changes with regards to AST, ALT and LDH following the acute exposure of the Ag NPs (Table 4). Most notably the IT exposure of the Ag NPs resulted in the biggest increase in ALT, AST and LDH in serum of expose animals which is indicative of liver-specific and general cellular damage. Following exposure *via* the IT route, the only significant change was in LDH levels, which was evident following exposure to the NPs at the highest dose. This finding suggests that IT exposure to Ag NPs does indeed result in cell death that might not necessarily be in organs that are the focus of this study. Interestingly, the oral exposure to the NPs did not result in any significant change in any of parameters investigated including LDH. This observation is important in corroborating the findings in other endpoints all suggesting that acute exposure to a relatively toxic NP *via* the oral route might not be associated with any meaningful adverse effects in locations other than the gastrointestinal tract.

### Liver biomarkers of damage

In an effort to identify potentially novel NP-induced biomarkers of liver damage, the changes in the expression of target proteins (CD36 and connexin 32) in the organ was analyzed by Western blotting (Figure 4) (the rationale for this is fully explained in the discussion section). The data demonstrated that Cx32 levels were decreased significantly following IV exposure to Ag NPs at the highest dose. The acute IT or oral exposure to the Ag NPs at either dose did not result in a change in the levels of the investigated proteins as compared to the PBS exposed animals. The novel finding here are further strengthened by *in vitro* observation of changes in Cx32 in nanoparticulate exposed quadruple cell human primary hepatic spheroids. The western blot data did not show any changes in the levels of





**Figure 3.** Reduced GSH (GSH) and total glutathione (Total GSH) measured in the (a) liver and (b) kidneys of control and Ag NPs-exposed animals at 24-hr postexposure. The values depict mean  $\pm$  SEM ( $n = 5$ ), significance indicated by \* $p < 0.05$  and \*\* $p < 0.005$ .

**Table 4.** Blood biomarkers of liver and systemic toxicity assessed in the serum of control and Ag NP (25  $\mu$ g or 100  $\mu$ g/per animal) exposed animals sacrificed 24-hr post-treatment following IV, IT and oral routes of exposure.

Biomarker	PBS	Low dose Ag NPs	High dose Ag NPs	AgNO <sub>3</sub>
<b>IV</b>				
ALT (U/l)	27.1 $\pm$ 2.5	26.8 $\pm$ 2.1	34.2 $\pm$ 1.8*	43.5 $\pm$ 1.9**
AST (U/l)	90.8 $\pm$ 15.1	110.9 $\pm$ 8.4	187.5 $\pm$ 5.6**	194.3 $\pm$ 4.9**
Albumin (g/l)	13.2 $\pm$ 0.1	13.4 $\pm$ 0.5	12.9 $\pm$ 0.3	13.1 $\pm$ 0.2
Cholesterol (mmol/l)	2.4 $\pm$ 0.1	2.3 $\pm$ 0.1	2.4 $\pm$ 0.1	2.4 $\pm$ 0.1
Triglycerides (mmol/l)	0.7 $\pm$ 0.1	0.7 $\pm$ 0.1	0.7 $\pm$ 0.1	0.6 $\pm$ 0.1
LDH (U/l)	645.2 $\pm$ 32.4	598.5 $\pm$ 23.7	795.2 $\pm$ 33.6*	745.8 $\pm$ 29.3*
<b>IT</b>				
ALT (U/l)	25.4 $\pm$ 4.3	23.6 $\pm$ 1.2	25.5 $\pm$ 3.4	24.7 $\pm$ 4.3
AST (U/l)	83.8 $\pm$ 6.4	90.6 $\pm$ 7.4	84.8 $\pm$ 9.5	100.6 $\pm$ 9.6
Albumin (g/l)	12.7 $\pm$ 0.4	10.7 $\pm$ 1.6	13.6 $\pm$ 2.4	11.8 $\pm$ 0.6
Cholesterol (mmol/l)	2.2 $\pm$ 0.3	2.1 $\pm$ 0.2	2.2 $\pm$ 0.2	2.0 $\pm$ 0.3
Triglycerides (mmol/l)	0.7 $\pm$ 0.2	0.9 $\pm$ 0.1	0.7 $\pm$ 0.1	0.7 $\pm$ 0.2
LDH (U/l)	598.6 $\pm$ 39.9	602.3 $\pm$ 11.8	645.8 $\pm$ 13.8*	687.3 $\pm$ 47.2*
<b>Oral</b>				
ALT (U/l)	23.6 $\pm$ 3.1	25.8 $\pm$ 1.8	26.6 $\pm$ 2.3	22.8 $\pm$ 1.7
AST (U/l)	76.8 $\pm$ 3.3	80.3 $\pm$ 2.4	77.5 $\pm$ 2.1	73.7 $\pm$ 3.7
Albumin (g/l)	10.8 $\pm$ 0.4	12.2 $\pm$ 0.2€	11.8 $\pm$ 0.1	11.1 $\pm$ 0.1
Cholesterol (mmol/l)	2.1 $\pm$ 0.2	2.2 $\pm$ 0.1	2.2 $\pm$ 0.1	2.2 $\pm$ 0.1
Triglycerides (mmol/l)	0.6 $\pm$ 0.1	0.6 $\pm$ 0.1	0.7 $\pm$ 0.1	0.6 $\pm$ 0.1
LDH (U/l)	631.2 $\pm$ 22.8	621.5 $\pm$ 46.9	640.2 $\pm$ 27.8	667.3 $\pm$ 66.2

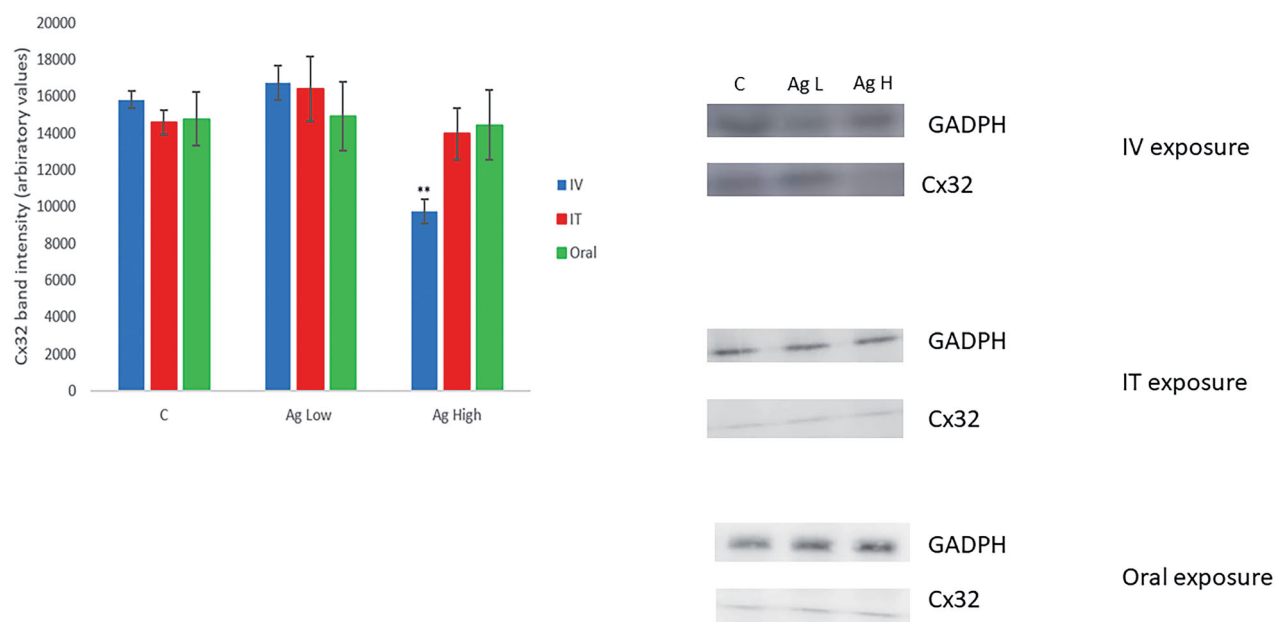
The values depict mean  $\pm$  SEM ( $n = 5$ ), significance indicated by \* $p < 0.05$  and \*\* $p < 0.005$ . €-  $n = 4$ .

CD36 for either of doses or routes of exposure (data not shown).

### Histology

The histopathological examination of the mice livers, kidneys and spleens revealed that exposure via the IV route resulted in the most notable pathological changes (Figure 5 and Tables 5–7). The exposure to the high dose of Ag NPs via the IV route resulted in comprehensive pathology in the liver manifested as numerous bi-nucleate hepatocytes, ballooning of hepatocytes, aggregation of inflammatory cells, necrotic hepatocytes, areas of

necrosis, granuloma formation and the destruction of the liver plates (Figure 5(b)). The exposure via IT or oral routes resulted in very little histological change in the livers as compared to PBS control animals (Table 5). Overall, the examination of the histopathology of the kidneys showed very little change in the normal renal cortex and glomeruli structure. However, the exposure via the IV route to the highest dose of the NPs resulted in a mild influx of inflammatory cells (Table 6). The histopathological examination of the control spleen sections showed normal splenic structure with normal lymphoid follicles and sinuses with well-defined white pulp. The IV exposure to the Ag NPs at the



**Figure 4.** The alteration in the levels of liver Cx32 protein level quantified by western blot analysis of PBS or Ag NP-exposed animals sacrificed 24-hr post-treatment following IV, IT, and oral exposure. The values represent mean  $\pm$  SEM with significance indicated by  $**p < 0.005$  compared to negative control ( $n = 3$ ).

dose of  $100 \mu\text{g}$  per animal resulted in minor changes, mostly visible as low-mild distorted lymphoid structures and detection of a few giant macrophages (Figure 5(f)). The histopathological changes in the spleens of oral and IT Ag NPs exposed animals were less noticeable (Table 7). Finally, it is important to state that the damage observed was not uniform across all regions of the lobes investigated and/or all the slides examined.

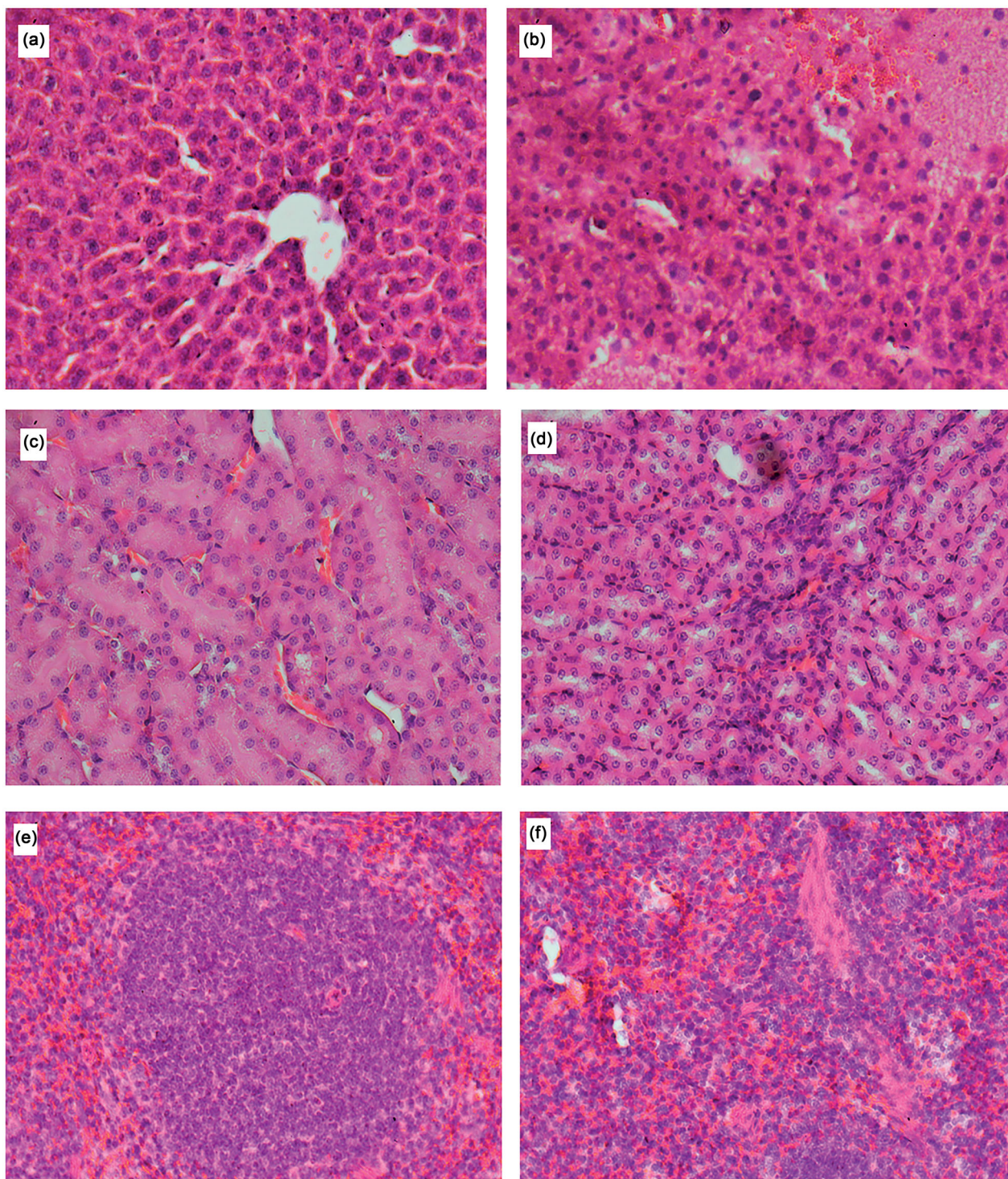
## Discussion

It is now clear that NPs entering the blood stream will accumulate within the liver (Sadauskas et al. 2009), kidneys (i.e. Recordati et al. 2016) and the spleen (Tassinari et al. 2021). It has previously been shown that the Ag NPs have adverse effects on hepatic and renal cell lines/models *in vitro* (i.e. Gaiser et al. 2013; Kermanizadeh, Vranic, et al. 2013) and induce an acute response in the liver and kidneys *in vivo* (i.e. Kermanizadeh et al. 2017; Recordati et al. 2016). In this study, we demonstrate that IV administration of Ag NPs resulted in NP-induced liver-specific inflammation, anti-oxidant depletion and an acute-phase response from the organ. The histopathological examination and analysis of a novel organ specific and general blood bio-markers further corroborated NP-induced liver damage. The

IV exposure at the dose of  $100 \mu\text{g}$  per animal also showed mild renal and spleen toxicity in the exposed animals. Interestingly, the IT exposure of Ag NPs also resulted in significant alterations in a number of end-points investigated which will be discussed in detail in turn. However, the oral exposure of Ag NPs did not result in any changes that could be interpreted as toxicity to the examined organs.

In this study as expected, the tissue distribution and disposition of Ag NPs following acute exposure of the nanoparticles was clear in the target organs, with the liver and the spleen accumulating largest quantities of Ag. The IT administration of the NPs resulted in smaller quantities of Ag, compared to IV administration, in all three target organs (Table 3). Of note and crucial in this acute study, was the observation that very little Ag was detected in any of the extra-pulmonary organs following oral exposure to the NPs. Numerous previous studies have demonstrated accumulation of NPs in the liver and spleen following IV exposure for Ag NPs (Gaiser et al. 2013), Silica NPs (Tassinari et al. 2021) and  $\text{TiO}_2$  NPs (Kreyling et al. 2017). Moreover, acute IT administration of Ag NPs was shown to result in small quantities of materials reaching the liver (Gosens et al. 2015). In an interesting study from 2018, long-term low-dose exposure to  $\text{CeO}_2$  and





**Figure 5.** The histopathological examination of H and E stained liver of mice exposed to (a) control (b) high dose NPs via the IV route with damage most evident by steatosis, vacuolar degeneration, inflammatory cell influx and hepatic necrosis; kidneys of mice exposed to (c) control (d) high-dose NPs via the IV route with small influx of immune cells and from spleen of mice exposed to (e) control (f) IV exposed to high-dose NPs resulting in observation in a number of giant macrophages.

TiO<sub>2</sub> NPs for 180 days via the IT route also resulted in accumulation of materials in liver, spleen, and kidneys, while the authors did not observe any absorption from the digestive tract to other organs

following oral gavage of the same materials (Modrzynska et al. 2018).

The analysis of hepatic organ inflammatory cytokines showed significant alterations in the levels of



**Table 5.** The histological score of liver pathology from five slides from three random animals for each treatment group ranked from 0–5.

IV	-PBS	Low Ag NP	High Ag NP
Steotosis	0, 1, 0	0, 0, 0	0, 0, 1
Inflammation	0, 0, 0	1, 0, 1	4, 4, 4
Necrosis	0, 0, 0	0, 0, 0	3, 1, 4
IT	-PBS	Low Ag NP	High Ag NP
Steotosis	0, 0, 0	0, 0, 1	0, 0, 0
Inflammation	0, 0, 0	0, 0, 0	1, 0, 2
Necrosis	0, 0, 0	0, 0, 0	0, 0, 0
Oral	-PBS	Low Ag NP	High Ag NP
Steotosis	1, 0, 0	1, 0, 1	0, 1, 0
Inflammation	0, 0, 1	0, 0, 1	0, 1, 0
Necrosis	0, 0, 0	0, 0, 0	0, 0, 0

**Table 6.** The histological score of kidney pathology from five slides from three random animals for each treatment group ranked from 0–5.

IV	-PBS	Low Ag NP	High Ag NP
Diminished and distorted glomeruli	0, 0, 0	0, 0, 0	0, 0, 1
Infiltration of inflammatory cells	0, 1, 0	0, 0, 1	2, 1, 0
Necrosis	0, 0, 0	0, 0, 0	0, 0, 0
IT	-PBS	Low Ag NP	High Ag NP
Diminished and distorted glomeruli	0, 0, 0	0, 0, 0	0, 0, 0
Infiltration of inflammatory cells	0, 0, 0	1, 0, 0	1, 1, 1
Necrosis	0, 0, 0	0, 0, 0	0, 0, 0
Oral	-PBS	Low Ag NP	High Ag NP
Diminished and distorted glomeruli	0, 0, 0	0, 0, 0	0, 0, 0
Infiltration of inflammatory cells	0, 0, 0	0, 0, 1	1, 0, 0
Necrosis	0, 0, 0	0, 0, 0	0, 0, 0

**Table 7.** The histological score of spleen pathology from five slides from three random animals for each treatment group ranked from 0–5.

IV	-PBS	Low Ag NP	High Ag NP
Distorted lymphoid architecture	0, 0, 0	0, 0, 0	2, 2, 2
Diffuse white pulp	0, 0, 0	1, 0, 1	2, 1, 2
Granular leukocytes, and giant macrophages	0, 0, 0	0, 0, 0	3, 3, 4
Vacuolation of red pulp	0, 0, 0	0, 0, 0	0, 0, 3
IT	-PBS	Low Ag NP	High Ag NP
Distorted lymphoid architecture	0, 0, 0	0, 0, 0	0, 0, 0
Diffuse white pulp	0, 1, 0	0, 0, 1	1, 1, 1
Granular leukocytes, and giant macrophages	0, 0, 0	0, 1, 0	1, 0, 0
Vacuolation of red pulp	0, 0, 0	0, 0, 0	0, 0, 0
Oral	-PBS	Low Ag NP	High Ag NP
Distorted lymphoid architecture	0, 0, 0	0, 0, 0	0, 0, 1
Diffuse white pulp	0, 0, 0	1, 0, 1	1, 1, 0
Granular leukocytes, and giant macrophages	0, 0, 0	0, 0, 0	1, 1, 0
Vacuolation of red pulp	0, 0, 0	0, 0, 0	0, 0, 0

IL10, MCP-1 and KC in liver tissue from mice treated with Ag NPs in a dose-dependent manner (Figure 1). For these cytokines, the data demonstrated that exposure via the IV route resulted in the most significant alterations compared to the negative control

mice. Moreover, smaller yet significant increases in the level of IL10 and MCP-1 were noted following IT exposure of the Ag NPs at the highest dose. Next, a significant increase in the levels of IL6 and IL1 $\beta$  were noted following exposure of the NPs via the IV route. Notably, the data showed no changes in any of the cytokines investigated following oral exposure to the Ag NPs.

Of note, one of the most significant changes in the overall hepatic cytokine response was increases in the anti-inflammatory IL10 levels following exposure to Ag NPs suggesting at an overall anti-inflammatory and immune-tolerant milieu of the healthy liver. It is understood that IL10 acts as an antagonist against the pro-inflammatory cytokines emphasizing that tolerance might be favored over an inflammatory response as a consequence of the acute NP challenge *in vivo*. Interestingly, similar observations in the fine counter-balance between anti versus pro-inflammatory hepatic cytokines have also been noted in complex multi-cellular *in vitro* models of the organ (Kermanizadeh et al. 2019). It is now understood that Kupffer cells are very important in dictating the overall liver immunity against xenobiotics including gut originated antigens and are known to contribute to a cytokine storm which can further activate hepatic T cells, in turn promoting phagocytosis and additional inflammatory response in a positive feedback loop (Kubes and Jenne 2018). These cells are also actively involved in progression of liver disease (Koyama and Brenner 2017). However, it is hypothesized that Kupffer cells in a nondiseased liver are in a constant semi-activated state and are essential in the maintenance of tolerance to food antigens in everyday life and one of the main reasons why there is not an extensive immune response to eating food (Heymann et al. 2015). Hence, the liver offers a unique environment in which the resident hepatic macrophages can both initiate an immune response or play an active role in retaining an immuno-tolerant state (Heymann et al. 2015). The data here clearly demonstrate the complexities of hepatic inflammation and reiterates the limitation of assessing this endpoint *in vitro* where single hepatocyte cell lines and IL8 alone are often used as a surrogate and representative for inflammation in the organ *in vivo*.

Acute-phase proteins are a range of blood proteins predominately produced in the liver (Jain,

Gautam, and Naseem 2011). The acute-phase response is a vital component of the innate immune system, which can be triggered by different stress stimuli including but not limited to infection, inflammation and physical trauma. To date, over 200 acute-phase proteins have been identified (Eklund, Niemi, and Kovanen 2012). The biological activities of these proteins are extremely important and varied and described previously in detail (Gruys et al. 2005). Serum amyloid A is a highly conserved protein produced by the liver. The plasma SAA concentration begins to increase 3–6 hr after an inflammatory stimulus, peaks on day 3, and returns to baseline levels after day 4 (this states that the peak of this response was not detected in this study). During an acute inflammatory response, the liver dictates a significant proportion of its synthesis capacity into producing SAA, which in mice comprised 2% of the total hepatic protein production (Eklund, Niemi, and Kovanen 2012). Important to this study is recognition that SAA is extremely immunologically active and plays a vital role in the recruitment of macrophages and neutrophils (Saber et al. 2014). In our experiments, the exposure to Ag NPs resulted in a dose-dependent increase in SAA3 levels most notable for the IV route of exposure but also following IT at lower levels. There was no evidence of a response following oral exposure at the specific doses and time-point. Similar observations have been observed following acute IV exposure to a different Ag NPs (Kermanizadeh et al. 2017) and IT exposure of multi-walled carbon nanotubes (Poulsen, Saber, Mortensen, et al. 2015).

As a means of assessing NP-induced oxidative stress, the levels of reduced and total glutathione content were measured in liver and kidney homogenates (Figure 3). The data in this study, showed a decrease in both reduced GSH and total GSH in the livers of Ag NP-exposed animals at 24 hr at the highest dose of Ag NPs after IV exposure. Next, a small yet significant decrease in reduced GSH levels were noted following IT exposure. Finally, the oral route of exposure resulted in a small yet significant decrease in total GSH in the liver following exposure to the highest dose of the NPs. The anti-oxidant depletion data are very much in line with other end-points in establishing the ranking of the route of NP exposure being vital in the adverse effects observed in extra-pulmonary organs. In a healthy

human adult, hepatocytes contain about 10% of the total human body pool of GSH (Loguercio and Federico 2003); therefore, in theory, the assessment of GSH could be a useful tool in assessing xenobiotic oxidative stress induced in the organ. This being said, it is important to state that the preservation of the redox balance is extremely complex and constantly is flux; hence, it is very difficult to identify an optimal time point for these measurements. It is conceivable that the assessment of GSH levels in the liver and kidneys a few hours before or after the 24-hr time point used within this study would have resulted in different outcomes. As a means of corroborating this point previous literature shows disparities in patterns of GSH depletion in the liver. As an example, the examination of antioxidant levels in the liver post IV administration of Ag NPs in rats had no significant effect in hepatic GSH levels 24 hr after exposure (Gaiser et al. 2013). However, the IT exposure to Ag NP resulted in a dose-dependent decrease in GSH levels in the liver of a mouse model (Gosens et al. 2015). Similarly, in the kidneys, the IV route of exposure resulted in the most evident decrease in the antioxidant levels in the organs. Moreover, the IT exposure to Ag NPs resulted in a small decrease in total GSH in the kidneys. The oral exposure to the Ag NPs at these doses and time-point did not alter kidney GSH levels. In a previous study on IV exposure of rats to 34 mg of  $\text{AlCl}_3/\text{kg}$  for 24 hr resulted in a clear decrease in reduced GSH in the kidneys in the exposed animals (Al Kahtani 2010). Elsewhere, acute 24-hr *in vitro* exposure of a kidney cell line to varying NPs have also resulted in decreased GSH levels in the exposed cells (Enea et al. 2020; Kermanizadeh, Vranic, et al. 2013).

In summary of blood biochemistry, the data showed statistically significant increases in the levels of AST, ALT and LDH following the acute exposure to the Ag NPs most notable for the IV route of exposure but also significant alterations in LDH levels following the IT route. Collectively, these biomarkers are indicative of Ag NP-induced cell death with AST and ALT being liver specific and LDH as a general systemic indicator of cell damage. Interestingly, there was no changes in any of the functional and metabolic biomarkers (albumin, triglycerides and cholesterol). Once again, these observations strongly suggest that the route of

exposure is critical in governing extra-pulmonary NP-induced adverse effects. Previous IV exposure to two differently sized PEG modified gold NPs at a dose of 4 mg per animal over 28 days, resulted in changes in blood biochemistry indicative of liver damage (Zhang et al. 2011). Elsewhere, 5 day repeated oral exposure of mice at a dose of 1000 mg/kg of amorphous silica NPs did not cause changes in blood chemistry (Cabellos et al. 2020). As another example, the IT exposure to 800 µg/kg of ZnO NPs four times over a week did not result in changes in AST/ALT levels in the serum of exposed animals (Wang et al. 2017). In a subacute study in which rats were intravenously co-administered with Au and Ag NPs, no changes in blood chemistry (Lee et al. 2018) were found. The variations between the data presented here and those observed by Lee and colleagues could be explained by the different species used in the studies, and the important consideration that acute transient changes in biochemistry might or might not manifest or be detectable in organ toxicity at a single time point following long-term exposure to NPs.

It is generally accepted that it is not always possible to make direct or meaningful comparisons between *in vitro* and *in vivo* hepatic toxicological responses. This statement is equally valid for all xenobiotics including chemicals, drugs and particulates (discussed in detail in Kermanizadeh, Powell, and Stone 2020). Amongst numerous reasons for such differences is the clear absence of appropriate organ-specific biomarkers and toxicological endpoints that can be measured both *in vitro* and *in vivo*. In an attempt to address this issue, in this study the expression of two target proteins (CD36 and Cx32) were investigated in the homogenates of the Ag NP-exposed animals (these proteins were identified from a pilot study looking at a total of 15 potential candidate biomarkers (data not shown)).

Gap junctions are intercellular channels consisting of connexin proteins that directly connect the cytosol of coupled cells and allow rapid communication of cellular signals and act as a unique route for amplification of innate immunity. The hepatic gap junction Cx32, is readily expressed throughout the organ and is understood to be most highly distributed in the pericentral region. Several previous studies have demonstrated the crucial role of Cx32 gap junctions in injury in various models of liver

disease (Guerra, Hadjihambi, and Jalan 2019; Luther et al. 2018; Willebrords et al. 2017). In this study, we show that Cx32 levels were decreased significantly following IV exposure to Ag NPs at the highest dose. The acute IT or oral exposure to the Ag NPs at either dose did not result in a change in the levels of the investigated proteins as compared to the PBS exposed animals. The novel findings here are further strengthened by *in vitro* observation of changes in Cx32 in nanoparticulate exposed quadruple cell human primary hepatic spheroids. Overall, the data within this study demonstrated Cx32 to be a promising candidate as a meaningful biomarker of NP-mediated liver damage which is quantifiable both *in vitro* and *in vivo* following exposure to physiologically relevant doses. In time, this will hopefully allow for more meaningful *in vitro* and *in vivo* data comparisons to be made which in turn will bridge the vast gap between data generated from *in vitro* and animal models and for better linking of the two different testing strategies. It is very important to state that despite early promise this biomarker still requires comprehensive validation in order to better understand the nature of the response, its mechanism and consequences. In order to address this, we have now analyzed over 200 pieces of liver tissue from animals exposed to a panel of NPs acutely and chronically via varying route of exposure at a range of doses (manuscript in preparation).

CD36 is a member of the class B scavenger receptor family with the ability to bind oxidized low-density lipoprotein (LDL). CD36 expression is relatively low in normal hepatocytes, but has been shown to increase in lipid-rich diets, hepatic steatosis, and nonalcoholic fatty liver disease (NAFLD) in animal models (Wilson et al. 2016). The western blot data here did not show any changes in the levels of CD36 for either Ag NP doses or any of the routes of exposure. Despite some encouraging *in vitro* observations, the reliability, and/or suitability of this protein as a meaningful biomarker of NP-induced hepatic injury is questionable (as mentioned above further investigations are in progress).

Finally, the histopathological examination of the mice livers, kidneys, and spleens revealed that exposure via the IV route resulted in the most notable pathological changes. The exposure to the high dose of Ag NPs via the IV route resulted in



comprehensive hepatic pathology manifested most notably as necrosis, changes in the structure of the organ and inflammatory cell infiltration. The degenerative changes in the Ag NP treated groups were observed in all zones of the hepatic lobules, but were not uniform across all regions of the lobes investigated and slices examined. The exposure via IT or oral routes resulted in almost no histopathological change in the livers as compared to PBS control mice. Overall, the examination of the histopathology of the kidneys only showed a mild influx of inflammatory cells following IV administration of NPs. In the spleen, the IV exposure of the Ag NPs at the highest dose resulted in low distortion in the lymphoid structure as well the detection of a number of giant macrophages. There were no histopathological changes from the controls in the spleens of oral and IT Ag NP-exposed animals. In the past, Ag NP exposure in mice has also shown varying degrees of similar hepatic histological pathologies (Ansari et al. 2016; Patlolla, Hackett, and Tchounwou 2015; Recordati et al. 2016). Elsewhere, the oral daily treatment of Ag NPs for three weeks at a dose of 50 mg/kg in rats resulted in increased number of abnormal glomeruli and necrotic tubules (Abdel-Wahhab et al. 2019). In a 2016 study, a single IV exposure to Ag NPs at a dose of 10 mg/kg resulted in severe hyperemia of the red pulp in the spleen of exposed animals (Recordati et al. 2016).

Ag NPs dispersed in any aqueous medium will release a degree of soluble Ag ions. Therefore, it might be important to distinguish between the toxic effects of Ag NPs and the dissolved Ag ion content. In this study, we included a AgNO<sub>3</sub> exposure group which was equivalent to 80% of NP dissolution. This being said, it is important to mention that it is almost impossible to state unequivocally what aspects of adverse effect observed in the organs of interest were attributed to the Ag ions, the Ag NPs or a contribution from both (which is most likely scenario at the 24-hr end-point).

Despite the many insights offered in this study and the comprehensive toxicological assessment in the extra-pulmonary organs, there is a major limitation in the study design that needs to be mentioned and considered in the interpretation of findings. In all reality, any realistic NP-induced adverse effects to extra-pulmonary organs in man would only occur following long-term exposure

(with the exception of intentional IV administration of nanomedicines). Hence in an ideal *in vivo* hazard assessment, studies should to be carried out with low intermittent repeated dosing and to incorporate recovery periods to allow for the assessment of clearance of NPs, manifestation of adverse effects and potential for organ recovery to be identified. That being said, this study is valuable in allowing for a direct comparison to be made between the different routes of toxicity and how this important variable affects the toxicity observed extra-pulmonary tissues. Any upcoming studies might also investigate a wider range of time points post material exposure to identify the optimum peak for certain endpoints (e.g. acute-phase response, cytokine production). It is also important to state that this is the first of two independent studies investigating NP-induced extra-pulmonary toxicity following different routes of exposure. In this paper, Ag NPs was selected as a high solubility material, while TiO<sub>2</sub> is utilized in the second study as a low solubility NP (manuscript in preparation).

## Conclusions

In this study, we carried out a comprehensive toxicity profiling of Ag NP-induced adverse effects in liver, kidneys, and spleen following acute exposure of the materials at identical doses via IV, IT and oral administration. The data clearly demonstrated that bioaccumulation and toxicity of the particles were most significant following IV, followed by IT. However, the oral exposure of the nanoparticles did not result in any changes that could be interpreted as toxicity in any of the organs of interest within confines of this investigation. The finding of this study clearly indicates the importance of the route of exposure in hazard assessment for NPs. Finally, we identify CX32 as a novel biomarker of NP-mediated hepatic damage which is quantifiable both (*in vitro*) and *in vivo* following exposure of physiologically relevant doses.

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