Acute toxicity, bioaccumulation and effects of dietary transfer of silver from brine shrimps exposed to PVP/PEI-coated silver nanoparticles to zebrafish

José María Lacave[1], Álvaro Fanjul[1], Eider Bilbao[1], Nerea Gutierrez[2], Irantzu Barrio[2], Inmaculada Arostegui[2], Miren P. Cajaraville[1], Amaia Orbea[1*]

[1] CBET Research group, Dept. of Zoology and Animal Cell Biology; Research Centre for Experimental Marine Biology and Biotechnology PIE and Science and Technology Faculty, University of Basque Country (UPV/EHU). Sarriena z/g, E-48940, Leioa, Basque Country, Spain.

[2] Dept. of Applied Mathematics, Statistics and Operations Research, Science and Technology Faculty, and Basque Center for Applied Mathematics – BCAM, University of the Basque Country UPV/EHU, Leioa, Spain

*Corresponding author: amaia.orbea@ehu.eus. CBET Research group, Dept. of Zoology and Animal Cell Biology; Research Centre for Experimental Marine Biology and Biotechnology PIE and Science and Technology Faculty, University of Basque Country (UPV/EHU). Sarriena z/g, E-48940, Leioa, Basque Country, Spain. Tel: 946012735; Fax: 946013500

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ABSTRACT

The extensive use and release to the aquatic environment of silver nanoparticles (NPs) could lead to their incorporation into the food web. Brine shrimp larvae of 24 h showed low sensitivity to the exposure to PVP/PEI-coated Ag NPs (5 nm), with EC_{50} values at 24 h of 19.63 mg Ag L^{-1}, but they significantly accumulated silver after 24 h of exposure to 100 µg L^{-1} of Ag NPs. Thus, to assess bioaccumulation and effects of silver transferred by the diet in zebrafish, brine shrimp larvae were exposed to 100 ng L^{-1} of Ag NPs as an environmentally relevant concentration or to 100 µg L^{-1} as a potentially effective concentration and used to feed zebrafish for 21 days. Autometallography revealed a dose- and time-dependent metal accumulation in the intestine and in the liver of zebrafish. Three-day feeding with brine shrimps exposed to 100 ng L^{-1} of Ag NPs was enough to impair fish health as reflected by the significant reduction of lysosomal membrane stability and the presence of vacuolization and necrosis in the liver. However, dietary exposure to 100 µg L^{-1} of Ag NPs for 3 days did not significantly alter gene transcription levels, neither in the liver nor in the intestine. After 21 days, biological processes such as lipid transport and localization, cellular response to chemical stimulus and response to xenobiotic stimulus were significantly altered in the liver. Overall, these results indicate an effective dietary transfer of silver and point out to liver as the main target organ for Ag NP toxicity in zebrafish after dietary exposure.

Keywords: bioaccumulation, brine shrimp, cellular effects, dietary transfer, silver nanoparticles, zebrafish

1. Introduction

Due to their antibacterial activity, silver nanoparticles (Ag NPs) are one of the most widely used nanomaterials, with a continuously growing production (Vance et al., 2015). This will lead to an increasing entry of silver into the environment (Yin et al., 2015). The current concentration of Ag NPs in the environment remains unknown, because of the lack of sensitive analytical methods to distinguish different metal forms in complex environmental matrices (Sun et al., 2014). Nevertheless, several studies have estimated the potential concentration of Ag NPs in diverse environmental compartments, using mathematical models (Fabrega et al., 2011; Chio et al., 2012; Hendren et al., 2013; Dumont et al., 2015). These studies have reported values ranging from 0.002 ng L^{-1} in the surface water of European rivers (Dumont et al., 2015) up to 40 µg L^{-1} in effluents of Taiwanese rivers (Chio et al., 2012).

Despite our limited knowledge on the fate and impact of Ag NPs in the environment, previous data on the environmental and physiological implications of the exposure of aquatic organisms to different silver compounds provides a baseline for the assessment of the potential effects of Ag NPs to the aquatic ecosystem (Fabrega et al., 2011). For Ag NPs, two different routes of entry into aquatic organisms have been defined: through the respiratory system, because the gills are directly exposed to the water column, and through the diet in animals that feed on suspended matter or on other organisms previously exposed to NPs (Schirmer et al., 2013). Most studies have focused on the effects provoked by waterborne exposure to Ag NPs (Aruvalsu et al., 2014; Massarsky et al., 2014), while few studies have been carried out to address dietary exposure. Merrifield et al. (2013) fed zebrafish (*Danio rerio*) for 14 days with artificial food containing Ag NPs and reported a toxic effect in the zebrafish microbiome which provoked changes in the digestive system function and organism health. Other authors have also reported toxic effects in fish fed with artificial food containing other nanomaterials. Blickley et al. (2014) fed the estuarine fish *Fundulus heteroclitus* for 85 days with diets containing 1 or 10 µg day^{-1} of lecithin-encapsulated CdSe/ZnS quantum dots and detected cadmium bioaccumulation in the liver. Ladhar et al. (2014) also measured cadmium bioaccumulation in the liver of zebrafish after 36 and 60 days and in brain and muscle after 60 days of feeding with artificial food contaminated with CdS NPs. Genotoxicity and oxidative stress was reported too after the experimental period.

Dietary transfer of metals can also occur from one organism to another, which may lead to bioaccumulation and biomagnification along the food web as well as to provoke

a long term negative impact on the ecosystem functions (Pakrashi et al., 2014). In order to study a potential transfer of NPs among aquatic organisms, diverse studies have tried to simulate a simple food chain of two trophic levels. In some cases, a primary producer (phytoplankton) is exposed to NPs and used to feed a primary consumer, such as zooplankton (Pakrashi et al., 2014). In both studies Al_2O_3 and Au NPs, respectively, were effectively transferred between species. In some other cases, a primary consumer (a crustacean) is exposed to NPs and used to feed a secondary consumer (fish), allowing to study biomagnification and toxic effects in the predator (Zhu et al., 2010). In these studies, TiO_2 NPs transfer and uptake of CdSe/ZnS QDs were reported, but biomagnification was not recorded after the dietary exposure. More recently, the toxicity of 10 and 20 µm long Ag nanowires was tested in the alga *Chlamydomonas reinhardtii*, the water flea *Daphnia magna* and the zebrafish in a 3-species food chain study. Results showed that Ag nanowires, especially the shortest ones, were accumulated in the body of fish (Chae and An, 2016).

In the present study, brine shrimps (*Artemia sp*), as the primary consumer, and zebrafish, as the secondary consumer, were selected in order to assess whether brine shrimps exposed to an environmentally relevant concentration (100 ng Ag L^{-1}) and to an effective concentration (100 µg Ag L^{-1}) of Ag NPs were able to transfer silver to zebrafish through the diet and to identify the effects provoked in different organs of zebrafish (liver and intestine) by the dietary exposure to Ag NPs. Brine shrimps, which serve for feeding many different fish species in culture, have been commonly used in ecotoxicological testing because of their capacity to adapt to different environments; moreover, they are emerging as a new biological model in nanoecotoxicology (Libralato, 2014). Zebrafish is a well established animal model for testing toxicological effects, being thoroughly used as a model for assessing the toxicity of nanomaterials (Chakraborty et al., 2016).

Before the dietary transfer experiment, the acute toxicity of the Ag NP suspension was analyzed in brine shrimp larvae, according to standardized OECD test guidelines for *Daphnia sp* (OECD TG202, 2004), where immobilization is used as mortality criteria. Silver bioaccumulation was also measured in brine shrimps exposed to different Ag NP concentrations and results were used to select the Ag NP concentrations for brine shrimp contamination in the zebrafish dietary experiment. Then, metal accumulation and effects of silver transferred through the diet were analyzed in zebrafish using brine shrimps exposed to two different Ag NP concentrations, an

environmentally relevant concentration and a potentially effective concentration. In zebrafish, accumulation of silver was measured through chemical analysis and metal distribution was assessed in the intestine and in the liver quantifying the volume density of black silver deposits (Vv_{BSDs}) after autometallography. Zebrafish liver and intestine transcriptomes were analyzed in individuals fed with brine shrimps exposed to the high Ag NP concentration. The general fish health status was studied through the lysosomal membrane stability test in the liver, as lysosomes are the central site for sequestration of toxic metals (Köhler et al., 2002). Finally, appearance of histopathological lesions in the intestine and in the liver was evaluated as an appropriate indicator of the general health of the individuals (Davies and Vethaak, 2012). The intestine is one of the major target tissues as it represents the main entrance of NPs into the organisms during the dietary exposure (Zhu et al., 2010; Piccinetti et al., 2014), while the liver is the principal organ involved in the detoxification of xenobiotics (Feist et al., 2004).

2. Materials and methods

2.1. Silver nanoparticles

Ag NPs were purchased from NANOGAP SUB-NM-POWDER, S.A. (A Coruña, Spain) as an aqueous dispersion containing 10 g L^{-1} of Ag NPs stabilized with poly-N-vynil-2-pirrolidone (PVP, Sigma-Aldrich, St. Louis, Missouri) and polyethylenimine (PEI, Sigma-Aldrich), being the concentration of the PVP/PEI mixture 104 g L^{-1} in a proportion of 77% PVP and 23% PEI. The NP size distribution measured by transmission electron microscopy was 5.08 ± 2.03 nm and the Z potential in distilled water measured by dynamic light scattering (DLS) was +18.6 ± 7.9 mV at pH 8.43, according to the information provided by the supplier.

Secondary characterization of these Ag NPs in artificial sea water has been recently published in Schiavo et al. (2017). At 10 mg Ag L^{-1}, Ag NPs immediately reached a mean size of about 100 nm (DLS measurement) and remained stable up to 24 h. After 48-72 h, aggregates were slightly smaller (around 90 nm). Zeta potential in sea water was -2.37 ± 2.61 mV. After 24 h, 20% of Ag^{+} was released and at 72 h, dissolution increased to 29.6%.

2.2. Brine shrimp culture and acute toxicity test

Cysts of brine shrimps (INVE Aquaculture, Salt Lake City, Utah, USA) were hatched and grown in reactors with artificial salt water (33‰ salinity). Brine shrimp cultures were maintained with continuous aeration and illumination in a temperature controlled room at 25ºC. After 24 h of hydration, most cysts hatched and, then, they were maintained for other 24 or 48 h. Brine shrimp nauplii were collected using a mesh of 150 µm.

The acute toxicity of the Ag NP suspension to brine shrimps was tested following a procedure based on the standardized OECD TG 202 (2004) for *Daphnia magna*. The test was carried out in covered 24-well polystyrene microplates placing 5-7 brine shrimp nauplii of 24 or 48 h post hatch (hph) per well in 2 mL of exposure medium, at a temperature of 18.5 ºC and continuous illumination. Brine shrimps were exposed to five different dilutions of the Ag NP suspension containing 1, 2.5, 5, 7.5 and 10 mg Ag L^{-1}. The toxicity of the PVP/PEI mixture alone was assayed in parallel by exposing the brine shrimps in the same conditions to the equivalent concentrations present in NP suspension dilutions. An unexposed control group was also run. The test was considered valid only when survival rate in the control group was ≥ 90% (OCDE TG 202, 2004). At 24 and 48 hours of exposure, brine shrimps were examined to determine mortality (percentage of immobilized larvae).

2.3. Exposure of zebrafish through contaminated brine shrimps

In order to establish the Ag NP concentrations to expose brine shrimps for the dietary experiment, 24 hph brine shrimp cultures were waterborne exposed for 24 h to five different concentrations of Ag NPs (0.1, 1, 10, 100 and 1000 µg Ag L^{-1}). Based on the results of the chemical analyses, two concentrations were selected (100 ng Ag L^{-1} and 100 µg Ag L^{-1}) and new brine shrimp (24 hph) cultures were daily exposed to obtain a continuous stock to feed zebrafish during the 21-day experimental period.

The experimental procedure involving adult zebrafish described herein was approved by the Ethics Committee in Animal Experimentation of the UPV/EHU according to the current regulations. During the experimental period, zebrafish of approximately 1 year were kept in 35 L aquaria containing 55-75 fish. These aquaria were equipped with biological filters and air pumps for water aeration and recirculation. Adult zebrafish were fed daily for 21 days with the brine shrimps exposed for 24 h to the selected Ag

NP concentrations. A control group fed with unexposed brine shrimps was run in parallel in identical experimental conditions. The daily amount of feeding was 2.5% of fish body weight (Lawrence et al., 2012) distributed in two doses.

During the experimental period, fish samples were taken at 3 or 21 days of exposure depending on the endpoint, after euthanasia by overdose of anesthetic (ethyl 4-aminobenzoate, Fluka, Steinheim, Germany). In addition, samples from the brine shrimp cultures were collected to quantify the silver content at four different days (1^{st}, 7^{th}, 14^{th}, and 21^{st}).

2.4. Chemical analyses of silver accumulation

Brine shrimp nauplii were collected from the cultures using a 150 µm mesh. The resulting samples were introduced into pre-weighted 25 mL Erlenmeyer flasks and weighted again. Then, flasks were placed into a 130 ºC oven overnight, and weighted again in order to calculate the dry weight of the brine shrimp samples. Then, the samples were digested using 6 mL of aqua regia, prepared as 25% nitric acid (65% extra pure quality, Scharlau, Barcelona, Spain) and 75% hydrochloric acid (36%, Tracepur®, Scharlau). The mouth of the Erlenmeyer flasks was blocked with a crystal ball to minimize evaporation. After finishing the digestion of the samples, the remnant liquid was evaporated in an 80 ºC hot plate inside an fume hood. After evaporation, 2.5 mL aqua regia was added to each flask and stored at 4 ºC.

For chemical analysis of zebrafish tissues, 20 individuals per experimental group were collected, frozen individually in liquid nitrogen and stored at -80ºC. Whole zebrafish were dried in an oven at 130 ºC for 24 h. Dry tissues were weighted, pooled (five pools of four zebrafish each), placed into 25 mL Erlenmeyer flasks and processed as described for brine shrimps. The content of each flask was then transferred into tubes and centrifuged for 4 min at 2000 rpm (Heraeus Labofuge 200 centrifuge, Hanau, Germany). The supernatants were moved to clean tubes and stored at 4 ºC.

In both cases, silver content was measured at the Advanced Research Facilities (SGIker-UPV/EHU) by inductively coupled plasma mass spectrometry (ICP-MS, 7700x, Agilent Technologies, California, USA) following US-EPA 6020A guideline, with a limit of detection of 0.01 µg L^{-1}.

2.5. Histological analysis

The visceral masses of 10 fish per experimental group were dissected after 21 days of dietary exposure. Tissues were placed in histological cassettes and immersed in 10% neutral buffered formalin for 24 h at 4ºC. Then, samples were transferred to 70% ethanol and stored at 4ºC until complete tissue processing under vacuum conditions in an ASP300 Tissue Processor (Leica Microsystems, Nussloch, Germany). Paraffin blocks were done using plastic molds. Sections (5 µm thick) were cut in a RM2125RT microtome (Leica Microsystems) for autometallography and histopathological analysis. For the histopathological analysis, slides were stained with hematoxylin/eosin (H/E) in an Auto Stainer XL (Leica Microsystems) and mounted in DPX (Sigma-Aldrich) by means of a CV5030 Robotic Coverslipper (Leica Microsystems).

H/E stained histological sections of the visceral mass were examined under a BX51 light microscope (Olympus, Tokyo, Japan). Sections were specifically examined for the determination of the presence of histopathological alterations, such as inflammatory responses, liver vacuolization and necrosis.

2.6. Autometallographic localization of metal accumulation

Paraffin sections were dewaxed in xylol (Fluka), hydrated in decreasing concentrations of ethanol and left until they were completely dry. A silver enhancement kit for light and electron microscopy (BBI Solutions®, Cardiff, UK) was used according to the manufacturer instructions. The reaction was stopped by rinsing the slides in tap water. Slides were mounted with Kaiser's glycerol gelatin (Sigma-Aldrich). Quantification of the volume density (Vv) of the developed black silver deposits (BSDs) in the intestine and liver tissues indicating the presence of metal was done over five different sections of each individual. Sections were examined under a Laborlux S microscope (Leica Microsystems) and quantification of Vv_{BSDS} was carried out by means of the Biological Measure System (BMS) software (Sevisan, Leioa, Spain).

2.7. Volume density (Vv) of goblet cells

Paraffin sections were dewaxed, hydrated and immersed into 1% Alcian blue (Sigma-Aldrich) pH 2.5 solution for 30 min, washed 2x30 s in deionized water, rinsed in tap water for 3 min, dehydrated in a graded series of ethanol and mounted in DPX.

Using a BX61 microscope (Olympus) equipped with a camera, two different microscopic fields on each individual were photographed at 10x magnification. Afterwards, using the Olympus CELL^D Software, a 50x50 μm^2 squared grid was superimposed onto the images. The number of the grid intersections over goblet cells and over the other intestinal cells was counted to calculate the Vv of goblet cells over the entire intestine using the following stereological formula: nº of intersections over goblet cells / (nº of intersections over goblet cells + nº of intersections over other intestinal cells).

2.8. Lysosomal membrane stability (LMS)

The liver of 5 individuals per experimental group was dissected after 3 and 21 days of dietary exposure, embedded in Cryo-M-Bed (Jung, Heidelberg, Germany) and frozen in liquid nitrogen. Frozen tissue sections (10 μm) were obtained in a CM3050S cryotome (Leica Microsystems) at a cabinet temperature of -24 ºC. The determination of LMS was based on the method used by Bröeg et al. (1999) as the time of acid labilization treatment required to produce the maximum staining intensity in hepatocyte lysosomes after demonstration of acid phosphatase activity. Time intervals used for acid labilization were 0, 2, 4, 6, 8, 10, 15, 20, 25, 30, 40 and 50 min according to Bilbao et al. (2010). Labilization period (LP) was determined under an Olympus BX51 light microscope. Four determinations were made for each individual liver by dividing each section in the acid labilization sequence into 4 approximately equal segments. A mean value was then obtained for each section, corresponding to an individual fish.

2.9. Microarray analysis

Intestine or liver total RNA was extracted following the Trizol® extraction method (Invitrogen, Life-Technologies). Concentration and purity of RNA were measured in a Biophotometer (Eppendorf, Hamburg, Germany). In addition, RNA quality was assessed in the Agilent 2100 Bioanalyzer (Agilent Technologies, California, USA). Only RNA samples with a RIN value above 8.5 were used for microarray and qPCR analysis.

100 ng of total RNA were retrotranscribed and labelled using the Low imput Quick Amp Labeling kit (version 6.5), One color (Agilent Technologies) following the

manufacturer's instructions. Hybridizations were performed on zebrafish 4x44k full genome microarrays (version V3, AMADID 026437 Agilent Technologies) containing 43,803 unique probes using the SuperHyb hybridization chamber (Agilent Technologies). Finally, slides were scanned using a G2565CA DNA microarray scanner (Agilent Technologies). Feature Extraction software v. 10.7.3.1 was used to feature signal intensity extraction and quantile normalization was applied to the raw intensities (log2 values) using Agilent GeneSpring GX software (v 11.2). Microarray analysis and main data treatment were carried out in the General Genomic Service – Gene expression Unit of the University of the Basque Country (SGiKer).

Gene transcription profiles were compared using the LIMMA analysis in the MultiExperiment Viewer (tMeV) vs. 4.7.1 (http://www.tm4.org/mev/) application. Benjamin-Hochberg method (FDR) for multiple test correction was employed to obtain the corrected *p* value. Significant differences were set at an adjusted *p* value $p<0.05$ and based on log2FC>1 or log2FC<-1 (log2 fold change). Then, significantly regulated transcripts were studied using the FatiGo analysis (Babelomics 5 software; Al-Shahrour et al., 2004) in order to decipher biological processes enriched after dietary exposure to Ag NPs. The Fisher exact test ($p<0.05$) was used to find statistically overrepresented functions. The significant regulation ($p<0.05$) of the KEGG pathways respect to the whole genome of *Danio rerio* was performed using the DAVID online tool .

The microarray data have been deposited in NCBI's Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo) and are accessible under the GEO series accession number GSE90457. [Data not yet released; reviewer read-only link: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE90457].

2.10. Quantitative Real Time PCR (qPCR)

Eight genes differentially regulated by the treatment were selected to validate microarray results using qPCR. Genes were selected based on two criteria: fold change (-1.95 <FC< 1.95, adj *p* value < 0.05) and known mechanism of toxicity for Ag NPs. Therefore, selected target genes were: metallothionein 2 (*mt-2*), vitellogenin 2 (*vtg2*), estrogen receptor 1 (*esr*), glutathione S-transferase theta 1b (*gstt1b*), vascular endothelial growth factor (*vegfaa*), peroxisome proliferator activated receptor alpha (*pparaa*), mitochondria associated apoptosis inducing factor (*aifm2*) and DNA J

(*dnajb9a*). Primers for the amplification of target genes were designed using the Primer Express 3.0 program (Applied Biosystems) (Table 1).

Total RNA (1 µg) was retrotranscribed to cDNA using the AffinityScript multi temperature cDNA synthesis kit (Agilent Technologies) following manufacturer's conditions in a 2720 Thermal Cycler (Applied Biosystems). cDNA was amplified in reactions (20 µl) containing 2 µl of diluted sample (Table 1), 10 µl of SYBR Green (Roche), primer pairs at set concentrations (Table 1) and RNAse free water. qPCRs were run in a ViiA7 Applied Biosystems thermocycler (Life Technologies) using 384 well plates. PCR conditions were set as: 50ºC for 2 min, 95ºC for 10 min; 40 cycles at 95ºC for 15 s followed by each melting temperature for 30 s (Table 1). Efficiency was determined running a standard curve and specificity of each reaction was certified by verifying the presence of a single peak in the melting curve plot. Three replicates of each sample and no template controls as well as retro-transcription minus controls were run. cDNA concentration of each sample was measured with the Quant it Oligreen ssDNA assay kit (Life Technologies). Briefly, diluted samples (50 µl) were placed in 96 well clear bottom dark microplates, the reagent was added (50 µl) and fluorescence was measured at 486/20 nm excitation and 528/20 emission in a FLx800 (Biotek) fluorimeter. Relative quantification (RQ) of transcription levels was calculated using the mean value of controls as calibrator in the following formula:

$$RQ= (1+E)^{-\Delta CT} /ng\ cDNA$$

2.11. Statistical analyses

Data recorded in both species from the acute toxicity tests were statistically analyzed by binomial logistic regression. EC_{50} values were calculated using a Probit model. Estimation of parameters was performed using the penalized maximum likelihood method proposed by Firth (Firth, 1993), whenever convergence was not obtained using the maximum likelihood method (Kosmidis, 2013). Data on metal accumulation and LMS were statistically analyzed by multivariate general linear regression models using R 3.1.0 (R Core Team, 2016). Histopathological results were analyzed by the Fisher exact test in the SPSS statistical package v23.0 (SPSS Inc, Microsoft Co, WA, USA).

Data on qPCR gene transcription levels data were tested for normality (Kolmogorov-Smirnov test) and homogeneity of variances (Levene´s test). Data following a normal distribution were analyzed by the Student´s t test, while in non normally distributed data

the non parametric Mann-Whitney U test was applied. In all cases, significance was established at $p<0.05$. Statistical analysis on gene transcription levels was carried out using the SPSS statistical package.

3. Results

3.1. Acute toxicity test in brine shrimps

After 24 h of exposure, significant effects on the surviving rate were recorded only for the 48 hph brine shrimps exposed to 10 mg L^{-1} of Ag NPs ($p=0.048$, Fig. 1C), while no significant effects were recorded for the 24 hph brine shrimps (Fig. 1A). After 48 h of exposure to the Ag NP suspension, a concentration dependent effect was detected for both the 24 hph and the 48 hph brine shrimps (Figs. 1B, 1D). Significant effects were recorded at 5 ($p=0.043$), 7.5 ($p<0.0001$) and 10 mg Ag L^{-1} ($p<0.0001$) for 48 hph brine shrimps and at 7.5 ($p=0.045$) and 10 mg Ag L^{-1} ($p=0.001$) for 24 hph brine shrimps. At 10 mg Ag L^{-1}, the percentage of surviving individuals decreased to 55% for the group of 24 hph brine shrimps and to 21.7% for the group of 48 hph brine shrimps. These decreases were also reflected in the odd ratios values, which indicate the increase of the risk of mortality in brine shrimps exposed to 10 mg Ag L^{-1} respect to the control group, with a value of 54.457 (confidence interval at 95% (CI) 6.628 - >999.99) in 24 hph brine shrimps and a value of 70.239 (CI: 19.445 - 384.71) in 48 hph brine shrimps. Exposure to the PVP/PEI mixture alone did not cause any significant effect in any of the assayed conditions (Fig. 1). Calculated EC_{50} values and their 95% confidence intervals are shown in Table 2.

3.2. Silver accumulation in brine shrimps and zebrafish and selection of exposure concentrations for zebrafish dietary experiment

Silver accumulation was measured in brine shrimps of 24 hph after exposure for 24 h to five different concentrations of the Ag NP suspension (Fig. 2A). At the two highest exposure concentrations (0.1 and 1 mg Ag L^{-1}) similar and significantly higher values of silver accumulation than in control fish were recorded ($p<0.0001$), with a bioconcentration factor (BCF) of about 78 for the exposure to 0.1 mg Ag L^{-1}. Exposure to 10 µg Ag L^{-1} resulted in an average accumulation of 1.8 µg Ag g^{-1} dw with a BCF of

3.5, although no significant differences were found in comparison with the control group. At lower Ag NP concentrations, similar values were measured in exposed and control brine shrimps.

Based on these results, the selected concentrations to expose brine shrimps for zebrafish feeding were 100 μg Ag L^{-1} as the high dose (HD) and 100 ng Ag L^{-1} as the low dose (LD), which is an environmentally relevant concentration of silver. Based on the accumulation data and the selected zebrafish diet of 2.5% body weight per day, nominal exposure concentrations of 2.1817 ng Ag $fish^{-1}$ day^{-1}, in the case of the HD exposure group, and 0.17025 ng Ag $fish^{-1}$ day^{-1}, in the case of the LD exposure group, were estimated.

During the dietary exposure experiment, a sample of the brine shrimp cultures was collected at four different days (days 1, 7, 14 and 21), for chemical analysis of silver in order to corroborate previous accumulation data. Accumulation of silver in brine shrimps was lower than in the previous experiment, but accumulation pattern was maintained (Fig. 2B). An average silver accumulation of 3.9 μg Ag g^{-1} dw was recorded for the HD exposure group, while a mean value of 0.3 μg Ag g^{-1} dw was obtained for the LD exposure group. Significant differences were found between de HD exposure group and the control group (p=0.002), as well as, between the HD and the LD exposure groups (p=0.003).

Chemical analysis of control zebrafish fed with unexposed brine shrimps and zebrafish fed for 21 days with brine shrimps exposed to the low concentration of the Ag NP suspension showed similar silver content. Zebrafish fed for 21 days with brine shrimps exposed to the high concentration of the Ag NP suspension showed higher silver content, although this increase was not statistically significant due to the high variability recorded between individuals (Fig. 2C).

3.3. Autometallography: volume density of BSDs

Intestine tissue showed higher metal accumulation (higher values of Vv_{BSDs}) than liver, although both organs showed the same pattern of dose- and time-dependent metal accumulation (Fig. 3).

In the intestine, despite the high mean values measured for Vv_{BSDs} in zebrafish fed with brine shrimps exposed to the HD of Ag NPs, no significant differences were detected at any exposure time due to the high variability recorded in individuals from

this group (Fig. 3A). As it can be seen in Figs. 4A and 4B, no BSDs were detected in intestine sections of the control fish, whereas in the intestine of fish fed with brine shrimps exposed to the LD, few BSDs were observed at three (Fig. 4C) and 21 days (Fig. 4D) resulting in low Vv_{BSDs} values (Fig. 3A). In the intestine of fish fed with brine shrimps exposed to the HD, an increase of the Vv_{BSDs} was already observed at three days which was further increased at 21 days (Fig. 3A). A high volume density of deposits was found in the epithelial cells of the intestine (Fig. 4F).

In the liver, differences in Vv_{BSDs} between treatments were observed after both exposure times, but these differences were statistically significant only after 21 days of exposure for fish fed with brine shrimps exposed to the HD of the Ag NP suspension (p=0.009, Fig. 3B). As in the case of the intestine tissue, a high variability was observed among the fish individuals of the HD treatment. No BSDs were detected in the liver of control fish (Figs. 5A, 5B). Few BSDs were detected after 3 days (Fig. 5C), increasing after 21 days in the liver of fish fed with brine shrimps exposed to the LD (Fig. 5D). In fish fed with brine shrimps exposed to the HD, few BSDs were present after 3 days (Fig. 5E), whereas large and abundant BSDs appeared homogeneously distributed in hepatocytes after 21 days (Fig. 5F).

3.4. General health status: Lysosomal membrane stability (LMS) and histopathological assessment

A significant decrease in the mean value of the labilization period of the hepatocyte lysosomal membrane was measured after both treatments and at both exposure times (p<0.0001, Fig. 6). No significant differences were found for this parameter between fish fed with brine shrimps exposed to the LD and those exposed to the HD of Ag NPs.

In the intestine, no significant differences among groups were detected in the volume density of goblet cells after Alcian blue staining of paraffin sections (data not shown). In the liver vacuolization and necrosis were detected in individuals fed with brine shrimps exposed to both doses of Ag NPs. Control fish showed a normal liver histology at both exposure times (Figs. 7A-B), only one individual sampled at 21 days of experiment showed vacuolization. After dietary exposure to both doses of Ag NPs, a significantly higher (p=0.033 for the low dose and p=0.030 for the high dose) prevalence of histopathological alterations was detected at 3 days of treatment in comparison to the control group but, in both cases, the prevalence of the lesions

diminished in fish sampled at 21 days (Table 3). The dietary exposure of zebrafish through brine shrimps treated with Ag NPs, even at the environmentally relevant concentration, provoked liver vacuolization, which prevalence increased significantly after 3 days of treatment (Fig. 7C). Necrotic foci were also observed in the liver of some zebrafish fed with brine shrimps exposed to both doses of Ag NPs (Fig. 7D).

3.5. Transcriptomics

No significant probes were regulated (LIMMA analysis, adj. *p* value < 0.05) in the intestine after 3 days of dietary exposure and only *pwp1* was significantly regulated (LIMMA analysis, adj. *p* value =0.017) after 21 days. In liver, only *atp2a2a* was significantly altered (LIMMA analysis, adj. *p* value =0.041) after 3 days of dietary exposure, whereas after 21 days, 261 probes were significantly altered, being 121 probes significantly up-regulated and 140 significantly downregulated. After the gene level analysis, 176 genes were found to be significantly regulated in liver after 21 days of exposure (LIMMA analysis, adj. *p* value < 0.05).

Lipid transport (GO:0006869) and lipid localization (GO:0010876), cellular response to chemical stimulus (GO:0070887) and response to xenobiotic stimulus (GO:0009410) (Table 4) were significantly regulated (adjusted p values < 0.05 obtained in the Fisher's exact test) in liver of zebrafish dietary exposed to AgNPs for 21 days. The KEGG pathways "protein processing in the endoplasmic reticulum" (*p* value= 7.8 e^{-4} and FDR = 3.8e^{-2}) and "glutathione metabolism" (*p* value = 4.6e^{-2} FDR = 6.9 e^{-1}) were significantly affected respect to the whole genome of zebrafish.

With the exception of *aifm2*, the rest of target genes selected for the validation of microarray results showed similar fold change levels after both microarray and qPCR analysis (Table 5).

4. Discussion

In the present study, transference of silver through the food web and derived effects were studied using brine shrimp larvae and adult zebrafish as a simple trophic chain. Brine shrimps were exposed to two different concentrations of a Ag NP suspension and, then, used to feed zebrafish for 21 days. Before the dietary exposure experiment, acute toxicity of the Ag NP suspension and of the PVP/PEI mixture present in the suspension

for the stabilization of the Ag NPs was tested in brine shrimp larvae. The PVP/PEI mixture was found to be nontoxic at any of the assayed exposure times or concentrations, while the Ag NP suspension showed significant acute toxic effects on brine shrimps at the highest tested concentrations (≥ 5 mg Ag L^{-1}, depending on the exposure conditions). As exposure time increased, the silver concentration causing significant effects decreased, in agreement with previously published results. Aruvalsu et al. (2014) reported an increase in the mortality rate of brine shrimp nauplii as exposure time to Ag NPs increased, detecting a higher amount of Ag NPs aggregates into the gut of brine shrimps exposed for 48 h than in the gut of brine shrimps exposed for 24 h. For the same exposure period, brine shrimps of 48 hph were more sensitive to Ag NPs than brine shrimps of 24 hph. The increased toxicity at more advanced stages of brine shrimp development, in comparison with the earlier stages, has been related to the fact that more developed brine shrimps feed more voraciously and, therefore, the ingestion of NPs may increase (Rajasree et al., 2011).

The Ag NP suspension used in the present study has been assayed in other test systems displaying a high toxicity. In zebrafish embryos, reported LC_{50} value at 120 h was 0.057 mg Ag L^{-1} (Orbea et al., this issue). Similar results were obtained in three different species of microalgae, with EC50 values ranging from 0.039 to 0.06 mg Ag L^{-1} (Schiavo et al., 2017). In contrast, EC50 values estimated in the present work for brine shrimps were much higher (7.39 to 19.63 mg Ag L^{-1}, depending on the exposure conditions). In comparison with other small crustaceans, such as *Daphnia magna*, brine shrimps also seem to be less sensitive to the exposure to Ag NPs. In *D. magna*, reported EC_{50} values at 24 h of exposure to Ag NPs prepared with different dispersion methods ranged between 0.004 and 3.844 mg Ag L^{-1} (Jo et al., 2012). This variability in the EC_{50} values for silver concentration reported for *D. magna* can be explained by the wide variety of treatments that Ag NPs can undergo and the diversity of coatings and sizes, which greatly affect their toxicity (Jo et al., 2012; Römera et al., 2013; Silva et al., 2014). Differences in the toxicity of Ag NPs to the different species can be related to several biotic and abiotic factors including the habitat and, therefore, the exposure medium used in the laboratory is a key factor. *D. magna* and zebrafish are freshwater organisms and brine shrimp is a salt water species. Salinity of the exposure medium can be a determinant factor for Ag NP toxicity. It is well described that the higher ionic strength may result in aggregation of NPs, as shown in the secondary characterization of the same Ag NPs reported by Schiavo et al. (2017), and create links between free silver

cations and anions present in the salt water, which may partially neutralize the toxicity of the silver ions in the Ag NP suspension (Kalbassi et al., 2011). This fact would not explain the difference in sensitivity compared to the microalgae species *Isochrysis galbana*, *Tetraselmis suecica*, and *Phaeodactylum tricornutum* that are also sea water species (Schiavo et al., 2017). Nevertheless, brine shrimps, as euryhaline species, have a great osmoregulatory capacity, which contributes to a greater resistance to the toxic effect of metal cations and allows them living in environments with a high salt concentration (Gajardo and Beardmore, 2012). In other crustacean species, such as the shore crab *Carcinus maenas*, it has also been reported that salinity strongly affects metal toxicity with lower toxicity at higher salinity (Blewett and Wood, 2015).

Although no acute toxicity was found, exposure of brine shrimps to 0.1 mg Ag L^{-1} led to significant silver accumulation and similar to that obtained after exposure to 1 mg Ag L^{-1}. Thus, 0.1 mg Ag L^{-1}, as a potentially effective concentration, and 100 ng Ag L^{-1}, which is considered as an environmentally relevant concentration according to literature data (Hendren et al., 2013), were selected to expose brine shrimps to be used as food for zebrafish.

According to the results obtained, dietary exposure resulted in a silver transfer from the brine shrimps exposed to the Ag NP suspension to zebrafish. Metal transference through the food chain has been previously detected in zebrafish fed with *D. magna* exposed to Ag NPs, causing changes in the microbiome structure of the zebrafish gut (Merrifield et al., 2013). Also, with other metal containing NPs, namely TiO_2 NPs, effective metal transfer has been measured from crustaceans to zebrafish in a simplified food web (Zhu et al., 2010). In this later study, metal accumulation was significantly higher in zebrafish exposed through the diet than in zebrafish waterborne exposed to the same original exposure concentration of TiO_2 NPs. In a waterborne exposure experiment performed in our laboratory, exposure of zebrafish for 21 days to 10 µg Ag L^{-1} of maltose-coated Ag NPs of 20 nm resulted in an accumulation value of 0.88 µg Ag g^{-1} dw (Lacave et al., in preparation). In the present study, dietary exposure for 21 days through brine shrimps exposed to 100 µg Ag L^{-1} resulted in an accumulation of 1.39 µg Ag g^{-1} dw. Therefore, zebrafish can take up silver directly from the medium and through the food, being necessary to consider both routes of entrance in order to assess the biological effects provoked by the exposure to Ag NPs in the environment.

The autometallographical staining performed in intestine and liver tissues of zebrafish manifested an effective dose- and time-dependent accumulation of metal in

the tissues. Autometallographical staining has been already employed to evidence the deposition of metals as appearance of BSDs, in the tissues of zebrafish after waterborne exposure to metals and metal bearing nanoparticles (Vicario-Parés et al., 2014; Osborne et al., 2015). Here, higher Vv_{BSD} values were found for the intestine than for the liver, in agreement with the exposure route used. Thus, the intestine seems to be the gate of entrance of the metal in zebrafish, as it was the site where the digestion of the contaminated brine shrimps took place. Many of the BSDs found in the intestine were located in the goblet cells, maybe due to the strong affinity that glycoproteins and proteoglycans present in the mucous exhibit for metals and other xenobiotics (Pawert et al., 1998), and their excretion function into the gut lumen. This novel excretion pathway of nanomaterials through the intestinal goblet cells has been proposed by Zhao et al. (2013) who injected zebrafish embryos with 30-200 nm activated carbon NPs directly into the yolk sac reporting that NPs can be excreted directly through intestinal tract without involving the hepato-biliary system. Despite the BSDs were detected in the goblet cells, no significant differences among treatment groups were detected in the volume density of these cells. In contrast, Osborne et al. (2015) detected an increased number of goblet cells in the epithelial layer, some reduction in microvilli, and partial damage to the lamina propria in zebrafish waterborne exposed to Ag NPs of 20 nm.

The amount (Vv_{BSDs}) of metal within the liver was lower than in the intestine. Similar results were also found in the liver of zebrafish waterborne exposed to Ag NPs (Lacave et al., in preparation). The presence of metal in the liver is mediated by the blood vessel transport after absorption through the intestine (Hadrup and Lam, 2014). The liver is the main organ involved in the detoxification of xenobiotics (Feist et al., 2004) and it is highly irrigated. Therefore, the transport through the blood vessels may be the main route for metals to access and to be accumulated in the liver. Yeo and Pak (2008) observed nanosilver accumulation in blood vessels after waterborne exposure zebrafish to Ag NPs.

Lysosomes have been described as a target organelle of metals and metal NP exposure (Köhler et al., 2002; De Matteis et al., 2015). The characteristic acidic environment of lysosomes can provoke the NP dissolution and, in turn, the release of silver ions to the cell cytoplasm increasing the production of oxyradicals (Wei et al., 2015). Vicario-Parés et al. (submitted) observed a desestabilization of the lysosomal membrane after 3 and 21 days of waterborne exposure of zebrafish to 10 µg Cu L^{-1} of ionic copper and CuO NPs. Also, waterborne exposure of zebrafish to 10 µg Ag L^{-1} of

ionic silver and Ag NPs caused the decrease of the time necessary to desestabilize the lysosomal membrane, although in a lesser extent than in the case of the exposure to CuO NPs (Lacave et al., in preparation). During the present study, although zebrafish fed for 3 days with brine shrimps exposed to the LD of Ag NPs did not accumulate silver significantly and few BSDs were detected in liver by autometallography, a significant decrease was measured in the stability of the lysosomal membrane, which can induce the formation of ROS when the NPs are exposed to the acidic environment of lysosomes (Chang et al., 2012). Thus, the dietary exposure of Ag NPs to zebrafish, even at environmentally relevant concentrations, and for a short-time provokes a toxic effect in the organisms.

Along with the accumulation of metal detected by autometallography and the decrease in the stability of the lysosomal membrane reported, some histopathological alterations were detected in the liver. Feeding with brine shrimps exposed to both Ag NP concentrations provoked similar alterations at both times of exposure. Fat vacuolization in the liver of fish has been proved to be provoked by the exposure to toxic compounds (Köhler et al., 2002; McHugh et al., 2011). This histopathological condition, which has also been demonstrated to be produced after the waterborne exposure (0.01 mg L^{-1}) to the same Ag NP suspension for 21 day (Orbea et al., this issue) is a symptom of metabolism disruption caused by the exposure of fish to metals (Bougas et al., 2016). Necrosis was also detected in liver of zebrafish fed with brine shrimps exposed to both concentrations, being in agreement with the results obtained by Devi et al. (2015), who detected extensive cell death, necrosis and degenerative changes in liver of adult zebrafish waterborne exposed to 0.1 mg L^{-1} of Ag NPs for 15 days. Similarly, in zebrafish exposed to higher concentrations (30 and 120 mg L^{-1}) for a shorter period of time (24 hours), histopathological lesions such as disruption of hepatic cells cords and apoptotic changes (chromatin condensation and pyknosis) have been reported (Choi et al., 2010). Devi et al. (2015) indicated the ability of Ag NPs to alter the biochemical functions associated with the liver which could provoke the toxic effect in the organism. Other authors have suggested that the exposure to Ag NPs provoke oxidative stress in liver of different fish, which may provoke the apparition of histopathological lesions in the liver (Chae et al., 2009; Choi et al., 2010; Wu and Zhou, 2013).

At the molecular level, high Vv_{BSDs} values measured in the intestine were not reflected as significant alterations in the intestinal transcriptome; in fact, significant

alterations in gene transcription levels were more marked in the liver than in the intestine, pointing out liver as the main target organ for Ag NPs dietary exposure of zebrafish. After 21 days of exposure, a single probe was significantly altered in the intestine (*pwp1*) while at the same period transcripts altered in the liver reached 261 (176 genes), 121 probes were significantly upregulated and 140 downregulated. In contrast only *atp2a2a* was significantly altered in liver after 3 days of exposure. This time dependent marked regulation of gene transcription levels was also reported after zebrafish waterborne exposure to Ag NPs (Lacave et al., in preparation).

Based on the FatiGO analysis, "lipid transport" (GO:0006869) and "lipid localization" (GO:0010876) and "cellular response to chemical stimulus (GO:0070887) and to xenobiotics" (GO:0009410) were the main affected biological processes in liver after 21 days of exposure. Among lipid transport and localization related genes, *apolipoprotein A IV 1 and 2* (*apo4b1 and apo4b2*), coding for the major component of HDL and chylomicrones, were significantly downregulated, which could significantly affect cholesterol biosynthesis. Cholesterol is required for membrane stability, formation of billiary acids or biosynthesis of steroid hormones (Santos et al., 2010) and thus, a significant decrease in the transcription of genes involved in the biosynthesis of cholesterol may have major implications in fish health status. Similarly, Lacave et al. (in preparation) reported significant alterations in the steroid biosynthesis metabolism after zebrafish waterborne exposure to Ag NPs and Lee et al. (2012) reported a decrease in the total concentration of cholesterol in *Cyprinus carpio* exposed to Ag NPs (12 nm) for 4 days. The nuclear receptor PPARα belongs to the steroid hormone receptor superfamily and is a key regulator of lipid metabolism. Thus significant decreased transcription levels on *pparaa* may lead to important alterations on lipid metabolism too, although this gene was grouped within the biological processes "cellular response to chemical stimulus and to xenobiotics". In addition, four different types of *vitellogenin (vtg1, vtg4, vtg6* and *vtg7),* considered marker of xenoestrogenicity, were also significantly altered in liver in relation to "lipid transport and lipid localization". Interestingly, upregulation of *vtg1* was also related to "cell response to chemical and xenobiotic stimulus" biological processes significantly affected after the exposure, in which upregulation of the nuclear receptor *estrogen receptor* (*esr*) was also found. This significant upregulation of *esr* and *vtg* genes could suggest an estrogenic effect of Ag NPs, which has also been reported in male medaka chronically exposed to Ag NPs (Pham et al., 2012).

As it was previously mentioned, the exposure to Ag NPs can provoke an overproduction of ROS that may cause oxidative stress (Christen et al., 2013) especially when the cellular antioxidant defense is deficient. Glutathione metabolism plays an important role in the antioxidant defense but, after the present Ag NPs dietary exposure it was one of the KEGG pathways significantly regulated in liver together with the "protein processing in the endoplasmic reticulum" whose deficient activity could lead to the production of misfolded proteins. Misfolded proteins are directed toward degradation through the proteasome; but in the present sudy, *keap1b* involved in protein ubiquitination (grouped in the biological processes "cellular response to chemical stimulus and to xenobiotics") and whose expression is inhibited by oxidative stress was significantly downregulated in liver after the Ag NP dietary exposure.

5. Conclusions

The suspension of PVP/PEI-coated Ag NPs of 5 nm tested in this work showed low toxicity to brine shrimp larvae, which seem to be much more sensitive than other species, such as zebrafish (embryos) and microalgae. The toxicity detected at high exposure concentrations could not be attributed to the presence of the stabilizer because the PVP/PEI mixture was not acutely toxic in any of the exposure conditions tested. Bioaccumulation of silver was detected in brine shrimps exposed to sublethal concentrations of Ag NPs, being effectively transferred through the diet to adult zebrafish. The silver transfer can cause toxic sublethal effects and act in detriment of the health of the fish as indicated by the significant reduction of the stability of the hepatocyte lysosomal membrane and the presence of histopathologies in the liver. At molecular level, transcription levels of genes involved in lipid localization and lipid transport and cellular response to chemical stimulus and xenobiotics were significantly altered in liver but not significant effects were recorded in the intestine. Therefore, the obtained results indicated that the food chain is an exposure route for nanomaterials in the aquatic environment and that the liver is a target organ for Ag NP toxicity when organisms are exposed through the diet.

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FIGURE LEGENDS

Figure 1.- Percentage of surviving brine shrimp nauplii exposed to different dilutions of the Ag NP suspension and the equivalent concentration of the PVP/PEI mixture present in the dilutions of the NP suspension. (A) 24 hph brine shrimps exposed for 24 h; (B) 24 hph brine shrimps exposed for 48 h; (C) 48 hph brine shrimps exposed for 24 h; (D) 48 hph brine shrimps exposed for 48 h. The empty symbols indicate significant differences (p<0.05) respect to the control group according to the binomial logistic regression models.

Figure 2.- Silver accumulation levels (µg Ag g^{-1} dry weight) measured by ICP-MS; (A) 24 hph brine shrimps exposed to five different concentrations for 24 h (n=4); (B) silver accumulation in brine shrimps cultured for the dietary exposure experiment and collected at four different days (n=4); (C) silver accumulation in whole zebrafish tissue after 21 days of dietary exposure (n=5). Results are given as mean ± standard deviation. Letters indicate statistically significant differences (p<0.05) according to the multivariate general linear regression models, groups bearing different letters differ significantly.

Figure 3.- Volume density of autometallographical black silver deposits (Vv_{BSDs}) indicating metal accumulation in the intestine (A) and in the liver (B) of zebrafish fed with brine shrimps exposed to different concentrations of the Ag NP suspension. Results are given as mean ± standard deviation (n=10). Letters indicate statistically significant differences (p<0.05) according to the multivariate general linear regression models, groups bearing different letters differ significantly. Numbers on the bars indicate the mean and standard deviation values reached by that group.

Figure 4.- Micrographs of paraffin sections of the intestine after autometallographical staining. Black silver deposits indicate metal accumulation in the tissue. (A-B) Zebrafish fed with unexposed control brine shrimps. (C) Zebrafish fed with brine shrimps exposed to the LD of the Ag NP suspension for 3 days. (D) Zebrafish fed with brine shrimps exposed to the LD of the Ag NP suspension for 21 days. (E) Zebrafish fed with brine shrimps exposed to the HD of the Ag NP suspension for 3 days. (F)

Zebrafish fed with brine shrimps exposed to the HD of the Ag NP suspension for 21 days. Scale bars: 20 µm.

Figure 5.- Micrographs of paraffin sections of the liver after autometallographical staining. Black silver deposits indicate metal accumulation in the tissue. (A-B) Zebrafish fed with unexposed control brine shrimps. (C) Zebrafish fed with brine shrimps exposed to the LD of the Ag NP suspension for 3 days. (D) Zebrafish fed with brine shrimps exposed to the HD of the Ag NP suspension for 3 days. (E) Zebrafish fed with brine shrimps exposed to the LD of the Ag NP suspension for 21 days. (F) Zebrafish fed with brine shrimps exposed to the HD of the Ag NP suspension for 21 days. Scale bars: 20 µm.

Figure 6.- Labilization period (in minutes) of the lysosomal membrane in liver cells. Results are given as mean ± standard deviation (n=5). Letters indicate statistically significant differences ($p<0.05$) according to the multivariate general linear regression models, groups bearing different letters differ significantly.

Figure 7.- Micrographs of paraffin sections of the zebrafish liver after H/E staining. (A) Liver of control zebrafish at 3 days. (B) Liver of control zebrafish at 21 days. (C) Liver of zebrafish fed for 3 days with brine shrimps exposed to the HD of Ag NPs, presenting vacuolization. (D) Liver of zebrafish fed for 21 days with brine shrimps exposed to the LD of Ag NPs, presenting a necrotic focus. Scale bar: 50 µm.

946 Table 1.- Selected target genes and Genbank accession codes are followed by specific
947 primers and qPCR conditions used for each fragment amplification.

Gene	Accession number	Forward (5'-3')	Reverse (5'-3')	cDNA dil.	[Primer] pmol/µl	Tm
Mt-2	NM_001131053	TGCACTAATTGCCAGTGTACTACCT	GCACACTTGCTGCAACCAGAT	1/5	0.625	60
vtg2	NM_001044913	CAATCAGCACCTTCAACAACAAA	GGGCTAAAACTTGGTAGCAGGAA	-	0.25	57
esr	NM_152959	CATGCAGACTGCGCAAGTGT	CGCCCTCCGCGATCTT	1/5	0.25	57
gsttib	NM_200584	CATTCGGATGCATGGAGCTA	GACCTCAGCTCCCAGCACTT	1/5	0.25	57
vegfaa	NM_001110349	CAACGCGTATCGCAGCATAA	CCTTTGGCCTGCATTCACA	-	0.25	57
pparaa	NM_001161333	TGCCGATTCCGCAAGTG	GCCCAAAACGAATAGCCGTTGT	1/5	0.25	57
aifm2	NM_001200010	GGCATTGCAGCAGCTCAAC	ACGCATCCAGGACGTCAATAA	1/5	0.375	58
dnajb9a	NM_001025184	CGACACGTAAAGAGGACAGATAGC	TCGCTGGGTCATCGTCCTA	1/5	0.375	59

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953

954 Table 2.- Estimated EC_{50} values and 95% confidence intervals at (in brackets) for brine
955 shrimps exposed to Ag NPs.

	24 hph		**48 hph**	
	24 h of exposure	**48 h of exposure**	**24 h of exposure**	**48 h of exposure**
Ag NP suspension (mg Ag L^{-1})	19.63 (3.81 - 35.45)	10.24 (8.96 - 11.52)	16.52 (10.83 - 22.21)	7.39 (6.63 - 8.15)
PVP/PEI mixture (mg PVP/PEI L^{-1})	277.07 (24.13 - 530)	500.73 (-486.77 - 1488.23)	206.73 (75.5 - 337.95)	212.7 (69.16 - 356.24)

956 hph: hours post hatch (at the beginning of the exposure).

957

958 Table 3.- Prevalence of histopathological alterations in liver of zebrafish after dietary
959 exposure to Ag NPs. Data are shown in percentages. Asterisks indicate statistically
960 significant differences between control and exposed groups (p<0.05) according to the
961 Fisher's exact test.

Group	Sampling	n	Vacuolization	Necrosis	Total
Control	3 days	10	0	0	0
	21 days	10	10	0	10
Low dose	3 days	10	40	10	50*
	21 days	10	0	20	20
High dose	3 days	10	60*	10	70*
	21 days	10	30	0	30

962 n: number of individuals per experimental group. Total: percentage of individuals per
963 group presenting any histopathological alteration.

964

965 Table 4.- Go biological processes and genes significantly altered in zebrafish liver 21
966 days after dietary exposure to Ag NPs. Adjusted p values obtained in the Fisher's exact
967 test (FatiGO) are also shown.

968

Go Process	Altered genes	Adj. p value
Lipid transport (GO:0006869) lipid localization (GO:0010876)	*vtg1, vtg4, vtg6, vtg7, apo4b.1, apo4b.2, tnks1bp1*	$1,2^{e-4}$
Cellular response to chemical stimulus (GO:0070887)	*keap1b, tsen15, bco21, dll4, sult1st5, er, pparaa, vtg1, pik3cg*	0,0029
Response to xenobiotic stimulus (GO:0009410)	*keap1b, tsen15, sult1st5, vtg1*	0,0039

969

970

Table 5.- Microarray and qPCR transcription levels of hepatic selected target genes after 21 days of exposure. Gene names are followed by log fold change (Fc) and adjusted p values for microarrays (LIMMA) as well as Fc and p values for qPCR data (Student´s t test or Mann Whitney U test). Asterisks indicate significant differences respect to controls.

Gene	Microarray			qPCR		
	Log Fc	**Adj P value**	**Reg.**	**Fc**	**P value**	**Reg**
mt-2	-2,63	0,048	DOWN*	-4,15	0,031	DOWN*
vtg2	12,65	$2,14\ e^{-5}$	UP*	11,6	0,008	UP*
esr	2,22	0,018	UP*	2,86	0,02	UP*
gsttib	-1,17	0,002	DOWN*	-1,25	0,14	DOWN
vegfaa	-1,5	0,011	DOWN*	-1,34	0,03	DOWN*
ppara	-1,29	0,028	DOWN*	-1,03	0,15	DOWN
aifm2	-2,29	0,003	DOWN*	0,67	0,29	UP
dnajb9a	1,15	0,011	UP*	2,5	0,01	UP*

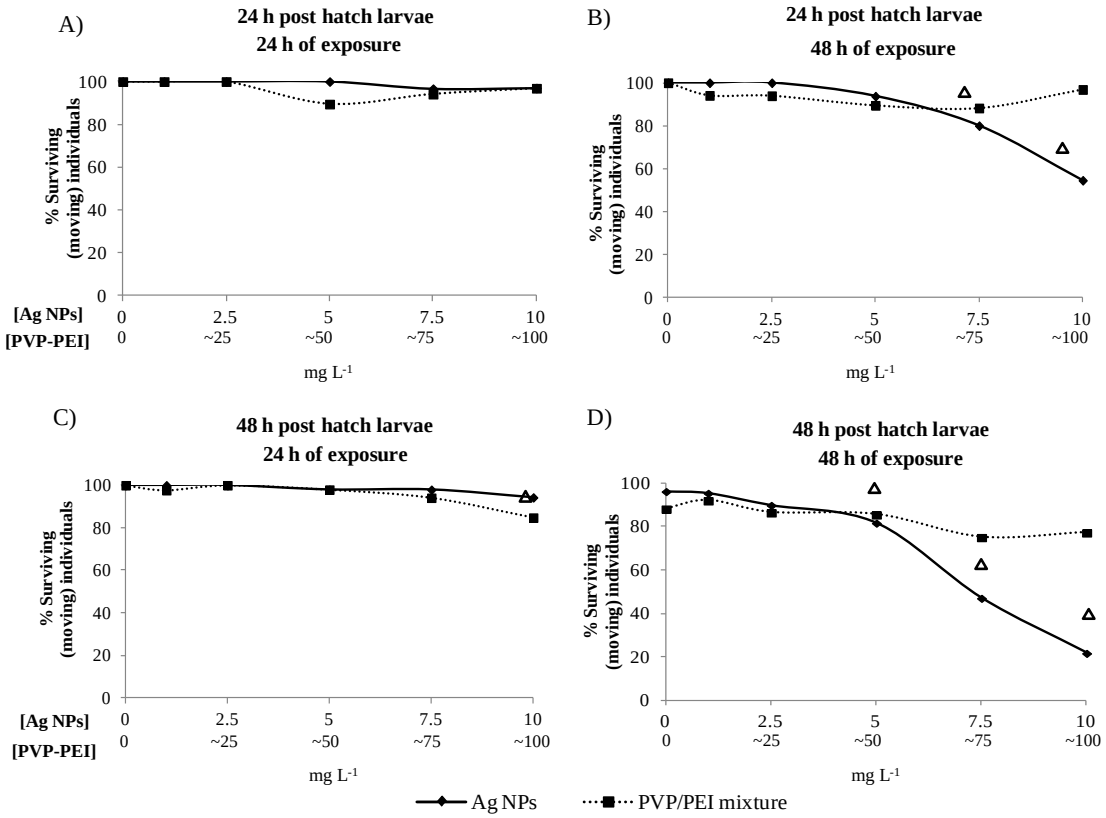
A)
24 h post hatch larvae
24 h of exposure
B)
24 h post hatch larvae
48 h of exposure
C)
48 h post hatch larvae
24 h of exposure
D)
48 h post hatch larvae
48 h of exposure
% Surviving (moving) individuals
[Ag NPs]
[PVP-PEI]
0 2.5 5 7.5 10
0 ~25 ~50 ~75 ~100
mg L-1
Ag NPs
PVP/PEI mixture

Figure 1.-

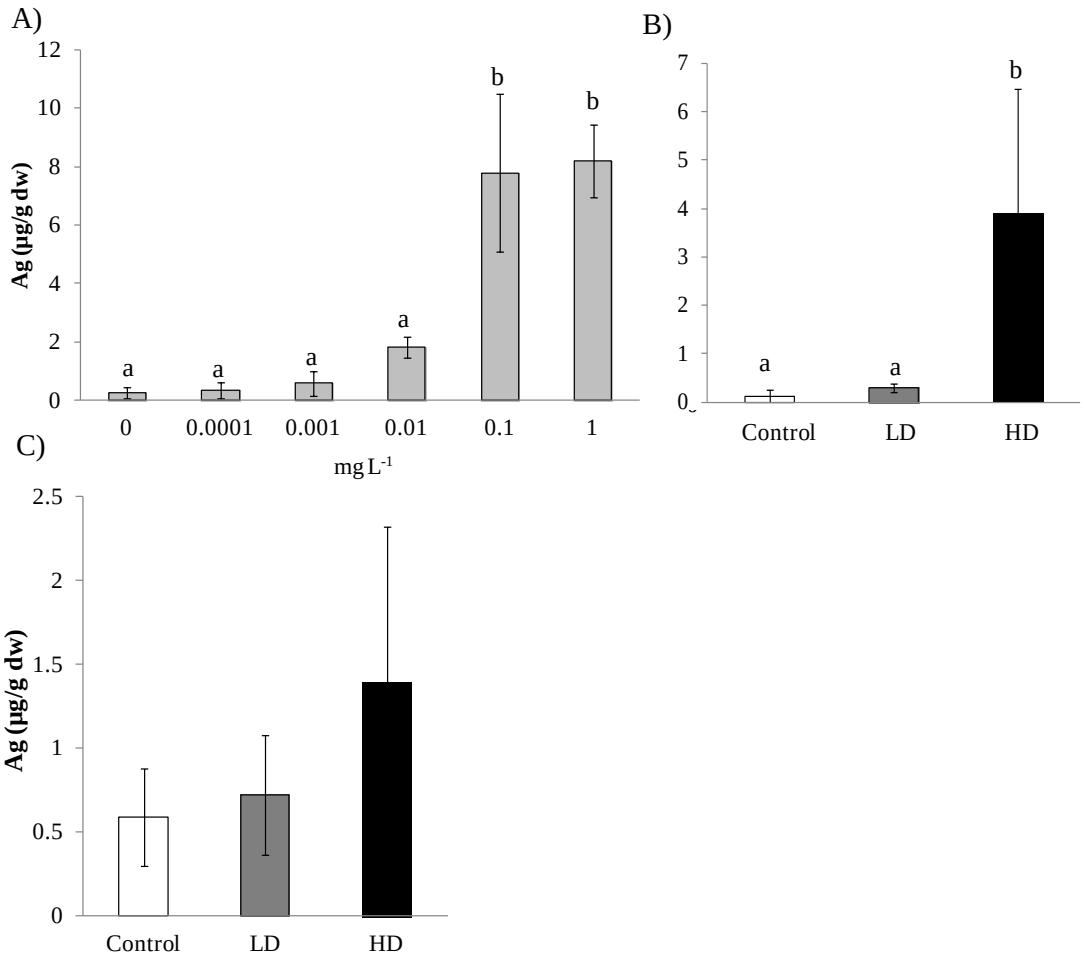
A)
Ag (µg/g dw)
12
10
8
6
4
2
0
a
a
a
a
b
b
0
0.0001
0.001
0.01
0.1
1
mg L-1
B)
7
6
5
4
3
2
1
0
a
a
b
Control
LD
HD
C)
Ag (µg/g dw)
2.5
2
1.5
1
0.5
0
Control
LD
HD

Figure 2.-

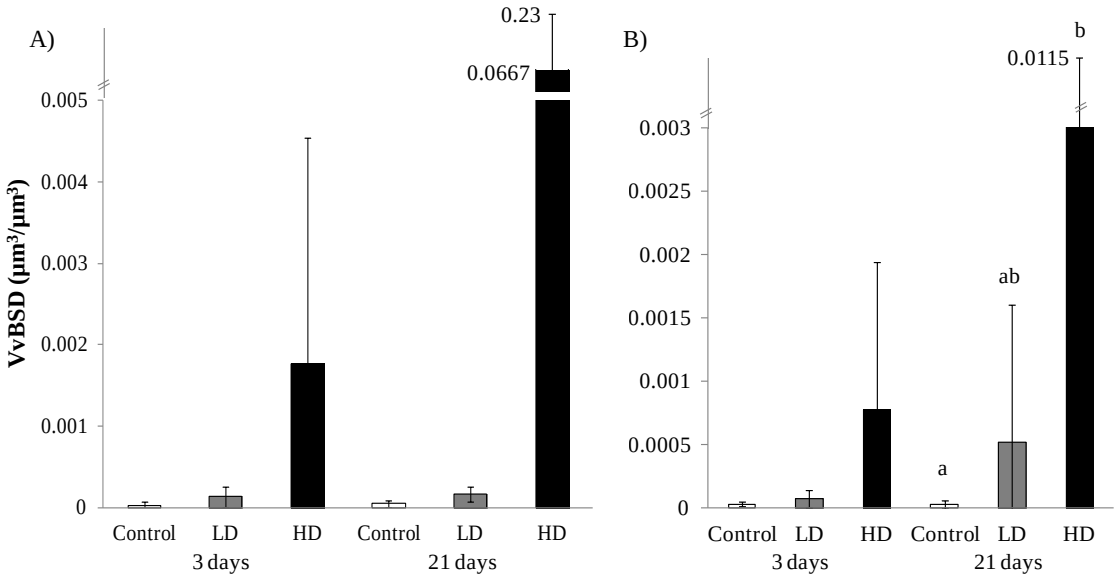
A)
B)
VvBSD (µm³/µm³)
0.23
0.0667
0.0115
b
ab
a
0
0.001
0.002
0.003
0.004
0.005
0.0005
0.0015
0.0025
Control
LD
HD
3 days
21 days

Figure 3.-

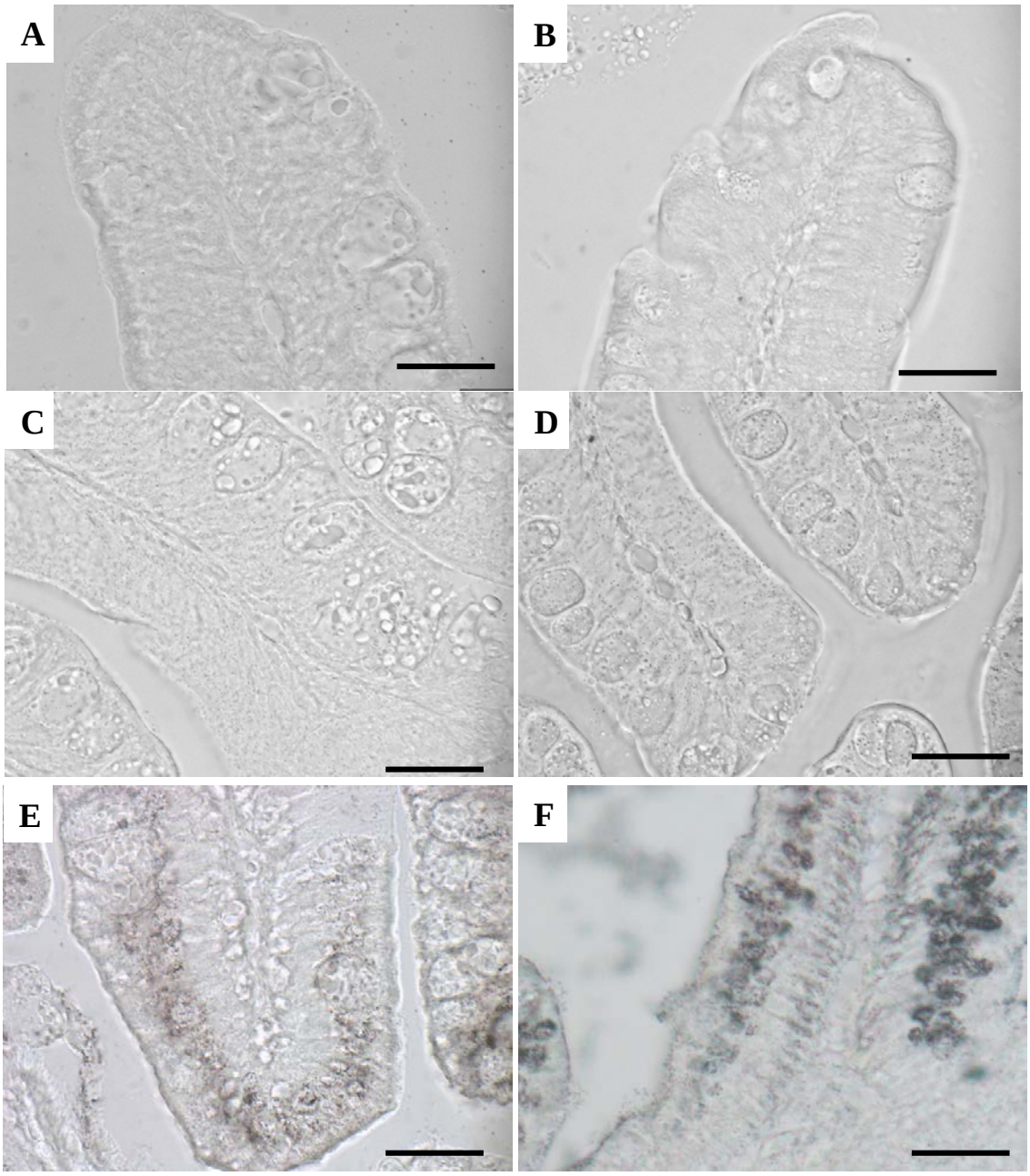

Figure 4.-

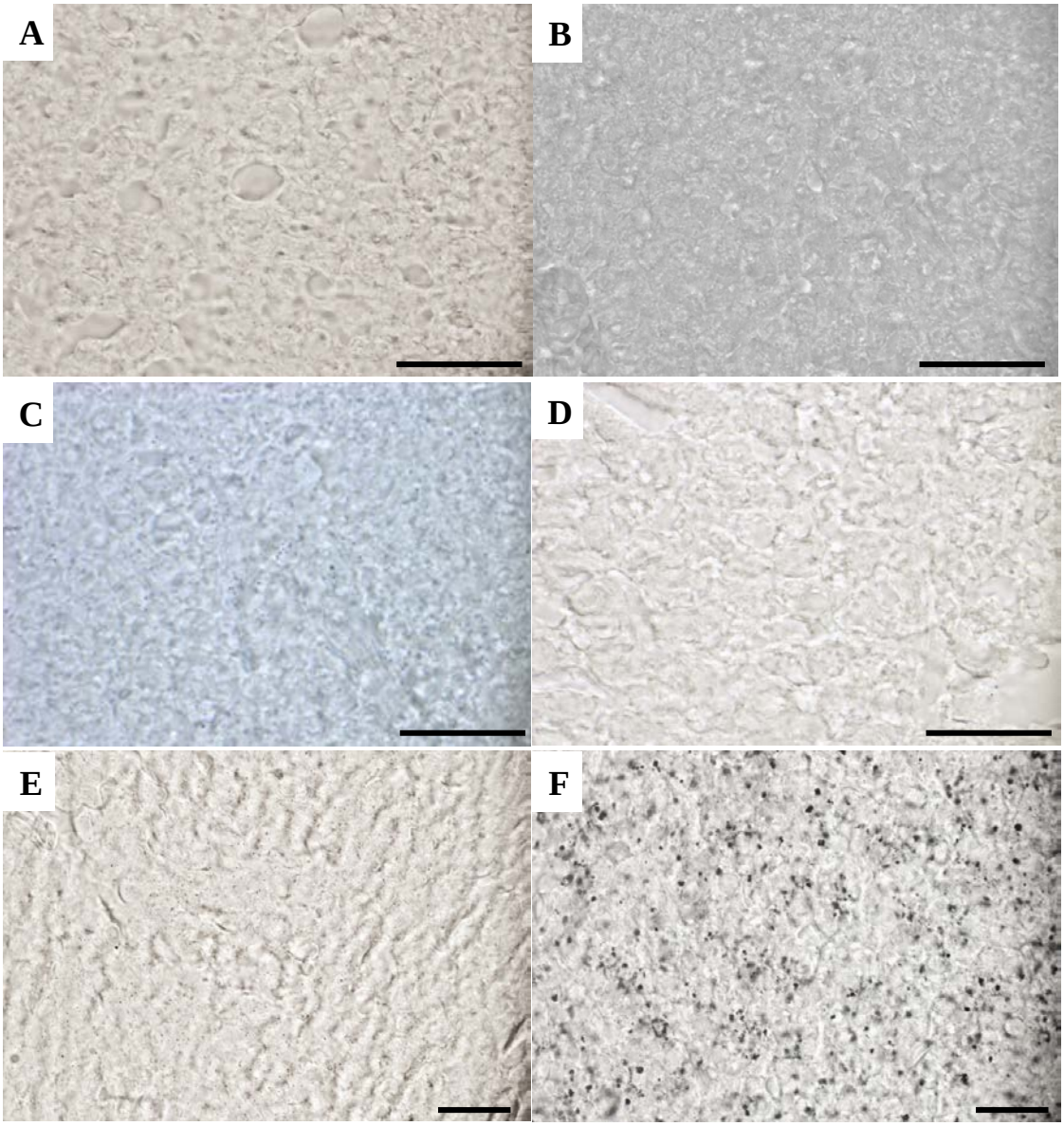

Figure 5.-

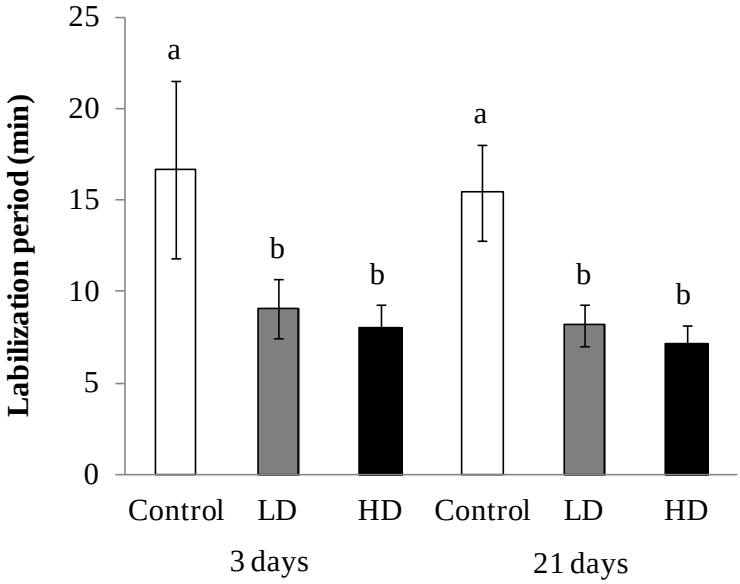
Labilization period (min)
25
20
15
10
5
0
a
b
b
a
b
b
Control
LD
HD
Control
LD
HD
3 days
21 days

Figure 6.-

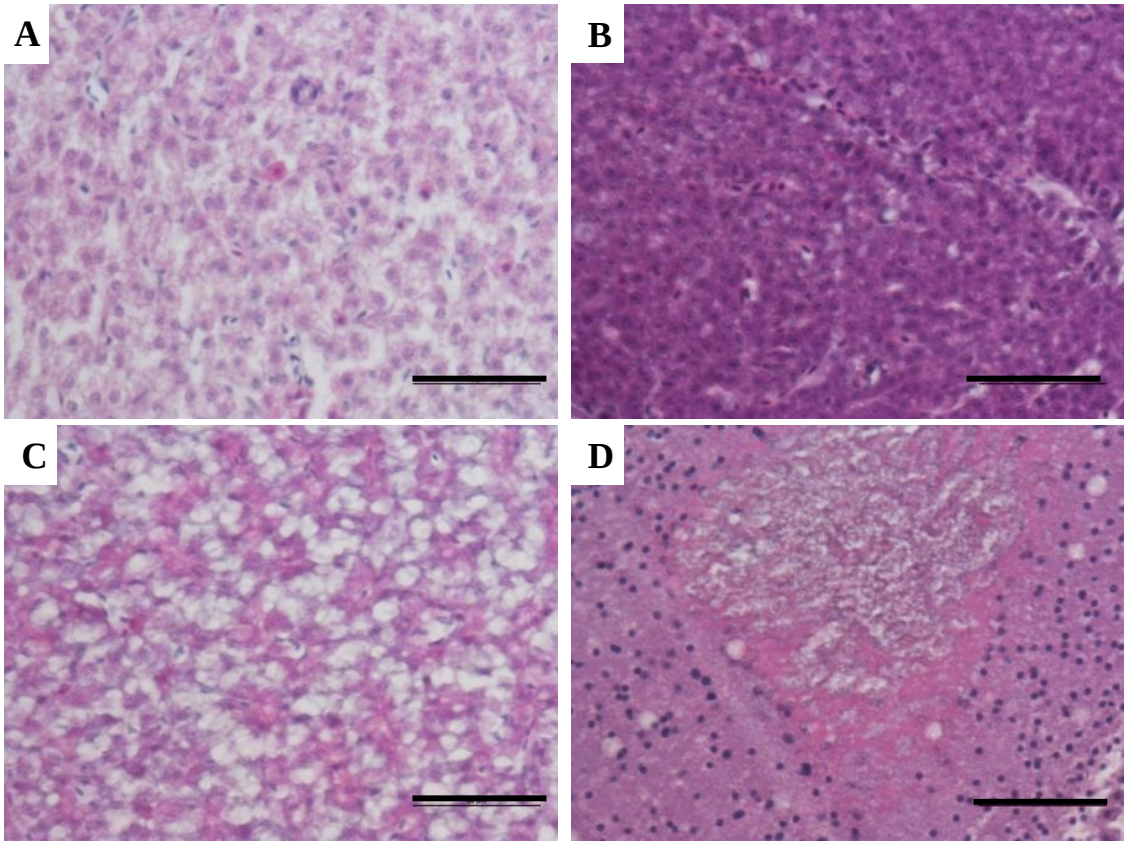

Figure 7.-