Evaluation of the effect of silver and silver nanoparticles on the function of selenoproteins using an *in-vitro* model of the fish intestine: the cell line RTgutGC

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EVALUATION OF THE EFFECT OF SILVER AND SILVER NANOPARTICLES ON THE FUNCTION OF SELENOPROTEINS USING AN *IN-VITRO* MODEL OF THE FISH INTESTINE: THE CELL LINE- RTgutGC

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Abstract:

The long-term adverse effects of silver (Ag) and silver nanoparticles (AgNP) on human and environmental health is a concern. Moreover, recent research in mammalian cells has clearly indicated detrimental inhibitory effects on vital selenoenzymes playing an important role in oxidative stress control. Therefore, any disruption of selenoprotein function can be detrimental to organismal health. Our primary objective is to test the inhibitory effects of Ag exposure in the form of silver salt ($AgNO_3$) and citrate coated silver nanoparticle (cit-AgNP) on selenoprotein function using a fish cell line derived from the Rainbow trout (*Oncorhynchus mykiss*) intestine (RTgutGC). Following exposure to non-toxic and toxic concentrations of $AgNO_3$ and cit-AgNP, glutathione peroxidase (GPx) and thioredoxin reductase (TrxR) function was evaluated by measuring their mRNA levels and enzymatic activity. Oxidative stress was measured using glutathione reductase (GR) mRNA levels and ROS formation by using the CM-H_2DCFDA assay. Metal bio-reactivity was evaluated by measuring Metallothionein b (MTb) mRNA levels. Intracellular accumulation of silver was measured by ICP-OES. Toxicity in RTgutGC cells was evaluated using the alamarBlue and CFDA-AM assay. Our results showed that cells exposed to equimolar amounts of $AgNO_3$ and cit-AgNP accumulated the same amount of silver intracellularly, however, $AgNO_3$ induced more cytotoxicity. Moreover, mRNA levels for MT increased at 24 hours (h) of exposure, confirming the uptake of silver by cells, while no induction was observed at 72 h suggesting scavenging of intracellular silver by MT. The mRNA levels for the target selenoenzymes (GPx and TrxR), however, remained unchanged, indicating that silver uptake had no effect on selenoproteins gene expression. However, an inhibition in activity of GPx was observed in cells exposed to toxic doses of $AgNO_3$ (1 µM) and cit-AgNP (5 µM), while the TrxR activity was inhibited in cells exposed to $AgNO_3$ (0.4 µM) and cit-AgNP (1, 5 µM). No induction of oxidative stress response was observed after $AgNO_3$ or cit-AgNP exposure. Overall, our study clearly established that silver exposure induces an inhibitory effect on target selenoenzymes (GPx and TrxR) activity level but not at the transcriptional level.

Keywords: $AgNO_3$, cit-AgNP, glutathione peroxidase, Thioredoxin reductase, RTgutGC, oxidative stress, selenoprotein enzymatic activity.

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CHAPTER I

INTRODUCTION

The importance and value of silver as a metal has been known for centuries. Due to its characteristics of conductivity, malleability and ductility it is used extensively in the electronic and jewelry industry. In addition to its conductive properties, silver (Ag) is well known for its anti-microbial properties and thus utilized extensively for medical applications (Franci et al., 2015). Salts of silver such as $AgNO_3$ have been used as topical medicines for disinfecting wounds and burns while silver nanoparticles (AgNP) have been found to be present in wound dressings, clothing, dietary supplements, coating on electric conductors etc. (Lemire et al., 2013; Medici et al., 2019). Moreover, AgNP has been proposed as an alternative to antibiotics in aquaculture operations as a disease control agent (Swain et al., 2014). This represent a potential for direct exposure to farmed fish. In addition, the wastes generated from these commercially available products can eventually permeate bodies of water (Petersen, 2017; Wood, 2011), which might result in a source of exposure for aquatic organisms.

It has been previously reported that exposure to water contaminated with AgNP results in accumulation of silver in fish tissues- specifically gill, gut and liver which are primary target organs (Scown et al., 2010; Swain et al., 2014; Yue et al., 2015). Silver is a known toxicant in

fishes due to its inhibitory effect on the basolateral Na^+/K^+-ATPase and intracellular carbonic anhydrase in the ionocytes. This effect depletes the Na^+ and Cl^- concentration in the blood plasma resulting in circulatory collapse and mortality (Wood, 2011). Hence, Ag could induce toxic effects on aquatic species in a similar way.

Furthermore, the bioavailability of silver in the environment is dependent on the chemical conversion of metallic silver into an oxidized form (Ag^+). Ag^+ has a tendency to bind and form complexes with other elements present in the water (e.g. Cl^-, S_2^-, etc.) which may mediate its toxicity (Behra et al., 2013). Moreover, the size of the nanoparticles determines the rate of oxidation to ionic form and the toxicity of silver in water (Akter et al., 2018; Park et al., 2011). This raises questions on whether a non-essential metal such as silver that is increasing exponentially in demand can actually have a deleterious effect on aquatic species health.

Moreover, Ag has been a known toxicant for several antioxidant enzymes such as catalase (CAT), glutathione peroxidase (GPx), superoxide dismutase (SOD) and thioredoxin reductase (TrxR), (Barros et al., 2019) which play an important role in protecting the cells against oxidative stress. Notably, GPx and TrxR belong to a group of proteins that incorporate selenium in form of selenocysteine as a catalytic co-factor called selenoproteins (Pacitti et al., 2013; Pilon-Smits et al., n.d.; Rotruck et al., 2016). Thus, inhibition of these enzymes by increase in Ag environmental concentration can be detrimental to the health of an organism (Choi et al., 2010; Kim et al., 2019). Indeed, Srivastava et al. (2012) observed that Ag ions and AgNP decreased selenium (Se) incorporation during TrxR synthesis in mammalian keratinocyte (HaCat) and lung (A549) cells in a dose-dependent manner. This observation indicated that antioxidant enzymes could be inhibited on interaction with Ag - a potential mechanism by which Ag cause detrimental intracellular effects.

Given that a dose-dependent effect was observed on TrxR, Ag could be inducing similar effects on other antioxidant enzymes such as glutathione (Habas and Shang, 2019; Srivastava et al., 2012).

This study therefore aimed to quantify the effects of intracellular Ag accumulation on two selenoenzymes, TrxR and GPx. We have used an *in vitro* model of the rainbow trout gut epithelium, the RTgutGC cell line. RTgutGC cells mimics the intestinal physiology of the rainbow trout (Minghetti et al., 2017) and was used as an *in vitro* model in this experiment to study toxic effects of silver (Minghetti et al., 2019; Minghetti and Schirmer, 2019, 2016). In fish, the gill and the intestine are the primary target organs for dissolved toxicants. Previously, silver nanotoxicology studies have been conducted on the gill epithelium *in-vivo* (Bruneau et al., 2016; Schultz et al., 2012) and *in-vitro* (Farkas et al., 2011; Yue et al., 2015) while the intestinal epithelium has not been investigated as much due to lack of appropriate model (Clark et al., 2019a; Minghetti and Schirmer, 2016). Moreover, the intestine is a major route of absorption of pollutants in fish living in brackish or sea water (Evans, 2008). It was shown previously that citrated coated AgNP induced toxicity in RTgutGC targeting specifically the lysosomes (Minghetti and Schirmer, 2016) and that dissolved silver is a potent disruptor of the homeostasis of essential trace elements such as zinc, iron and copper (Minghetti and Schirmer, 2019). Previous studies have also shown evidence of detrimental effects of AgNP on selenium including cases of accumulation as Ag_2S in human tissues (Srivastava et al., 2012). Therefore, in this study, the impact of both $AgNO_3$ and AgNP was evaluated specifically as a disruptor of selenoprotein (GPx, TrxR) function in RTgutGC. Moreover, as selenoprotein inhibition might affect the cellular antioxidant machinery (Lu and Holmgren, 2009) the role of these compounds on oxidative stress was also evaluated. The effect of silver on cellular oxidative stress was evaluated indirectly by determining the mRNA levels of GR which was previously shown to be a good marker of oxidative stress (Minghetti et

al., 2008) and directly by detection of reactive oxygen species (ROS). Moreover, intracellular bio-reactivity of silver was determined by measuring the MTb mRNA levels, a known biomarker of metal exposure (Coyle et al., 2002; Minghetti et al., 2014)

CHAPTER II

MATERIALS AND METHODS

2.1 Preparation of stock solutions

Citrate coated AgNPs (cit-AgNP; nominal size: 19 nm, NanoSys GMbH, Switzerland) were purchased as aqueous suspension with a concentration of total silver of 1g/L (9.27 µM, pH 6.46). $AgNO_3$ stock solution was freshly prepared before each experiment at a concentration of 10 mM in ultrapure water (16–18 $M\Omega cm^{-1}$; Gen pure, Thermo Scientific, USA). The stock solutions were prepared as previously described (Minghetti and Schirmer, 2016). Cit-AgNPs hydrodynamic diameter in water and in the exposure medium L-15/ex (see section 2.3) was measured at 30 nm and ~800 nm by dynamic light scattering (DLS; Nano ZS, Malvern Instruments, Malvern, UK) with a zeta potential of -35 mV and -18 mV, respectively (Minghetti and Schirmer, 2019, 2016).

2.2 RTgutGC cell culture

RTgutGC cells were cultured as previously described (Minghetti et al., 2017). RTgutGC cells were routinely cultured in the commercial cell culture medium Leibovitz' L-15 (Gibco/Thermofisher, Waltham, MA, USA) supplemented with 5% FBS (Sigma, USA) and 1% Gentamicin (L-15/FBS; Life Technologies, USA) in 75 cm^2 flasks (Greiner Bio-One, USA) at 19 °C and split into two flasks once they reached approximately 80-90% confluency. For cell splitting,

confluent flasks were washed twice with Versene (Life Technologies, USA) and cells were detached using trypsin (0.25% in phosphate-buffer saline) in phosphate buffer (PBS; Life

Technologies, USA). Cell suspensions were then seeded in two flasks. When seeding cells for exposure experiments, cells were counted using an automated cell counter (Thermofisher, Countess II automated cell counter, Waltham, USA), and seeded at 74,000 cells per cm^2.

2.3 Preparation of exposure solutions for cytotoxicity assay: $AgNO_3$ and AgNP

RTgutGC cells were exposed in a modified medium, L-15/ex (Schirmer et al., 1997). L-15/ex has an identical composition of L-15 but without amino acids or vitamins. L-15/ex is used for preparing exposure solutions in order to remove any chance of amino acids or proteins scavenging Ag^+ and protecting cells against toxicity (Minghetti and Schirmer, 2016). The $AgNO_3$ and cit-AgNP solutions were prepared to contain identical nominal concentrations of total silver, nominal concentrations were confirmed by ICP-OES measurement (Table 1). A side by side comparison of silver toxicity and intracellular accumulation was conducted by exposing RTgutGC cells to 1 µM, 2 µM and 5 µM of $AgNO_3$ and cit-AgNP respectively, followed by incubation at 19° C for 24 h in the dark. For evaluating gene expression, cells were exposed to $AgNO_3$ at 0.4 µM (non-toxic) and 1 µM (corresponding to the EC20 value) and to cit-AgNP at 0.4 µM and 1 µM, which are both non-toxic (Minghetti and Schirmer, 2016). To measure oxidative stress, cells were exposed to $AgNO_3$ at 0.4 µM (non-toxic), 1 µM (corresponding to EC20 value), 2 µM (corresponding to EC70 value) and 5 µM (corresponding to EC 90) and to cit-AgNP at 1 µM (non-toxic), 5 µM (corresponding to EC15 value) and 10 µM (corresponding to EC35 value) respectively. To measure selenoenzyme activity, RTgutGC cells were exposed to $AgNO_3$ at 0.4 µM (non-toxic) and 1 µM (corresponding to EC20 value) and to cit-AgNP at 1 µM (non-toxic) and 5 µM

(corresponding to EC50 value) respectively. Non-toxic and toxic concentrations of $AgNO_3$ and cit-AgNP were calculated by averaging alamarBlue and CFDA-AM toxicity data previously reported (Minghetti and Schirmer, 2016).

2.4 Cell viability assay

Cytotoxicity in RTgutGC cells was evaluated in 24 multi-well plates (Greiner Bio-One, USA). The test plate contained three technical replicates of each exposure concentration, one blank well (empty and free of cells), three L-15/ex controls and two L-15/FBS controls. Both L-15/ex and L-15/FBS served as negative controls in the cytotoxicity assay. RTgutGC cells were seeded in L-15/FBS complete media at a density of 74,000 cells/cm^2 and incubated at 19° C for 48 h to allow attachment of the cells and development of a confluent monolayer on the multi-well plate surface. Following the incubation, the media was aspirated and cell monolayers were washed twice with L-15/ex and the exposure solutions 1 µM, 2 µM and 5 µM of $AgNO_3$ and cit-AgNP were applied. Cells were then incubated for 24 hours. The exposure solution was aspirated from the wells and the cells were washed with L-15/ex. Following this, 400 µL of a 5% (v/v) alamarBlue (AB; Thermofisher, Waltham, MA, USA) and 0.1% of CFDA-AM (CFDA-AM; Thermofisher, Waltham, MA, USA) working solutions were prepared and added to each well. AlamarBlue measures cell metabolic activity while CFDA-AM measures membrane integrity (Bloch et al., 2017; Minghetti and Schirmer, 2016). The plates were incubated in the dark at 19 ºC for 30 minutes. Fluorescence was then recorded for both AB and CFDA-AM simultaneously with a Cytation 5 multi-well plate reader (Biotek, USA), at excitation/emission wavelengths of 530/595 nm and 485/530 nm for AB and CFDA-AM, respectively. Results obtained were reported as % viability based on fluorescent units (FU) of the L-15/ex control and calculated using following equation:

$$\% \text{ of control} = (FU_{ex.cells} - FU_{ex.no\ cells}) \times 100 / (\text{Average } [FU_{con.} - FU_{con.no\ cells}])$$

Effect concentrations were determined by the non-linear regression sigmoidal dose-response curve fitting module using the hill slope equation (Minghetti and Schirmer, 2016).

2.5 Quantification of intracellular Silver

RTgutGC cells were seeded in 6 well plates at a density of 74,000 cells per cm^2. After cells were exposed to 1 µM, 2 µM and 5 µM doses of $AgNO_3$ and cit-AgNP, respectively, the exposure media was then aspirated and transferred to an eppendorf tube for quantifying total Ag. The cells were washed twice with 0.5 mM cysteine solution to remove loosely bound Ag. Cells were then lysed by adding 1 ml of 50 mM NaOH and incubated for 2 h in a shaker. An aliquot (100 µL) of the cell lysate was used for protein quantification using the modified Lowry assay (Thermo Sceintific, Waltham, USA). The remaining 900 µL of the lysed sample was desiccated using a SpeedVac (Eppendorf, Germany). Samples were digested overnight by adding 800 µL of 69% HNO_3. 200 µL of H_2O_2 was added and incubated for at least one more hour, then the entire mixture was transferred to a 15 mL falcon tube and diluted 10 times with ultrapure water thus bringing the concentration of acid to 5% HNO_3. Total Ag was determined by ICP-OES analyzer (iCAP 7400; Thermo Scientific, Waltham, MA, USA). Validation and calibration of the ICP-OES was achieved by using multi-element external reference standards (CPI International, Santa Rosa, CA, USA). Additionally, an in-line Yttrium internal standard (Peak Performance Inorganic Y Standard, CPI International, Santa Rosa, CA, USA) was used to correct for any instrument drift or matrix effects. Digestion blanks for all digestions, consisting of just diluted HNO_3 with no sample, were also run to correct for background concentrations. The Ag concentrations in the exposure medium L-15/ex was also determined for quality control purposes and is reported in Table 1.

2.6 Cloning of target selenoprotein genes

The genes encoding for GPx and TrxR were identified from previously cloned genes from rainbow trout (Pacitti et al., 2015, 2013). Primers were designed using NCBI website primer BLAST (Table 2), while published primers for Metallothionein b, glutathione reductase, ubiquitin and elongation factor 1 alpha were previously described (Minghetti et al., 2014). The PCR was performed using the Platinum^{TM} Green Hot Start PCR Master Mix (2X) (Thermofisher, USA) and the following thermocycler setup: activation, 3 minutes at 95 °C followed by 35 cycles of denaturation (95 ºC for 30 seconds), annealing (52 ºC for 30 seconds for GPx and TrxR and 55 ºC for MTb and GR), extension (72 °C for 30 seconds), and final extension (72 ºC for 5 minutes). After the PCR products were obtained, they were checked by gel electrophoresis. Cloning was performed using TOPO® TA Cloning® Kit following the manufacturer instructions (Invitrogen, Thermo scientific, USA). The plasmids were linearized after cloning using the Not 1 restriction enzyme and were used to prepare the standard curve used for copy number quantification.

2.7 RNA extraction, cDNA synthesis and determination of gene expression by qPCR

RTgutGC cells were seeded at a density of 74,000 cells /cm^2 in 6 well plates for 48 h. The cells were then exposed to 0.4 µM $AgNO_3$, 0.4 µM cit-AgNP and 1 µM cit-AgNP (non-toxic) and 1 µM $AgNO_3$ (corresponding to EC20 value) prepared in L-15/ex. Then after 24 and 72 hours of exposure the exposure medium was removed and total RNA was extracted using TRIzol reagent (Invitrogen) following manufacturer instructions. RNA quantity and quality was then determined by spectrophotometry using the Cytation 5 plate reader and by gel electrophoresis using 0.5 µg of total RNA in 1% agarose gel. Messenger RNA levels of target genes were measured by quantitative PCR (qPCR) (SyBr Green iTaq Universal, Biorad, USA). The qPCR was performed using the

thermocycler setup indicated in Table 3. Messenger RNA levels are reported as the fold change of the treated from the untreated sample. Normalization was based on the geometric mean expression of two reference genes (ubiquitin and elongation factor 1α) as previously described (Minghetti et al., 2014). RNA extraction, DNAse treatment, cDNA synthesis, measurement of mRNA levels by quantitative PCR (qPCR) were performed as described in (Minghetti and Schirmer, 2016).

2.8 Measurement of oxidative stress using H_2DCFDA dye

RTgutGC cells were seeded at a density of 31,000 cells/ cm^2 in 96-well TPP plates (Greiner, flat bottom). After 48 h, cells were exposed to 0.4 µM, 1 µM, 2 µM and 5 µM of $AgNO_3$, 1 µM, 5 µM and 10 µM of cit-AgNP H_2O_2 was used as a positive control at 15% and 6% (w/w). All exposure solutions were prepared in L-15/ex. The exposure time points were 1h, 5h and 24 h. At each time point, the cells were washed with L-15/ex and then incubated with 50 µl of 50 µM H_2DCFDA dye in L-15/ex for 30 minutes. Finally, the cells were washed gently with 50 µl of L-15/ex and read using Cytation-5 plate reader at absorbance/emission wavelength of 485/520 nm. The ROS levels measured are reported as percentage of the negative control (L-15/ex).

The H_2DCFDA (2',7'-dichlorodihydrofluorescein diacetate) dye is a chemically reduced form of fluorescein which is used to indicate presence of ROS in cells. Upon cleavage of the acetate groups by intracellular esterases and oxidation, the non-fluorescent H_2DCFDA is converted to highly fluorescent 2',7'-dichlorodihydrofluorescein (DCF) (Thermofisher, USA).

2.9 Enzymatic activity

RTgutGC cells were seeded at a density of 80,000 cells/cm^2 in 75 cm^2 flasks (Greiner) for 6 days. After a monolayer was formed, the cells were exposed to non-toxic (0.4 µM of $AgNO_3$ and 1 µM of cit-AgNP) and toxic (1 µM of $AgNO_3$ and 5 µM of cit-AgNP) doses for 24 h. Following

exposure, cells were washed with 5 mL L-15/ex in the flask. Then, the cells were scrapped using a cell scraper and collected into a 15 mL tube. The cells were centrifuged at 1000 x g for 10 minutes and homogenized in enzyme assay buffer (50 mM Tris-HCl, pH 7.6, containing 5 mM EDTA) using an ultrasonic homogenizer (Vibra-Cell™ Ultrasonic Liquid Processors, Sonics and Materials Inc., CT, USA). The cell lysate was centrifuged again at 10,000 x g for 15 min to obtain a supernatant containing the cytoplasmic proteins which were separated from pellet containing the membrane-bound proteins. The enzymatic activity was then measured from a 20 µl aliquot by following the manual instructions in the assay kit (Glutathione peroxidase and Thioredoxin reductase assay, Cayman chemicals, MI, USA). The GPx activity was based on the oxidation of NADPH (Nicotinamide adenine dinucleotide phosphate) to $NADP^+$ that is accompanied by a decrease in absorbance at 340 nm. This decrease was directly proportional to the GPx activity of the sample. While TrxR activity was based on the reduction of DTNB (Ellman's reagent) with NADPH to TNB. It was a colorimetric reaction producing a yellow color measured at 405 nm.

An aliquot (100 µL) of the cell lysate was used for protein quantification using the modified Lowry assay (Thermo Scientific, Waltham, MA). The enzymatic activity was normalized by the total protein content and reported as nmol/min/mg of protein for GPx activity and µmol/min/mg of protein for TrxR activity respectively.

2.10 Data analysis

Statistical analysis was performed using GraphPad Prism Version 8.0 (GraphPad Software Inc., San Diego, CA). For multiple groups, statistical analysis was performed by the analysis of variance (ANOVA) followed by Tukey's *post hoc* test and Dunnet post hoc multiple comparison test when comparing to a control group. Values of $p < 0.05$ were considered statistically significant.

CHAPTER III

RESULTS

3.1 Effect of silver exposure on RTgutGC cells:

a) Cytotoxicity of AgNP and $AgNO_3$

RTgutGC cells were exposed to equal doses of $AgNO_3$ or cit-AgNP (1 µM, 2µM and 5 µM) for 24 h in order to evaluate the toxicity as indicated by the metabolic activity and membrane integrity assays. Microscopy observation of controls and exposed RTgutGC cells show that exposure to toxic doses (1, 2 and 5 µM) of $AgNO_3$ induces a reduction in cell nuclei while exposure to cit-AgNP (5 µM) shows accumulation and cit-AgNP agglomerates on top of the cells. However, no visible signs of toxicity or reduction of cell nuclei were observed in cells exposed to cit-AgNP (Figure 1).

The toxicity induced by $AgNO_3$ was found to be dose-dependent as 1 µM of $AgNO_3$ induced a 50% decrease in metabolic activity and 40% decrease in membrane integrity, while 2 µM and 5 µM of $AgNO_3$ induced higher toxicity on the two endpoints tested (60% and 30% decrease at 2 µM, and 90% and 95% decrease at 5 µM for metabolic activity and membrane integrity, respectively). Exposure to cit-AgNP at 1 µM, 2 µM and 5 µM induced lower toxicity. The 1µM, 2 µM and 5 µM of cit-AgNP did not induce any toxicity on metabolic activity. The 1 µM and 2

μM of cit-AgNP, however, induced 5% decrease in membrane integrity, while 5 μM induced a 40% decrease in membrane integrity (Figure 2, A and B). Therefore, when comparing the toxic effects on both metabolic activity and membrane integrity, $AgNO_3$ was found to be more toxic than cit-AgNP.

b) Intracellular uptake and accumulation

RTgutGC cells were exposed to identical concentrations of $AgNO_3$ or cit-AgNP at 1 μM, 2μM and 5 μM for 24 h in order to evaluate the intracellular accumulation of total silver. Cells exposed to equimolar amount of $AgNO_3$ or cit-AgNP accumulated the same amounts of silver intracellularly (Figure 2 C).

3.2 Messenger RNA levels of target selenoprotein genes and other biomarkers

Messenger RNA levels of target selenoproteins genes (GPx and TrxR), MTb and GR were determined in cells exposed to 0.4 μM and 1 μM of $AgNO_3$ and cit-AgNP respectively for 24 and 72 h. At 24 h exposure, only the highest doses of $AgNO_3$ and cit-AgNP (1 μM) induced a significant increase of MTb levels (13.67 ± 7.07 and 10.43 ± 5.62-fold increase), whereas all other tested genes showed no alteration of their mRNA levels when compared to control (Figure 3). After 72 h exposure, MTb mRNA levels were reduced in cells exposed to 1 μM $AgNO_3$ and cit-AgNP and were not statistically different from controls. All other genes in exposed cells showed mRNA levels not statistically different from control (Figure 3).

3.3 Oxidative stress induction by $AgNO_3$ and cit-AgNP over time

Fluorescence based assays to measure oxidative stress occurring in RTgutGC cells exposed to increasing doses of $AgNO_3$ (0.4 μM, 1 μM, 2 μM and 5 μM) and cit-AgNP (1 μM, 5 μM and 10 μM) is reported in Figure 4.

RTgutGC cells exposed to 6% (w/w) H_2O_2 showed an increase of ROS at 24 hours, whereas cells exposed to 15% (w/w) H_2O_2 showed an increase in H_2O_2 at 1, 5 and 24 hours (Two-way ANOVA, Dunnett's Test, $p < 0.05$). Apart from the positive control (H_2O_2), none of the tested $AgNO_3$ and cit-AgNP exposure concentrations induced any statistically significant increase in the ROS response when compared to control. The decrease in ROS level observed in cells exposed to 5 μM $AgNO_3$ at 1 h is due to cell mortality at such high exposure dose (Figure 4).

3.4 Activity of selenoenzymes in response to toxic and non-toxic concentrations of $AgNO_3$ and cit-AgNP

The higher concentrations of silver 1 μM $AgNO_3$ induced a decrease of more than 50% the activity of GPx, while 5 μM cit-AgNP induced more than 60% decrease in of the GPx enzyme activity when compared to the control. There was no significant difference observed in the GPx activity in samples exposed to lower silver concentrations (0.4 μM $AgNO_3$ and 1 μM cit-AgNP). Similarly, 0.4 μM $AgNO_3$ induced decrease of 50% TrxR activity, while 1 μM and 5 μM of cit-AgNP induced a decrease of 35% and a decrease of 30% TrxR activity, respectively when compared to control (Figure 5).

CHAPTER IV

DISCUSSION

It was recently shown that silver disrupts the homeostasis of essential trace elements such as zinc, iron and copper in fish intestinal cells (Minghetti and Schirmer, 2019). Another essential element, selenium, which plays an important role as a catalytic co-factor of a vital group of proteins called selenoproteins (Hefnawy and Tórtora-Pérez, 2010; Pacitti et al., 2013; Pilon-Smits et al., n.d.; Rotruck et al., 2016), was shown to be affected by silver and AgNP exposure in mammalian systems. In this study, $AgNO_3$ and cit-AgNP exposure in fish intestinal cells (RTgutGC) resulted in an inhibitory effect on selenoenzymes activity (GPx and TrxR) while no significant effects were observed on mRNA levels of selenoproteins and on oxidative stress. Moreover, RTgutGC cells accumulated the same amount of intracellular silver when exposed to equal amount of $AgNO_3$ or cit-AgNP. However, $AgNO_3$ resulted to be more toxic than cit-AgNP, a result that is supported by previous studies (Minghetti and Schirmer, 2019, 2016). In addition, intracellular silver bio-reactivity was shown by the increase in MTb mRNA levels after 24 hours of exposure. Clearly, our data supports the hypothesis that silver disrupts selenoprotein function. This correlates with the study conducted by Srivastava et al. (2012) in mammalian cell lines where a dose-dependent inhibitory effect of silver on TrxR was observed (Srivastava et al., 2012). However, the focus of their study was only on TrxR activity, so the effect of Ag on other important selenoprotein such as Glutathione peroxidase broadens the impact of silver and silver nanoparticles and suggests that

silver might be a direct inhibitor of several selenoenzymes. Overall, our study strengthens the role of silver as a disruptor of essential trace elements.

While some research has been done on the effect of silver on selenoenzymes, especially on mammalian systems (Srivastava et al., 2012), the role of silver on selenoenzymes in environmental species, such as fish, is less well understood. Previously, Thummabancha et al. (2016) characterized the effects of AgNP exposure on two genes involved in oxidative stress metabolism, Selenoprotein P and thioredoxin-interacting protein (TXNIP) *in vivo* in Nile Tilapia (*Oreochromis niloticus*). A downregulation in the selenoprotein P expression and an upregulation in TXNIP suggested that AgNP suppressed the expression of the key antioxidant proteins in target organs like liver, intestine, peripheral blood leucocytes, brain and gills (Thummabancha et al., 2016). This study indicates that $AgNO_3$ and AgNP induce a possible alteration of antioxidant pathways related to selenium metabolism but it does not present a direct evidence of selenoenzyme inhibition. Previous studies have shown that the intestine is an important site of accumulation in fish (Clark et al., 2019b, 2019a; Johari et al., 2015). However, the effect of silver and AgNP specifically on selenoenzyme function is not been investigated previously.

In this study, equimolar amounts of intracellular $AgNO_3$ was found to be more toxic than cit-AgNP. A similar effect was shown previously *in vivo* (Clark et al., 2019a) and *in vitro* (Minghetti et al., 2019). Given that cit-AgNP was shown to remain in nano form for up to 72 hours in RTgutGC (Minghetti et al., 2019), and considering our toxicity data, we can hypothesis that Ag ions but not AgNP are the major contributors of toxic effects on the cell. Yue et al. (2017) also demonstrated that $AgNO_3$ was more toxic compared to AgNP in algal and fish cells (Yue et al., 2017).

The intracellular bio-reactivity of silver following exposure to $AgNO_3$ and cit-AgNP (1 μM) was confirmed by the induction of Metallothionein b (MTb) levels. Metallothionein is a cysteine rich protein. Cysteine possess a thiol (–SH) group which is known for its affinity to different metals including silver (Liu et al., 2017). Previous research by Minghetti et. al. (2016) showed a significant increase in Metallothionein b (MTb) mRNA levels in RTgutGC, a known biomarker of metal bio-reactivity. Metallothionein is regulated by the MTF1 transcription factor and a rise in the mRNA levels indicates cellular uptake of silver from the outside environment (Minghetti and Schirmer, 2016). Interestingly, the mRNA levels of MTb increased in cells exposed for 24 h, but decreased at 72 h. Our hypothesis is that when the cells are exposed to silver, MTb is rapidly induced within the first 24 hours and binds to the metal to provide protection against silver toxicity but at 72 h, most of the intracellular Ag has been scavenged by MT and there is no further induction of MTb mRNA levels. This effect was shown previously (Minghetti et al., 2019; Minghetti and Schirmer, 2016).

Glutathione (GSH) and its associated enzymes, GPx and TrxR, are controlled by the nuclear factor-2 related erythroid factor-2 (Nrf2) transcription factor (Li et al., 2016; Tindell et al., 2018). Leurquin et al. (2018) recently tested the effects of AgNP on the Nrf2 pathway in Caco-2 (human colo-rectal adenocarcinoma) cell line. However, no effect was observed indicating that Nrf2 was not regulated by the presence of silver in cells (Leurquin, 2018; Tebay et al., 2015). In our study, mRNA levels of GPx, TrxR and GR (glutathione reductase- a marker of oxidative stress) were also not induced after exposure to $AgNO_3$ and cit-AgNP supporting the study of Leurquin et al., (2018).

Metals with multiple oxidation states like iron, copper, cobalt and nickel have been shown to take part in the Fenton reaction by converting hydrogen peroxide, produced in cells, to hydroxyl

radicals (•OH) (Rubino, 2015). For example, copper (+1 to +2) and iron (+2 to +3) cycle between two oxidation states and undergo two-electron redox chemistry in the cells. The non-essential metal- silver maintain a stable oxidation state of +1 (Beer et al., 2012; Font et al., 2014) and is not able to take part in the Fenton reaction. Therefore, silver is not directly inducing ROS formation. In our experiment, exposure to $AgNO_3$ or cit-AgNP did not result in the direct measurement of ROS or induction of GR mRNA levels in RTgutGC cells which could be explained by silver oxidation state. Therefore, our results together with previous studies (Böhmert et al., 2015; Leurquin, 2018) suggest that silver is not directly inducing oxidative stress in intestinal cells but a different mechanism of toxic action is occurring. In RTgutGC the main toxic effect measured was the inhibition of GPx and TrxR. The possible mechanism behind the disruption of selenoenzyme activity could be the affinity of silver towards seleno-cysteine-rich domains of proteins (Srivastava et al., 2012). Selenocysteine is an amino acid analogous to cysteine containing a selenol group in place of the sulfur-containing thiol group in cysteine (Turanov et al., 2011). The cysteine rich domain in proteins shows metal specificity and binds to metals using the thiol (-SH) group (Belmonte et al., 2016). Hence, a metal with high affinity for the thiol group such as silver (Behra et al., 2013) can directly displace the Se form the selenocysteine domain leading to inhibition of selenoenzyme function. Concluding, the GPx and TrxR enzyme inhibition, suggests that main mechanism of toxic action of silver is the binding of silver to the thiol group of proteins which alters the function of enzymes, a mechanism previously described (Rubino, 2015).

Moreover, the effect of silver on the gene expression and enzymatic activity of the target selenoproteins observed in our data suggests that silver directly interact with the protein at their catalytic site such as that of selenocysteine but does not affect the transcriptional regulation of selenoenzymes (Leurquin, 2018). A similar effect, has been observed in mammals exposed to

metals such as mercury, cadmium, arsenic and lead due to their chemical affinity for thiol groups (i.e. selenocysteine) (Rubino, 2015) which suggest that metals affect directly selenoenzymes activity but do not induce direct transcriptional regulation.

The important role of selenium in fish nutrition was emphasized in a study conducted on cultured fish (Khan et al., 2017). Any disturbance in the selenium supplementation impaired antioxidant activity resulting in susceptibility of the fish to oxidative stress. Gao et al. (2019) demonstrated an exacerbation in injury and inflammation in carp liver due to deficiency in dietary selenium which affected growth and survival (Gao et al., 2019). Thus, the recent increase in use of AgNP for disinfecting bodies of water in aquaculture practices (Khanh and Cu, 2019; Swain et al., 2014) can be potentially detrimental to aquatic organism health. According to a study by Chris Wood, dissolved concentration of Ag in freshwater bodies ranged from 0.5 to 5 ng/L and rising almost 1000 folds in highly contaminated sites. Although the concentration of AgNP used in aquaculture practices have not been studied, laboratory experiments have shown that a concentration of 1400 µM (150 µg/ml) is potent and induce anti-microbial activity (Khanh and Cu, 2019; Palanisamy et al., 2017). The concentration of AgNP dissolved in water for aquaculture practices has not been clearly documented yet and could possibly be similar to the potency range of microbes. A study by Clark et al. (2019) demonstrated that rainbow trout can survive acute dietary exposure of 927 µM (100 µg/ml) AgNP without effects on growth or toxicity. The ingested Ag accumulated over time in internal organs such as liver, intestine and kidney leading to sub-lethal effects, but no mortality (Clark et al., 2019a). Our study shows cit-AgNP at non-cytotoxic concentrations (1µM) induced an inhibition of TrxR while 5 µM of cit-AgNP inhibited GPx and TrxR.

This study establishes a clear pattern on the detrimental effects of cellular exposure to $AgNO_3$ and cit-AgNP on selenoproteins in fish intestinal cells. Environmental effects of silver in bodies of

water have been documented to cause bioaccumulation and alterations in tissue morphology (Johari et al., 2015; Scown et al., 2010) and biochemistry of target organs in cases of acute exposures (Schultz et al., 2012) However, the effect on chronic exposure in fishes are not well understood. Future studies should focus on the consequences of chronic exposures of silver and AgNP at concentrations similar to our *in vitro* experiment which showed cellular biochemical alterations. The use of lower concentrations and chronic exposures would be more environmentally relevant bringing attention of environmental agencies on the possible consequences of long term exposures to silver in fish.

CHAPTER V

CONCLUSION

Due to the protective function of selenoproteins in cells against oxidative stress, a depletion in their intracellular levels will be detrimental for the organism. With the extensive use of silver in daily life products, but also in medical and anti-microbial applications, the potential for an environmental exposure to Ag-waste has also increasing rapidly. Presence of Ag in different body of water is a concern and measurement of silver in organs of aquatic organisms including cetaceans, fishes and gastropods have been reported. Moreover, the emergence of antibiotic resistance in aquatic microbes has contributed to a renewed interest in developing alternative methods of control and prevention of diseases in aquaculture. Among the alternative methods, AgNP has been considered due to their potential in controlling pathogens and have been applied in aquaculture. However, the possible detrimental effects of silver on fish health should also considered.

Previously, inhibition of selenoprotein function and depletion in mRNA levels following exposure to silver have been recorded using mammalian cell lines. Our data confirm that silver exposure induces a dose-dependent inhibition of target selenoenzymes activity in fish cells as well. The detrimental effects on selenoprotein function can have serious implication on the overall health of an organism and thus, needs to be further investigated on the influence of silver behind the inhibition. These findings may have significant implications, as the outcome of this research may

be instrumental in weighing the beneficial and adverse effects of incorporating Ag in commercial products.

CHAPTER VI

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Table 1: Silver concentrations in exposure solutions measured by ICP-OES.

Nominal	Measured (µg/L)	% Ag recovery in L-15/ex
1 µM $AgNO_3$	91.84 ± 9.64	91.84
1 µM AgNP	97.231.12	97.23

Values are reported as mean and standard deviation % recovery of Ag in L-15/ex. Measurements were used to determine stability of Ag dissolved in L-15/ex exposure medium and exposed to cells.

Table 2: List of primers designed using NCBI website primer-BLAST software.

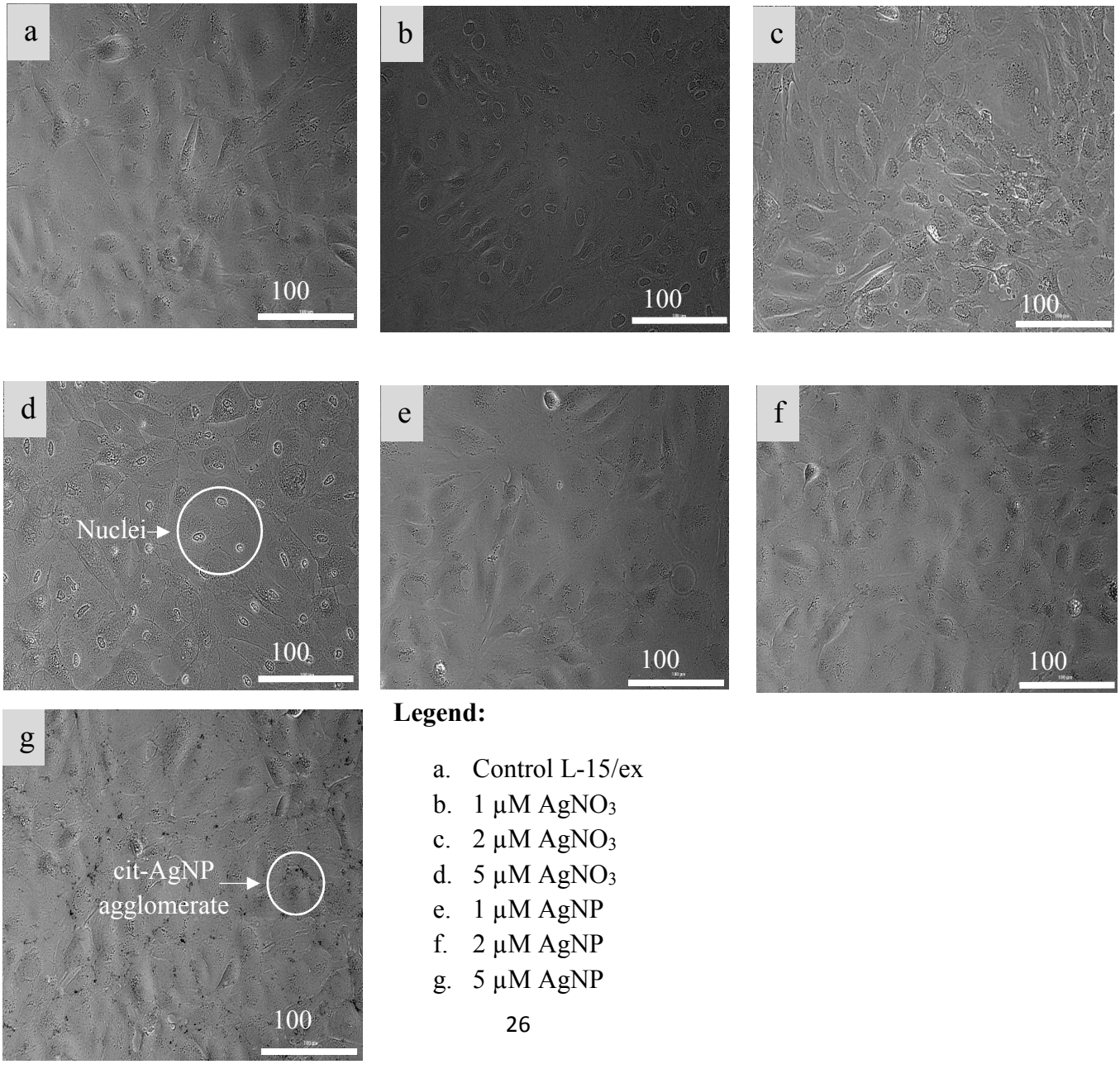
Gene name	Gene Id	Accession No.	Forward primer	Reverse primer
Glutathione peroxidase 1a	GPx 1a	HE687021	GAGAAGCGGGGAGTGACATTTA	CTGCCTGGACGGTTTATTTCAC
Glutathione peroxidase 1b1	GPx 1b1	HE687022	TTCAAACGCTACAGCAGGAGAT	TGCCCATACATGCAAGGTATCA
Glutathione peroxidase 1b2	GPx 1b1	HE687023	GAGAAGCGGGGAGTGACATTTA	GATCAGCACCACTTTACCCTGA
Thioredoxin reductase 3a	TrxR 3a	HF969246	GTAGCGTGGACATACAAGGTGA	GTCAAACAGCACTCACACATCC
Thioredoxin reductase 3b	TrxR 3b	HF969247	TGGATGTGACAGTCATGGTACG	CTTCAGCCTCCCAGGAGTACCT

Table 3: qPCR thermocycler setup.

qPCR	GPX, TrxR and GR	Cycles	MTb	Cycles
Activation	95°C for 30 sec	1	95°C for 3 minutes	1
Denaturation	95°C for 5 sec	39	95°C for 10 sec	39
Annealing	55°C for 30 sec		55°C for 30 sec	
Extension	72°C for 30 sec,		72°C for 30 sec	
Melting Curve	65°C for 5 sec and 95°C for 5 sec	1	65°C for 5 sec and 95°C for 5 sec	1

CHAPTER VII

LIST OF FIGURES



Legend:

a. Control L-15/ex
b. 1 µM $AgNO_3$
c. 2 µM $AgNO_3$
d. 5 µM $AgNO_3$
e. 1 µM AgNP
f. 2 µM AgNP
g. 5 µM AgNP

Figure 1: Images of RTgutGC cells exposed to 1, 2 and 5 μM of $AgNO_3$ and cit-AgNP for 24 h in comparison with control cells in L-15/ex. Reduction in nuclei size was observed in cells exposed to 2 μM and 5 μM of $AgNO_3$ in d. indicating intracellular toxic effect of high ionic Ag concentration and cit-AgNP agglomeration was observed in g.

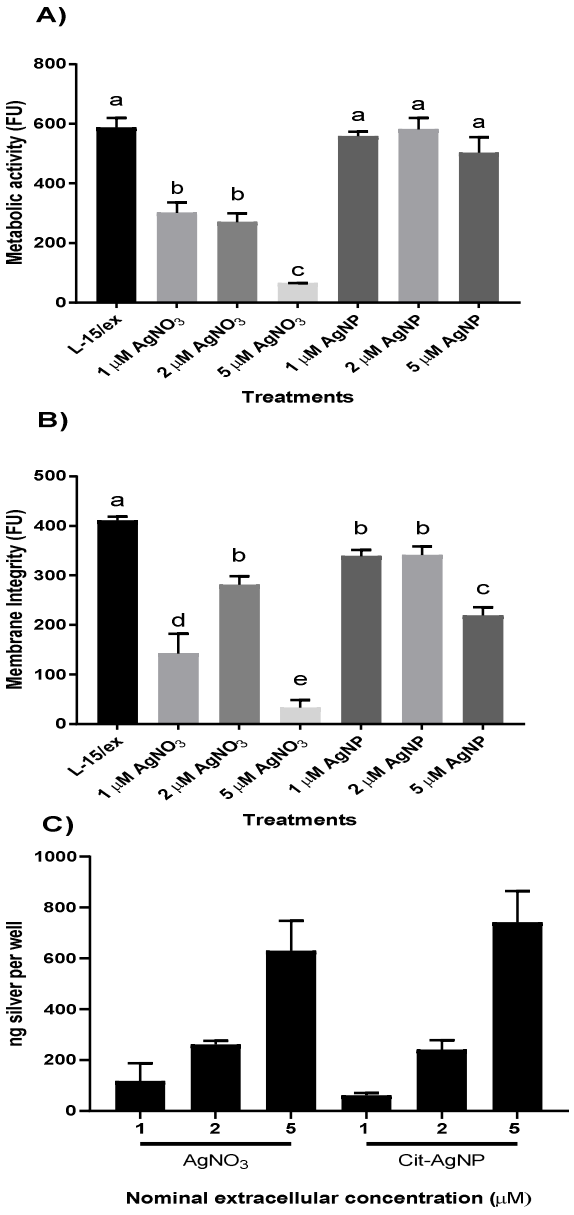


Figure 2: Cytotoxicity and accumulation of $AgNO_3$ and cit-AgNP in RTgutGC. A) metabolic activity measured using alamarBlue assay and B) membrane integrity was measured by CFDA-AM assay. Values are represented as mean ± standard deviation. Difference in metabolic activity and membrane integrity in cells exposed to equimolar concentrations of $AgNO_3$ and cit-AgNP are indicated by different lettering. (One-way ANOVA, Tukey's *post hoc* test, $p < 0.05$). C) Intracellular concentration of Ag was determined by ICP-OES in RTgutGC cells exposed to 1, 2 and 5 µM $AgNO_3$ and cit-AgNP for 24 h.

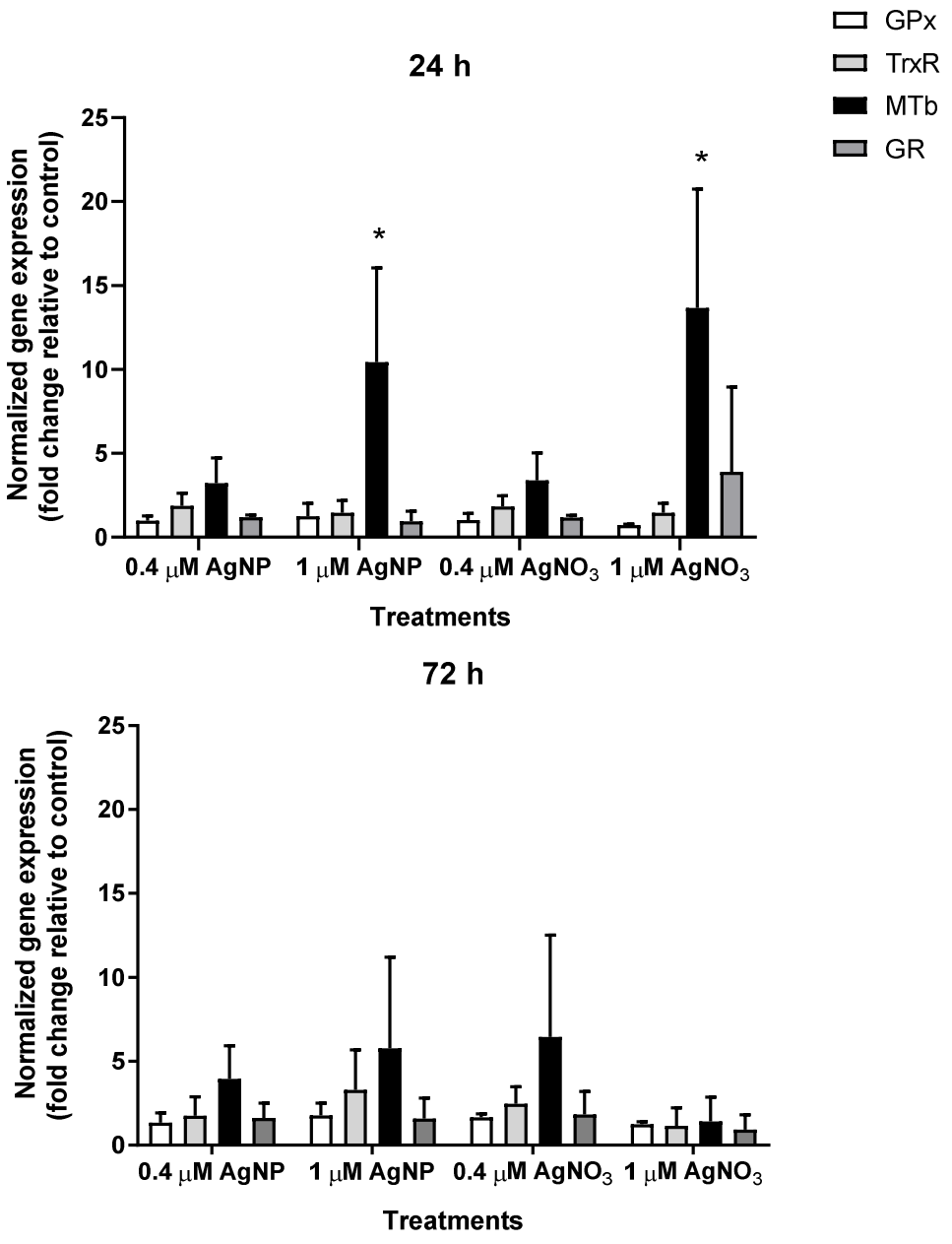


Figure 3: Normalized mRNA levels of GPx, TrxR, GR and MTb genes measured in RTgutGC cells exposed to 0.4 μM and 1μM of $AgNO_3$ and cit-AgNP. Target genes normalized expression is reported as ratio of the expression in cells exposed to control media L-15/ex (Figure S1). Values represent mean normalized fold change ± standard deviation, n = 3-5. Statistical difference from respective control, i.e. untreated cells at each time point is indicated by an asterisk (One-way ANOVA, Dunnett's Test, $p < 0.05$).

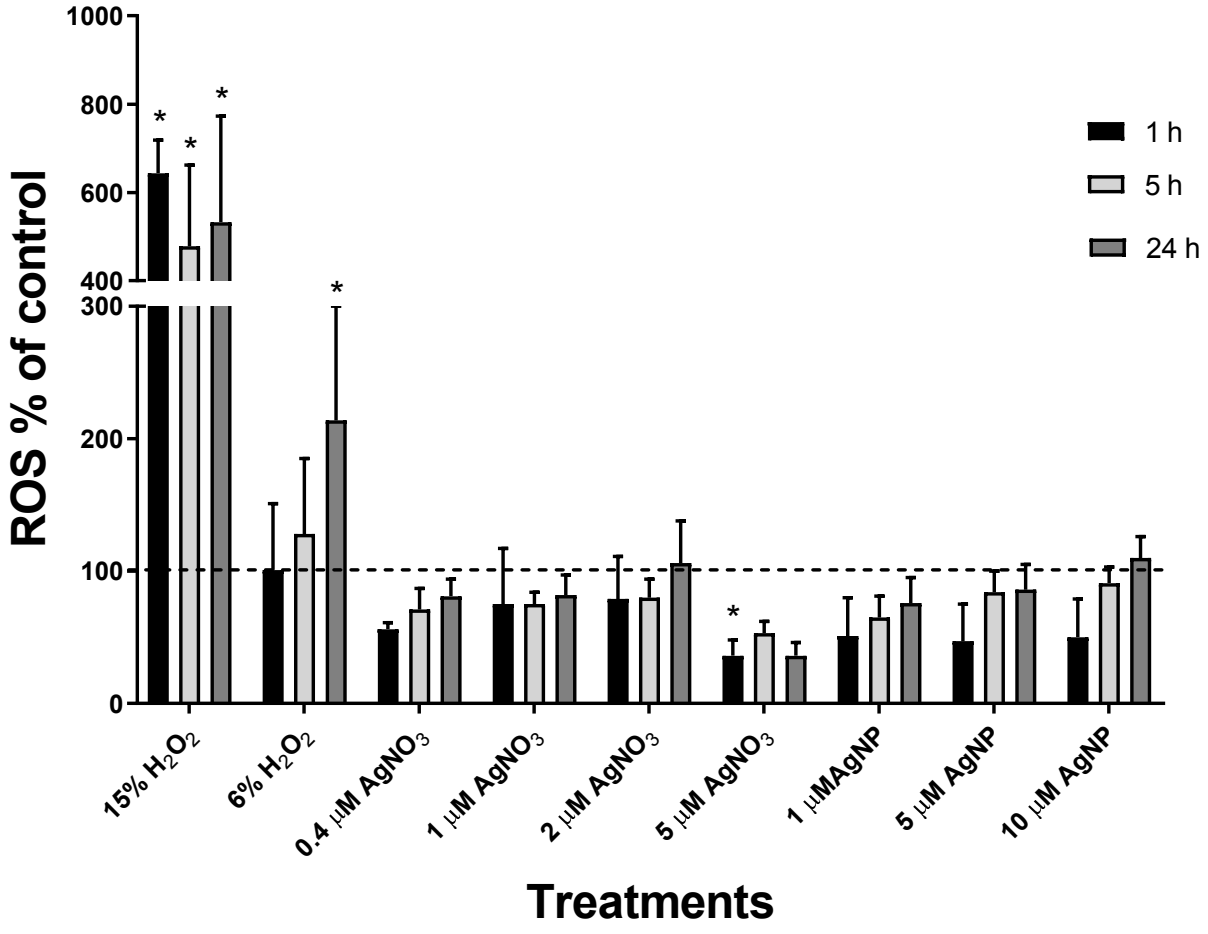


Figure 4: Determination of oxidative stress in RTgutGC cells exposed to $AgNO_3$ and cit-AgNP at 1 h, 5 h and 24 h. 6% and 15 % H_2O_2 were used as positive control. Oxidative stress was detected using the H_2DCFDA assay. Each experiment included at least three technical replicates for each condition. Values are means ± standard deviation of three independent experiments, n=3. Bars bearing asterisks indicate significant difference from control (Two-way ANOVA, Dunnett's Test, $p < 0.05$).

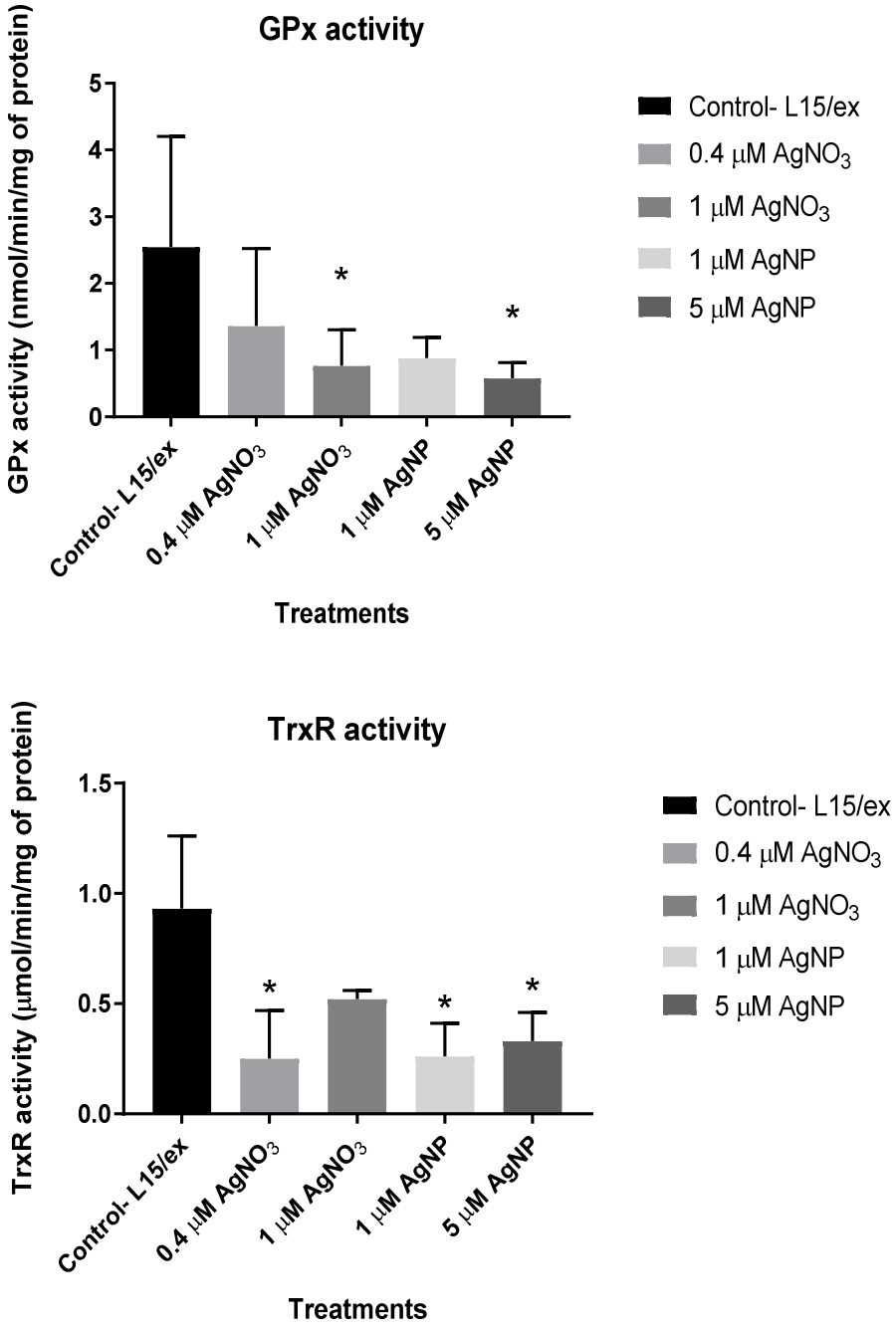


Figure 5: GPx and TrxR enzymatic activity measured in RTgutGC cells exposed to 0.4 μM and 1 μM $AgNO_3$ and 1 μM and 5 μM of cit-AgNP. All values were normalized per mg of total protein. Values are represented as mean ± standard deviation, n = 3. Statistical difference from respective control, i.e. untreated cells at each time point is indicated by an asterisk (One-way ANOVA, Dunnett's Test, $p < 0.05$).

CHAPTER VIII

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CHAPTER IX

SUPPLEMENTARY MATERIAL

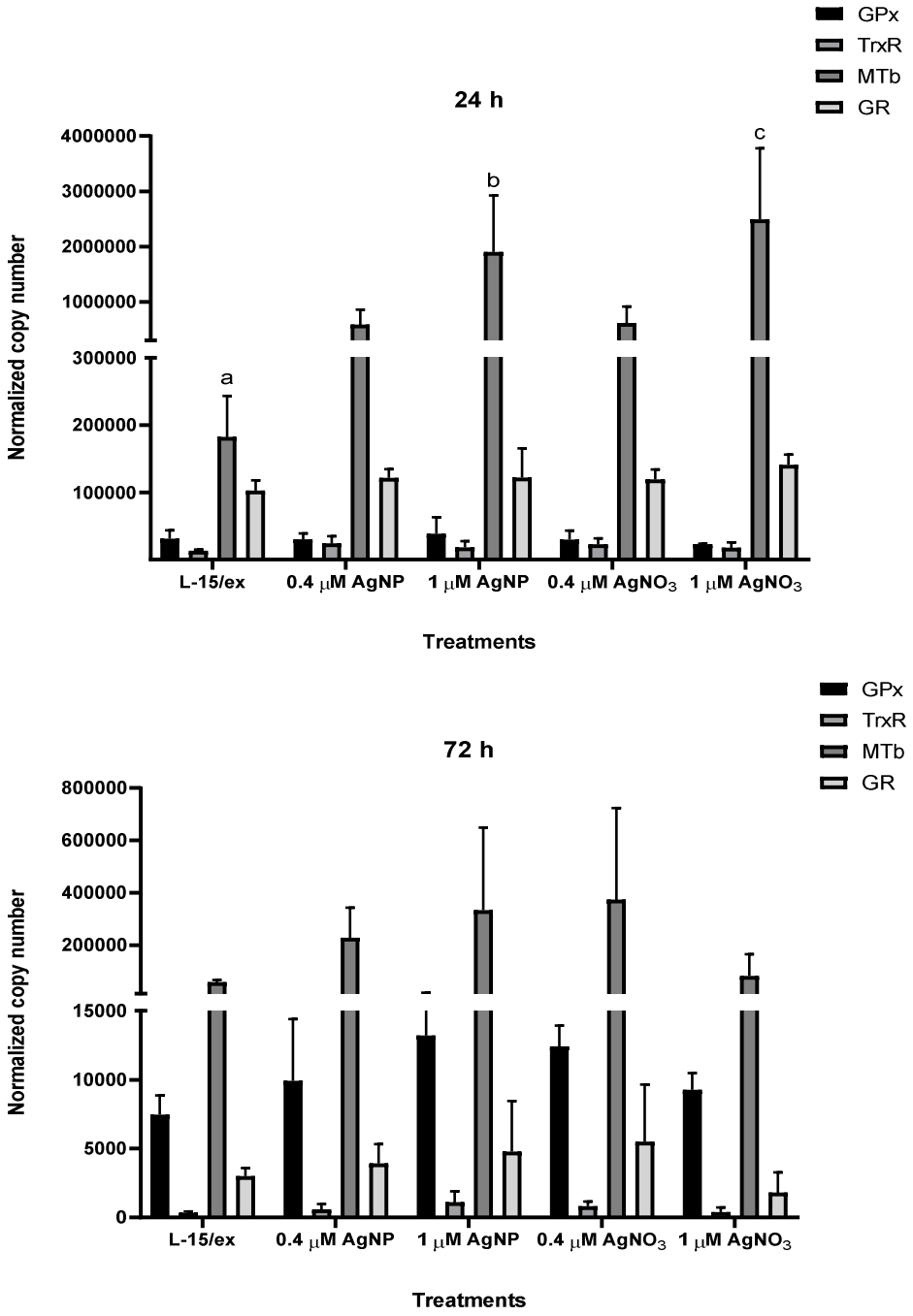

Figure S1: Normalized mRNA levels of GPx, TrxR, MTb and GR measured in RTgutGC cells exposed to non-toxic and toxic concentrations of $AgNO_3$ and cit-AgNP at 24 h and 72 h. The values are mean ± SD (n= 3-5). Values bearing different letters indicate statistical significance among different groups (One-way ANOVA, Tukey's *post hoc* test, $p < 0.05$).

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Master of Science

Thesis: EVALUATION OF THE EFFECT OF SILVER AND SILVER NANOPARTICLES ON THE FUNCTION OF SELENOPROTEINS USING AN *IN-VITRO* MODEL OF THE FISH INTESTINE: THE CELL LINE- RTgutGC

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