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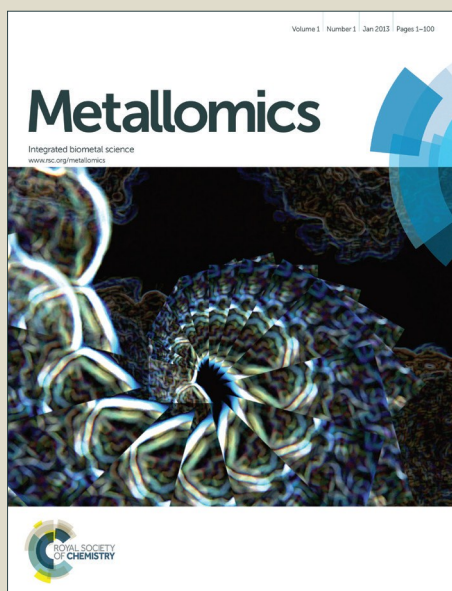## Metallomics

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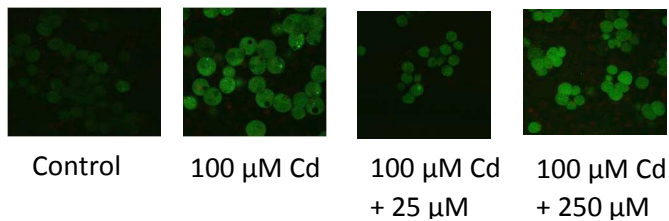
Confocal fluorescent images of isolated rainbow trout hepatocytes exposed to 100μM Cd, alone or in combination with low (25μM) or high (250μM) concentration of selenium

An *in vitro* examination of selenium-cadmium antagonism using primary cultures of rainbow trout (*Oncorhynchus mykiss*) hepatocytes

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Abstract

The present study evaluated the ameliorative properties of selenium (Se) against cadmium (Cd)-induced oxidative stress, using isolated rainbow trout (*Oncorhynchus mykiss*) hepatocytes in primary culture as the model experimental system. Cadmium (Cd) is known to induce cytotoxic effects by disrupting cellular oxidative homeostasis. On the other hand, selenium (Se) is an essential component of biological antioxidative machinery, and thus may provide protection against the toxic insults of Cd by augmenting cellular antioxidant response. However, Se, when present above the threshold concentration, can also induce reactive oxygen species (ROS) generation and cause oxidative damage. In this experiment, trout hepatocytes in primary culture were exposed to 100 µM Cd, alone or in combination with different concentrations (25 - 500 µM) of selenite (SeO_3^{2-}) or selenomethionine (SeMet) for 48 hrs. Our findings indicated that both chemical forms of Se, at the lowest concentration used (25 µM), significantly reduced Cd-induced cytotoxicity (measured as cell viability). In contrast, Se at higher concentrations (≥50 µM) did not offer any protection against Cd induced decrease in cell viability. Reduced cytotoxicity of Cd in the presence of 25 µM selenite or SeMet was associated with reduced intracellular ROS production, recovery of cellular thiol status (ratio of reduced and oxidized glutathione), and amelioration in the activities of major enzymatic antioxidants (superoxide dismutase, catalase, and glutathione peroxidase). Co-treatment of hepatocytes with Cd and pharmacological antioxidants (TEMPO and NAC) also reduced Cd-induced oxidative stress in trout hepatocytes. This provided further evidence that Se likely ameliorates Cd toxicity *via* different antioxidative mechanisms.

Keywords: cytotoxicity, cadmium, selenite, selenomethionine, antagonism, thiol redox, oxidative stress.

Introduction

Cadmium (Cd) has been categorized as a global priority pollutant because of its ubiquitous presence, bio-accumulative nature and potential to induce toxic effects at relatively low concentrations.[1] Cadmium is a non-essential metal, and toxic to all life forms, including fish. Cadmium is a calcium antagonist, and known to cause toxicity in fish by disrupting branchial calcium uptake and homeostasis, especially during acute exposure.[2] The toxicity of Cd has also been attributed to the disruption of oxidative homeostasis.[3, 4] Cellular oxidative homeostasis is primarily maintained by various enzymatic antioxidants such as catalase (CAT), glutathione peroxidase (GPx), superoxide dismutase (SOD), as well as by multiple non-enzymatic antioxidants such as glutathione, ascorbate and metallothionein.[5] However, exposure to Cd often leads to the disruption of antioxidative enzymes and/or depletion of the non-enzymatic antioxidant pool, resulting in a concomitant cellular accumulation of reactive oxygen species (ROS) and oxidative damage.[6, 7]

The intracellular handling of Cd can be influenced by the crosstalk of cellular pathways involved in the metabolism and regulation of essential/nutrient elements, which may elicit additive, synergistic or antagonistic effects. Selenium (Se) is one such essential element that has been suggested to influence the toxicity of Cd in different organisms, including mammals and fish (see Zwolak and Zaporowska for review).[8] Selenium is essential for the synthesis of selenoproteins, which have various critical adaptive and housekeeping functions in organisms, including the maintenance of cellular oxidative homeostasis.[8] Among all the living organisms, fish are known to have the highest requirement of Se, as they possess the largest selenoproteome.[9] For example, fish are known to have 30-37 selenoproteins, whereas mammals contain only 23-25 selenoproteins.[9] Selenium exists in the environment in both organic and

inorganic forms.[10] Selenomethionine (SeMet) is the most common form of organic Se found in fish diet, whereas selenite (SeO_3^{2-}) is usually the most abundant soluble form of inorganic Se found in natural waters under normal conditions.[11] Since Se is known to have antioxidative properties, it can be assumed that Se may ameliorate Cd-induced cellular oxidative stress, and thus provide protection against the toxicity of Cd. However, it is also interesting to note here that Se is also an important aquatic pollutant,[12] and when present above the threshold level in biological systems, can rapidly turn into a pro-oxidant.[10] It has been demonstrated that exposure to high levels of both selenite and SeMet causes cellular oxidative stress in fish, essentially by disrupting thiol redox and inducing ROS generation.[13, 14] Thus, the protective effects of Se against Cd-induced cytotoxicity might be influenced by Se exposure dose, with antagonistic effect at low exposure levels and additive or synergistic effects at high exposure levels.

Previous mammalian studies have demonstrated the protective effects of both selenite and SeMet against Cd cytotoxicity,[8, 15, 16] however the precise mechanistic underpinnings of this antagonism are not fully understood. The protective effects of Se against the organismal toxicity of Cd have also been reported in fish exposed to sub-lethal Cd in the presence of Se.[17, 18] However, all of these previous studies have investigated the ameliorative effects of relatively low exposure levels of Se against Cd toxicity, and how this effect is modulated by high Se exposure level is yet to be characterized. Moreover, it is important to note that the chemical speciation of Se may also influence cellular effects of Cd exposure, since inorganic and organic Se are known to be metabolized through different cellular pathways.[19-21] This is particularly important in fish since they acquire Se primarily through their diet as SeMet.[11] Although the antagonistic behaviour of selenite and Cd has been suggested to be mediated by the amelioration of oxidative

stress,[16, 22] it is not known whether the cytoprotective effect of SeMet against Cd cytotoxicity occurs through a similar mechanism.

The main objectives of this study were twofold: (i) to examine how the chemical speciation [inorganic (selenite) vs organic (SeMet)] and exposure dose of Se influences Cd induced cytotoxicity at the cellular level, and (ii) to provide a deeper insight into the cellular pathways underlying the antagonism of Se and Cd in fish. Rainbow trout (*Oncorhynchus mykiss*) hepatocytes in primary culture were used as the model *in vitro* experimental system in the present study, since hepatocytes are the functional units of liver and one of the main sites of Se and Cd metabolism.[23-25]

Methods

Chemicals

High purity, cell culture tested sodium selenite (Na_2SeO_3, purity ~ 98%), seleno-L-methionine (purity>98%), cadmium chloride ($CdCl_2$, purity~99.99%), cell dissociation solution non-enzymatic (cat# C1419), CelLytic MT^{TM} solution, Dulbecco's phosphate buffered saline and trichloroacetic acid (TCA) were purchased from Sigma Aldrich, USA. Antibiotic and antimicotic solution, and L-15 media were purchased from Invitrogen, Canada. Aquacalm^{TM} (Metomidate hydrochloride) was purchased from Syndel Laboratories Ltd, Canada. All other chemicals were purchased from VWR International, Canada.

Experimental animals

Rainbow trout weighing 600-700g were used for the experiments. Fertilised eggs from reference rainbow trout females were hatched in the department of biology, University of Saskatchewan. The spawn were reared until they reached 600-700g in dechlorinated Saskatoon

City water at a rate of 2 l/min under constant aeration. Fish were maintained at a photoperiod of 16 h light: 8 h dark and a water temperature of 12 ± 1 $^{\circ}$C. The fish were fed once daily with commercial diets at a ration of 2% of body weight.

Hepatocyte isolation and culture

Trout hepatocytes were isolated using a two-step collagenase perfusion technique as described by Mommsen et al, (1994) with slight modifications.[14, 26] Briefly, fish were euthanized with an overdose of Aquacalm (0.5 g/l) in dechlorinated water. The hepatic portal vein was cannulated with PE-50 tubing and perfused with ice-cold modified Hank's Media (136.9 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4.7H_2O$, 0.33 mM $Na_2HPO_4.7H_2O$, 0.44 mM KH_2PO_4, 5.0 mM HEPES, 5.0 mM Na-HEPES, pH 7.63). Perfusion with Hank's media was continued until the liver was completely blanched, after that the perfusion line was switched to the medium containing 0.2 mg/ml collagenase in Hank's Media. Perfusion with collagenase was performed until liver was fully digested. Digested liver was chopped into small pieces with a razor blade and the dissociated cells were filtered, first through 260 μm and then through 73 μm mesh size strainers. The cells obtained in the filtrate were centrifuged at 100xg for 5 minutes at 4°C and washed twice in Hank's media. This was followed by a single washing with the Hank's media containing BSA (2%) and $CaCl_2$ (1.5 mM). The cells were then incubated for 30 min in L-15 media (pH 7.63) containing antimicrobial and antimicotic solution on an ice bath. The settled down cells were collected by aspirating out the media on top. The cells were then re-suspended in 25 ml of L-15 media and their viability was determined by trypan blue exclusion test.[27] The suspensions showing more than 85% cell viability were used for the experiments. The cells were plated in 6-well Primaria plates (BD Falcon, USA) at a density of 0.3 x 10^6 cells/cm^2 and

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incubated, in dark at 15°C for 24 h using a low temperature incubator (Fisher Scientific, Canada), to form monolayer before their use in the experiment.

Exposure of hepatocytes to Cadmium, alone or in combination with Selenium

At first, the hepatocytes were exposed only to an increasing range of Cd concentrations (0-500µM) in order to determine the dose-dependent effect of Cd on cell viability. After 24 h of culture, following isolation, the media from culture plates was aspirated out and the hepatocytes were exposed to the media containing different concentrations of CdCl$_2$. Cadmium exposures were conducted for 48 h with a change of exposure media at 24 h. A consistent 30% reduction in cell viability was observed at 100 µM Cd exposure dose (n=4; data not shown). This concentration was therefore chosen for all of the subsequent experiments described below.

To determine how Se influences Cd-induced cytotoxicity, hepatocytes were exposed to 100 µM Cd, alone or in combination with different concentrations (25, 50, 100, 250 and 500 µM) of selenite (SeO$_3^{2-}$) or selenomethionine (SeMet) for 48 hrs. Our preliminary work revealed that Se did not influence Cd-induced loss of cell viability at concentrations below <25 µM (selenite or SeMet) (data not shown). The hepatocytes in the control group were treated similarly with the media without any added Cd or Se. The exposure media was spiked with appropriate amounts of freshly prepared solutions of CdCl$_2$, selenite or SeMet prior to each exposure. The exposure media was also changed after 24 h of exposure as described above. The osmolality of the exposure media was measured using a 5100C vapour pressure osmometer (Wescor Inc., USA), and no change was recorded in any treatment due to the addition of CdCl$_2$, SeO$_3^{2-}$ or SeMet. At the end of the exposure period, cells were collected from the culture plate using a non-enzymatic cell dissociation solution. Cell viability was measured immediately by the trypan blue

exclusion test. The experiment was performed five times using hepatocytes isolated from an individual fish at each time.

For the measurement of enzymatic activities, the harvested cells were centrifuged at 500xg for 5 min and washed with Dulbecco's Phosphate Buffer Saline three times and then lysed with 500 µl of CelLytic-M reagent. The lysate was centrifuged at 25,000xg for 20 min at 4°C to pellet the cellular debris. The supernatant was collected and stored at -80°C for the enzymatic analysis. For the measurement of oxidized and reduced glutathione, cells were treated as before, except that an ice-cold 5% TCA solution was used along with CelLytic-M reagent during the cell lysis. The cell lysate was split into two fractions (250 µl each). One fraction was stored as such at -80°C for measurement of reduced glutathione (GSH). A 20 µL aliquot of 0.04 M of N-ethylmaleimide was added immediately to the other fraction in order to prevent the oxidation of GSH, and stored at -80°C for measurement of oxidised glutathione (GSSG). The Bradford method was used for estimation of protein content of the samples.[28]

Exposure of hepatocytes to pharmacological antioxidants in the presence of Cadmium

Pharmacological antioxidants, (2,2,6,6-tetramethylpiperidin-1-yl)oxidanyl (TEMPO) and *N*-acetyl-*L*-cysteine (NAC), were used to compare with the antioxidative effects of Se in trout hepatocytes. Hepatocytes were exposed to 100 µM Cd, alone as well as with TEMPO or NAC (100 µM) for 48 h, as described previously for exposures with Se. At the end of the exposure, cells were harvested and cell viability was assessed as mentioned above.

Measurement of cellular thiol redox balance (GSH:GSSG ratio)

Cellular thiol redox balance is assessed traditionally by measuring GSH to GSSG ratio.[29-31] The concentration of the reduced (GSH) and oxidized (GSSG) glutathione in the cell lysates was measured using a fluorometric method[32], modified to a 96-well microplate based assay.[14] In

order to confirm the linearity of the reaction rate in the adopted method, commercially purified GSH and GSSG were used to calibrate the standard curve. The measurement of GSH was performed in a final reaction mixture volume of 200 µl, which contained 180 µl of phosphate–EDTA buffer (0.1 M sodium phosphate–0.005 M EDTA, pH 8.0), 10 µl of o-Phthalaldehyde (OPT, 100 µg per 100 µl methanol) and 10 µl of sample. The reaction mixture was incubated for 15 min at room temperature, and the fluorescence was measured in a multimode microplate reader (Varioskan Flash, Thermo Fisher Scientific, Finland) at excitation and emission wavelengths of 350 nm and 450 nm, respectively. The GSH content was expressed as µg per mg of protein. GSSG was measured similarly, except the final reaction mixture volume (200µl) contained 140 µl of 0.1 N NaOH, 20 µl of o-Phthalaldehyde (OPT, 100 µg per 100 µl methanol) and 40 µl of sample. The GSSG content was also expressed as µg per mg of protein. Finally, GSH content of each replicate was divided by its corresponding GSSG content and expressed as a ratio.

Measurement of antioxidant enzyme activities

We measured the activities of three antioxidant enzymes, SOD, CAT, and GPx using 96-well microplates and a multimode plate reader (Varioskan Flash, Thermo Fisher Scientific, Finland). Enzyme activities were measured in the hepatocytes exposed to 100 µM Cd alone or in combination with 25 or 250 µM selenite or selenomethionine. The enzyme activities were measured using SOD (Catalogue #706002), CAT (Catalogue #707002), and GPx (Catalogue #706002) activity kits as per the manufacturer's (Cayman chemical company, USA) instructions. SOD activity was expressed as % of control. Activities of CAT and GPx were expressed as nmol/min/mg protein. One unit of CAT was defined as the amount of enzyme that will cause the

formation of 1.0 nmol formaldehyde at 25°C. One unit of GPx was defined as the amount of enzyme that will cause the oxidation of 1.0 nmol of NADPH to NADP$^+$ per minute at 25°C.

Measurement of intracellular reactive oxygen species (ROS) generation using Confocal Microscopy

Confocal microscopy was used to investigate the effect of selenite and SeMet on ROS production in hepatocytes exposed to 100 µM Cd. An ROS sensitive fluorescent dye, 50,6-chloromethyl-20,70-dichlorodihydro-fluorescein diacetate (CM-H$_2$DCFDA) was used for this purpose. CM-H$_2$DCFDA passively diffuses into cells, where its acetate groups are cleaved by intracellular esterases. Following the cleavage of acetate groups the dye gets oxidized by intracellular ROS to yield a fluorescent adduct which can be evaluated under a confocal microscope with maximum excitation and emission spectra of 495 nm and 529 nm, respectively. Fluorescent intensity can be measured and quantified, thus, providing a measure of intracellular ROS generation.

A 2 mM stock of CM-H$_2$DCFDA was prepared in anhydrous dimethylformamide (DFO) which was diluted to 5 µM in L-15 media for use in the experiments. Final concentration of DFO in the exposure media was less than 1%. For this experiment, hepatocytes were cultured on glass bottom dishes coated with poly-D-lysine (Mat Tek Corporation, USA) for 24 h. The 24 h culture was then exposed to 5 µM of CM-H$_2$DCFDA for 45 min at 15°C and washed three times with L-15 media without phenol red. Subsequently, the hepatocytes were exposed to media containing 100 µM Cd, alone or in combination with a low concentration (25 µM) or a high concentration (250 µM) of selenite or SeMet for 2 h. An exposure period of 2 h was employed instead of 24 h, primarily to capture the effects during the early exposure phase and also to prevent any leakage of the dye from the cells due to potential ROS induced membrane damage. At the end of the

exposure period, intracellular ROS production in each treatment were measured at room temperature (21°C) using the 488 nm excitation Argon laser beam and emission was collected using 505–530 nm band pass filter of the confocal microscope (Zeiss Axiovert LSM 510 Meta Confocal System, Carl Zeiss, Germany). Similar microscopic settings for the imaging were maintained throughout to allow conformity of the results.

ImageJ software (National Institutes of Health, Bethesda, Maryland, USA) was used to quantify the fluorescence intensity of the cells. Integrated density of all the cells under the view of microscope was measured from which mean background intensity was subtracted. The final fluorescent intensity was expressed as corrected total cell fluorescence (CTCF) using the following formula:

CTCF = Integrated Density - (Area of selected cell X Mean fluorescence of background readings)

Statistical analysis

All data are presented as mean ± standard error of mean (S.E.M.). Sample size 'n' indicates the number of true independent evaluations, each conducted with cells isolated from a different fish. The experiment involved manipulation of only Se as an independent variable therefore significant differences among the treatment groups were analyzed by one-way analysis of variance (1-WAY ANOVA) followed by Tukey's multiple comparison test (SigmaPlot, version 11, Systat Software, Inc., USA). The assumptions of ANOVA, normality of distribution and homogeneity of variances, were verified using the Shapiro-Wilk and Levene's tests, respectively. A p-value of ≤ 0.05 was considered to be significant while comparing different treatments.

Results

Effects of selenite and SeMet on Cd induced cytotoxicity

Hepatocyte viability after 48 h of exposure to 100 µM Cd, alone or in combination with selenite or SeMet (25, 50, 100, 250, and 500 µM) is illustrated in Fig. 1. There was a significant effect of Cd exposure, independently or in combination with different concentrations of Se, on cell viability ($F_{11,48}=13.29$; $p<0.001$) . Exposure to Cd alone reduced hepatocyte survival by 30% ($p<0.001$). Co-exposure of hepatocytes to Cd with selenite or SeMet reduced Cd induced cytotoxicity. However, the protective effect of Se against Cd toxicity was observed only at 25 µM Selenite or SeMet concentration, where the cell viability was restored and did not differ from the control group ($p=0.65$). No such protective effect of Se against the cytotoxicity of Cd was observed at 50 - 250 µM of selenite or SeMet ($p>0.05$). Co-exposure of 100 µM Cd and 500 µM Se (selenite or SeMet) was found to be more toxic than 100 µM Cd alone, as the hepatocyte viability decreased significantly in the former treatment relative to the latter ($p=0.025$).

Effects of pharmacological antioxidants on Cd induced cytotoxicity

The effects of pharmacological antioxidants on Cd-induced cytotoxicity are presented in Fig. 2. Exposure of hepatocytes to Cd, alone or in combination with pharmacological antioxidants, had a significant effect on cell viability ($F_{3,16}=13.92$; $p<0.001$). Co-exposure of hepatocytes to 100 µM Cd and 100 µM TEMPO or NAC completely restored the cell viability ($p<0.001$). Exposure to only 100 µM Cd reduced cell viability by about 30% ($p<0.001$) in comparison to the control. However, there was no statistically significant difference in cell viability between the control and the co-exposure of Cd with TEMPO or NAC.

Effects of selenite and SeMet on Cd induced changes in cellular thiol redox balance

Changes in GSH:GSSG ratio in hepatocytes exposed to 100 µM Cd alone or in combination with selenite or SeMet are illustrated in Fig. 3. Cellular thiol redox balance was significantly influenced by Cd exposure, alone or in combination with different concentrations of Se ($F_{11,36}$=8.65; $p<0.001$). Exposure to Cd alone resulted in a significant decrease in GSH:GSSG ratio ($p<0.001$). Similar to the effect of Se on Cd induced loss in cell viability, 25 µM of Se, either as selenite or SeMet, was able to fully restore the GSH:GSSG ratio as no difference relative to the control was observed. However, exposure of hepatocytes to ≥50 µM selenite or SeMet did not alter the Cd induced decrease in cellular thiol redox ($p>0.05$).

Effects of selenite and SeMet on Cd induced changes in antioxidative enzyme activities

Cadmium exposure, independently or in combination with Se, had a significant effect on SOD activity ($F_{5,22}$=42.0; $p<0.001$). Exposure to 100 µM Cd alone reduced SOD activity by 40%, which was significantly different relative to the control ($p<0.001$) (Fig. 4A). A similar decrease in the activity was also observed when hepatocytes were co-exposed to Cd and 250 µM Se ($p<0.001$). However, the SOD activity recovered back to the control level when hepatocytes were exposed to Cd in the presence of 25 µM SeMet, whereas a treatment with 25 µM selenite resulted in a partial recovery of Cd induced decrease in SOD activity.

Cadmium exposure, independently or in combination with Se, had a significant effect on CAT activity ($F_{5,24}$=9.24; $p<0.001$) Exposure of hepatocytes to 100 µM Cd alone resulted in a >50% decrease in the activity of CAT, which was significantly lower relative to the control (Fig. 4B). The enzyme activity, however, was similar to the control in hepatocytes exposed to Cd in combination with 25 µM selenite or SeMet. In contrast, co-exposure to 100 µM Cd and 250 µM

of selenite or SeMet resulted in a similar decrease in CAT activity as caused by 100 µM Cd alone.

GPx activity exhibited a similar pattern as observed with CAT, when treated with 100 µM Cd alone or together with 25 µM or 250 µM selenite or SeMet ($F_{5,25}$=6.77; $p<0.001$) (Fig. 4C). The GPx activity decreased significantly (~35%), in hepatocytes treated with Cd alone or in combination with 250 µM selenite or SeMet, relative to the control. However, a partial recovery of GPx activity was recorded in hepatocytes exposed to 100 µM Cd in combination with 25 µM selenite or SeMet.

Effects of selenite and SeMet on Cd induced intracellular ROS generation

The fluorescence intensity from ROS generation was measured as corrected total cell fluorescence (CTCF) and is illustrated in Fig. 5 A and B. There was a significant increase in intracellular ROS generation in hepatocytes exposed to 100 µM Cd alone relative to the control ($F_{5,18}$=13.72; $p<0.001$) (Fig. 5A i and ii, and 4B). However, co-exposure of hepatocytes to Cd with 25 µM of selenite or SeMet resulted in a significant decrease in intracellular ROS level, and no difference in ROS generation was recorded in comparison to the control (Fig 5A iii and iv, and 5B). In contrast, no difference in intracellular ROS generation was observed between the treatments of 100 µM Cd alone, and in combination with 250 µM selenite or SeMet (Fig. 5A iv and vi, and Fig 5B). Intracellular ROS level remained significantly high in hepatocytes exposed to Cd, with or without 250 µM Se, relative to the control ($p=0.001$).

Discussion

In the present study, we have examined the mechanistic underpinnings of Se-Cd antagonism at the cellular level in rainbow trout hepatocytes in primary culture. Two different seleno-compounds, selenite (inorganic) and SeMet (organic), over a broad range of exposure

dose (25-500 µM), were used to understand the chemical species specific and dose dependent effects of Se on Cd cytotoxicity. In general, our findings supported our original hypothesis that Se, irrespective of its chemical form, ameliorated Cd induced oxidative stress by augmenting cellular antioxidative machinery, albeit the protective effect was evident only at the lowest Se exposure dose (25 µM) used in this study.

Our results demonstrated that a low dose of Se (25 µM) restored the Cd induced loss in cell viability, however Se at concentrations >50 µM did not elicit any protective effect. Treatment with both selenite and SeMet produced similar protective effect against the cytotoxicity of Cd. Similar protective effects of selenite and/or SeMet (10-50 µM) against Cd-induced loss in cell viability were reported previously in mammalian *in vitro* studies with human erythroleukemia K-562 cells,[33] and porcine LLC-PK1 renal epithelial cells.[34] The findings of the present study are also consistent with previous *in vivo* observations which suggested that supplementation of fish diet with low to moderate levels of inorganic or organic Se could reduce waterborne Cd toxicity in fish.[17, 35] Interestingly however, our study also revealed that an exposure to 100 µM Cd in conjunction with the highest dose of selenite or SeMet used (500 µM) was more toxic to the trout hepatocytes than 100 µM Cd alone. Although Se is an essential element and has antioxidative functions,[36-38] it can rapidly turn into a pro-oxidant when its concentration exceeds a certain threshold.[10, 12] Previous studies have demonstrated that both selenite and SeMet induce oxidative stress and markedly decrease viability of trout hepatocytes when their exposure concentrations exceed 200 µM.[13, 14] Thus, the co-exposure of Cd with selenite or SeMet at high dose levels elicited an additive adverse effect on cell viability, likely because of the oxidative stress induced by both elements.

Previous experimental evidences suggest that one of the primary mechanisms of Cd cytotoxicity is the induction of oxidative stress, mediated mainly by the depletion of glutathione,[39, 40] and increased accumulation of intracellular of ROS.[3, 4] In our study, a complete recovery in cell viability was observed when the hepatocytes were exposed to Cd in the presence of pharmacological antioxidants, TEMPO or NAC (Fig. 2). This strongly suggests that the decrease in the viability of trout hepatocytes exposed only to Cd occurred due to the induction of oxidative stress. TEMPO and NAC are both pharmacological antioxidants, but they act through different mechanisms. TEMPO is known to be a ROS scavenger,[41] whereas NAC restores cellular thiol redox balance by facilitating GSH synthesis.[42] Since both TEMPO and NAC were able to ameliorate Cd induced cytotoxicity, it is reasonable to suggest that Se at a low dose level (25 μM) was able restore the Cd induced loss in cell viability *via* mechanisms similar to both of these two pharmacological antioxidants.

Analysis of the GSH:GSSG ratio in the present study revealed that exposure to 100 μM Cd alone caused a significant drop in cellular thiol redox potential (Fig. 3). Again, a complete recovery of GSH:GSSG ratio was observed when trout hepatocytes were exposed to 100 μM Cd in conjunction with 25 μM of selenite or SeMet. In contrast, no significant improvement in thiol redox was noticed when cells were exposed to Cd along with higher concentrations of Se, especially >100 μM (Fig. 3). It has been reported previously that the exposure to toxic concentrations of Cd causes a decline in cellular pool of GSH and thiol containing proteins in mollusc,[40, 43] and freshwater fish.[44] A dose dependent decline in GSH:GSSG ratio in response to Cd exposure was also observed in a mammalian renal cell line.[45] GSH can scavenge free ionic species of Cd (Cd^{2+}), which is the main driver for Cd cytotoxicity.[7] In addition to this, the thiol group of GSH in its reduced state is able to donate an electron to ROS and thereby neutralize it.[46]

During this process, GSH itself becomes oxidized and readily reacts with another oxidized glutathione to form GSSG. Cellular pool of GSH is replenished by two cellular processes: (i) *de novo* synthesis of GSH, catalysed by γ-glutamylcysteine ligase,[47] and (ii) reduction of GSSG to GSH by glutathione reductase enzyme.[46] During continuous exposure to Cd, *de novo* synthesis of GSH by γ-glutamylcysteine ligase and GSH regeneration from GSSG recycling could get overwhelmed, leading to the depletion of cellular pool of GSH and build-up of GSSG, resulting in reduced cellular thiol redox potential.[48] This might have occurred in the present study which resulted in the reduction of GSH:GSSG ratio in trout hepatocytes exposed to Cd alone. A cell with reduced redox potential is more susceptible to Cd toxicity because of its negative implications on several biochemical pathways that depend on reduced intracellular environment.[7, 49]

Selenium at optimal concentrations is known to upregulate *de novo* synthesis of GSH.[50, 51] An increase in GSH:GSSG ratio was reported by Fontagné-Dicharry *et al.,* when rainbow trout were fed with SeMet/selenite supplemented diet.[52] Moreover, it has been demonstrated by Chung and Maines, that Se could upregulate the activity of γ-glutamylcysteine ligase in rat liver, which can lead to increased GSH synthesis.[50] This could be a likely mechanism by which Se, at a low exposure dose (25 μM), was able to augment cellular GSH:GSSG ratio observed in the present study. The improved cellular thiol ratio, in turn, probably played an important role in ameliorating Cd induced oxidative stress and loss of cell viability. On the other hand, exposure to high doses of Se (both selenite and SeMet) can lead to a reduced thiol ratio.[13, 14] It has been suggested that the cellular metabolism of selenite, when present in excess, occurs via its reaction with GSH, which leads to the generation of superoxide anion ($O_2^{\cdot-}$).[20, 53] This causes a depletion of cellular GSH pool, and thereby induce cytotoxicity. In contrast, SeMet, when present in

abundance, is metabolised into methylselenol by the enzyme, L-methionine-γ-lyase. Subsequently, methylselenol undergoes redox cycling, which requires GSH, and produce $O_2{}^{\cdot-}$ in the process.[19] Therefore, the decrease in GSH:GSSG ratio, observed in the present study when trout hepatocytes were exposed to high doses of selenite or SeMet, was likely mediated by the cellular metabolism of selenite and SeMet, which also resulted in increased intracellular ROS generation and oxidative stress.

In the present study, we analysed the activities of three major enzymatic antioxidants, SOD, CAT, and GPx. These enzymes represent the first line of defence against ROS. SOD is responsible for dismutation of $O_2{}^{\cdot-}$ into H_2O and H_2O_2,[54] whereas CAT or GPx reduce H_2O_2 to non-toxic H_2O and O_2.[55, 56] These enzymes are also used as oxidative biomarkers because their activities are usually induced in response to mild oxidative stress as a compensatory mechanism. However, a rapid increase in intracellular ROS generation can overwhelm the antioxidative mechanisms, resulting in the suppression of antioxidant enzymes.[57] In our study, a short-term (2h) exposure to Cd was found to cause a marked increase in intracellular ROS level, which could have overwhelmed the cellular antioxidative response capacities, leading to an apparent decline in the activity of these enzymes. The reduced activities of these key antioxidative enzymes, in turn, would reduce the capacity to neutralize ROS, leading to various cytotoxic effects. Therefore, the suppressed activity of enzymatic antioxidants could be one of the major cellular implications of Cd toxicity. Furthermore, metalloenzymes such as SOD, CAT and GPx require an essential metal as cofactor to function, and Cd is known to inhibit metalloenzymes by substituting metal cofactors.[58, 59] It is possible that Cd at the dose used in our study might have been able to impair the functionality of these enzymes by replacing essential metals from their active sites. Reduced activity of enzymatic antioxidants following exposure to Cd has been

reported in several *in vitro* mammalian studies, using hamster ovarian cell line,[60] and in cultures of rat pneumocytes,[61] male gonadal cells,[62] and hepatocytes.[63] Similarly, *in vivo* studies conducted with different mammalian and piscine species also reported decreased activities of these antioxidant enzymes during exposure to Cd.[7, 17, 64-67]

We have also demonstrated in this study that a low exposure dose of Se (25 μM of selenite or SeMet) was able to alleviate the activities of these antioxidant enzymes. Our results are in agreement with previous *in vivo* studies that reported upregulation of antioxidative enzymes, when fish were treated with low doses of Se.[68, 69] This effect is probably attributable to the role of Se in the maintenance of enzymes involved in redox reactions. Selenium causes this effect indirectly through GSH, which maintains the redox status of the enzymes.[70] As discussed previously, low concentration of Se was found to increase the cellular GSH:GSSG ratio in our study. It is perhaps this elevated cellular thiol status that facilitated the increase of antioxidative enzyme activities. We have also demonstrated in this study that when hepatocytes were exposed to Cd along with high Se (250 μM), no induction of antioxidant enzymes was observed. This occurred likely because Se, when present in excess, can interact with the thiol moieties of antioxidative enzymes and impair their functions. For example, Se, when present in high concentrations, has been reported to inhibit GPx activity in mammals, by the formation of selenotrisulfide (S-Se-S), selenenylsulfide (S-Se), and diselenide bonds (Se-Se), and also by the catalysis of disulfide bond (S-S).[71]

As discussed above, Cd exposure can increase intracellular ROS generation indirectly by depleting thiol levels as well as suppressing enzymatic antioxidants. In addition, Cd is known to inhibit and uncouple complex III of the mitochondrial electron transport chain (ETC) and cause proton leak, which ultimately leads to the generation of ROS.[3, 4] Cadmium can also replace

essential metals like Fe, Zn, or Cu from various intracellular sites that binds them and keep their cytosolic concentrations low.[17, 58] Increased concentration of these pro-oxidative metals in the cytosol promote generation of ROS through Fenton's reaction.[72] It has been demonstrated previously that increased ROS production is linked to reduced cell viability.[73, 74] In the present study we also demonstrated that exposure to Cd significantly increased intracellular ROS production, which corresponded with a reduction in cell viability. However, co-exposure of 100 μM Cd with 25 μM selenite or SeMet was found to reduce ROS production back to the level observed in the control, along with a full recovery of cell viability. It is apparent that the reduction in intracellular ROS generation during treatment with the lowest dose of Se occurred due to increased ROS scavenging capacity, as a result of elevated cellular thiol redox and antioxidative enzyme activities. In contrast, we found that co-exposures of Cd with 250 μM selenite or SeMet did not alter the intracellular ROS generation as well as cell viability, relative to that in the cells exposed to Cd alone. This was to be expected since high Se treatment did not produce any improvement in cellular thiol redox or antioxidative enzyme activities.

Conclusion

The present study demonstrated that both selenite and SeMet could protect the hepatocytes of rainbow trout against toxicity of Cd, but this cytoprotective effect of Se occurs only at a low/non-toxic exposure dose (25 μM). Cadmium was found to decrease cell viability, which corresponded with increased intracellular ROS generation, and decreased cellular thiol redox and antioxidative enzyme activities. Selenium at a low exposure concentration was found to alleviate Cd-induced intracellular ROS generation by restoring the cellular thiol redox potential and capacity of enzymatic antioxidants. This indicates that Se at low exposure levels could act as an antidote for Cd poisoning in fish and potentially in other organisms including

humans. In contrast, Se at a high exposure dose was not found to be protective against Cd toxicity, as it did not induce any change in cellular thiol redox status or capacity of enzymatic antioxidants to overcome the oxidative stress caused by Cd exposure. Overall, our study demonstrated that the antagonistic effects of Se on Cd-induced cytotoxicity occur *via* both enzymatic and non-enzymatic antioxidative mechanisms.

Acknowledgements

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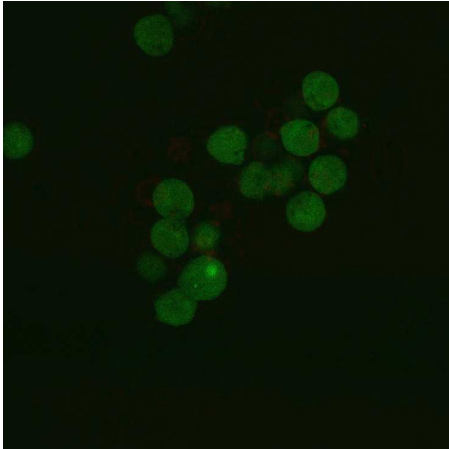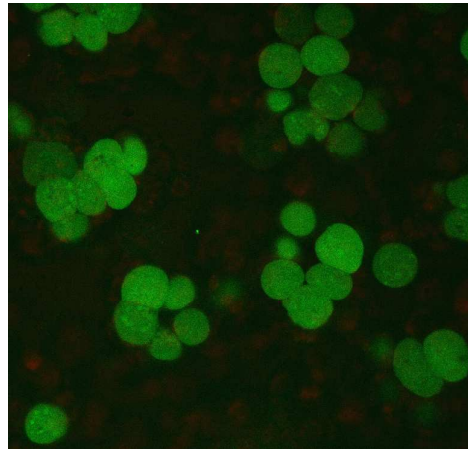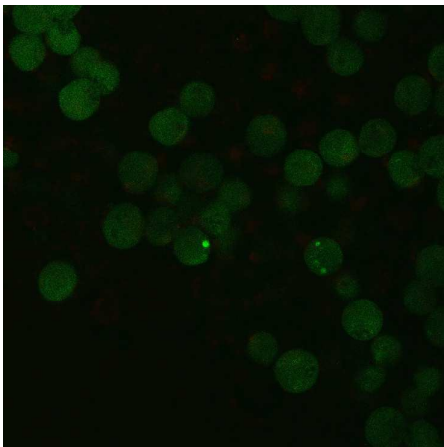
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i) Control
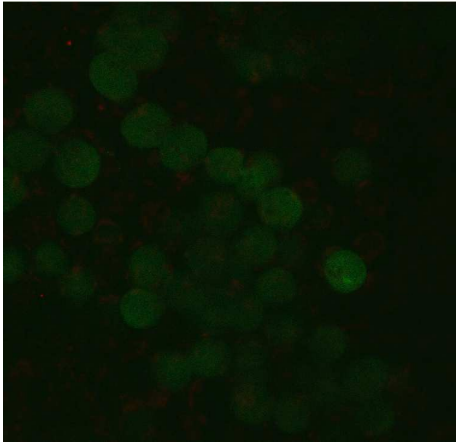
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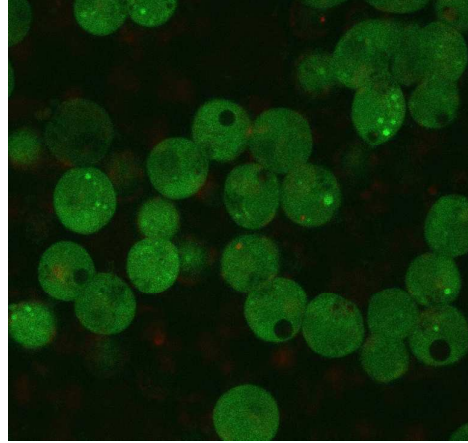
iii) 25 μM SeO$_3^{2-}$+100 μM Cd

iV) 250 μM SeO$_3^{2-}$+100 μM Cd

v) 25 μM SeMet+100 μM Cd

vi) 250μM SeMet+100 μM Cd
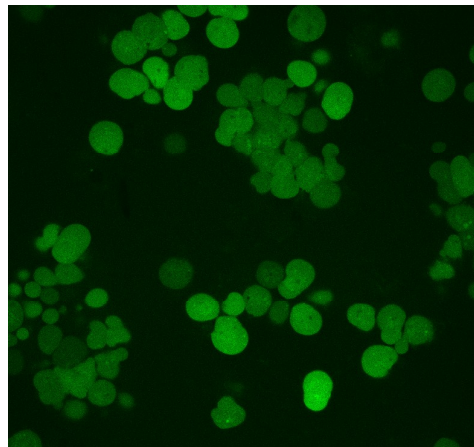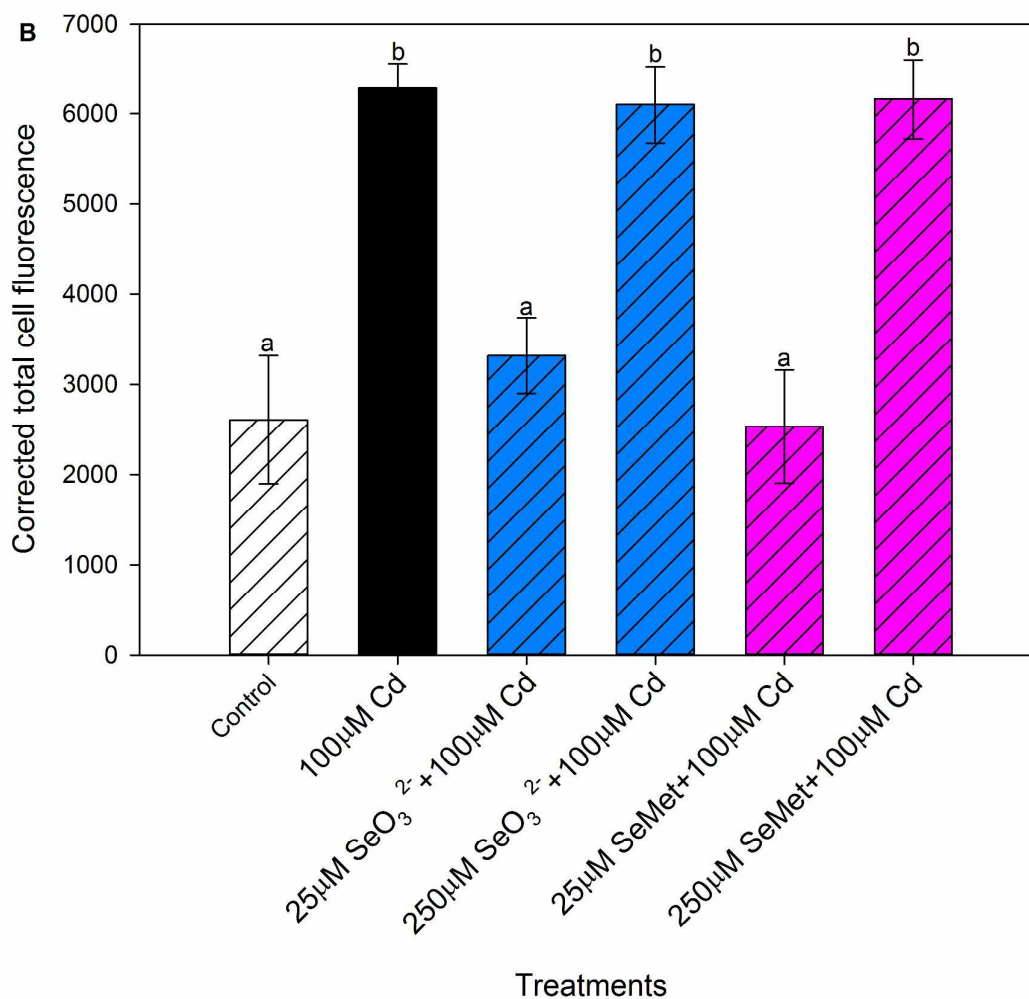
Fig. 5 Representative confocal fluorescent images (A) and corrected total fluorescent intensity (B) of isolated rainbow trout hepatocytes exposed to 100μM Cd, alone or in combination with low (25μM) or high (250μM) concentration of selenite (SeO_3^{2-}) or selenomethionine (SeMet) for a period of 2 h. The cells were loaded with CM-H2DCFDA for 45 min followed by exposure to various treatments. The intensity of fluorescent signals was measured using ImageJ software (National Institutes of Health, Bethesda, Maryland, USA). Data are presented as mean ± S.E.M. of average fluorescence intensity of 15–20 cells from each replicate and the experiment was repeated four times using four different fish. Mean values with different letters are statistically significant ($p < 0.05$).