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Impact of selenium co-administration on methylmercury exposed eleutheroembryos and adult zebrafish (*Danio rerio*): Changes in bioaccumulation and gene expression

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HIGHLIGHTS

- Se co-administration significantly decreases MeHg accumulation in eleutheroembryos.
- Se protects from MeHg accumulation in muscular tissue and the intestine of adult fish.
- Se does not protect from MeHg accumulation in brain and liver of adult fish.
- Gene expression analysis demonstrates the protective role of Se in MeHg toxicity.
- Results from gene expression correlates well with the Hg found in different organs.

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ABSTRACT

Mercury still represents one of the most hazardous threats for the aquatic ecosystem due to its high toxicity, and the fact that it can be easily incorporated into the food chain by accumulation in fish as MeHg. On the other hand, selenium is a micronutrient that is part of different antioxidant enzymes that regulate the cellular redox state, and whose complex interaction with Hg has been extensively studied from a toxicological point of view. In order to evaluate the protective effect of Se(IV) co-administration against MeHg accumulation and toxicity, we have selected an *in-vivo* model at two developmental stages: zebrafish eleutheroembryos and adult fish. Embryos were exposed during 48 h to MeHg (5 or 25 μg/l) and a concentration of Se (IV) representing a molar ratio close to one (2.5 or 12.5 μg/l), while adult zebrafish were exposed during 72 h to either 25 μg/l of MeHg alone or co-exposed with 12.5 μg/l of Se (IV). A significant decrease in MeHg bioaccumulation factor was observed in eleutheroembryos co-exposed to Se(IV). A time-dependent accumulation of MeHg was observed in all the analyzed organs and tissues of adult fish, which was significantly reduced in the muscular tissue and the intestine by Se(IV) co-administration. However, such protection against MeHg bioaccumulation was not maintained in the brain and liver. The data derived from the gene expression analysis also demonstrated the protective effect of Se(IV) against MeHg-induced oxidative stress and the activation of different defense mechanisms by Se(IV) co-administration.

1. Introduction

Elemental mercury (Hg) can be easily biotransformed into methylmercury (MeHg), which due to its capacity to penetrate into lipid bilayers, especially in the central nervous system (Chang et al., 2013), is considered one of the most toxic pollutants in the world (Joshi et al., 2014). MeHg can be assimilated into the food chain and biomagnify up to a 10-million-fold. For this reason, the consumption of contaminated fish constitutes a universal health risk to humans. Investigation of MeHg intake, accumulation, biotransformation, and excretion in fish can help to understand the specific toxic effects on organs and tissues, and how that toxicity could

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translate into humans.

Selenium (Se) is an essential nutrient for all vertebrates. It is a crucial part of many antioxidant enzymes such as glutathione peroxidase (Arteel and Sies, 2001), thioredoxin reductase (Branco et al., 2011) and several other selenoproteins, which modulate the cellular redox and antioxidant status (Glaser et al., 2010). However, Se can also be toxic at levels slightly above homeostatic requirement (Zhang et al., 2014). Its level of toxicity may depend on its chemical form, as inorganic and organic species have distinct biological properties. Overexposure to Se has been positively associated with several disorders such as type 2 diabetes, high-grade prostate cancer, and neurodegenerative diseases (Vinceti et al., 2018).

The interaction between Se and Hg has been extensively studied in many models (Suzuki, 1997; Whanger, 2001; Beijer and Jernelov, 1978; Culvin-Aralar and Furness, 1991; Beyrouty and Chan, 2006; Ralston et al., 2007; Bjerregaard et al., 2011) in order to elucidate the effects of different species of Se in protecting organisms from Hg-induced toxicity. Although the results have demonstrated that Se can indeed minimize the toxic effects induced by Hg, the interaction between the two elements has been proven to be extremely complex. Thus, the specific mechanisms of interaction Hg-Se as well as which Se species are more effective in which systems are still not clear (Luque-Garcia et al., 2013). Getting a deeper insight into such interaction mechanisms at the molecular level seems to be crucial to better understand the potential of Se in protecting, preventing or even reversing the damage induced by Hg in exposed organisms.

Zebrafish (*Danio rerio*) has emerged as a key model for studies on biological processes of environmental and medical relevance. The use of this model is increasing due to a wide range of advantages that zebrafish provides, such as low cost, ease of management and accommodation, short life cycle, comprehensive reproductive performance in laboratory, and extensive homology at genetic, neural and endocrine levels with mammals (Maximino et al., 2011). Additionally, zebrafish is characterized by a high reproductive rate, a fast embryonic development. Thus, the OECD has recommended zebrafish for the bioconcentration test 305 (OECD, 2012), used for evaluating the bioconcentration factors (BCFs) of chemical compounds. An alternative to the OECD 305 test has been recently developed using zebrafish eleutheroembryos (Sanz-Landaluze et al., 2015) that allows for a significant reduction in time, reagents and animal suffering during experiments, and provides high correlation with bioconcentration data experimentally obtained with adult fish.

With the aim of evaluating the potential protective effect of Se against MeHg-induced toxicity, and to establish whether such protective effect varied during eleutheroembryos development as compared to adult fish, we measured the differences in MeHg bioaccumulation in eleutheroembryos and in liver, brain, intestine and muscular tissue of adult fish co-exposed to Se(IV). We selected Se(IV) for being the most extensively studied Se species as potential agent against Hg toxicity. Additionally, we evaluated changes in the level of expression of a number of selected genes in all the above-mentioned samples. A correlation between the protective role of Se(IV) against MeHg accumulation and the differences observed in gene expression was finally assessed.

2. Material and methods

2.1. Zebrafish exposure and sampling

Two stock solutions of MeHg and Se(IV), at 500 and 100 mg/l respectively, were prepared by dissolving methylmercury(II) chloride (Alfa Aesar, Karlsruhe, Germany) in DMSO and inorganic sodium selenite (Merck, Damstadt, Germany) in 0.1% nitric acid. Both stock solutions were stored in the dark at 4 °C. Exposures were performed with zebrafish eleutheroembryos (72 h post fecundation, hpf) and adult zebrafish (1 year old). Eleutheroembryos were obtained from wild type adult zebrafish bred and maintained in the AZTI Zebrafish Facility (REGA ES489010006105) under standard conditions (Westerfield, 2007).

Eleutheroembryos were suspended in embryo water solution (294 mg of $CaCl_2 \cdot 2H_20$, 123.3 mg of $MgSO_4 \cdot 7H_2O$, 63 mg of $NaHCO_3$ and 5.5 mg of KCl in 1 l distilled water) containing different concentrations of MeHg or MeHg with Se(IV). The nominal concentrations for the exposure were established on the basis of previous studies (Cuello et al., 2012a, 2012b) using the highest concentration at 1% of the LC_{50} value and the second concentration differing in a factor of ten from this previous value. LC_{50} values were taken from the literature and the concentrations tested were the following: 5 and 25 μg/l of MeHg and 2.5 and 12.5 μg/l of Se(IV). Before exposures, the pH of all solutions was adjusted to 7.5 with sodium hydroxide. Exposures were performed at 27 °C using a 12h light/12h dark cycle. Exposures aimed at estimating MeHg bioaccumulation were carried out in two phases according to test OECD 305 (OECD, 2012) and an alternative protocol using eleutheroembryos (Sanz-Landaluze et al., 2015). Basically, embryos were exposed during 48 h to MeHg and a concentration of Se(IV) representing a molar ratio close to one at the two selected concentrations (uptake phase). Subsequently, they were kept in a clean media in the absence of MeHg and Se(IV) during 24 h (depuration phase). About 20 embryos and their corresponding exposure media were collected in triplicate at different exposure times (0, 24, 48 and 72 h) for total Hg determination.

Gene expression experiments were carried out by exposing groups of 25 embryos to MeHg (25 μg/l) and to a mixture of MeHg (25 μg/l) and Se(IV) (12.5 μg/l) during 48 h. After the exposure, embryos were collected deep frozen in liquid nitrogen and used for RNA extraction.

Adult zebrafish were exposed during 72 h to either 25 μg/l of MeHg alone or co-exposed with 12.5 μg/l of Se(IV). Before exposures, the pH of all solutions was adjusted to 7.5 with sodium hydroxide. Experiments were performed in a 5 l tank with 10 fish/lat 27 °C on a 12h light/12h dark cycle. Exposure media was replaced every 24 h to ensure the nominal exposure concentration constant. Fish were sampled at 8, 24, 48 and 72 h. From each fish, liver, brain, intestine and a portion of muscular tissue were extracted. Each organ or tissue was weighted individually and then, groups of 4 organs were pooled together. Pools were weighted and homogenized. Half of the homogenized pool was used for MeHg determination and the remaining sample was used for RNA extraction and gene expression analysis. For each time point, 3 replicates of pools originated from 4 organs/tissues were obtained. At each time point, an aliquot of the media was also taken. All experimental procedures were approved by the Regional Animal Ethics Committee and designed according to the OECD guidance (OECD, 2012).

2.2. Total mercury determination

Total MeHg content in embryos, the exposure media and in the different fish organs/tissue was determined using a DMA-80 direct mercury analyzer (Milestone Inc, Shelton, CT, USA), which combines the techniques of thermal decomposition, catalytic conversion, amalgamation and atomic absorption spectrophotometry following US EPA method 7473 (EPA, 2007). Samples in a nickel boat were dried and thermally decomposed. MeHg was reduced to elemental Hg and trapped in a gold amalgam. The amalgam was then heated to release the Hg vapors to the atomic absorption spectrophotometer, and the absorbance was measured at 253.7 nm.

All samples were analyzed in triplicate, and a control measure was performed every 20 samples with a certified reference material (CRM), to ensure the quality of the results. Blank samples were also analyzed every 20 analysis or following a high concentrated sample. Recoveries for CRM-029 (sewage sludge) and CRM-710 (oyster tissue) were within 5% of the certified value. The limit of detection (LOD) was 0.5 ng, while the limit of quantification (LOQ) was 1.68 ng of MeHg.

2.3. Estimation of the bioconcentration factors

The bioconcentration factor (BCF) is the parameter most widely used for evaluating the accumulation capability of contaminants by living organisms (Tsuda et al., 1998). Bioconcentration values can be calculated as the ratio between concentration of analytes found in fish and their concentration in the exposure media, once the equilibrium is reached (OECD, 2012), or by fitting these data to a first-order kinetic equation to obtain the uptake (k_1) and depuration (k_2) rate constants. Details about how the uptake and the depuration processes as a first-order kinetic (Eq. (1)) are calculated have been previously described (López-Serrano Oliver et al., 2011 and Sanz-Landaluze et al., 2015).

$$\begin{aligned} \frac{dC_f}{dt} &= k_1.C_W - k_2.C_f \quad \text{(uptake)} \\ \frac{dC_f}{dt} &= -k_2.C_f \quad \text{(depuration)} \end{aligned} \tag{1}$$

where C_f is the concentration in fish (ng/g), t is the exposure time (h), k_1 is the first order uptake constant (liter per kilogram dry weight per hour), C_w is the concentration of the chemical in the exposure media (ng/ml) and k_2 is the first order elimination rate constant (per hour). Assuming that at t_0 the concentration of the test compound in fish is negligible and in the medium is constant, equation (2) is obtained:

$$\begin{aligned} C_f &= \frac{k_1}{k_2}.C_w\left(1 - e^{-k_2 t}\right) \quad \text{(uptake)} \\ C_f &= C_{f,0}.\, e^{-k_2 t} \quad \text{(depuration)} \end{aligned} \tag{2}$$

where $C_{f,0}$ denotes the analyte concentration in the organism when the depuration phase begins. k_1 and k_2 values can be obtained if the experimentally determined concentration values in the bioconcentration test fit to this equation. When the equilibrium is reached (steady-state), equation (2) may be reduced to equation (3).

$$C_f/C_w = BCF_k = \frac{k_1}{k_2} \tag{3}$$

The software NONLIN 5.1 was used for kinetic calculations, which is specific for no linear adjustments (Nashville, TN) (Sherrod, 1995). No significant differences were detected by application of the *t*-test ($p \leq 0.05$).

2.4. RNA extraction and gene expression analysis

RNA from pools of 25 embryos or from homogenized organs/tissues of adult fish was extracted using TRIzol® (Sigma-Aldrich) following the instructions of the manufacturer. RNA concentration and quality were determined using a BioAnalyzer 2100 (Agilent, Santa Clara, CA, USA). Reverse transcription reactions were carried out using 20 ng of total RNA using a TaqMan Reverse Transcriptase Reagents kit (Applied Biosystems, Carlsbad, CA, USA). Reaction conditions were as follows: 25 °C for 10 min, 48 °C for 30 min and 95 °C for 5 min. The corresponding cDNA was used as a template for qRT-PCR.

Quantitative PCR was carried out with a LightCycler® 480 Real-Time PCR System (Roche, Mannheim, Germany). Eleven genes were tested. Primers are shown in Table 1. Gene selection was made on the basis of results obtained with a transcriptomic assay performed on zebrafish embryos exposed to 9 μg/l of MeHg (unpublished results). Different genes were selected based on their implications in different toxicity response pathways. Special attention was paid to genes related to oxidative stress, as previous studies have shown this effect in zebrafish larvae exposed to MeHg (Cuello et al., 2012a, b; Zhang et al., 2016). Specifically, genes *gpx1* (glutathione peroxidase 1), *gstp1* (glutathione S-transferase P), *txn1* (thioredoxin 1) and *prdx1* (peroxiredoxin 1) are involved in oxidative stress; *mt2b* (metallothionein-2) encodes for a detoxifying enzyme with a specificity for metals and it is also activated by oxidative stress; *hsp70* (70 kDa heat shock protein) encodes for a general stress response enzyme; *mrps31* (mitochondrial ribosomal protein S31) is a structural constituent of ribosomes involved in protein translation; *msna* (radixin) is a moesin encoding for a membrane organizing extension spike protein, important for cell-cell recognition and signaling and for cell movement; *fos* belongs to a group of genes that encodes for a family of transcription factors implicated in the regulation of cell proliferation, differentiation, and transformation; *btbd10b* (BTB (POZ) domain containing 10b) encodes for theta defensin b, a peptide with antimicrobial activity against a variety of microorganisms; *irg1l* (immunoresponsive gene 1, like) is a homolog of the human pro-inflammatory cytokine induced gene *irg1* and it is involve in antimicrobial response of macrophages.

Each reaction was performed in a 10 μl solution, containing

Table 1
Primers used for the gene expression analysis in this study.

Gene	Accession Number	Forward Primer (5′-3′)	Reverse Primer (5′-3′)
β-actin	AF057040	TGCTGTTTTCCCCTCCATTG	TTCTGTCCCATGCCAACCA
gpx1	NM_001007281.2	GGTTCTGGGCGCTCCCT	TTCTTGCAGTTCTCCTGGTGC
gstp1	AB194127	ATGAAGGGCGACTTGAAAGC	CATGGCGTTGGACTGAAACA
prx1	BC091459	TGCCTGATGGACAGTTTGGAG	TGAAGGCGATGATCTCAGTGG
mt2	AY514791	TGTGGATACTCTCTGGAAAAATGG	AGGTAGCACCACAGTTGCAAGTT
hsp70	AF210640	CCATCGAAGACGGCATCTTT	TCACCGCCCAGATGAGTGT
mrps31	AL928875	TGCACAGAAGACACGAACACC	GTTCCTAATGGCCTGTGGCTT
msna	BC081551	AGGAACTGATTCAGGAGGCCA	GCATAAGAGGCCAGCAAAACC
fos	BC065466	CTCACCGATACACTGCAAGCTG	GGATTTTTCATCCTCAAGCTGGT
btbd10b	XM_682338.5	CCAACTCACACCCTGTATTCGA	TCTTGTAGGTGCCCTCTGAGCT
irg1l	BC125936	AACTGCCACAACAGAAAGACACTG	CATCAAAGTCCATCGAGTGAGC
txn1	BI 864190	TCATCTTGATCATCATTCAACAACA	CTGAGAGTGATGGAGCCTTCATC

300 mM of each pair of primers, 5 µl SYBR Green PCR master mix (Roche) and 4 µl of the cDNA obtained in the previous step. Reaction conditions were as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. A final cycle at 95 °C for 1 min and 65 °C for 1 min, followed by 40 °C for 30 s was performed. The β-actin gene was used as a constitutive control to normalize all samples. The expression levels of RNAs were calculated according to Livak and Schmittgen (2001). The significance of the results was measured using the Relative Expression Software Tool (REST) of Qiagen (Hilden, Germany; http://www.REST.de.com) based on the pair wise fixed reallocation randomization test (Pfaffl et al., 2002). Gene expression data were the results of three independent replicates.

To assess if the addition of Se(IV) had any protective action on MeHg-induced cellular damage detectable by gene expression data, the reading of the cycle number at detection threshold (Cp) of samples exposed to MeHg were compared to those of samples exposed to MeHg and Se(IV). Significant differences were detected by application of the *t*-test ($p \leq 0.05$).

3. Results and discussion

3.1. Effect of Se(IV) co-exposure on the bioaccumulation of MeHg in zebrafish eleutheroembryos

In order to study the potential of Se as a protective agent against MeHg toxicity, we analyzed the total Hg bioaccumulation in zebrafish eleutheroembryos co-exposed to MeHg and Se(IV). In our previous work, carried out with identical exposure conditions (Cuello et al., 2012a), it was observed that zebrafish did not biotransform MeHg, therefore we assumed that the total Hg detected corresponds to MeHg. As mentioned above, zebrafish eleutheroembryos were exposed to two different nominal concentrations of each metal (5 µg/l of MeHg with 2.5 µg/l of Se(IV), and 25 µg/l of MeHg with 12.5 µg/l of Se(IV)). To calculate the bioaccumulation factor, according to the OECD guideline (OECD, 2012), also total MeHg concentration in the exposure media was analyzed. Test 305 requires that the chemical concentration in the exposure solution remains constant within, at least, a 20% of the nominal value during the uptake phase. In our case, MeHg concentration in the exposure media was maintained constant during 48 h (uptake phase).

The results for the MeHg bioaccumulation study in zebrafish eleutheroembryos, exposed to the two selected conditions, are shown in Fig. 1. Around 2000 and 3000 ng/g (wet weight) of MeHg were detected in eleutheroembryos co-exposed to MeHg and Se(IV) in the two tested conditions within the uptake phase, respectively. In agreement with a previous study (Cuello et al., 2012a), our results confirmed a dose and time dependency in the bioaccumulation of MeHg in eleutheroembryos. The highest concentration of MeHg was reached during the uptake phase and a slow elimination was detected in the depuration phase. This behavior has also been observed by other authors (Amlund et al., 2007; Liao et al., 2005) and might be explained by the capacity of MeHg to accumulate in lipids and by its high affinity with S and Se present in proteins. Actually, this fact makes MeHg a real threat during embryonic development, since it may get easily incorporated into muscular tissues or the brain during protein synthesis (Harris et al., 2003).

BCF_k values obtained in this study for the two tested conditions (Table 2) were 1500 and 350 for condition 1 (5 µg/l MeHg + 2.5 µg/l Se(IV)) and 2 (25 µg/l MeHg + 12.5 µg/l Se(IV)), respectively. Previous results obtained for MeHg exposure using the same alternative bioconcentration protocol with zebrafish eleutheroembryos provided a BCF_k of 5000 and 2333 for MeHg exposure at 1 and 10 µg/l, respectively (Cuello et al., 2012a). Thus, we can conclude

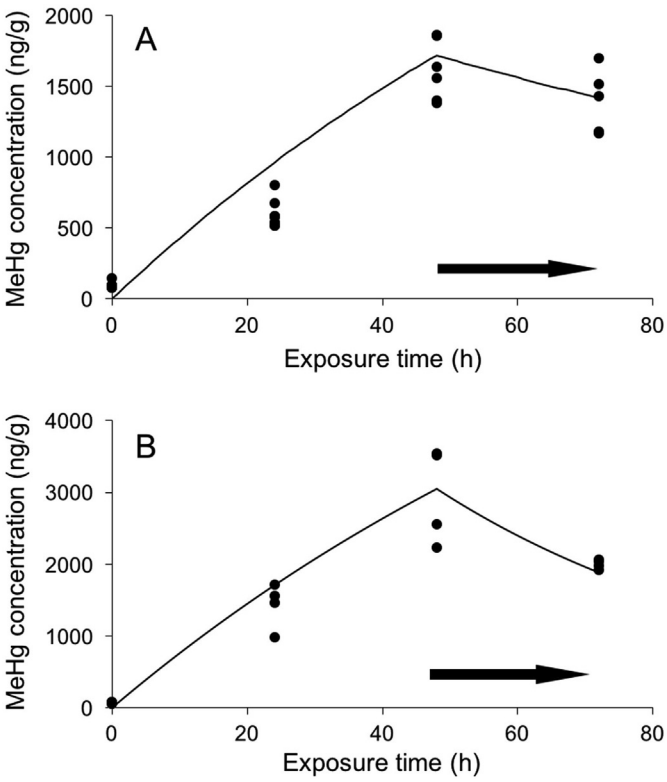

Fig. 1. Accumulation of MeHg in eleutheroembryos exposed to (A) 5 µg/l MeHg + 2.5 µg/l Se(IV) and (B) 25 µg/l MeHg + 12.5 µg/l Se(IV). Solid circles represent the experimental points and lines the expected values based on the model calculations.

Table 2
Bioconcentration factors obtained for eleutheroembryos exposed to different concentrations of MeHg and Se (IV).

Parameters	Condition 1[a]	Condition 2[b]	Condition 1[c]	Condition 2[d]
Cw (ng/mL)	3.45 ± 053	13.31 ± 0.43	0.31 ± 0.09	6.7 ± 0.8
K_1 (ngmL/h)	15	3,5	150	35
$K_{2\ (acum)}$ (ng/mL)	0.01	0.01	0.03	0.015
$K_{2\ (dep)}$ (ng/ml)	0.008	0.02	0.02	0.008
BCF_k	**1500**	**350**	**5000**	**2333**

[a] Condition 1: 5 µg/l MeHg + 2.5 µg/lSe(IV).
[b] Condition 2: 25 µg/l MeHg + 12.5 µg/lSe(IV).
[c] Condition 1: 1 µg/l MeHg [a] Cuello et al. (2012a).
[d] Condition 2: 10 µg/l MeHg [a] Cuello et al. (2012a).

that there is indeed a protective role of Se(IV) in this case, since addition of Se(IV) to zebrafish eleutheroembryos exposed to MeHg in a molar ratio close to 1, resulted in a significant decrease of MeHg accumulation during the uptake phase, and a better elimination of MeHg at the depuration phase (Fig. 2).

3.2. Se(IV) co-exposure affects MeHg bioaccumulation and distribution in adult fish

The potential protective effect of Se(IV) against MeHg toxicity was also evaluated in adult zebrafish. We measured the total amount of MeHg in liver, intestine, brain and muscular tissue of adult fish exposed to 25 µg/l MeHg alone or in combination with 12.5 µg/l Se(IV). We also calculated the BCFs by fitting the experimental data collected after 24, 48 and 72 h of exposure to a first-order kinetic equation as previously described (Table 3). As expected, in all the organs analyzed in fish exposed to MeHg alone, Hg concentration increased with increasing exposure times. The

Table 3
Bioconcentration factors obtained for adult zebrafish co-exposed to different concentrations of MeHg and Se (IV).

Organ	BCF_k Condition 1[a]	BCF_k Condition 2[b]
Liver	1400	2000
Intestine	3100	2300
Brain	980	1050
Muscular Tissue	200	500

[a] Condition 1: 25 μg/LMHg.
[b] Condition 2: 25 μg/LHg +12.5 μg/LSe(IV).

highest Hg concentrations after 72 h of exposure were found in the intestine followed by the liver. Significantly lower concentrations were found in brain and muscular tissue. Comparative results were obtained for the BCFs calculated for each of the organs/tissues evaluated.

It has been previously described that MeHg is mainly incorporated into aquatic organisms by oral route (Vazquez et al., 2014). Thus, the gastrointestinal wall can be considered the form of entry of MeHg into the systemic bloodstream. This fact could explain the high concentration of Hg found in the intestine in our experiment. As for the liver, such high concentrations were somehow expected, because of the well-known role of this organ in detoxification processes for most organisms (Liao et al., 2005). However, considering the well-documented neurotoxic effect of MeHg, which is due to its ability to penetrate the blood brain barrier (Amlund et al., 2007), the amount of Hg found in the brain was low if compared to other organs and to previously published data (Korbas et al., 2008). This could be explained by the relatively short exposure time (72 h). As commented before, the high concentration of Hg found in the intestine might be due to accumulation on the enterocytes, with little transport over the intestinal barrier, suggesting that this exposure time was not enough for MeHg to cross the gastrointestinal tract and reach the brain (Fig. 3).

Se(IV) is used as a nutritional source of Se due to its ability to become part of amino acids, proteins and enzymes (Whanger, 2002). Besides this beneficial effect, several studies have been previously carried out to elucidate the protective effect of Se(IV) against MeHg-induced toxicity (Luque-Garcia et al., 2013). In this way, it has been suggested the formation of mercuric selenide (HgSe), a stable biological complex between Se(IV) and MeHg (Yang et al., 2008). In order to evaluate the impact of this effect on the accumulation of Hg in different organs of adult fish, we compared the data obtained after MeHg single exposure, with the amount of Hg found in the liver, intestine, brain and muscular tissue of zebrafish co-exposed to MeHg and Se(IV) (Fig. 3). Our results pointed out that while at shorter exposure times (24 h) the accumulation of MeHg was lower in most organs when Se(IV) was co-administered, at the longest exposure time tested (72 h) the results significantly differed upon the organ studied.

Besides ingestion, MeHg can enter fish through muscular tissue, since MeHg can form lipid soluble compounds and therefore bind (Andres et al., 2002) or diffuse across epithelia membranes (Aschner and Clarkson, 1988). Our results showed a significant reduction of MeHg found in intestine and muscular tissue in the co-exposure experiment as compared to zebrafish exposed to MeHg alone (Fig. 3B and D). One of the possible explanations to this behavior could be the reduction in bioavailable MeHg due to the formation of insoluble MeHg-Se complexes, which can translate into a decreased MeHg uptake (Wyatt et al., 2016). Actually, at 24 h, the total Hg found in zebrafish exposed to MeHg and Se(IV), was 30% lower as compared to fish exposed to the same concentration of MeHg alone.

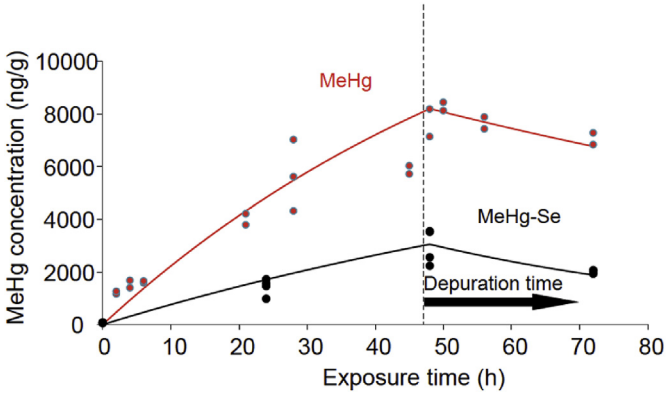

Fig. 2. Comparison of bioaccumulation profiles of experiments carried out only with MeHg 10 μg/l (in red) and 25 μg/l MeHg + 12.5 μg/l Se(IV) (in black). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

The most significant difference was found in the muscular tissue (Fig. 3D). A reduction of more than a 50% of MeHg concentration was found in this tissue at 72 h in the co-exposure experiment. Although some authors have proposed the muscular tissue as a storage compartment for MeHg as a protective mechanism (Wiener and Spry, 1996), it seems that Se(IV) co-administration drastically reduces the accumulation of MeHg in this organ, which is in agreement with previous studies (Mailman et al., 2014). This decrease is especially important because muscular tissue represents more than 65–70% of total fish weight, being the tissue where more MeHg is accumulated. Actually, this fact has generated interest to study the possible molecular ratios between Se and Hg in tissues and the necessary amount of Se to protect the adverse effect of MeHg (Bjerregaard et al., 2011).

After 72 h of exposure, total Hg concentration found in the intestine was reduced by approximately a 30% in zebrafish exposed to MeHg and Se(IV) as compared to those exposed to MeHg alone (Fig. 3B). Taking together the hypothesis that glutathione (GSH) participates in the formation of the Se-Hg complex (Balthrop and Braddon, 1985) and the fact that fish intestines possess the enzymes necessary to produce GSH (Huang et al., 2013), the reduction of Hg observed in the intestine upon Se(IV) co-exposure, could be explained by the formation of the MeHg-Se complex, which would promote MeHg excretion. Additionally, it is known that Se(IV) is reduced by the glutaredoxin and thioredoxin system to selenide, which is used to synthesize selenocysteine (Sec) (Ralston and Raymond, 2010). A possible increase in the concentration of Sec and the high affinity of MeHg for Se, could explain the formation of a Sec-MeHg complex able to get through different membrane barriers and reach other organs, thus modifying MeHg distribution within the fish in the MeHg-Se(IV) co-exposure experiment.

The results obtained for the total Hg found in the brain showed that, although there was a marked reduction in Hg accumulation after 24 h in the co-exposure experiment, the concentration of Hg after 72 h exposure were quite similar in both experiments (Fig. 3C). Actually, we found a slight increase in Hg accumulation in the brain of zebrafish exposed to MeHg-Se(IV). This results are in accordance with previous authors that demonstrated that neither Se(IV) nor selenomethione reduced the accumulation of Hg in the brain of cod (Bjerregaard et al., 2011) and white sturgeon (Huang et al., 2013), respectively. Similar results were obtained in a study carried out in rats, where co-exposure to MeHg and Se(IV) increased the concentration of the Hg found in the brain as compared to rats exposed to MeHg alone (Magos and Webb, 1980). Thus, it can be concluded that Se(IV) is not able to preclude

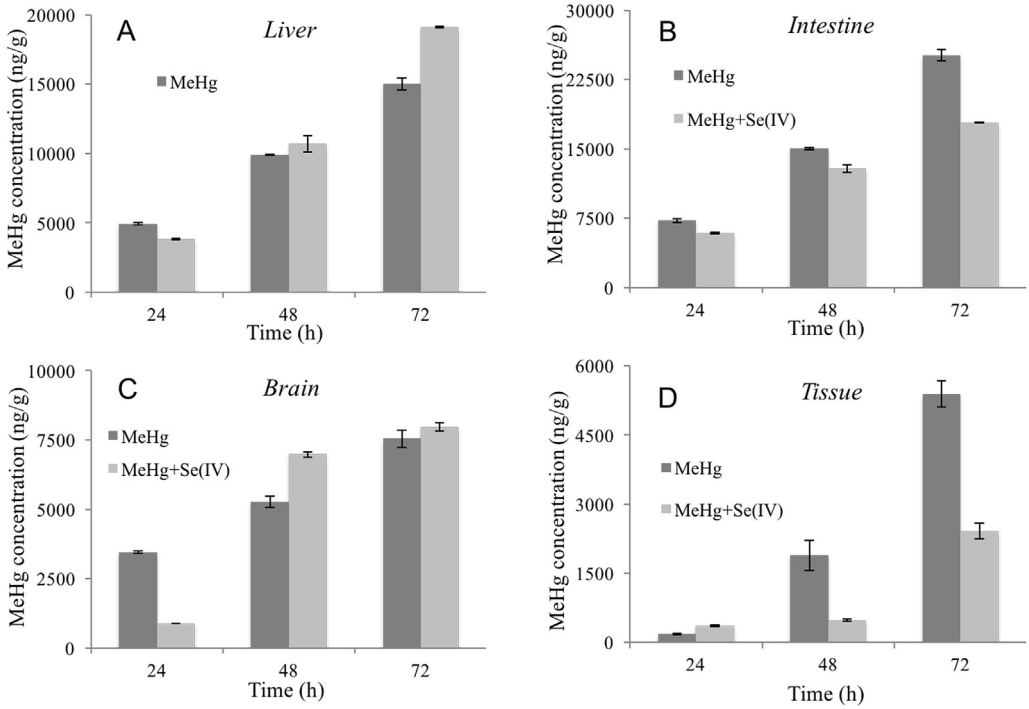

Fig. 3. Concentration of MeHg (ng/g) found in (A) liver, (B) intestine, (C) brain and (D) muscular tissue of adult zebrafish exposed to either MeHg alone (25 μg/l MeHg) or MeHg and Se(IV) (25 μg/lMeHg + 12.5 μg/l Se(IV)). Bars show mean ± s.d.(n = 3). $P < 0.05$ Student's *t*-test.

accumulation of MeHg in the brain of adult fish after long-term exposure, but rather to increase its concentration, at least in certain cases.

In the case of the liver of zebrafish exposed to either MeHg or MeHg-Se(IV), we found a similar situation as in the brain. Far from reducing the accumulation of Hg, Se(IV) co-exposure significantly increased the amount of total Hg found in this organ after 72 h of exposure. Since liver is involved in detoxification processes, this result could be explained by an accumulation of the MeHg-Se complex in this organ, as a final product for MeHg detoxification (Yang et al., 2008). However, although our results are in agreement with previous works (Bjerregaard et al., 2011), some studies showed opposite or different results. While Se(IV) seemed to reduce MeHg accumulation in the liver of zebra sea breams (Branco et al., 2012), it did not protect against the accumulation of MeHg or Hg(II) in the liver of Atlantic cod (Ringdal and Julshamn, 1985; Olsvik et al., 2015) or zebra seabreams (Branco et al., 2012), respectively. These contradictory data show the extreme complexity of the interaction between MeHg and Se, especially in the liver.

3.3. Differential gene expression in zebrafish eleutheroembryos and adult fish exposed to MeHg and MeHg-Se(IV)

The differential expression of 11 genes selected from previous experiments was assessed to determine the toxic effect of MeHg exposure in eleutheroembryos and in different organs of adult zebrafish. The potential protective effect of Se(IV) on the MeHg-induced toxicity was also evaluated as the selected genes are involved in different toxicity response pathways.

Exposure to MeHg caused significant repression of most of the tested genes in zebrafish eleutheroembryos (Fig. 4): *mt2*, *hsp70*, *gpx1*, *mrps31*, *btbd10b*, *fos*, *irg1l* and *txn1*. Only *prdx1* showed a significant increased level of expression. On the other hand, the gene expression analysis of organs of adult fish exposed to MeHg evidenced a tissue-specific expression rate; while most genes were repressed in liver and brain, they were mainly induced in the intestine and the muscular tissue (Fig. 5).

As expected, the results were consistent with previous reports in which Hg species have been found to induce oxidative stress in zebrafish embryos exposed to MeHg and Hg(II). This induction of oxidative stress has been proven to affect antioxidant enzyme activities, endogenous GSH and MDA contents (Zhang et al., 2016), mRNA levels of several genes (Ho et al., 2013) and the proteins themselves (Cuello et al., 2012a, b). In our study, we found a MeHg-induced downregulation of *gpx1* in eleutheroembryos and in the three organs and muscular tissue of adult zebrafish after 72 h exposure, which constitutes the perfect signature of the onset of oxidative stress (Cambier et al., 2012). Since MeHg exposure induces Se deficiency in cells, which leads to the degradation of *gpx1*

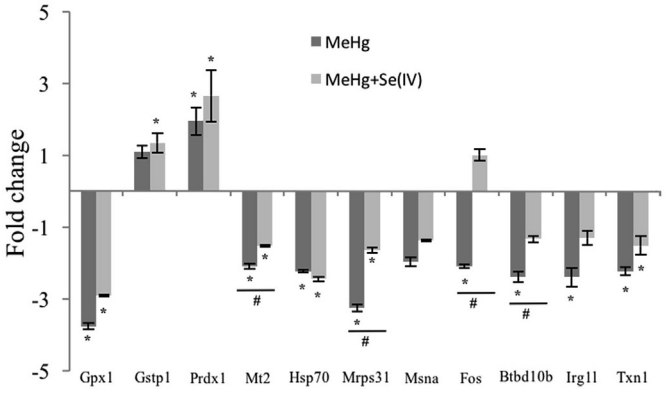

Fig. 4. Differential gene expression analysis of the selected 11 genes in eleutheroembryos exposed to 25 μg/lMeHg or to 25 μg/lMeHg +12.5 μg/lSe(IV). Stars (*) represent statistical differences between the tested condition and the control sample, while (#) represent statistical differences within the same gene upon Se(IV) co-administration.

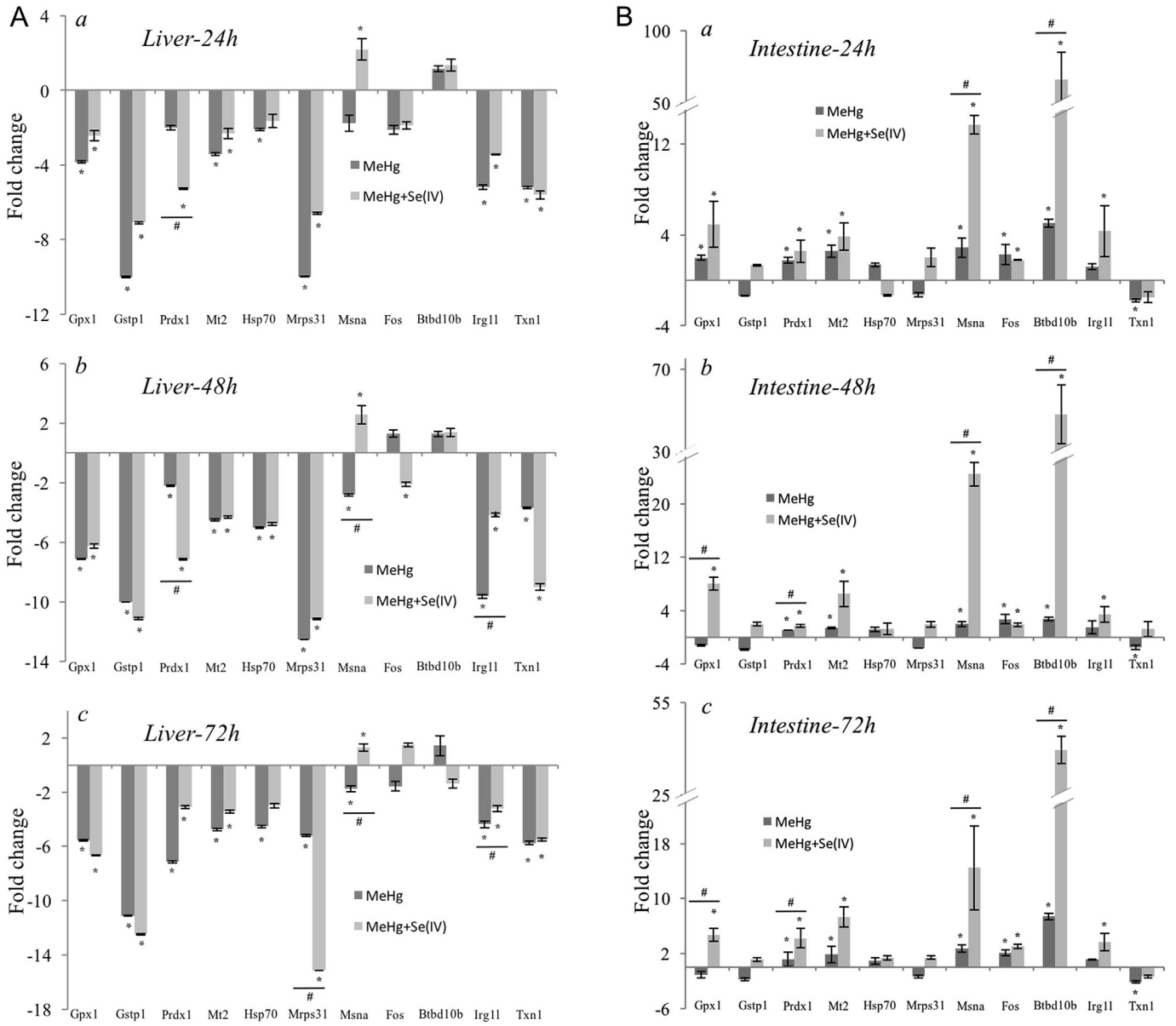

Fig. 5. Differential gene expression analysis of the selected 11 genes in (A) liver, (B) intestine, (C) brain and (D) muscular tissue of adult zebrafish exposed to 25 μg/l MeHg or to 25 μg/l MeHg + 12.5 μg/l Se(IV). Stars (*) represent statistical differences between the tested condition and the control sample, while (#) represent statistical differences within the same gene upon Se(IV) co-administration.

mRNA by nonsense-mediated mRNA decay (Usuki et al., 2010), it would be expected that Se(IV) supplementation could recover *gpx1* expression to basal levels or even induce its overexpression. In fact, our results have pointed out that addition of Se(IV) to adult zebrafish exposed to MeHg, was enough to recover to basal level the expression of *gpx1* in brain, and to produce an overexpression in intestine and muscular tissue after 72 h MeHg-Se(IV) exposure (Fig. 5). A similar reduction of the repression of this gene was observed in eleutheroembryos; *gpx1* was closer to the basal levels after Se(IV) addition, as compared to embryos exposed to MeHg alone; however, Se(IV) did not induce overexpression of this gene. As for the expression of *gpx1* in the liver of exposed adult zebrafish, the behavior was different. Not only Se(IV) was unable to reverse the downregulation of *gpx1*, but actually enhanced it (Fig. 5A). However, this different behavior is in good agreement with the data obtained for MeHg accumulation in the different organs and muscular tissue (Fig. 4). While Se(IV) significantly reduced the amount of MeHg in the intestine and muscular tissue, it increased its accumulation in the liver. Thus, it seems to be a logic correlation between the amount of MeHg found in the different organs after addition of Se(IV), and the levels of expression of *gpx1*. If down-regulation of *gpx1* constitutes a sign of the presence of oxidative stress, so does the overexpression of *gstp1* and *prdx1* (Cambier et al., 2012; Tahmasbpour Marzont et al., 2016). *Gstp1* is an enzyme that catalyzes the conjugation of GSH to xenobiotic substrates for detoxification, while *prdx1* plays an antioxidant protective role in cells and reduce H_2O_2 and alkyl hydroperoxides (Tahmasbpour Marzont et al., 2016). In fact, *gstp1* is considered a biomarker for assessing the environmental impact of xenobiotics that generates oxidative stress (Livingstone, 1998). We found significant induction

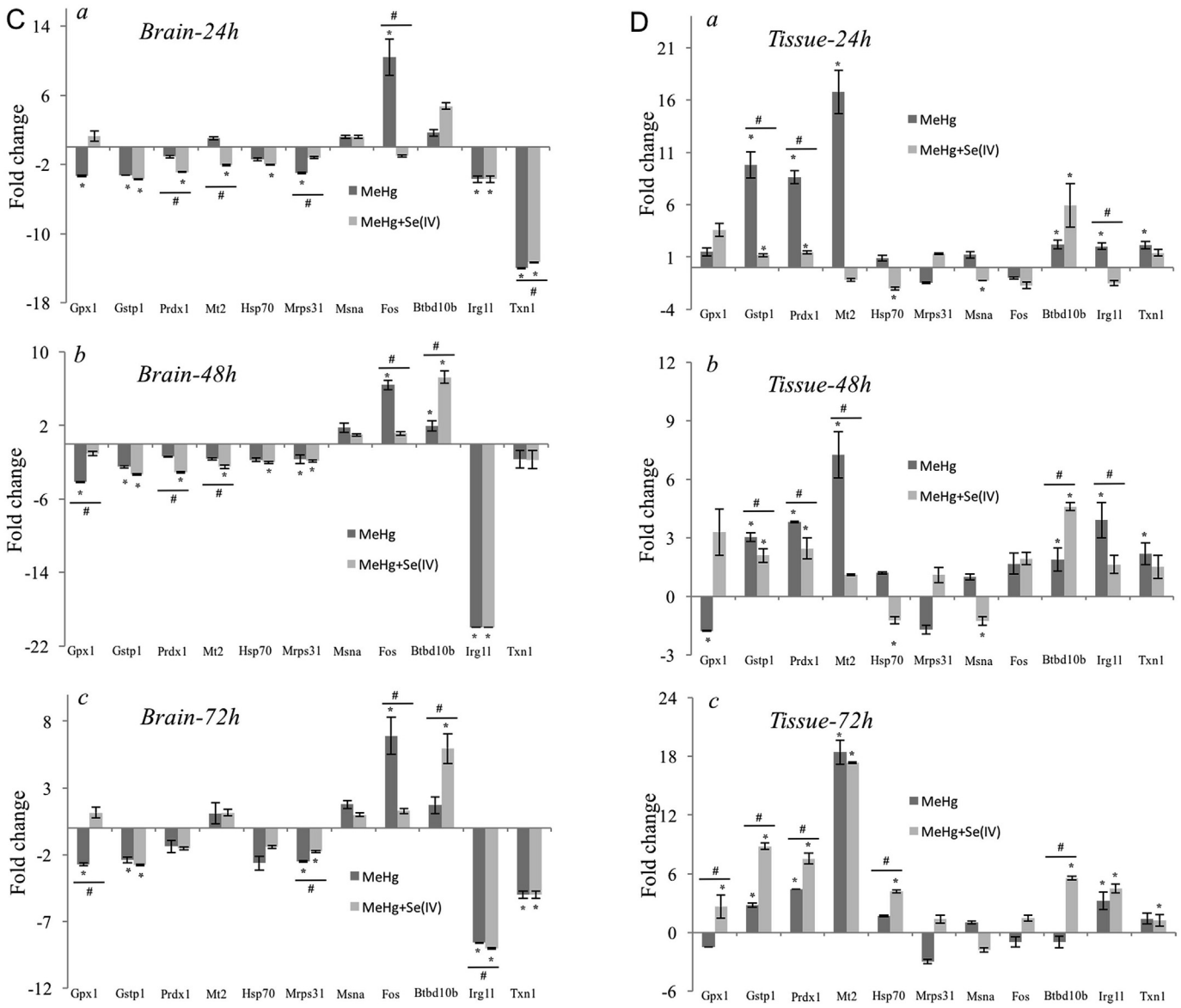

Fig. 5. (*continued*).

of *gstp1* and *prdx1* in eleutheroembryos exposed to MeHg-Se(IV), even though the difference in the induction of this gene between embryos exposed to MeHg alone and those co-exposed to Se(IV) are not significant, this trend could indicate a greater detoxifying activity after addition of Se(IV) (Fig. 4). A similar behavior was observed in the intestine and the muscular tissue of adult zebrafish (Fig. 5B and D). The expression levels of *gstp1* and *prdx1* increased with the addition of Se(IV) after 72 h exposure, and in these cases, the induction was significantly different for the two types of exposure. In the case of the muscular tissue, it is remarkable to see how the addition of Se(IV) was only effective after 72 h. We found *gstp1* and *prdx1* downregulated in the liver and the brain of adult fish (Fig. 5A and C); the addition of Se(IV) did not ameliorate the repression of these mRNAs but enhanced it as in the case of *gpx1*. The different levels of *gstp1* and *prdx1* in the brain and the liver in comparison to the intestine and the muscular tissue, could also be explained by the different protective effect of Se(IV) observed in terms of MeHg accumulation (Fig. 3).

The expression of *mt2*, which encodes for metallothionein isoform 2, a protein involved in detoxification processes, was affected by the addition of Se(IV) to MeHg exposed eleutheroembryos. A repression of this gene was observed after MeHg exposure but such repression was significantly diminished in the co-exposure experiment (Fig. 4). A different behavior was observed in the organs of adult fish, in which Se(IV) did not significantly varied the expression of *mt2* after 72 h exposure (Fig. 5). While *mt2* appeared significantly repressed in the liver (Fig. 5A), it showed an induction in the intestine and the muscular tissue (Fig. 5B and D), with no significant changes in the brain as compared to its basal level (Fig. 5C). It is worth mentioning the changes observed in the brain, which depended on the exposure time. While at 24 h the addition of Se(IV) caused a repression of this gene that was maintained at 48 h exposure, at 72 h there were no significant differences in comparison to control values (Fig. 5C). This behavior could also be explained based on the role of Se(IV) in the accumulation of MeHg in the brain (Fig. 3). At 24 h Se(IV) did protect against MeHg accumulation in the brain but at longer exposure times, it actually increased the accumulation of MeHg. Oxidative stress generally leads to increased synthesis of heat-shock proteins in the cells including *hsp70*, which acts as an apoptosis inhibitor (Yurinskaya

et al., 2015). However, in our experiment, *hsp70* appeared repressed in eleutheroembryos (Fig. 4) and in the brain and the liver of adult zebrafish (Fig. 5A and C) exposed to either MeHg alone or MeHg-Se(IV); thus suggesting increased levels of apoptosis. However, in the muscular tissue the expression of *hsp70* was very different depending on the addition of Se(IV) and the exposure time (Fig. 5D). After 24 and 48 h exposure, Se(IV) did actually induce repression of *hsp70* in this tissue as compared to fish exposed to MeHg alone. However, after 72 h we observed a significant induction of *hsp70* in the tissue; thus showing a Se(IV) induced-protection against apoptosis (Fig. 5D). These results show the complexity of the mechanisms underlying toxicity of MeHg, and the relevance of considering the exposure time (Zeeshan et al., 2016).

The expression of *mrps31* was also very different depending on the organ or tissue tested. There were no significant differences in the mRNA expression in the muscular tissue and the intestine when comparing adult fish exposed to MeHg or to MeHg-Se(IV) with control fish (Fig. 5B and D). On the contrary, a significant repression was observed in the liver (Fig. 5A), which was actually more pronounced after addition of Se(IV) at 72 h exposure. However, the repression of *mrps31* observed in the brain of adult fish and in eleutheroembryos exposed to MeHg was minimized by Se(IV) (Figs. 4 and 5C). Although the molecular function of *mrps31* is not clear, its implication in the oxidative phosphorylation system by which mitochondria produce ATP has been identified (Sylvester et al., 2004). Since Hg and MeHg induce mitochondrial dysfunction, which reduces ATP synthesis and increases lipid, protein and DNA peroxidation (Carocci et al., 2014), that could be the explanation of the *mrsp31* repression found in eleutheroembryos and in most of the tested organs in exposed adult zebrafish. The levels of *msna* found in eleutheroembryos (Fig. 4) and in the brain and muscular tissue of adult zebrafish (Fig. 5C and 5D) exposed to either MeHg or MeHg-Se(IV) were not significantly different compared to the controls. However, in both the intestine and the liver, *msna* was significantly induced, especially in the MeHg-Se(IV) co-exposure experiments (Fig. 5A and 5B). This induction could be related with the amount of MeHg accumulated in the different organs and tissues, being the liver and the intestine the organs with higher concentrations after 72 h exposure. Additionally, it is known that *msna* plays a relevant role in an anti-apoptotic machinery (Schelling and Abu Jawdeh, 2008), being also required for the maintenance of the normal oxidative stress in cells (Chang et al., 2011), which might explain its significant induction observed in the co-exposure experiments, thus showing another explanation for the protective role of Se(IV) against MeHg-induced toxicity.

The evaluation of the expression levels of *fos* showed a significant repression in eleutheroembryos, which was recovered by addition of Se(IV) to its basal levels (Fig. 4). However, in the brain of adult zebrafish exposed to MeHg, *fos* was found highly induced (Fig. 5C), as in the brain of rats exposed to MeHg (Cheng et al., 2006), thus showing the role of *fos* in the process of brain injury induced by MeHg. It is important to mention that such induction was not observed in the co-exposure experiments, which might indicate one of the protective mechanisms of Se(IV) against MeHg-induced neurotoxicity. Although the molecular function of the protein encoded by *btbd10b* has not been fully elucidated, we decided to study the expression of this gene based on a previous microarray analysis carried out in zebrafish eleutheroembryos exposed to MeHg, in which this gene was highly altered (data not shown). Our results pointed out that exposure to MeHg induced the expression of *btbd10b* in all the organs tested except the liver (Fig. 5). Additionally, such induction was much more pronounced in the co-exposure experiment with Se(IV). Actually, the induction of this mRNA in the intestine was the highest found in all the experiments (Fig. 5B). Although, as stated before, little is known about the molecular functions associated to *btbd10b*, its over-expression has been linked to the suppression of superoxide dismutase 1 (*sod1*) (Furuta et al., 2013), which is a well-known protein involved in oxidative stress and apoptosis (Corsetti et al., 2016). Thus, based on our results, we can hypothesize that a high induction of *btbd10b* induced by Se(IV) is going to be linked to a decrease of *sod1*, therefore acting as a defense mechanism against MeHg-induced cellular damage. These results, especially considering the extremely high induction observed in *btbd10b* and the fact that the same behavior was observed in the intestine, the muscular tissue and the brain, open up a new and interesting door to better understand the protective role of Se(IV) against MeHg-induced toxicity.

Irg1l is a homolog of the human pro-inflammatory cytokine induced gene *irg1*, which is highly induced under pro-inflammatory conditions, such as bacterial infections, therefore indicating a protective effect on the immune system (Degrandi et al., 2009). In this study exposure to MeHg caused a significant repression of *igr1l* in eleutheroembryos (Fig. 4), and in the liver and the brain of adult fish at all the exposure times tested (Fig. 5A and C), indicating a possible repression of the innate immune system. Repression of the innate immune system in zebrafish embryos exposed to arsenic has already been reported by detecting the repression of two cytokines involved in inflammation (Nayak et al., 2007). Such repression was partially ameliorated by Se(IV) co-exposure in eleutheroembryos and in the liver of adult fish. However, Se(IV) did not show any protective effect against the repression of *irg1l* in the brain. Gene *txn1*, appeared downregulated in eleutheroembryos (Fig. 4) and in all the organs tested except for the muscular tissue (Fig. 5). These results are in agreement with previously published studies in which *txn1* has been demonstrated to be a target for Hg compounds (Branco et al., 2014; Rodrigues et al., 2015). Since *txn1* plays an important role in the suppression of oxidative stress and endoplasmic reticulum stress (Zeng et al., 2015), its repression exacerbates oxidative stress damage (Li et al., 2015). Our results also showed that in none of the cases Se(IV) was able to significantly reduce the repression of this gene (Figs. 4 and 5), pointing out that the Se(IV) protective role against MeHg induced toxicity is not related to *txn1*.

4. Conclusions

The present study has been carried out to evaluate the impact of Se(IV) co-administration in MeHg-exposed eleutheroembryos and adult zebrafish. The potential protective role of Se(IV) has been tested in terms of: (i) its ability to reduce the amount of Hg accumulated in either the embryos or the different organs and muscular tissue of adult fish, and (ii) its ability to modify the expression of genes affected (repressed or induced) upon MeHg exposure. Our results have demonstrated that Se(IV) does clearly reduce the BCFs in eleutheroembryos and thus, the total amount of Hg found in exposed ones. However, such protection against MeHg bioaccumulation was not maintained throughout all the organs of adult zebrafish tested. While Se(IV) was able to significantly reduce Hg concentration in the muscular tissue and the intestine, it did actually increase the amount of Hg in the brain and liver. These results reveal the complexity of the MeHg-Se interaction. The data derived from the gene expression analysis also point out significant differences depending on the gene and the sample tested. In general, the results obtained for eleutheroembryos and adult fish demonstrate MeHg-induced oxidative stress and different defense mechanisms activated by Se(IV) co-administration. Besides the well-known genes involved, we also tested other genes that appeared highly induced upon Se(IV) co-exposure such as *msna*

and *btbd10b*. The study of the function of the proteins encoded by these genes may open up a new door for understanding the role of Se(IV) against MeHg-induced toxicity. In most cases, the different behavior observed in gene expression analysis, correlated well with the amount of Hg found in each organ, and the role of Se(IV) co-administration in such accumulation.

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