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Inhibitory effect of Cadmium on estrogen signaling in zebrafish brain and protection by Zinc

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Abstract

The present study was conducted to assess the effects of cadmium (Cd) exposure on the estrogen signaling in the zebrafish brain, as well as the potential protective role of zinc (Zn) against Cd-induced toxicity. For this purpose, effects on the transcriptional activation of estrogen receptors (ERs), aromatase B (Aro-B) protein expression and molecular expression of related genes were examined *in vivo* using wild-type and transgenic zebrafish embryos. For *in vitro* studies, an ER-negative glial cell line (U252-MG) transfected with different zfER subtypes (ER α , ER β 1 and ER β 2) was also used. Embryos were exposed either to estradiol (E2), Cd, E2+Cd or E2+Cd+Zn for 72 hours and cells were exposed to the same treatments for 30 hours. Our results show that E2-treatment promoted the transcriptional activation of ERs and increased Aro-B expression, at both the protein and mRNA levels. Although exposure to Cd, does not affect the studied parameters when administered alone, it significantly abolished the E2-stimulated transcriptional response of the reporter gene for the three ER subtypes in U251-MG cells, and clearly inhibited the E2-induction of Aro-B in radial glial cells of zebrafish embryos. These inhibitory effects were accompanied with a significant down-regulation of the expression of *esr1*, *esr2a*, *esr2b* and *cyp19a1b* genes compared to the E2-treated group used as a positive control. Zn administration during simultaneous exposure to E2 and Cd strongly stimulated zfERs transactivation and increased Aro-B protein expression, whereas mRNA levels of the three ERs as well as the *cyp19a1b* remained unchanged in comparison with Cd-treated embryos. In conclusion, our results

clearly demonstrate that Cd acts as a potent anti-estrogen *in vivo* and *in vitro*, and that Cd-induced E2 antagonism can be reversed, at the protein level, by Zn supplement.

Keywords: Cadmium, Estradiol, Zinc, Estrogen receptor, Aromatase

Short Abstract for Table of Contents

This study was conducted to assess the effects of cadmium (Cd) exposure on the estrogen signaling in the zebrafish brain, and the potential protective role of zinc (Zn) against Cd-induced toxicity. Effects on the transcriptional activation of estrogen receptors, aromatase B protein expression and molecular expression of related genes were examined. Our results demonstrate that Cd acts as a potent anti-estrogen *in vivo* and *in vitro*, and that Cd-induced estradiol antagonism can be reversed, at the protein level, by Zn supplement.

Introduction

Cadmium (Cd) is a widespread environmental toxicant that harmfully affects humans and animals. Its effects as an endocrine disruptor (ED) have been extensively documented. This metal can act by altering the homeostatic balance of a variety of hormones in both mammals (Lafuente *et al.*, 2003 ; Piasek and Laskey, 1999 ; Piasek *et al.*, 2001) and fish (Kime, 1984 ; Mukherjee *et al.*, 1994). Gonadal damage and interference with gametogenesis were also observed after Cd exposure. In testis, Cd can cause hemorrhagic necrosis (Parizek and Zahor, 1956 ; Thompson and Bannigan, 2008) and reduced sperm count (Amara *et al.*, 2008), whereas in ovaries, it increases the proportion of atretic follicles, reduces the number of vitellogenic oocytes and decreases yolk formation (Chouchene *et al.*, 2011). Several other studies have also associated Cd with a wide range of reproductive disorders, including growth retardation, sterility and embryotoxicity (Fahmy and Aly, 2000).

While the effects of Cd on the endocrine system and its potential deleterious effects on living organisms are no longer in doubt, the underlying mode of actions remained unclear. However, many of the toxic effects of Cd have been suggested to result from interaction with essential elements, such as zinc (Zn) (Brzóška and Moniuszko-Jakoniuk, 2001 ; Lansdown *et al.*, 2001 ; Waalkes, 1986), via the mechanism of ionic mimicry (Bridges and Zalups, 2005). Zn is a trace element of great importance for the organism, involved in many metabolic processes and well known as a Cd antagonist. Indeed, numerous studies have shown that Zn strongly reversed Cd-induced toxicity in various organs and tissues such as liver (Banni *et al.*, 2011), kidney (El Heni *et al.*, 2010), blood (Jemai *et al.*, 2007) and testis (Messaoudi *et al.*, 2010). Using adult female zebrafish, we have recently reported similar results in the ovarian tissue (Chouchene *et al.*, 2011). Zn treatment during Cd exposure reversed Cd-induced Zn depletion, as well as Cd and metallothionein accumulation, thus reducing ovarian histopathological damages (Chouchene *et al.*, 2011). Cd has been shown to exert its toxicity by disturbing Zn uptake via changes in the gene expression pattern of Zn transporters, mainly by downregulating ZnT1 (as exporter) and overexpressing ZIP10 (as importer). We also concluded that the improving effects of dietary Zn supplementation on Cd-induced toxicity are mediated, in part, by the increase of Zn availability and subsequently the induction of

ZnT1 gene expression. These results in ovary have prompted us to further investigate the effect of these compounds in the endocrine system and especially on estrogen signaling.

Estradiol (E2) is a female sexual steroid best known for its roles in reproductive functions. This hormone exerts crucial effects on the development and functioning of the gonadotropic axis at the level of the gonads, pituitary and brain (Carreau, 2002). Its actions are mediated by the estrogen receptor (ER), a member of the superfamily of nuclear receptors, acting as ligand-dependent transcription factors (Moggs and Orphanides, 2001). ER is composed of three functional domains: the N-terminal domain involved in transcriptional activation of gene expression; the DNA-binding domain (DBD) thought to coordinate two Zn ions with cysteine residues into Zn finger motifs; and the C-terminal domain carrying the ligand-binding domain (LBD) (Evans, 1988 ; Pike, 2006). Upon E2 binding, ER dimerizes so that it may bind to specific DNA sequences called estrogen responsive elements (EREs) and may stimulate the transcription of specific genes (Kerdivel *et al.*, 2013). A number of studies demonstrated that Cd can mimic the effects of E2 in rats and estrogen-responsive breast cancer cell line (MCF-7) (Johnson *et al.*, 2003 ; Martin *et al.*, 2003 ; Stoica *et al.*, 2000), whereas other groups reported a lack of estrogenicity and an inhibitory effect of this heavy metal on E2-induced reporter activity and cell proliferation (Le Guével *et al.*, 2000 ; Silva *et al.*, 2006).

Since Zn is well known to protect against Cd toxicity in many tissues, as previously documented, we wondered if this oligoelement could have an effect on reversing Cd-agonism or antagonism versus E2. To our knowledge, there is no information regarding the effects of combined exposure to Cd and Zn on estrogen signaling. Therefore, the present work was undertaken to investigate the estrogenic or anti-estrogenic potency of Cd and the ability of Zn to reverse Cd-induced toxicity in zebrafish. Both *in vivo* and *in vitro* approaches were combined in order to obtain a better understanding of the molecular actions of Cd. For this, wild-type and transgenic zebrafish were used, as well as a cell-based transcription assay based on an ER-negative glial cell line U251-MG transfected with ER subtypes and an E2-responsive reporter gene. First, the impact of several concentrations of Cd and Zn, alone or in mixture with E2, on the transcriptional activity of the three distinct zebrafish ERs (zfERs) was tested, as well as the molecular expression of the related genes. Second, the effects of Cd alone or in combination with Zn were monitored, upon E2-treatment, on the expression of an estrogen-sensitive gene, *cyp19alb*, that encodes brain aromatase (Aro-B) in fish (Diotel *et al.*, 2010).

Materials and methods

***In vitro* experiments**

Cell culture and transient transfection

Human astrocyte U251-MG cells (ECACC) were maintained at 37°C under a 5% CO₂ humidified atmosphere in Dulbecco's modified Eagle medium (DMEM; Gibco) supplemented with 10% fetal calf serum (FCS, Biowest) and 1% antibiotic (penicillin/streptomycin). One day before transfection, cells were washed and seeded in fresh medium on 24-well plates at a density of 0.2 x 10⁵ cells/ml. After 24 hours, the medium was replaced with fresh phenol red-free DMEM containing 2.5% FCS. Transient transfections of U251-MG cells were carried out with plasmid-DNA using JetPEI™ reagent, as indicated by the manufacturer (Polyplus-transfection, France). The DNA templates contained 50 ng of

zebrafish estrogen receptor expression vector (*esr1*, *esr2a*, and *esr2b*), 150 ng of *ERE-TK-Luc* reporter plasmid and 25 ng of internal β -galactosidase control vector (*cytomegalovirus [CMV]- β -gal*). Following an overnight incubation, cells were replaced in fresh DMEM 2,5% desteroided FCS medium and treated during 30 hours with either 17 β -estradiol (10^{-9} M; Sigma, St. Louis, MO), cadmium chloride (CdCl_2 , Fluka 20899), zinc chloride (ZnCl_2 , LOBA Chemie 280410), E2 + Cd, or E2 + Cd + Zn. Dose-response experiments for Cd (10^{-8} M to 10^{-5} M) and Zn (10^{-9} M to 10^{-5} M) were performed in order to determine the Cd concentration that inhibited 50% of the E2 effect (IC_{50}) and the effective concentration of Zn. Ethanol (EtOH) was used as a vehicle.

Cells were lysed 48 hours later using a luciferase assay system (Promega, France). The reporter gene activity was quantified from the luciferase activity with Veritas Luminometer (Turner biosystems) and then the absorbance of the β -galactosidase activity was determined with iMark microplate reader (Biorad). The luciferase activity was then normalized by β -galactosidase activity and expressed as fold induction relative to control group (EtOH).

All transfections were performed in triplicate in at least three independent experiments.

Plasmids

Expression vector-encoding regions of zebrafish *esr1*, *esr2a*, and *esr2b*, respectively for zfER α , zfER β 1 and zfER β 2, were previously described (Menuet *et al.*, 2002), as well as the estrogen response element (ERE)-thymidine kinase-luciferase (*ERE-TK-luc*) and *CMV- β -gal* reporter plasmids (Métivier *et al.*, 2002). The plasmid *CMV- β -gal* was used for the normalization of the transfection efficiency and the cytomegalovirus promoter led independent the expression of the β -galactosidase enzyme.

In vivo experiments

Ethics

This study was approved by the ethics committees INERIS (Institut National de l'Environnement Industriel et des Risques) and CREEA (Comité Rennais d'Ethique en matière d'Expérimentation Animale) under permit number EEA B-35-040. All steps have been taken to reduce suffering of animals. Experiments were performed in accordance with the European Union regulations concerning the protection of experimental animals (Directive 86/609/EEC).

Animals and treatments

Experiments were performed on wild-type and transgenic (*cyp19a1b-GFP* line) (Tong *et al.*, 2009) zebrafish (*Danio rerio*). Animals were housed in the Biosit zebrafish facility (INRA, LPGP, Rennes, France) and were treated in agreement with the European Union regulations concerning the protection of experimental animals. Immediately after spawning, zebrafish eggs were divided into seven groups. Two groups were used as controls and were treated with distilled water for the first group and ethanol (EtOH) for the second one. The remaining five experimental groups were treated either with E2 (10^{-9} M), Cd (10^{-6} M as CdCl_2), Zn (10^{-5} M as ZnCl_2), E2 + Cd (E2 10^{-9} M + Cd 10^{-6} M), or E2 + Cd + Zn (E2 10^{-9} M + Cd 10^{-6} M + Zn 10^{-5} M). In all conditions, 80 and 20 eggs, respectively, for wild-type and transgenic zebrafish line, were treated for 72 hours in Petri dishes at 28.5°C. The chemicals were renewed every 24 hours. Each substance was tested at least in three independent experiments.

Aromatase B immunohistochemistry

After 72 hours of treatment, wild-type and transgenic embryos were fixed overnight at 4°C in a saline phosphate buffer (PBS, pH 7.4) containing 4% paraformaldehyde (Sigma), and then immersed in sucrose. Samples were embedded in Tissue-Tek (Neg 50, Thermo Scientific, France) and the entire head was then processed for cryostat sections (8 µm of thickness). Immunohistochemistry experiments were performed on the brain of wild-type embryos. Tissue sections were first rinsed in PBS 1X, and then nonspecific binding was blocked for 1 hour at room temperature in PBS containing 0.2% Triton and 0.5% milk powder. Sections were then incubated with a specific affinity-purified antibody directed against zebrafish aromatase B (Menuet *et al.*, 2005 ; Pellegrini *et al.*, 2007) diluted 1:800. Slides were kept overnight at room temperature in a humidified chamber. After three washes in PBS 0.2% Triton, tissue sections were incubated with the appropriate secondary antibody (Goat anti rabbit Alexa 594, 1:500 in PBS containing 0.5% milk) for 2 hours before washing in PBS. At the end of the immunohistochemistry protocol, sections were mounted on glass slides in Vectashield containing 4',6-diamidino-2-phenylindole (DAPI) counterstaining (Vector, USA). Brain sections of wild-type and transgenic zebrafish embryos, obtained from three independent exposure experiments performed in duplicate, were then observed with an Olympus Provis microscope equipped with a digital camera. Images were obtained with the Olympus Cell software.

RNA Extraction and Reverse Transcription

Following 72 hours of treatment, pools of wild-type embryos (50) exposed to the same treatment were rapidly frozen and total RNA was extracted using the Trizol reagent (Invitrogen) by sonication. Reverse transcription was performed in presence of Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT) according to the manufacturer's procedure (Promega). Briefly, two micrograms of total RNA were reverse transcribed for 1 hour at 37°C in 25 µl reaction mixture containing 0.5 µg random hexamers (Promega, France), 25 mM dNTPs, 5X MMLV-RT buffer, and 50 U MMLV-RT (Promega).

Quantitative Real-Time polymerase chain reaction

Quantitative polymerase chain reaction (qPCR) was carried out on an iCycler MyiQ (Bio-Rad, France) using iQ SYBR Green supermix (Bio-Rad). The primers used are shown in Table 1. In our laboratory, we have previously shown that the most commonly used reference genes including β -actin, GAPDH and RNA 18S, classically used for standardization in such experiments, showed variations during zebrafish development (Mouriec *et al.*, 2009b). However, the elongation factor 1 (EF1) was found to be appropriate for normalisation in zebrafish embryos since its expression was not affected by treatments and remained stable in control and treated embryos (Mouriec *et al.*, 2009a). Therefore, the *ef1* gene was chosen to normalize the expression levels of the mRNAs. Samples not exposed to reverse transcriptase were used as negative control to monitor DNA contamination. Specific amplification of single products was controlled by performing melting curves and analysing PCR efficiency. PCR reactions were run at least in triplicate. The MyiQ (version 1.0) optical system software (Bio-Rad) was used to analyze PCR amplifications. Results were expressed according to the Ct method (ddCt) for relative quantification of gene expression (Schmittgen and Livak, 2008). For each sample, the difference (dCt) was calculated between Ct values obtained for target and reference amplicons. Comparative ddCt was then determined using as a reference the dCT calculated for the vehicle control samples (EtOH for E2-treated samples and H₂O for Cd

and Zn ones), and absolute values for comparative expression level were determined as equal to 2^{ddCt} .

Statistical Analysis

The statistical analyses were performed using the Statview 4.57 software (Abacus Concepts Inc, Berkeley, CA). Kolmogorov-Smirnov test was used to check the normality assumption. For zfERs transactivation, differences among the experimental groups were assessed by one-way ANOVA, followed by protected least significant difference Fisher's test. To calculate the normalized relative gene expression levels (fold induction), and due to the absence of normal distribution, data were analyzed using the non-parametric Mann-Whitney U test with Bonferroni correction for multiple comparisons. Values were considered to be significant when $P < 0.05$.

Results

Dose–response effect of Cadmium Chloride on transcriptional activation of zfERs in U251-MG cells

The effect of Cd was analyzed using the three ER isoforms and is represented as fold induction of luciferase activity in treated versus control samples. The maximum transcriptional response corresponding to 100% was assigned to the E2-treated group. As shown in Figure 1, the three zebrafish ERs were unable to induce the transcriptional activity of the reporter gene in the presence of Cd alone (Figure 1A). However, Cd caused a clear dose-dependent inhibition of the E2-induced luciferase activity in the U251-MG cells transfected with the three ER subtypes (Figure 1B). A Cd concentration of 10^{-6} M, corresponding to a 50% inhibition of the maximal E2 effect, was chosen for subsequent analyses and studies.

Effect of Zn supplementation on zfERS transactivation in U251-MG cells

The effects obtained with the different Zn concentrations are reported in Figure 2A. The data show that Zn is able to reverse, in a dose-dependent manner, the inhibitory effects of Cd on E2 up-regulation of the reporter gene. As shown in the Figure 2A, the induction of the reporter gene *ERE-TK-luc* was stronger for the highest concentration of Zn (10^{-5} M) causing a 70 to 100 fold induction depending on the ER subtype compared to the E2+Cd-treated cells. Treatment with Zn alone had no effect (Figure 2B).

Effects of Cd and Zn on the E2-induced aromatase B protein expression in zebrafish

Results regarding *in vivo* Aro-B expression in the zebrafish brain are shown in Figure 3. E2-treated embryos, used as a positive control in these experiments, exhibited a very strong expression of the aromatase B in the radial glial cells (RGC) (Figure 3B). In contrast, control EtOH-treated group displayed no significant signal (Figures 3A). However, as seen in Figure 3C, co-treatment with Cd strongly decreased the intensity of the aromatase B staining. Indeed, only a few labeled cells were observed corresponding to RGC based on their typical

morphology: small nucleus along the ventricles and long cytoplasmic processes. Interestingly, Zn supplementation was able to reverse this effect resulting in a strong staining in RGC immunoreactive cells. The pattern of aromatase B labeling observed in E2+Cd+Zn-treated embryos was very similar to that observed in E2-treated group (Figure 3D).

To confirm these data the E2-responsive transgenic tg(*cyp19a1b*-GFP) zebrafish line was used. Figure 3F shows that E2 treatment of embryos caused a massive expression of GFP in RGC compared to the EtOH control group (Figure 3E). As previously, Cd treatment inhibits GFP E2-induced expression in RGC (Figure 3G) and Zn was able to reverse this effect (Figure 3H).

Effects of Cd and Zn on the E2-induced *cyp19a1b* transcripts in zebrafish embryos

Figure 4 shows the mRNA levels of the *cyp19a1b* gene measured by reverse transcription real-time quantitative experiments (RT-qPCR) in zebrafish embryos after treatment with E2, Cd and/or Zn. There was no significant effect of Zn or Cd alone (Figure 4B). However, as expected, E2 robustly stimulated the expression of the *cyp19a1b* gene (Figure 4A), resulting in a fold induction of thirty-eight times compared to the control condition (EtOH). Co-treatment with E2 and Cd resulted in a significant inhibition of the E2-induced *cyp19a1b* mRNA levels (Figure 4A) while, surprisingly, supplementation with Zn has no significant effect.

Effects of Cd and Zn on *esr1*, *esr2a*, and *esr2b* mRNAs in zebrafish embryos

Figure 5A shows that a significant increase in *esr1*, *esr2a* and *esr2b* transcripts occurred after E2 treatment. However, this effect was more obvious on *esr1* messengers (3.53 fold) compared to *esr2a* (1.54 fold) and *esr2b* (1.80 fold). These actions were totally abolished by co-treatment with Cd, but Zn supplementation did not reverse the inhibitory impact of Cd (Figure 5A). Exposure to Cd and Zn alone did not affect ERs gene (Figure 5B).

Discussion

During the past years, many scientists have reported evidence that environmental contaminants such as organochlorine pesticides, polychlorinated biphenyls and heavy metals could bioaccumulate in different areas of the brain and significantly affect neuroendocrine systems. This led to the concept of neuroendocrine disruptors which are defined as environmental pollutants capable of modulating the synthesis, metabolism or action of neurohormones, impacting then physiological or behavioral processes in humans or animals (León-Olea et al., 2014). The brain is considered as an organ highly sensitive to heavy metals such as mercury, lead and Cd, which are well known to affect brain development in mammals including humans (Genuis and Kelln, 2015 ; Grandjean and Landrigan, 2014). However little is known about the potential effects of heavy metals in fish. Thus, the aim of the present work was to study the effects on the transcriptional activation of zfERs as well as the Aro-B expression after exposure of culture cells and zebrafish embryos to Cd, individually or in mixture with E2, and supplemented or not with Zn.

Our results showed that zfERs were unable to promote a transcriptional response of the E2-target gene, *in vitro* nor *in vivo*, after Cd exposure. However, a number of previous reports showed that Cd exerts characteristic effects of the endogenous steroid hormone, E2, such as activation of the human ER α and induction of proliferation of MCF7 breast cancer cells (Brama *et al.*, 2007 ; Garcia-Morales *et al.*, 1994). Stoica *et al.* (2000) found that cadmium chloride at 10⁻⁶ M was as potent as 10⁻⁹ M E2 in activating the ER ; result which we and others (Silva *et al.*, 2006) failed to obtain for the same concentrations tested. Moreover, according to the data here presented, this heavy metal seems to completely abolish the E2-induction of the reporter gene in a dose-dependent manner and for the three ER subtypes, thus suggesting an E2-antagonism. These findings are in perfect agreement with those obtained by Le Guével *et al.* (2000) who observed that Cd inhibited the transcriptional activity of E2-activated ER in yeast cells transfected either with the rainbow trout or the human ER and who showed that this metal did not induce activation of any receptor when tested alone.

However, it is important to emphasize that not all estrogenic chemicals disrupt the endocrine system through binding to specific nuclear ERs since estrogens can also signal through a non classical genomic pathway. EDs-indirect effects can thus be considered as the disruption of the action of enzymes involved in steroidogenesis (Kerdivel *et al.*, 2013). For instance, different substances from diverse chemical families have been shown to disrupt aromatase (Monod *et al.*, 1993 ; Rocha Monteiro *et al.*, 2000 ; Sanderson *et al.*, 2002), a crucial enzyme which converts androgens into estrogens. In fact, the brain of adult teleost fish is well known for its high aromatase activity (Pellegrini *et al.*, 2005) corresponding to the strong expression of the *cyp19a1b* gene, that encodes CYP19A1B (or Aro-B), the brain form of aromatase in fish (Tchoudakova *et al.*, 2001). It was indicated that this gene can be used as a sensitive marker of the effects of xenoestrogens in zebrafish early life stages (Hinfrey *et al.*, 2006 ; Le Page *et al.*, 2006). In our investigation, we demonstrated, by both RT-qPCR and immunohistochemistry, that E2 treatment causes strong induction of Aro-B in RGC of zebrafish embryos. This result was further strengthened by the use of the *cyp19a1b-GFP* transgenic zebrafish carrying the green fluorescent Aro-B as indicator of protein expression in live embryos. This line strongly and faithfully expresses Aro-B in the same cellular context after E2 treatment (Tong *et al.*, 2009). Accordingly, several studies have also noted such observations reporting that *cyp19a1b* gene in fish brain is up-regulated by E2 through a positive feedback loop (Callard *et al.*, 2001), an effect that involves ER and an ERE sequence localized on the proximal promoter of the *cyp19a1b* gene (Menuet *et al.*, 2005 ; Le Page *et al.*, 2008). Additionally, our results further confirm that aromatase expression is extremely cell specific since it is strictly restricted to RGC as previously documented (Forlano *et al.*, 2001 ; Menuet *et al.*, 2003). These cells are well known for being crucial in brain development or brain repair. They are considered as progenitor cells ultimately capable of generating all brain cell types during embryogenesis before disappearing in mammalian adults (Bonfanti and Peretto, 2007 ; Malatesta *et al.*, 2008) whereas they largely persist in adult fish (Arochena *et al.*, 2004). This characteristic could be related to the capacity of the fish brain to grow and regenerate during adulthood (Ekström *et al.*, 2001). After exposure of wild-type and transgenic zebrafish to both E2 and Cd, Aro-B protein and transcripts were found to be significantly down-regulated comparing to E2-treated embryos. Such a result was noted by Menuet *et al.* (2005) who showed that the E2-dependent induction of the *cyp19a1b* was entirely blocked by co-treatment with E2 and an excess of the pure antagonist, ICI₁₈₂₋₇₈₀ indicating that functional ERs were involved. This was further evidenced by the fact that the promoter of the zebrafish *cyp19* gene was shown to contain a 1/2 ERE and an ERE putative site (Callard *et al.*, 2001 ; Tong and Chung, 2003).

Based on these observations and considering our results on transcriptional activation and Aro-B expression, we may speculate that Cd could have a direct effect on zfERs, thus affecting Aro-B expression. As well known, many effects of this heavy metal action result from interaction with essential elements, such as Zn (Brzóska *et al.*, 2007 ; Waalkes, 1986), a metal of great importance required for gene transcription and cofactor of many essential enzymes (Coleman, 1992 ; Vallee and Falchuk, 1993). It is also known as an ER-component. Predki and Sarkar (1992) showed that the Zn finger motif can be affected by some other metals having the ability to substitute for Zn such as copper, Cd, cobalt and nickel. In this regard, it has been reported that the effects of Cd could reflect a disruption of Zn binding in the DBD by a competition process (Le Guével *et al.*, 2000). Hence, given the ability of this heavy metal to compete and to replace Zn, we thought that the supplement of this oligoelement could provide some corrections. Indeed, several studies, in fish and mammals, have pointed that the treatment with Zn during Cd exposure prevented or decreased the harmful effects of Cd in different tissues (Banni *et al.*, 2011 ; Chouchene *et al.*, 2011 ; El Heni *et al.*, 2010 ; Messaoudi *et al.*, 2010). Consistent with these reports, the current study has shown that Zn supplement totally reversed the complete abolition by Cd of the E2-stimulatory effects on *ERE-TK-luc* activity as well as Aro-B protein expression in wild-type and transgenic zebrafish. Furthermore, quantification of the GFP expression in the *tg-(cyp19a1b-GFP)* zebrafish in multiple field of view mode was performed as previously reported (Brion *et al.*, 2012) and confirmed the protective effect of Zn (data not shown). Although Zn concentrations were not measured in fish and culture cells used in this study, it is possible that the protection provided by this oligoelement against Cd-induced toxicity might result from an increase of Zn availability due to Zn supplementation as reported by some authors (Chemek *et al.*, 2015 ; Chouchene *et al.*, 2011). The correction observed at the zfERs transactivation level leads to think that the Zn finger motifs could be involved at first in Cd toxicity and later in Zn protection. Indeed, the DBD coordinates two Zn ions with cysteine residues into Zn finger motifs (Evans, 1988 ; Pike, 2006). We hypothesize that Cd is able to interact with the ER, after its activation by E2, by competing with Zn in the DNA binding domain, suggested as a putative site of Cd action (Le Guével *et al.*, 2000), impeding thus the transformation of the ER to a DNA-binding form. This then reduces the interaction of activated ER with DNA and subsequent transcriptional activity. This idea was also supported by Silva *et al.* (2006), while other investigators reported a second mechanistic explanation suggesting that Cd binds with a high affinity to the LBD of ER α by forming a coordination complex with amino-acid residues, which blocks the binding of E2 (Stoica *et al.*, 2000). Additionally, our results demonstrate that Zn at 10^{-5} M did not induce activation of any receptor when tested alone. This shows that Zn-protective effect could not have been masked by its ability to be E2-mimetic, and further proves that Zn effect requires interaction with Cd.

Parallely and as previously stated, E2-dependent regulation of aromatase involves a direct transcriptional action of ERs requiring the synergistic effect of ERE and $\frac{1}{2}$ ERE in the promoter region of the *cyp19b* gene (Menuet *et al.*, 2005). On this basis and given Cd effects reported herein, it can be hypothesized that this heavy metal is able to affect Aro-B expression in the brain, indirectly, through competing with Zn at the ER level. This assumption is further reinforced by the fact that the improvement of ERs transactivation level after Zn exposure was accompanied by a strong Aro-B protein expression. But surprisingly, the ameliorative effect of Zn supplement was not observed using RT-qPCR, whilst a significant up-regulation was expected. This result leads us to suggest a second hypothesis supporting the idea that Zn could also exert its protective effect by interacting with the toxic metal Cd at the protein level stabilizing thus the protein. Indeed, it is well known that the presence of Cd in the organism decreases the iron (Fe) level in several tissues (Jurczuk *et al.*, 2004). Since Aro-B is a

hemeprotein with Fe-containing site (Favia *et al.*, 2006 ; Hong *et al.*, 2009), the decreased induction of the enzyme in the zebrafish brain exposed to Cd might be a result of protein inactivation due to Fe deficiency. The strong induction of Aro-B revealed by immunohistochemistry after Zn supplementation could thus be explained by a protein stabilization since the activity of a large number of enzymes depends on a sufficient supply of trace elements, such as Zn, selenium and copper (Rükgauer *et al.*, 2001). Given the results herein presented and in addition to the well-documented effects of heavy metals, notably Cd, on thyroid signaling and brain development (Grandjean and Landrigan, 201), it is also possible that neuroendocrine disruption occurs through interactions with estrogen signaling.

In summary, the present study describes Cd as a potent anti-estrogen *in vivo* and *in vitro* and provides evidence that this heavy metal inhibits estrogen action in the developing zebrafish brain, as demonstrated by inhibition of E2-induction of Aro-B. The effect of Cd on the Aro-B expression in the radial glial cells at early developmental stages can therefore affect the neuroendocrine system and the reproductive function. While the precise mechanisms remain to be elucidated, our data would suggest that Cd effects on estrogen signaling in the brain could be, in part, due to interference with classical nuclear ER subtypes affecting then Aro-B expression. Our results further demonstrated that Zn supplement provides an ameliorative effect against E2-antagonism of Cd in zebrafish embryos and U251-MG cells as evidenced by the improvement of the protein expression of Aro-B and the enhancement of ERs-transcriptional activation level. However, the exact mechanisms underlying the protective effect of Zn on the neuroendocrine system need to be fully clarified.

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Table 1: Sequences of the oligonucleotides used in Real Time Polymerase Chain Reaction experiments.

Gene	Accession number	Sequence
<i>esr1</i>	NM_152959.1	Fw: 5'-CTGGAGATGCTGGACGCTCA-3' Rev: 5'-GCTGCAGCTCCTCCTCCTGG-3'
<i>esr2a</i>	NM_180966.2	Fw: 5'-GATCCTCCTGAACTCCAACATG-3' Rev: 5'-CCAGCAGACACAGCAGCTTGGA-3'
<i>esr2b</i>	NM_174862.3	Fw: 5'-GATCCTGCTCAACTCTAATAAC-3' Rev: 5'-CCAGCAGATTCAGCACCTTCCC-3'
<i>cyp19a1b</i>	NM_131642.1	Fw: 5'-TCGGCACGGCGTGCAACTAC-3' Rev: 5'-CATACCTATGCATTGCAGACC-3'
<i>efl</i>	NM_131263.1	Fw: 5'-AGCAGCAGCTGAGGAGTGAT-3' Rev: 5'-CCGCATTTGTAGATCAGATGG-3'

Figure Legends

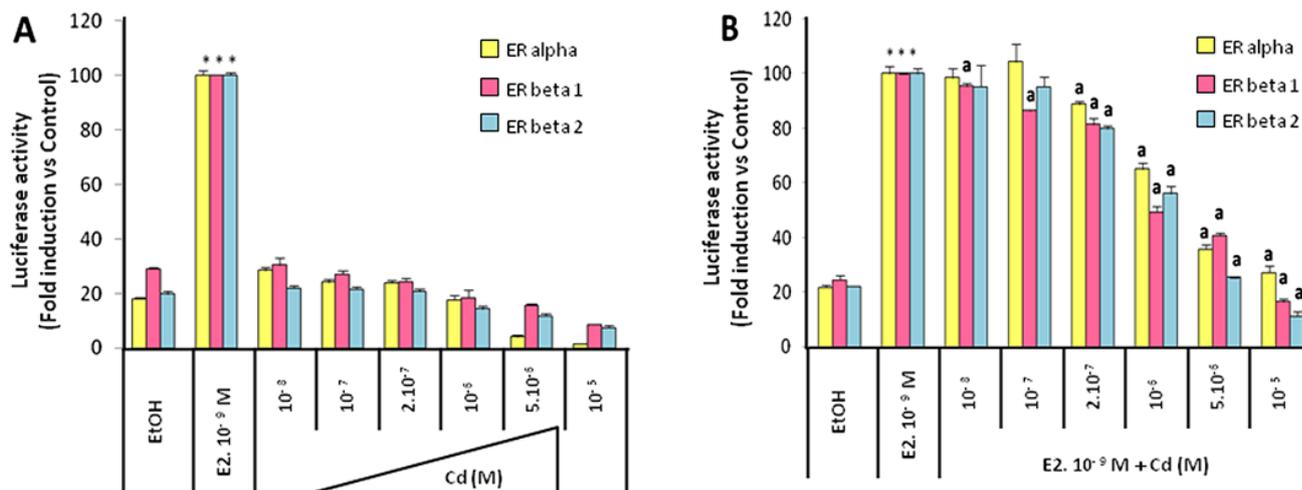


Figure 1: Evaluation of the transcriptional activation of the *ERE-TK-luc* reporter gene by the three zebrafish estrogen receptors in the presence of ethanol (EtOH), estradiol (E2) and cadmium alone (Cd) in figure (A) and ethanol (EtOH), estradiol (E2) and estradiol + cadmium together (E2+Cd) in figure (B). Data are the mean of at least three separate transfection experiments performed in triplicate. * and a indicate significant differences comparing to EtOH and E2-treated groups respectively ($P < 0.05$ according to ANOVA test).

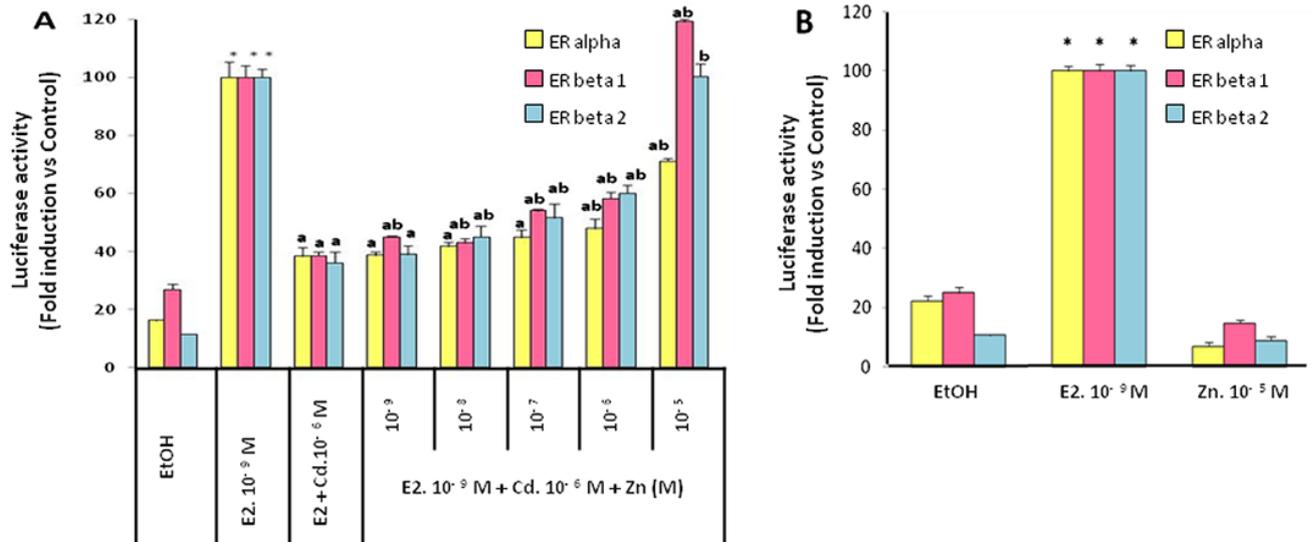


Figure 2: Evaluation of the transcriptional activation of the *ERE-TK-luc* reporter gene by the three zebrafish estrogen receptors in the presence of ethanol (EtOH), estradiol (E2), estradiol + cadmium (E2+Cd) and estradiol + cadmium + zinc (E2+Cd+Zn) in figure (A) and ethanol (EtOH), estradiol (E2) and zinc alone (Zn) in figure (B). Data are the mean of at least three separate transfection experiments performed in triplicate. *, a and b indicate significant differences comparing to EtOH, E2 and E2+Cd-treated groups respectively (P<0.05 according to ANOVA test).

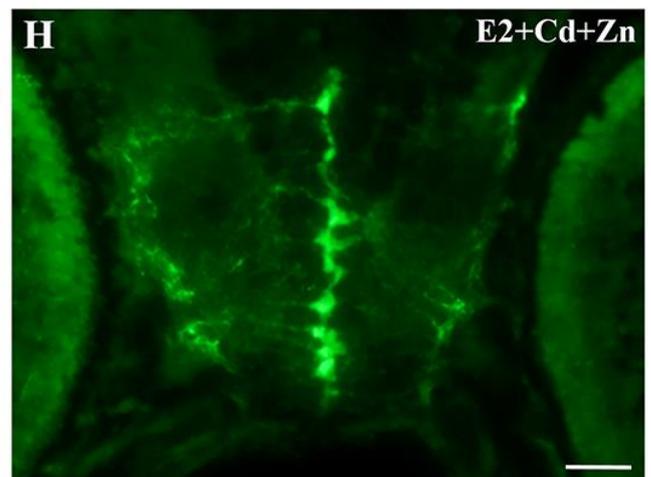
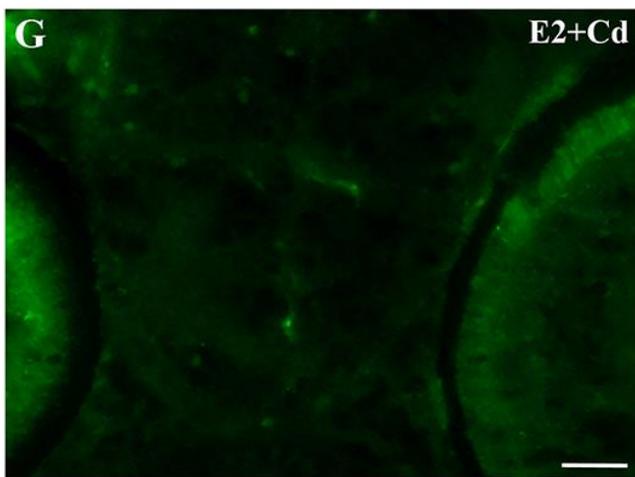
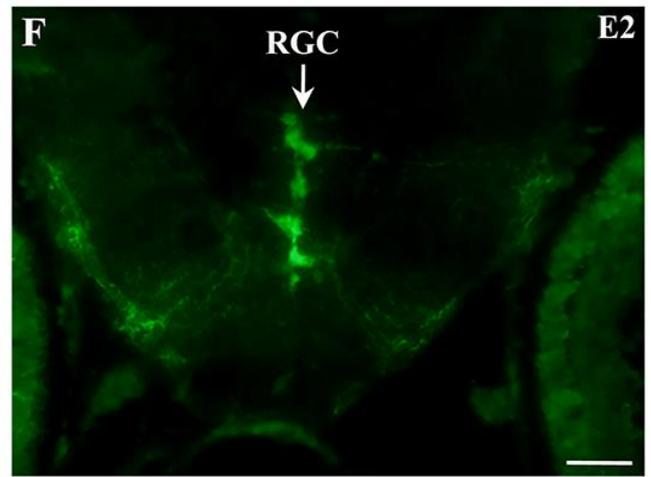
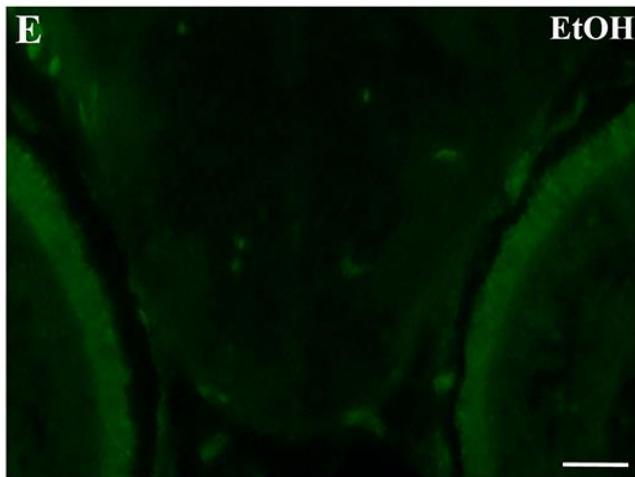
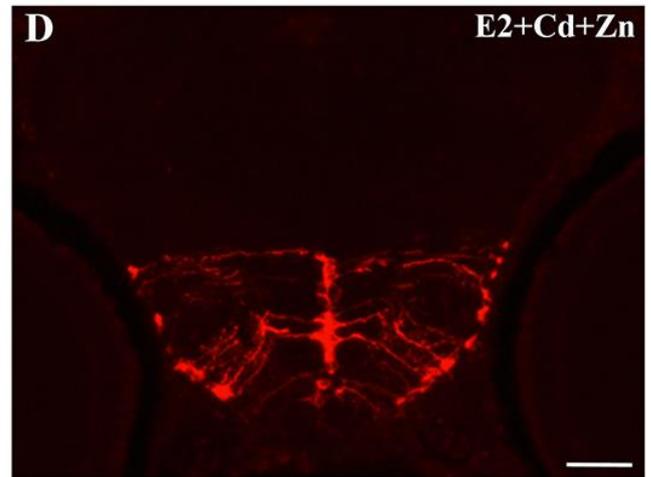
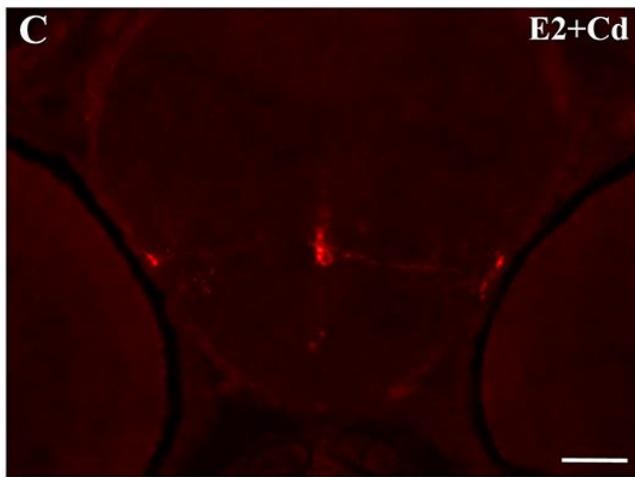
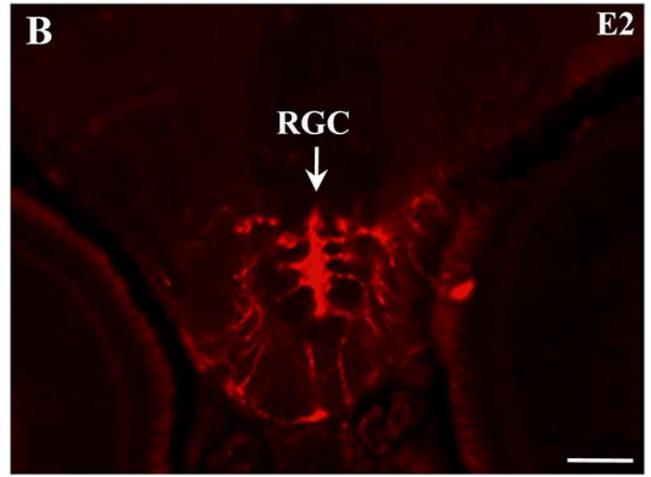
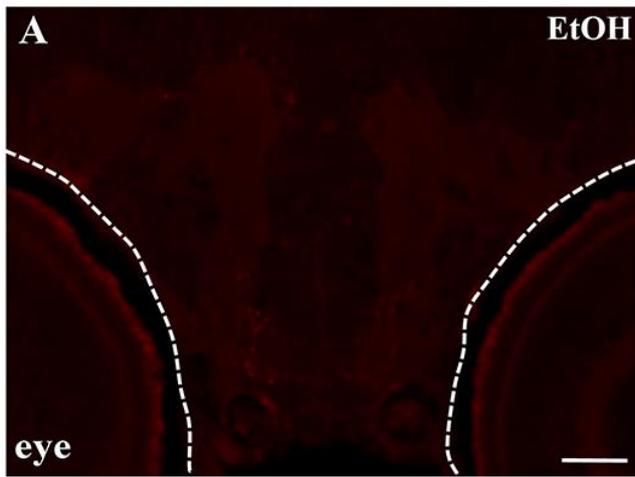


Figure 3: Transversal sections of the anterior hypothalamus of 3 days embryos showing aromatase B expression.

A, B, C and D : aromatase B immunoreactivity in radial glial cells (RGC) after exposure of wild-type zebrafish to ethanol (A, EtOH), estradiol (B, E2), estradiol + cadmium (C, E2+Cd) and estradiol + cadmium + zinc (D, E2+Cd+Zn). Scale bar = 100 μ m

E, F, G and H: GFP expression in RGC after exposure of *cyp19a1b-GFP* transgenic zebrafish to ethanol (E, EtOH), estradiol (F, E2), estradiol + cadmium (G, E2+Cd) and estradiol + cadmium + zinc (H, E2+Cd+Zn). Scale bar = 50 μ m

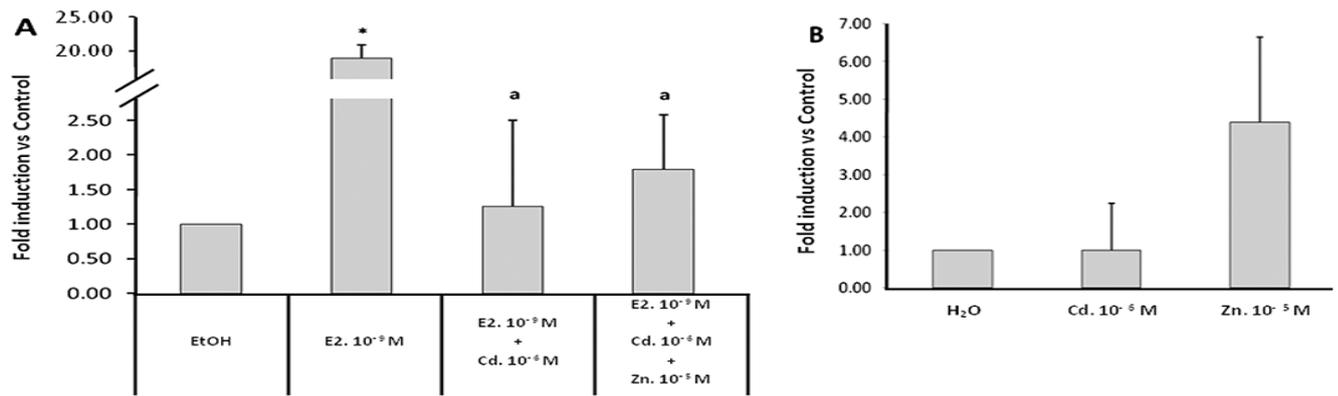


Figure 4: Expression of *cyp19a1b* gene in zebrafish embryos exposed to ethanol (EtOH), estradiol (E2), estradiol + cadmium (E2+Cd) and estradiol + cadmium + zinc (E2+Cd+Zn) in figure (A) and to distilled water (H₂O), cadmium (Cd) and zinc (Zn) in figure (B). The levels of Aro-B and *efl* transcripts were determined by quantitative reverse transcriptase-polymerase chain reaction (RT-qPCR) in 72 h postfertilization. The expression of *efl* mRNA was used as reference to normalize the expression levels of the studied gene mRNA. Data are expressed as fold induction relative to control and corresponds to the median of five independent exposure experiments performed in triplicate. * and **a** indicate significant differences comparing to EtOH and E2-treated groups respectively (P<0.05 according to Mann-Whitney test corrected by Bonferroni correction).

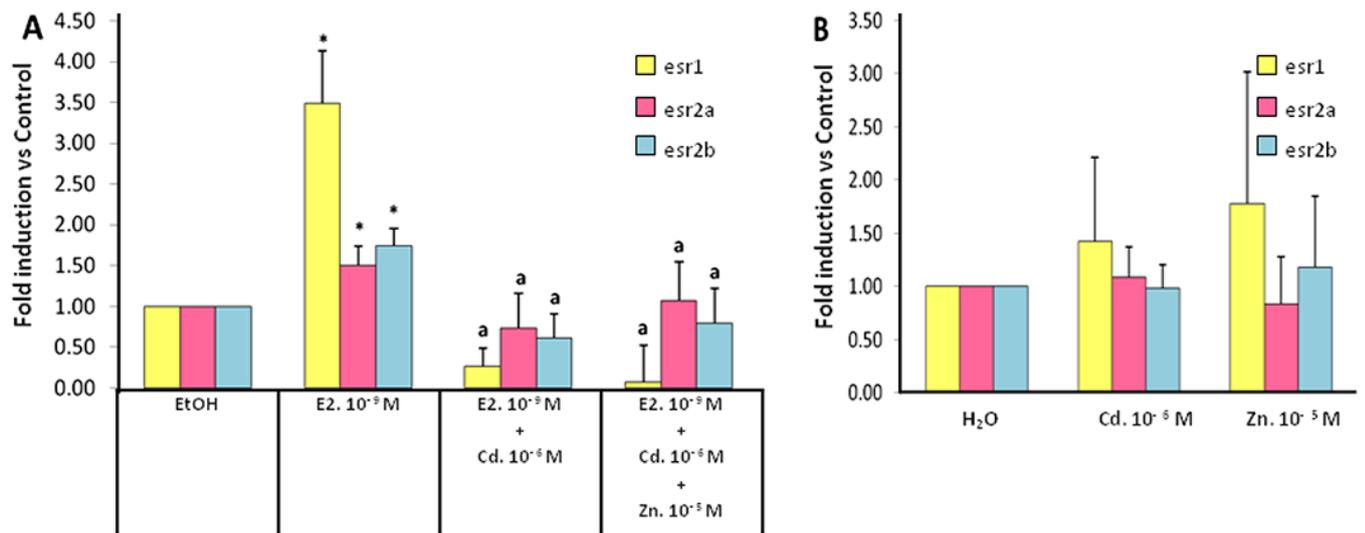


Figure 5: Expression of *esr1*, *esr2a* and *esr2b* genes in zebrafish embryos exposed to ethanol (EtOH), estradiol (E2), estradiol + cadmium (E2+Cd) and estradiol + cadmium + zinc (E2+Cd+Zn) in figure (A) and to distilled water (H₂O), cadmium (Cd) and zinc (Zn) in figure (B). The levels of *esr1*, *esr2a*, *esr2b* and *ef1* transcripts were determined by quantitative reverse transcriptase-polymerase chain reaction (RT-qPCR) in 72 h postfertilization. The expression of *ef1* mRNA was used as reference to normalize the expression levels of the studied gene mRNA. Data are expressed as fold induction relative to control and corresponds to the median of five independent exposure experiments performed in triplicate. * and **a** indicate significant differences comparing to EtOH and E2-treated groups respectively (P<0.05 according to Mann-Whitney test corrected by Bonferroni correction).