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ABSTRACT

Cadmium (Cd), a worldwide occupational pollutant, is an extremely toxic heavy metal, capable of damaging several organs, including the brain. Its toxicity has been related to neurodegenerative diseases such as Alzheimer's and Parkinson's diseases. The neurotoxic potential of Cd has been attributed to the changes induced in the brain enzyme network involved in counteracting oxidative stress. On the other hand, it is also known that trace elements, such as zinc (Zn) and selenium (Se), required for optimal brain functions, appears to have beneficial effects on the prevention of Cd intoxication.

Based on this protective effect of Zn and Se, we aimed to investigate whether these elements could protect neuronal cells from Cd-induced excitotoxicity. The experiments, firstly carried out on SH-SY5Y catecholaminergic neuroblastoma cell line, demonstrated that the treatment with 10 μM cadmium chloride ($CdCl_2$) for 24 hours caused significant modifications both in terms of oxidative stress and neuronal sprouting, triggered by endoplasmic reticulum (ER) stress. The evaluation of the effectiveness of 50 μM of zinc chloride ($ZnCl_2$) and 100 nM sodium selenite (Na_2SeO_3) treatments showed that both elements were able to attenuate the Cd-dependent neurotoxicity. However, considering that following induction with retinoic acid (RA), the neuroblastoma cell line undergoes differentiation into a cholinergic neurons, our second aim was to verify the zinc and selenium efficacy also in this neuronal phenotype.

Our data clearly demonstrated that, while zinc played a crucial role on neuroprotection against Cd-induced neurotoxicity independently from the cellular phenotype, selenium is ineffective in differentiated cholinergic cells, supporting the notion that the molecular events occurring in differentiated SH-SY5Y cells are critical for the response to specific stimuli.

Key words: Cadmium; Selenium; Zinc; Neurotoxicity; Neuronal phenotype.

INTRODUCTION

Cadmium (Cd) is the seventh most toxic heavy metal as per Agency for Toxic Substances and Disease Registry - ATSDR ranking (ATSDR, 2017) among the environmental pollutants with which humans and animals can potentially come in contact. Given that Cd is widely distributed in natural and industrial sources (Mead, 2010), exposure to cadmium can occur in occupations such as mining, electroplating or in the vicinity of Cd-emitting industries or incinerators where it is produced or used. In fact, Cd levels in ambient range from 0.1 to 5 ng/m^3 in rural areas, 2-15 ng/m^3 in urban areas, and

15-150 ng/m^3 in industrialized areas (ToxGuide^{TM} for Cadmium, 2012). Nevertheless, numerous studies have reported health effects of daily cadmium exposure in the general population also in the absence of specific industrial exposure, the main source of exposure being food and tobacco smoke. It was reported that the average Cd intake from food generally varies between 8 and 25 µg per day (Bérglund et al., 1994; MacIntosh et al., 1996; Thomas et al., 1999; Ysart et al., 2000; Larsen et al., 2002; Olsson et al., 2002; Llobet et al., 2003; Egan et al., 2007), and that normal smokers present twice the levels in their body than non-smokers and this values are four times higher in heavy smokers (Järup and Akesson, 2009; ATSDR, 2017). The half-time for Cd in the whole body in humans is >26 years and in general population the Cd normal human level in the blood (indicative for a recent exposure) is 0.315 µg/L, whereas the urine level (indicative for previous exposure) is 0.185 µg/L (ToxGuide^{TM} for Cadmium, 2012).

Many evidences highlighted the correlation between environmental pollutant (in particular heavy metals) and chronic brain inflammation and neurodegeneration (Calderon-Garciduenas et al., 2002; Calderon-Garciduenas et al., 2003). In particular, Cd is included among the etiopathogenetic factor of some neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), autism spectrum disorder (ASD), myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) (Thatcher et al., 1982; Marlowe et al., 1983; Panayi et al., 2002; Barnham and Bush, 2008; Pacini et al., 2012).

Cadmium-induced neurotoxicity involves the generation of reactive oxygen species (ROS) and free radicals, disturbances in calcium/zinc-dependent processes, dysregulation of cell repair systems, epigenetic modifications and oestrogen-mimicking effects (Wang et al., 2004; Bertin and Averbeck, 2006; Monroe and Halvorsen, 2009; Kim et al., 2013; Yuan et al., 2016). Indeed, Cd is known to block calcium channels in mitochondria, inducing a membrane potential decrease and the consequent release of cytochrome c, eventually leading to apoptosis cascade (Fern et al., 1996; Xu et al., 2011; Yuan et al., 2013). Furthermore it has been demonstrated that Cd induces ER stress (Chen et al., 2015), leading to cell death by a non-mitochondrial dependent pathway (Hitomi et al., 2004).

The most commonly used therapeutic strategy for heavy metal poisoning is chelation therapy to promote metal excretion. However chelators are reported to have a number of different safety and efficacy concerns, and none of these therapies have yet been approved for clinical use (Goyer and Clarkson, 2001;McCarty, 2012). Recent studies have shown that essential metals dietary supplements play important roles in protecting against Cd even because they are expected to have very few side effects compared to the chelators (Zhai et al., 2015).

One of the most well studied essential metal is zinc (Zn), possessing similar chemical and physical properties to Cd, competing for the binding sites of metal absorptive and enzymatic proteins (Bridges

and Zalups, 2005). Moreover, Zn induces the synthesis of the CNS specific metallothionein III (Suzuki et al., 1990; Aschner et al., 1997; Jin et al., 1998; Hidalgo et al., 2008), a low molecular weight, cysteine-rich protein that has high affinity for Cd and causes detoxification by binding Cd (Nordberg and Nordberg, 2000; Hartwig, 2001). Moreover, Zn intake has been reported to alleviate the oxidative stress caused by Cd and lead exposure (Amara et al., 2008; Prasanthi et al., 2010).

On the other hand, a considerable number of studies have shown that selenium (Se) administration is protective against Cd toxicity within a range of different organs of mice, including the brain (Newairy et al., 2007; Cardoso et al., 2015). Selenium is a cofactor of the antioxidant enzyme glutathione peroxidase (GPx) and it contributes to the antioxidant defence system, reducind the Cd-induced oxidative stress and enhancing the antioxidant capacity of the host (Luchese et al., 2007; Liu et al., 2013).

Therefore, the first aim of this study was to investigate the neuroprotective properties of Zn and Se against Cd-induced neurotoxicity in SH-SY5Y neuroblastoma cell line, a widely used catecholaminergic *in vitro* model for studies on neurotoxicity of compounds affecting the nervous system (Faria et al., 2016; Heusinkveld and Westerink, 2017). However, in addition to the catecholaminergic system (Gupta et al., 1990), Cd has been shown also to affect glutamatergic (Borges et al., 2007; Borisova, 2011), monoaminergic (Ali et al., 1990; Gutierrez-Reyes et al., 1998; Abdel Moneim et al., 2014), as well as cholinergic system where it blocks the cholinergic transmission inducing a more pronounced cell death (Del Pino et al., 2014). Furthermore, many studies have evidenced significant degree of interplay between catecholaminergic and cholinergic system in the regulation of CNS activity (Raevskii et al., 1993). Since undifferentiated dopaminergic SH-SY5Y human neuroblastoma cells can be differentiated by retinoic acid (RA) in mature cholinergic neurons (Presgraves et al., 2004; Lopes et al., 2010; Kovalevich and Langford, 2013), the second aim of the present study was to evaluate if the treatments with Zn and Se show different efficacy against Cd-induced neurotoxicity in undifferentiated catecholaminergic cells with respect to the cholinergic neuronal phenotype.

MATERIALS AND METHODS

2.1 Cell line and treatments

Human neuroblastoma SH-SY5Y cell line, was purchased by Istituto Zooprofilattico dell'Emilia e della Romagna (Brescia, Italy). Cells were routinely cultured in DMEM High Glucose/Ham's F12 Mixture Medium (1:1) supplemented with 10% foetal bovine serum (FBS), 2 mM L-Glutamine

(EuroClone S.p.a., Milano, Italy) at 37°C, 5% CO_2 in humidified atmosphere. The growth medium was changed every 2-3 days.

In order to reproduce in vitro conditions that could mimic a chronic human Cd intoxication, we decide to use a concentration of 10 µM of $CdCl_2$ (Sigma Aldrich, Milano, Italy) and a time of exposure of 24 hours as reported by Del Pino (Del Pino et al., 2014) and further confirmed by dose-response curves (supplementary figure S1 – Panel A).

Aimed to evaluate the effect of Zn and Se supplementation at doses corresponding to the human physiological levels, the concentration of 100 nM Na_2SeO_3 and of 50 µM $ZnCl_2$ (Sigma Aldrich, Milano, Italy) were chose on the basis of previously reported data (Szuster-Ciesielska et al., 2000; Barayuga et al., 2013; Hendrickx et al., 2013) and of dose-response curves performed for both essential metals (supplementary figure S1 – Panels B and C).

All treatments were performed in starvation medium because manipulating Se and Zn content of culture medium is impaired by the presence of these essential elements in FBS. The timeline with the entire experimental procedures were reported in supplementary Table S1.

2.2 SH-SY5Y differentiation

Human neuroblastoma SH-SY5Y cell line was differentiated with 10 µM all-*trans* RA (Sigma Aldrich, Milano, Italy) for 48 h in their appropriate medium (DMEM High Glucose/Ham's F12 Mixture Medium (1:1), 2 mM L-Glutamine) supplemented with 1% FBS. Briefly, in all the experiment reported below, the cells were seeded in each support for 24 h in their complete growth medium. The day after, cells were starved in 1% FBS medium for 48 h and differentiated by adding RA 10 µM. After two days of differentiation, the cells were starved in 0% FBS medium for 24 h and then stimulated for 24 h in starved medium (0% FBS) as reported above. The stimuli and the different time of each treatment, was the same described for each experiment both for undifferentiated and differentiated SH-SY5Y.

2.3 Cell viability assay

Cell viability was evaluated by the reduction of 3-(4,5-di-methylthiozol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as an index of mitochondrial functional activity. Briefly, SH-SY5Y cells were seeded into 96 well plates at a density of 20,000 cells/well in complete growth medium for 1 day. Differentiated and undifferentiated cells were treated with $CdCl_2$ (for 24 h) in the absence or after the pre-treatment with $ZnCl_2$ or Na_2SeO_3 (for 24 h). After removing the medium with different stimuli, 1 mg/ml MTT was added into each well and incubated for at least 20 min at 37 °C. Following the removing of the chromogenic solution, the formazan crystals were dissolved in 50 µl

of dimethyl sulfoxide (DMSO) and the absorbance was measured at 595 nm by a Multiscan FC photometer (ThermoScientific, Milan, Italy). Three independent experiments were conducted and each experiment was performed in quintuplicate.

2.4 Measurement of intracellular ROS

To detect intracellular ROS production, SH-SY5Y cells seeded on glass cover slips were loaded with 10 μM 2,7-dichlorodihydrofluorescein diacetate for 10 min (CM-H_2DCFDA, Thermo Fisher Scientific, Waltham, MA, USA), as previously described (Capitini et al., 2014). Cell fluorescence was analysed by the motorized Leica DM6000 B microscope equipped with a DFC350FX camera (Leica, Mannheim, Germany). The full-specimen thicknesses were acquired as z-stack series, deconvolved using Huygens Professional software (SVI, Hilversun, The Netherlands), and displayed using ImageJ software. The microscope was set at optimal acquisition conditions, and settings were maintained constant for each analysis. Five microscopic fields for each experimental point were analyzed. Three independent experiments were conducted and each experiment was performed in triplicate.

2.5 Western blotting analysis

SH-SY5Y cell line, at the density of 10^7 cells/well, were plated in Petri dishes in complete growth medium and treated as reported. After each treatment, the medium was removed and two washes with PBS (phosphate buffered saline) were performed. The cells were scraped from the surface of the dishes and the cell suspensions were centrifuged at 1,000 rpm for 10 min at room temperature (RT). The supernatant was discarded and the pellets were treated with lysis buffer (TRIS 50 mM, pH 7; NaCl 150 mM; 1% TRITON X-100; EDTA 1.5 mM; 0.25% SDS) containing protease inhibitors cocktail (Sigma Aldrich, Milan, Italy) for 30 min at 4°C. The homogenates were centrifuged at 4 °C for 10 min at 12,000 rpm and the supernatants were used to evaluate the protein concentration by Bradford method. Equal amounts of proteins (30 μg) were analysed on a 12% polyacrylamide gel and then transferred onto nitrocellulose membrane (Porablot NPC, MACHEREY-NAGEL, Carlo Erba Reagents, Milano, Italy). After 1 h blocking with 3% bovine serum albumin (BSA) in Tris-buffered saline containing 0.1% Tween 20 (T-TBS) at RT, the blot was incubated overnight at 4 °C with 1:500 mouse monoclonal anti- GAP-43 (B-5), 1:300 rabbit polyclonal anti-BAX (P-19), 1:300 rabbit polyclonal anti-pro-caspase-3 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 1:500 rabbit polyclonal anti-GRP78 antibody (ThermoFischer Scientific, Milan, Italy), and then with 1:5,000 goat anti-mouse (for GAP-43) and goat anti-rabbit (for BAX, GRP78 and pro-caspase-3) HRP secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at RT. GAP-

43 as well as BAX were detected with the Amersham ECL Plus Western Blotting Detection Reagent (GE Healthcare, EuroClone, Milan, Italy). Protein expression levels were quantified by ImageJ64 analysis software (ImageJ, National Institute of Health, USA, http://imagej.nih.gov/ij, 1.47t) and expressed as percentage of control. For cytochrome c immunofluorescent quantification, five microscopic fields for each experimental point were analysed.

All data were obtained by three independent experiments; each experiment was performed in triplicate.

2.6 Caspase-3 enzymatic activity

SH-SY5Y cell line was plated in 6-well plates ($7x10^5$/well) in appropriate complete growth medium. The following day, cells were treated as reported in Table S1. After each treatment, cells were scraped in 100 mM lysis buffer (200 mM Tris–HCl buffer, pH 7.5, containing 2 M NaCl, 20 mM EDTA, and 0.2% Triton X-100). Fifty µl of the supernatants were incubated with 25 µM fluorogenic peptide caspases 3 substrate rhodamine 110 bis-(N-CBZ-L-aspartyl-L-glutamyl-L-va- lyl-L-aspartic acid amide) (EnzChek® Caspase-3 Assay Kit, Molecular Probes) at 25 °C for 30 min. The amount of cleaved substrate in each sample was measured in a 96-well plate fluorescent spectrometer (Perkin-Elmer; excitation at 496 nm and emission at 520 nm). Three independent experiments were conducted and each experiment was performed in triplicate.

2.6 Immunofluorescent staining

SH-SY5Y cells were seeded in 6 well plates at the density of $7x10^4$ cells/well or 10^5 cells/well (differentiated and undifferentiated respectively) on cover slip slides. As described above, differentiated and undifferentiated cells were treated with different concentration and exposure time of $CdCl_2$ and/or $ZnCl_2$ or Na_2SeO_3. At the end of each treatment, the starvation medium containing stimuli was removed and two washes with cold PBS were performed. The cells were then fixed with 1 ml of 4% paraformaldehyde for 10 min at RT. After three washes with cold PBS (5 min. each wash), the cells were permeabilized with 1% TRITON X-100 in PBS for 10 min. at RT. Cells were then washed three times with PBS and incubated for 15 min in a blocking solution (1% BSA in PBS) at RT. Each cover slip was incubated overnight at 4°C with 1:200 mouse anti-β3 tubulin or rabbit anti-cytochrome c antibodies (Santa Cruz Biotechnology, Santa Cruz CA). The day after, cells were washed three times with PBS and each cover slip was incubated with 1:200 Alexa Fluor 647 goat anti-mouse (for β3 tubulin) or goat anti-rabbit (for cytochrome c) immunoglobulin G (IgG) secondary antibodies, respectively (Thermo Fisher Scientific, Waltham, MA, USA) for 1 h at RT. After secondary antibody incubation, two washes were performed with PBS and DAPI (4',6-diamidin-2-

fenilindolo; 1:2,000 dilution; Thermo Fisher Scientific, Waltham, MA, USA) was added for 5 min at RT to each cover slip. Eventually, after two more washes in cold PBS and one more in distilled water, cover slip glasses were mounted using Fluoromount anti-fade solution (Life Technologies-Thermo scientific) on cover slides. Digitalized images were collected at 200x or 400x total magnification (five microscopic fields for each experimental point) by a motorized Leica DM6000B microscope equipped with a DFC350FX. Five microscopic fields for each experimental point were analysed.
Image analysis of β3 tubulin staining was performed counting the total number of neurite. Fluorescence intensity in cytochrome c immunostained cells was processed by ImageJ64 analysis software and the results were expressed as percentage of control.
All data were reported after normalization for the total number of cells *per* field. Three independent experiments were conducted and each experiment was performed in triplicate.

2.7 Statistical analysis

Statistical analyses were performed by Two-way ANOVA followed by the Mann–Whitney test. All assessments were made by researchers blinded to treatments. Data were analysed using "Origin 9" software (OriginLab, Northampton, USA). Differences were considered significant at $p<0.05$.

RESULTS

3.1 SH-SY5Y differentiation

In order to determine the RA-dependent differentiation of SH-SY5Y cells, an immunofluorescent staining against β3 tubulin, a neuronal marker known to be overexpressed in differentiated neurons (Hernandez-Martinez et al., 2017), was performed at different exposure time. As shown in supplementary figure S2, the neurite sprouting from cell bodies increased in a time-dependent manner comparing to untreated cells (control). As previously reported (Cheung et al., 2009; Sallmon et al., 2010; Dwane et al., 2013; Pak et al., 2014) 10 μM RA increased the number and the length of cytoplasmic elongation in a time-dependent manner up to 72 h. At prolonged exposure time (120 h) a RA-dependent increase of mitochondrial permeability and the consequent cytochrome c release into the cytoplasm (Rigobello et al., 1999; Xun et al., 2012) were observed (Supplementary figure S3). Thus, we decided to induce SH-SY5Y differentiation for 48 h.

3.2 Evaluation of cell viability

To validate SH-SY5Y cell viability after treatments with $CdCl_2$ in the absence or in the presence of $ZnCl_2$ and/or Na_2SeO_3, the MTT assay was performed. As shown in figure 1 (panel A), cell viability

was significantly ($p<0.05$) decreased after treatment with 10 μM $CdCl_2$ for 24 h. On the other hand, both Na_2SeO_3 and $ZnCl_2$ were able to prevent the decrease of SH-SY5Y viability when used as pre-treatment for 24h before $CdCl_2$ addition (figure 1 – panel A, right histograms). When Na_2SeO_3 and $ZnCl_2$ were tested as co-treatment with $CdCl_2$ stimulation, only $ZnCl_2$ was able to counteract the decrease in cell viability induced by the heavy metal (figure 1 – panel A, left histograms).
Furthermore, in RA differentiated SH-SY5Y cells (figure 1, panel B) while $ZnCl_2$ was able to prevent the effects induced by $CdCl_2$ both during pre-treatment and co-treatment, the presence of Na_2SeO_3 did not induce any significant change in cell viability (figure 1 – Panel B, left and right histograms). Taking into account these results, we decided to perform the other sets of experiments only with $ZnCl_2$ or Na_2SeO_3 pre-treatment (24 h) conditions.

3.3 Cd-dependent ROS production

In order to evaluate the effects of Se and Zn on Cd-induced oxidative stress and ROS generation, undifferentiated and RA differentiated SH-SY5Y cells were treated with 10 μM $CdCl_2$ for 24 h in the presence or in the absence of 50 μM $ZnCl_2$ or 100 nM Na_2SeO_3. The quantitative analysis of ROS levels (figure 2 – histograms) revealed that Cd-treated cells exhibited a significative ($^{*}p<0.05$ *vs* control) increase in reactive oxygen species in respect to control cells, confirming that Cd neurotoxicity may be associated with its induction of ROS as previously reported (Chen et al., 2008; Yuan et al., 2013; Liu et al., 2014; Chen et al., 2014; Xu et al., 2017). However, the results clearly show that only a pre-treatment with Zn is effective in the prevention of Cd-induced ROS formation ($^{\#}p<0.05$ *vs* $CdCl_2$ treated cells) in both undifferentiated and differentiated cells. On the other hand Se is effective only in differentiated cells.

3.4 Cd-dependent apoptotic pathway

To better understand the Cd-induced ER stress, we evaluated the expression of GRP78, a chaperone well known to be induced by ER stress (Lee, 2005), in RA differentiated and undifferentiated cells. Figure 3A (left panel) clearly shows how $CdCl_2$ treatment is able to evoke the over-expression of GRP78 in undifferentiated SH-SY5Y. Such adverse events were prevented by the presence of Na_2SeO_3 or $ZnCl_2$ pre-treatment.
Furthermore, since Cd is well known to induce oxidative stress and to activate the apoptotic pathway (Watjen and Beyersmann, 2004), the expression level of the pro-caspase-3 (figure 3A, middle panel) the caspase-3 enzymatic activity (figure 3B), as well as the expression levels of the pro-apoptotic protein BAX (figure 3A, right panel), were investigated in the presence of 50 μM $ZnCl_2$ or 100 nM Na_2SeO_3 in undifferentiated cells. Our results showed that Cd induced a two-fold increment in BAX

expression level, and the decrease of pro-caspase-3 expression, paralleled by the increase of the caspase-3 activity, that were partially prevented by the presence of Zn and Se (figures 3A and 3B). In RA differentiated SH-SY5Y cells (figures 4A and 4B), the treatment with $CdCl_2$ induced similar results to those evidenced in undifferentiated cells. Interestingly, while $ZnCl_2$ was able to partially restore GRP78, pro-caspase-3 and BAX levels, Na_2SeO_3 did not show any protective effect against Cd-induced neurotoxicity (figures 4A and 4B).

To verify the effect of BAX protein overexpression during the treatment with 10 µM $CdCl_2$ for 24 h, the immunofluorescent staining of cytochrome c was performed (figure 3C). In undifferentiated cells, 10 µM $CdCl_2$ clearly increased the expression of cytochrome c that was poorly labelled around the nuclei in untreated cells (control). When cells were pre-stimulated with $ZnCl_2$ and Na_2SeO_3, before $CdCl_2$ addition, cytochrome c remained barely expressed nearby the nuclei indicative for a mitochondrial membrane integrity.

As regards Zn-dependent prevention of $CdCl_2$-dependent cytochrome c mitochondrial extrusion, similar results were obtained on RA differentiated cells. Figure 4C shows the high diffusion and expression of cytochrome c in the soma of cells treated with 10 µM $CdCl_2$ for 24 h. On the contrary, in differentiated cells Na_2SeO_3 pre-treatment did not prevent cytochrome c overexpression, evidenced by immunofluorescence peri-nuclear localization.

3.5 Cd effect on neurite outgrowth: evaluation of GAP-43 and β3 tubulin

In order to determine the effects of Cd on neuronal sprouting, we evaluated the expression of GAP-43 in RA differentiated and undifferentiated SH-SY5Y cells. As shown in figure 5A Cd-treated cell extracts exhibited decreased cross-reactivity with the GAP-43 antibody when compared to control extracts ($^{*}p<0.05$ *vs* control). Nevertheless, when SH-SY5Y cell line was pre-treated for 24 h with $ZnCl_2$ or Na_2SeO_3, the densitometric analysis revealed a significantly ($^{\#}p<0.05$ *vs* $CdCl_2$ treated cells) higher expression of GAP-43. These data suggest that both $ZnCl_2$ and Na_2SeO_3 are key elements directly involved in neuronal branching and neurite regeneration during Cd-induced neuronal damage.

Immunofluorescent staining of β3 tubulin (figure 5B) validated and reinforced the data obtained by western blotting analysis concerning GAP-43 expression. The untreated cells (control), as well as cells treated with 50 µM $ZnCl_2$ or 100 nM Na_2SeO_3 for 24 h, clearly showed that, sprouting from the cell soma, cytoplasmic elongation extended, reaching close neuronal clusters. The neuronal branches significantly ($^{*}p<0.05$ *vs* control) decreased when the cells were treated with 10 µM $CdCl_2$ for 24 h (figures 5B and 6B). Interestingly, concerning $CdCl_2$ treatment figure 5B and 6B shows that, even though the neurite was not present and the cell number was decreased, β3 tubulin signal was clearly

evident and abundant in the neuronal soma, alongside the nuclei. When SH-SY5Y cells were pre-treated with 50 μM $ZnCl_2$ or 100 nM Na_2SeO_3 for 24 h before the stimulation with 10 μM $CdCl_2$, the β3 tubulin distributed along neurite length, partially preventing the alteration of the neuronal networks. Similar results were observed on RA differentiated SH-SY5Y cells (figure 6 panels A and B). As expected, both the number and the length of neurite was increased in control, $ZnCl_2$, and Na_2SeO_3 treated cells. As undifferentiated cells, 10 μM $CdCl_2$ induced a visible retraction of cytoplasmic elongations as well as a high localization nearby the cell nucleus; the pre-treatment with 50 μM $ZnCl_2$ prevented neuronal damage, maintaining almost intact the neuronal network. On the other hand, the pre-treatment with 100 nM Na_2SeO_3, poorly prevented neurite loss during $CdCl_2$ treatment.

DISCUSSION

Cadmium is found in the earth crust primarily and is released to the biosphere form both natural sources and anthropogenic sources. Since Cd is not degraded in the environment, the risk of human exposure is constantly increasing because Cd also enters the food chain (ATSDR, 2017). Chronic exposure to Cd has been found associated with diseases of the lung, prostate, pancreas and kidney (Howard, 2002; Beyersmann and Hartwig, 2008). In the central nervous system, it has been demonstrated that cadmium can increase permeability of the BBB in rats (Shukla et al, 1987; Shukla et al., 1996) and accumulate in the brain of developing and adult rats (Méndez-Armenta and Rios, 2007; Gonçalves et al., 2010), leading to cellular dysfunction, and cerebral oedema. For this latter feature, Cd has been taken into consideration as a possible etiopathogenic factor for neurodegenerative disorders such as AD, PD and ASD (Thatcher et al., 1982; Marlowe et al., 1983; Panayi et al., 2002; Barnham and Bush, 2008).

Cadmium permeates the cell membrane mainly through the same pathways of $Ca2^+$ influx, interacting with voltage gated calcium channels (VGCC) on the membrane surface (Snutch et al., 2000-2013; Usai et al., 1999). Once inside neuronal cells, Cd induces oxidative stress by ROS production triggering a molecular cascade passing through mitochondria dysregulation and leading to apoptosis. Furthermore, it induces a membrane depolarization as demonstrated by Polson et al., (2011) causing the release of cytochrome c from mitochondria. These latter data were also supported and better explained by data demonstrating that the regulation of BAX protein by JNK pathway is essential for mediating the apoptotic release of cytochrome c from mitochondria (Papadakis et al., 2006; Zhang et al., 2017).

Our results confirmed the role of Cd-induced ROS production, leading to the upregulation of BAX protein passing through the mitochondrial membrane depolarization and the consequent cytochrome c release.

Although mitochondria may play a central role in stress induced neuronal apoptosis, growing evidences suggest that endoplasmic reticulum may also regulate neuronal apoptosis in stress conditions (Hitomi et al., 2004; Galehdar et al., 2010). GRP78, which is an unfolded protein response (UPR)-related protein, sensor of ER stress, is highly upregulated during ER stress (Hitomi et al., 2004; Lee, 2005; Kim et al., 2006; Yokouchi et al., 2008).

We observed a Cd-induced upregulation of GRP78 protein level which paralleled with caspase-3 activation and BAX expression levels increment, suggesting a linkage between ROS-induced ER stress, caspase activation and apoptosis. Moreover, these results strongly suggest that the Cd-induced ROS-dependent apoptosis could involve both the mitochondrial and the ER stress apoptotic pathway. On the other hand, increasing evidences suggest also that Cd alters a plethora of signalling cascades (Thévenod, 2009), taking place directly or indirectly by ROS formation, that affects also gene transcription. In fact, Pak and coauthors (Pak et al., 2014) have demonstrated a Cd-dependent downregulation of GAP43 expression, a crucial player during neurite outgrowth, during SH-SY5Y cells differentiation. Here we showed that this downregulation, did not occur only during neuroblastoma differentiation, but that it is also evident in both undifferentiated and differentiated cells. In addition, our results on the immunodistribution of β3 tubulin, a protein involved in neurogenesis and axon guidance and maintenance, showed that the consequence of lowering the GAP43 levels caused an altered (cytoplasmic instead of axonal) distribution of this protein in both neuroblastoma and RA differentiated cells. Moreover, compared to the work of Pak (Pak et al., 2014), we can hypothesized that the GAP43 downregulation can be ascribed not only to a Cd-dependent transcriptional failure, but also indirectly to an impairment of ER functionality.

Interestingly, our experiments evidenced a Se-dependent significant upregulation of GAP43 expression both in undifferentiated and RA differentiated cells. According with these results, showing that sodium selenite is able *per se* to induce a GAP43 overexpression, there are evidences that Se, as well as RA, can induce a neuronal differentiation of SH-SY5Y, presumably through TrxR1 (thioredoxin reductase 1 splice variant) activation that regulates the expression of genes associated with differentiation and adhesion (Nalvarte et al., 2015). It is important to underlie that the Se-induced differentiation results in a phenotype more closely resembling dopaminergic neurons (Barayuga et al., 2013), whereas the RA differentiated cells, as well as the RA differentiated cells treated with sodium selenite, showed a cholinergic neuronal phenotype (Nalvarte et al., 2015; Kovalevich, 2013). On the other hand, it was also reported that RA differentiation, in addition to genomic action, also occurs by the activation of extranucler RA receptors, eliciting a signaling pathway (Liao et al., 2004; Cañon et al., 2004; Chen et al., 2008) that results in the growth of axonal cone (Farrar et al., 2009). Therefore, upon RA action GAP43 is highly expressed and translocated into the growing neurite (Lu

et al., 2013; Buizza et al., 2013). These results are in agreement with our results showing a GAP43 overexpression in differentiated cells during $ZnCl_2$ treatment, where the presence of RA is sufficient to increase GAP43 expression levels in respect to the undifferentiated cells.

With regard to the capacity of Zn to revert the cadmium-dependent toxicity it has been demonstrated that, given the chemical similarity to Cd (Cotzias et al., 1961), they compete with one another for a variety of ligands (Pulido et al., 1966; Gunn et al., 1968). Moreover, Zn possess known anti-oxidant properties, which meet to cadmium-induced oxidative stress (Ebadi et al., 1995; Volpe et al., 2011). Following this reasoning and given the fully demonstrated selenium-dependent inhibition of oxidative stress, we should expect a similar response in the presence of selenium; on the contrary, this metal has protective effects against cadmium only in undifferentiated cells. In our opinion, this behavior is strictly dependent on the cellular phenotype. In fact, seleno-glutathione peroxidase (GPx) is one of the most abundant antioxidant enzymes in the brain (Flohé, 1982) in which Se is incorporated; moreover, it has been demonstrated that it is expressed in undifferentiated SH-SY5Y cells, and that in serum-free media with defined supplement of Se there is a GPx upregulation (Barayuga et al., 2013). However, Trepanier and coauthors (Trépanier et al., 1996) revealed that catecholaminergic neurons express GPx protein in the adult mouse brain, whereas some subsets of cholinergic neurons lack this expression and are more susceptible to oxidative stress. These findings suggest that the reason for which Se is not effective in preventing Cd neurotoxicity could be related to the low expression of GPx. Nevertheless, further studies are needed to address this hypothesis. Anyway, our results are further supported by the results of Becker and coauthors (Becker et al., 2012) which evidenced a RA-dependent downregulation of apoER2 (apolipoprotein E receptor 2), a receptor predominantly expressed by neurons throughout the brain (Clatworthy et al., 1999) that serves to selenium for entering the cell.

In conclusion, our results strongly suggest the efficacy of Zn and Se in counteracting the effects of Cd neurotoxicity and the resultant oxidative stress. These results also suggest that these metals have an ability to inhibit the Cd-dependent intracellular signalling pathway leading to oxidative stress and neuronal disfunction. Nevertheless, given that Se is ineffective in counteracting the Cd neurotoxicity in cholinergic neurons, our results show that the efficacy of essential metals is closely related to the neuronal phenotype. Therefore, further studies are required to better elucidate the relationship between intracellular signalling pathways triggered by Cd and the different neuroprotective agents in order to devise new preventive therapeutic strategies.

References

Abdel Moneim A.E., Bauomy A.A., Diab M.M., Shata M.T., Al-Olayan E.M., El-Khadragy M.F. 2014. The protective effect of Physalis peruviana L. against cadmium-induced neurotoxicity in rats. Biol Trace Elem Res. 160, 392-399.

Ali M.M., Mathur N., Chandra S.V. 1990.Effect of chronic cadmium exposure on locomotor behaviour of rats. Ind J Exp Biol. 28, 653-656.

Amara S., Abdelmelek H., Garrel C., Guiraud P., Douki T., Ravanat J.L., Favier A., Sakly M., Ben R.K. 2008. Preventive effect of zinc against cadmium-induced oxidative stress in the rat testis. J Reprod Dev. 54, 129–134.

Aschner, M., Cherian, M.G., Klaassen, C.D., Palmiter, R.D., Erickson, J.C., Bush, A.I., 1997. Metallothionein in brain—the role in physiology and pathology. Toxicol. Appl. Pharmacol. 142, 229–242.

ATSDR Agency for Toxic Substance and Disease Registry, U.S. Toxicological Profile for Cadmium. Department of Health and Humans Services, Public Health Service, Centers for Disease Control, Atlanta, GA, U.S.A., 2017.

Barayuga, S.M., Pang, X., Andres, M.A., Panee, J., Bellinger F.P., 2013. Methamphetamine decreases levels of glutathione peroxidases 1 and 4 in SH-SY5Y neuronal cells: protective effects of selenium. Neurotoxicology 37, 240-246.

Barnham, K.J., Bush, A.I. 2008. Metals in Alzheimer's and Parkinson's diseases. Curr. Opin. Chem. Biol. 12, 222–228.

Becker, J., Fröhlich, J., Perske, C., Pavlakovic, H., Wilting, J., 2012. Reelin signalling in neuroblastoma: Migratory switch in metastatic stages. Int J Oncol. 41, 681-689.

Bérglund M., Lind B., Lannerö E., Vahter M. 1994. A pilot study of lead and cadmium exposure in young children in Stockholm, Sweden: methodological considerations using capillary blood microsampling. Arch Environ Contam Toxicol. 27, 281-287.

Bertin, G., Averbeck, D., 2006. Cadmium: cellular effects, modifications of biomolecules, modulation of DNA repair and genotoxic consequences (a review). Biochimie. 88, 1549-1559.

Beyersmann, D., Hartwig, A. 2008. Carcinogenic metal compounds: recent insight into molecular and cellular mechanisms. Arch. Toxicol. 82, 493–512.

Borges V.C., Santos F.W., Rocha J.B., Nogueira C.W. 2007. Heavy metals modulate glutamatergic system in human platelets. Neurochem Res. 32, 953-958.

Borisova N. Krisanova R. Sivko L. Kasatkina A. Borysov S. Griffin M. Wireman. 2011. Presynaptic malfunction: the neurotoxic effects of cadmium and lead on the proton gradient of synaptic vesicles and glutamate transport. Neurochem Int. 59, 272-279.

Bridges C.C., Zalups R.K. 2005. Molecular and ionic mimicry and the transport of toxic metals. Toxicol Appl Pharmacol. 204, 274–308.

Buizza. L., Prandelli. C., Bonini. S.A., Delbarba. A., Cenini. G., Lanni. C., Buoso. E., Racchi. M., Govoni. S., Memo. M., Uberti. D. 2013. Conformational altered p53 affects neuronal function: relevance for the response to toxic insult and growth-associated protein 43 expression. Cell Death Dis. 4, e484.

Calderon-Garciduenas, L., Azzarelli, B., Acuna, H., Garcia, R., Gambling, T.M., Osnaya, N., Monroy, S., DEL Tizapantzi, M.R., Carson J.L., Villarreal-Calderon, A., Rewcastle, B., 2002. Air pollution and brain damage. Toxicol. Pathol. 30, 373–389.

Calderon-Garciduenas, L., Maronpot, R.R., Torres-Jardon, R., Henríquez-Roldán, C., Schoonhoven, R., Acuña-Ayala, H., Villarreal-Calderón, A., Nakamura, J., Fernando, R., Reed, W., Azzarelli, B., Swenberg, J.A., 2003. DNA damage in nasal and brain tissues of canines exposed to air pollutants is associated with evidence of chronic brain inflammation and neurodegeneration. Toxicol. Pathol. 31, 524–538.

Cañón, E., Cosgaya, J.M., Scsucova, S., Aranda, A., 2004. Rapid effects of retinoic acid on CREB and ERK phosphorylation in neuronal cells. Mol Biol Cell 15, 5583–5592.

Cardoso, B.R., Roberts, B.R., Bush, A.I., Hare, D.J., 2015. Selenium, selenoproteins and neurodegenerative diseases. Metallomics 7, 1213-1228.

Chen, L., Liu, L., Huang, S., 2008. Cadmium activates the mitogen-activated protein kinase (MAPK) pathway via induction of reactive oxygen species and inhibition of protein phosphatases 2A and 5. Free Radic. Biol. Med. 45, 1035-1044.

Chen, N., Onisko, B., Napoli, J.L., 2008. The nuclear transcription factor RARα associates with neuronal RNA granules and suppresses translation. J Biol Chem 283, 20841-20847.

Chen, S., Ren, Q., Zhang, J., Ye, Y., Zhang, Z., Xu, Y., Guo, M., Ji, H., Xu, C., Gu, C., Gao, W., Huang, S., Chen, L. 2014. N-acetyl-L-cysteine protects against cadmium-induced neuronal apoptosis by inhibiting ROS-dependent activation of Akt/mTOR pathway in mouse brain. Neuropathol. Appl. Neurobiol. 40, 759-777.

Chen, C.Y., Zhang, S.L., Liu, Z.Y., Tian, Y., Sun, Q., 2015. Cadmium toxicity induces ER stress and apoptosis via impairing energy homoeostasis in cardiomyocytes. Biosci Rep. 35, e00214.

Cheung, Y.T., Lau, W.K., Yu, M.S., Lai, C.S., Yeung, S.C., So, K.F., Chang, R.C., 2009. Effects of all-trans-retinoic acid on human SH-SY5Y neuroblastoma as in vitro model in neurotoxicity research. Neurotoxicology 30, 127-135.

Clatworthy. A.E., Stockinger. W., Christie. R.H., Schneider. W.J., Nimpf. J., Hyman. B.T., Rebeck. G.W., 1999. Expression and alternate splicing of apolipoprotein E receptor 2 in brain. Neuroscience. 90, 903-911.

Cotzias, G.C., Borg, D.C., Selleck, B., 1961. Virtual absence of turnover in cadmium metabolism: Cd109 studies in the mouse. Am. J. Physiol. 201, 927-930.

Del Pino J., Zeballos G., Anadon M.J., Capo M.A., Díaz M.J., García J., Frejo M.T. 2014. Higher sensitivity to cadmium induced cell death of basal forebrain cholinergic neurons: a cholinesterase dependent mechanism. Toxicology. 325, 151-159.

Dwane, S., Durack, E., Kiely, P.A., 2013. Optimising parameters for the differentiation of SH-SY5Y cells to study cell adhesion and cell migration. BMC Res. Notes 6, 366.

Ebadi, M., Iversen, P.L., Hao, R., Cerutis, D.R., Rojas Castaneda, P., Happe, H.K., Murrin, L.C., Pfeiffer, R.F., 1995. Expression and regulation of brain metallothionein, Neurochem. Int. 27, 1–22.

Egan SK., Bolger P.M., Carrington C.D. 2007. Update of US FDA's Total Diet Study food list and diets. J Expo Sci Environ Epidemiol. 17, 573-582.

Faria J., Barbosa J., Queirós O., Moreira R., Carvalho F., Dinis-Oliveira R.J. 2016. Comparative study of the neurotoxicological effects of tramadol and tapentadol in SH-SY5Y cells. Toxicology. 359, 1-10.

Farrar, N.R., Dmetrichuk, J.M., Carlone, R.L., Spencer, G.E., 2009. A novel, nongenomic mechanism underlies retinoic acid-induced growth cone turning. J Neurosci. 29, 14136-14142.

Fern, R., Black, J.A., Ransom, B.R., Waxman, S.G., 1996. Cd^{2+}-induced injury in CNS white matter. Journal of Neurophysiology 76, 3264–3273.

Flohé L. 1982. Glutathione peroxidase brought into focus. In Free Radicals in Biology Ed. Pryor W. A. 5, 223–254. Academic Press, New York.

Galehdar Z., Swan P., Fuerth B., Callaghan S.M., Park D.S., Cregan S.P. 2010. Neuronal apoptosis induced by endoplasmic reticulum stress is regulated by ATF4-CHOP-mediated induction of the Bcl-2 homology 3-only member PUMA. J Neurosci. 30, 16938–16948.

Gonçalves, J.F., Fiorenza, A.M., Spanevello, R.M., Mazzanti, C.M., Bochi, G.V., Antes, F.G., Stefanello, N., Rubin, M.A., Dressler, V.L., Morsch, V.M., Schetinger, M.R., 2010. N-acetylcysteine prevents memory deficits, the decrease in acetylcholinesterase activity and oxidative stress in rats exposed to cadmium. Chemico-Biological Interactions. 186, 53–60.

Goyer R.A., Clarkson T.W. 2001. Toxic effects of metals. In: Klaassen C., editor. Casarett and Doull's Toxicology: The Basic Science of Poisons. 6th ed. McGraw-Hill Health Professions Division; New York, NY, USA, pp. 822–826.

Gunn, S.A., Gould, T.C., Anderson, W.A., 1968 Mechanisms of zinc, cysteine and selenium protection against cadmium induced vascular injury to mouse testis. J. Reprod. Fertil. 15, 65-70.

Gupta A., Murthy R.C., Thakur S.R., Dubey M.P., Chandra SV. 1990. Comparative neurotoxicity of cadmium in growing and adult rats after repeated administration. Biochem Int. 21, 97–105.

Gutierrez-Reyes E.Y., Albores A., Rios C. 1998. Increase of striatal dopamine release by cadmium in nursing rats and its prevention by dexamethasone-induced metallothionein. Toxicology. 131, 145-154.

Hartwig A. 2001. Zinc finger proteins as potential targets for toxic metal ions: differential effects on structure and function. *Antioxid Redox Signaling*. **3**, 625–634.

Hendrickx W., Decock J., Mulholland F., Bao Y., Fairweather-Tait S. 2013. Selenium biomarkers in prostate cancer cell lines and influence of selenium on invasive potential of PC3 cells. Front Oncol. 3, 239.

Hernandez-Martinez, J.M., Forrest, C.M., Darlington, L.G., Smith, R.A., Stone, T.W., 2017. Quinolinic acid induces neuritogenesis in SH-SY5Y neuroblastoma cells independently of NMDA receptor activation. Eur. J. Neurosci. 45, 700-711.

Heusinkveld H.J., Westerink R.H.S. 2017. Comparison of different in vitro cell models for the assessment of pesticide-induced dopaminergic neurotoxicity. Toxicol In Vitro. 45, 81-88.

Hidalgo, J., Aschner, M., Zatta, P., Vasa´k, M., 2001. Roles of the metallothionein family of proteins in the central nervous system. Brain Res. Bull. 55, 133–145.

Hitomi, J., Katayama, T., Taniguchi, M., Honda, A., Imaizumi, K., Tohyama, M., 2004. Apoptosis induced by endoplasmic reticulum stress depends on activation of caspase-3 via caspase-12. Neurosci Lett. 357, 127-30.

Howard, H., 2002. Human health and heavy metals exposure, in: McCally, M., (Ed.) Life Support: The Environment and Human Health. MIT press, Cambridge, pp. 1-13.

Järup L., Akesson A. 2009. Current status of cadmium as an environmental health problem. Toxicol Appl Pharmacol. 238, 201-208.

Jin, T., Lu, J., Nordberg M., 1998. Toxico kinetics and biochemistry of cadmium with special emphasis on the role of metallothionein. NeuroToxicology 19, 529–536.

Kim R., Emi M., Tanabe K., Murakami S. 2006. Role of the unfolded protein response in cell death. Apoptosis. 11, 5–13.

Kim, S., Cheon, H.S., Kim, S.Y., Juhnn, Y.S., Kim, Y.Y., 2013. Cadmium induces neuronal cell death through reactive oxygen species activated by GADD153. BMC Cell Biol. 14, 4.

Kovalevich J., Langford D. 2013. Considerations for the use of SH-SY5Y neuroblastoma cells in neurobiology. Methods Mol Biol. 1078, 9-21.

Larsen E.H., Andersen N.L., Møller A., Petersen A., Mortensen G.K., Petersen J. 2002. Monitoring the content and intake of trace elements from food in Denmark. Food Addit Contam. 19, 33-46.

Lee, A.S., 2005. The ER chaperone and signaling regulator GRP78/BiP as a monitor of endoplasmic reticulum stress. Methods. 35, 373-81.

Liao, Y.P., Ho, S.Y., Liou, J.C., 2004. Non-genomic regulation of transmitter release by retinoic acid at developing motoneurons in Xenopus cell culture. J Cell Sci 117, 2917-2924.

Liu M.C., Xu Y., Chen Y.M., Li J., Zhao F., Zheng G., Jing J.F., Ke T., Chen J.Y., Luo W.J. 2013. The effect of sodium selenite on lead induced cognitive dysfunction. Neurotoxicology. 36, 82–88.

Liu, W., Zhao, H., Wang, Y., Jiang, C., Xia, P., Gu, J., Liu, X., Bian, J., Yuan, Y., Liu, Z., 2014 Calcium-calmodulin signaling elicits mitochondrial dysfunction and the release of cytochrome c during cadmium-induced apoptosis in primary osteoblasts. Toxicol. Lett. 224, 1-6.

Llobet J.M., Falcó G., Casas C., Teixidó A., Domingo J.L. 2003. Concentrations of arsenic, cadmium, mercury, and lead in common foods and estimated daily intake by children, adolescents, adults, and seniors of Catalonia, Spain. J Agric Food Chem. 51, 838-842.

Lopes F.M., Schroder R., da Frota M.L. Jr, Zanotto-Filho A., Muller C.B., Pires A.S., Meurer R.T., Colpo G.D., Gelain D.P., Kapczinski F., Moreira J.C., Fernandes Mda C., Klamt F. 2010. Comparison between proliferative and neuron-like SH-SY5Y cells as an in vitro model for Parkinson disease studies. Brain Res. 1337, 85–94.

Lu. J., Zhang. F., Yuan. Y., Ding. C., Zhang. L., Li. Q., 2013. All-trans retinoic acid upregulates the expression of p53 via Axin and inhibits the proliferation of glioma cells. Oncol Rep. 29, 2269-2274.

Luchese C., Brandão R., de Oliveira R., Nogueira C.W., Santos F.W. 2007. Efficacy of diphenyl diselenide against cerebral and pulmonary damage induced by cadmium in mice. Toxicol Lett. 173, 181–190.

Marlowe, M., Errera, J., Jacobs, J., 1983. Increased lead and cadmium burdens among mentally retarded children and children with borderline intelligence. American Journal of Mental Deficiency 87, 477–483.

MacIntosh D.L., Spengler J.D., Ozkaynak H., Tsai L., Ryan P.B. 1996. Dietary exposures to selected metals and pesticides. Environ Health Perspect. 104, 202-209.

McCarty M.F. 2012. Zinc and multi-mineral supplementation should mitigate the pathogenic impact of cadmium exposure. Med. Hypotheses. 79, 642–648.

Mead, M.N., 2010. Cadmium confusion: do consumers need protection? Environ. Health Perspect. 118, a528-a534.

Méndez-Armenta, M., Rios, C., 2007. Cadmium neurotoxicity. Environ. Toxicol. Pharmacol. 23, 350–358.

Monroe, R.K., Halvorsen, S.W., 2009. Environmental toxicants inhibit neuronal Jak tyrosine kinase by mitochondrial disruption. Neurotoxicology. 30, 589-598.

Nalvarte, I., Damdimopoulos. A.E., Rüegg. J., Spyrou. G., 2015. The expression and activity of thioredoxin reductase 1 splice variants v1 and v2 regulate the expression of genes associated with differentiation and adhesion. Biosci. Rep. 35, e00269.

Newairy, A.A., El-Sharaky, A.S., Badreldeen, M.M., Eweda, S.M., Sheweita, SA., 2007. The hepatoprotective effects of selenium against cadmium toxicity in rats. Toxicology 242(1-3), 23-30.

Nordberg M., Nordberg G. 2000. Toxicological aspects of metallothionein. Cell Mol Biol. 46, 451–463.

Olsson I.M., Bensryd I., Lundh T., Ottosson H., Skerfving S., Oskarsson A. 2002. Cadmium in blood and urine--impact of sex, age, dietary intake, iron status, and former smoking--association of renal effects. Environ Health Perspect. 110, 1185-1190.

Pacini S., Fiore M.G., Magherini S., Morucci G., Branca J.J., Gulisano M., Ruggiero M., 2012. Could cadmium be responsible for some of the neurological signs and symptoms of Myalgic Encephalomyelitis/Chronic Fatigue Syndrome. Med Hypotheses. 79, 403-407.

Pak, E.J., Son, G.D., Yoo, B.S., 2014. Cadmium inhibits neurite outgrowth in differentiating human SH-SY5Y neuroblastoma cells. Int. J. Toxicol. 33, 412-418.

Panayi, A.E., Spyrou, N.M., Iversen, B.S., White, M.A., Part, P., 2002. Determination of cadmium and zinc in Alzheimer's brain tissue using inductively coupled plasma mass spectrometry. J. Neurol. Sci. 195, 1-10.

Papadakis, E.S., Finegan, K.G., Wang, X., Robinson, A.C., Guo, C., Kayahara, M., Tournier, C., 2006. The regulation of Bax by c-Jun N-terminal protein kinase (JNK) is a prerequisite to the mitochondrial-induced apoptotic pathway. FEBS Lett. 580, 1320-1326.

Polson, A.K., Sokol, M.B., Dineley, K.E., Malaiyandiet L.M., 2011. Matrix cadmium accumulation depolarizes mitochondria isolated from mouse brain. Impulse 2011, 1-8.

Prasanthi R., Devi C.B., Basha D.C., Reddy N.S., Reddy G.R. 2010. Calcium and zinc supplementation protects lead (Pb)-induced perturbations in antioxidant enzymes and lipid peroxidation in developing mouse brain. Int J Dev Neurosci. 28, 161–167.

Presgraves S.P., Ahmed T., Borwege S., Joyce J.N. 2004. Terminally differentiated SH-SY5Y cells provide a model system for studying neuroprotective effects of dopamine agonists. Neurotox Res. 5, 579–598.

Pulido, P, Kägi, J.H., Vallee, B.L., 1966. Isolation and some properties of human metallothionein. Biochemistry 5, 1768-1777.

Raevskii V.V., Tegetmayer C., Trifonov A.A. 1993. Does the destruction of catecholaminergic neurons in newborn rats influence the modulator function of the cholinergic system? Neurosci Behav Physiol. 23, 529-533.

Rigobello, M.P., Scutari, G., Friso, A., Barzon, E., Artusi, S., Bindoli, A., 1999. Mitochondrial permeability transition and release of cytochrome c induced by retinoic acids. Biochem. Pharmacol. 58, 665-670.

Sallmon, H., Hoene, V., Weber, S.C., Dame, C., 2010. Differentiation of human SH-SY5Y neuroblastoma cells by all-trans retinoic acid activates the interleukin-18 system. J. Interferon Cytokine Res. 30, 55-58.

Shukla, G.S., Chandra, S.V., 1987. Concurrent exposure to lead, manganese, and cadmium and their distribution to various brain regions, liver, kidney, and testis of growing rats. Archives of Environmental Contamination and Toxicology. 16, 303–310.

Shukla, A., Shukla, G.S., Srimal, R.C., 1996. Cadmium-induced alterations in blood-brain barrier permeability and its possible correlation with decreased microvessel antioxidant potential in rat. Hum. Exp. Toxicol. 15, 400-405.

Snutch, T.P., Peloquin, J., Mathews, E., McRory, J.E., 2000-2013. Molecular Properties of Voltage-Gated Calcium Channels, Madame Curie Bioscience Database, Landes Bioscience, Austin (TX).

Suzuki C.A., Ohta H., Albores A., Koropatnick J., Cherian M.G. 1990. Induction of metallothionein synthesis by zinc in cadmium pretreated rats. Toxicology. 63, 273–284.

Szuster-Ciesielska A., Stachura A., Słotwińska M., Kamińska T., Sniezko R., Paduch R., Abramczyk D., Filar J., Kandefer-Szerszeń M. 2000. The inhibitory effect of zinc on cadmium-induced cell apoptosis and reactive oxygen species (ROS) production in cell cultures. Toxicology. 145, 159-171.

Thatcher, R.W., Lester, M.L., McAlaster, R., Horst, R., 1982. Effects of low levels of cadmium and lead on cognitive functioning in children. Archives of Environmental Health 37, 159–166.

Thévenod F. 2009. Cadmium and cellular signaling cascades: to be or not to be? Toxicol Appl Pharmacol. 238, 221-239.

Thomas K.W., Pellizzari E.D., Berry M.R. 1999. Population-based dietary intakes and tap water concentrations for selected elements in the EPA region V National Human Exposure Assessment Survey (NHEXAS). J Expo Anal Environ Epidemiol. 9, 402-413.

ToxGuide™ for Cadmium, CAS# 7440-43-9, U.S. Department of Health and Human Services, Public Health Service, Agency for Toxic Substances and Disease Registry (www.atsdr.cdc.gov) October 2012.

Trépanier G., Furling D., Puymirat J., Mirault M.E. 1996. Immunocytochemical localization of seleno-glutathione peroxidase in the adult mouse brain. Neuroscience. 1996 75, 231-243.

Usai C., Barberis A., Moccagatta L., Marchetti C., 1999. Pathways of cadmium influx in mammalian neurons. J. Neurochem. 72, 2154-2161.

Volpe, A.R., Cesare, P., Almola, P., Boscolo, M., Valle, G., Carmignani, M., 2011. Zinc opposes genotoxicity of cadmium and vanadium but not of lead. J. Biol. Regul. Homeost. Agents 25, 589-601.

Wang, Y., Fang, J., Leonard, S.S., Rao, K.M., 2004. Cadmium inhibits the electron transfer chain and induces reactive oxygen species. Free Radic. Biol. Med. 36, 1434-1443.

Watjen, W., Beyersmann, D., 2004. Cadmium-induced apoptosis in C6 glioma cells: influence of oxidative stress. Biometals 17, 65-78.

Xu, B., Chen, S., Luo, Y., Chen, Z., Liu, L., Zhou, H., Chen, W., Shen, T., Han, X., Chen, L., Huang, S., 2011. Calcium signaling is involved in cadmium-induced neuronal apoptosis via induction of reactive oxygen species and activation of MAPK/mTOR network. PLoS One 6, e19052.

Xu, C., Huang, S., Chen, L., 2017. An insight of rapamycin against cadmium's neurotoxicity. Oncotarget 8, 9013-9014.

Xun, Z., Lee, D.Y., Lim, J., Canaria, C.A., Barnebey, A., Yanonne, S.M., McMurray, C.T., 2012. Retinoic acid-induced differentiation increases the rate of oxygen consumption and enhances the spare respiratory capacity of mitochondria in SH-SY5Y cells. Mech. Ageing Dev. 133, 176-185.

Yokouchi, M., Hiramatsu, N., Hayakawa, K., Okamura, M., Du, S., Kasai, A., Takano, Y., Shitamura, A., Shimada, T., Yao, J., Kitamura, M., 2008. Involvement of selective reactive oxygen species upstream of proapoptotic branches of unfolded protein response. J Biol Chem. 283, 4252-60.

Ysart G., Miller P., Croasdale M., Crews H., Robb P., Baxter M., de L'Argy C., Harrison N. 2000. 1997 UK Total Diet Study--dietary exposures to aluminium, arsenic, cadmium, chromium, copper, lead, mercury, nickel, selenium, tin and zinc. Food Addit Contam. 17, 775-786.

Yuan, Y., Jiang, C.Y., Xu, H., Sun, Y., Hu, F.F., Bian, J.C., Liu, X.Z., Gu, J.H., Liu, Z.P., 2013. Cadmium-induced apoptosis in primary rat cerebral cortical neurons culture is mediated by a calcium signaling pathway. PLoS One 8, e64330.

Yuan, Y., Wang, Y., Hu, F.F., Jiang, C.Y., Zhang, Y.J., Yang, J.L., Zhao, S.W., Gu, J.H., Liu, X.Z., Bian, J.C., Liu, Z.P. 2016. Cadmium activates reactive oxygen species-dependent AKT/mTOR and mitochondrial apoptotic pathways in neuronal cells. Biomed Environ Sci. 29,117-126.

Zhai Q., Narbad A., Chen W. 2015. Dietary Strategies for the Treatment of Cadmium and Lead Toxicity. Nutrients. 7, 552–571.

Zhang, M., Zheng, J., Nussinov, R., Ma, B., 2017. Release of cytochrome C from Bax pores at the mitochondrial membrane. Sci Rep. 7, 2635.

FIGURE LEGEND

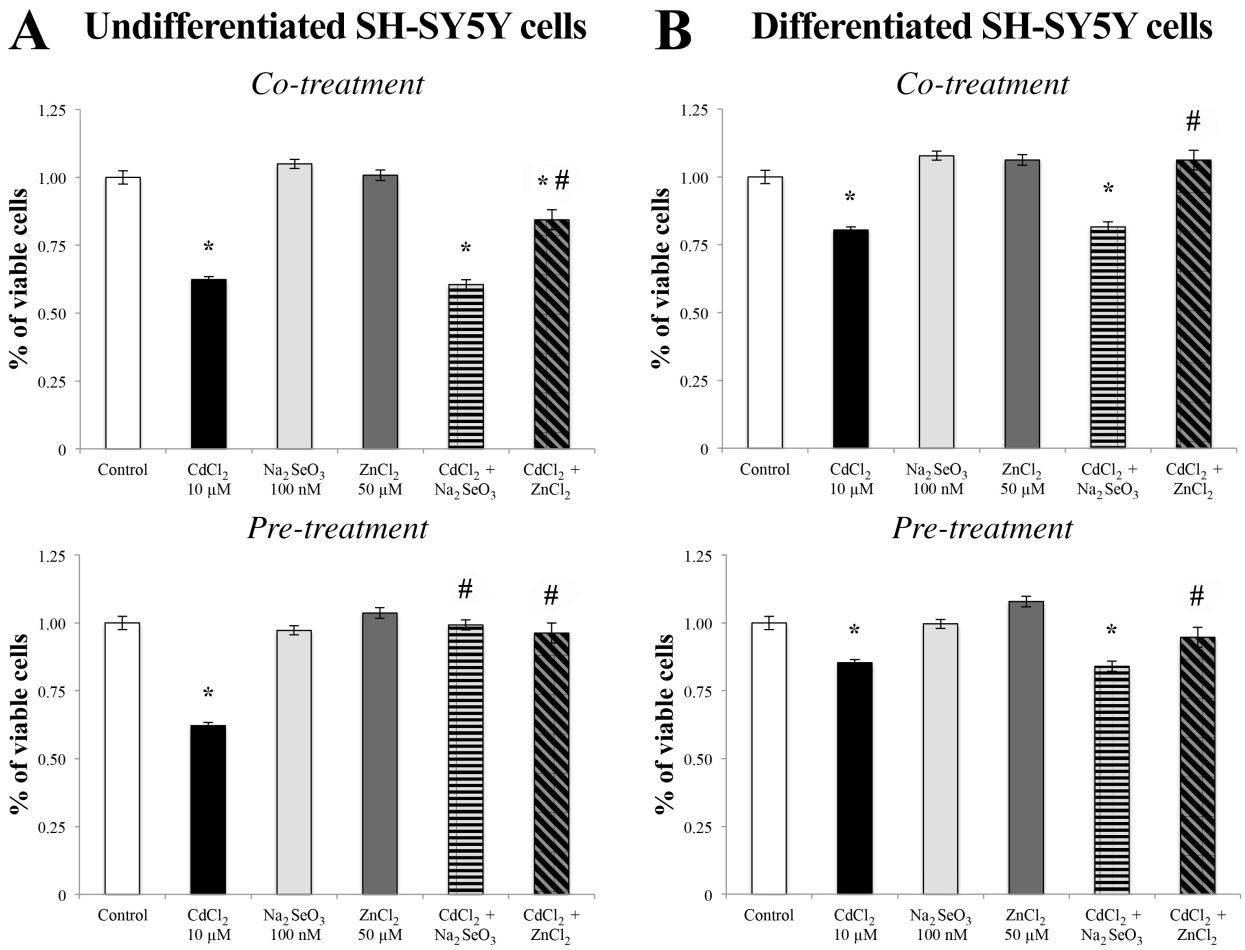


Figure 1. SH-SY5Y cell viability (MTT) assay.

Effect of $CdCl_2$, $ZnCl_2$, and Na_2SeO_3 on SH-SY5Y cell viability. ***Panel A***. cell viability was determined by MTT assay after co-treatment (left histograms) and pre-treatment (right histograms) of undifferentiated SH-SY5Y cells with 10 µM $CdCl_2$ for 24 h in the presence of 50 µM $ZnCl_2$ or 100 nM Na_2SeO_3. A Cd-dependent significant reduction of cell viability was seen ($^{*}p<0.05$ vs control cells). A Zn significant prevention on Cd neurotoxicity was evidenced in both treatment types, whereas Se significantly counteracted the Cd-dependent decrease of cellular viability only during pre-teratment ($^{\#}p<0.05$ *vs*. 10 µM $CdCl_2$ treated cells). ***Panel B***. Cell viability in RA differentiated cells. Left histograms (co-treatment) reflected the same results evidenced for undifferentiated cells, as well as Se showed no effect in preserving the decrease in cell viability ($^{\#}p<0.05$ *vs*. 10 µM $CdCl_2$ treated cells). Untreated cells were taken as 100%. Results are expressed as mean value ± S.E.M. The data shown represent the typical data from three independent experiments that yielded similar results. Each experiment point was performed in quintuplicate.

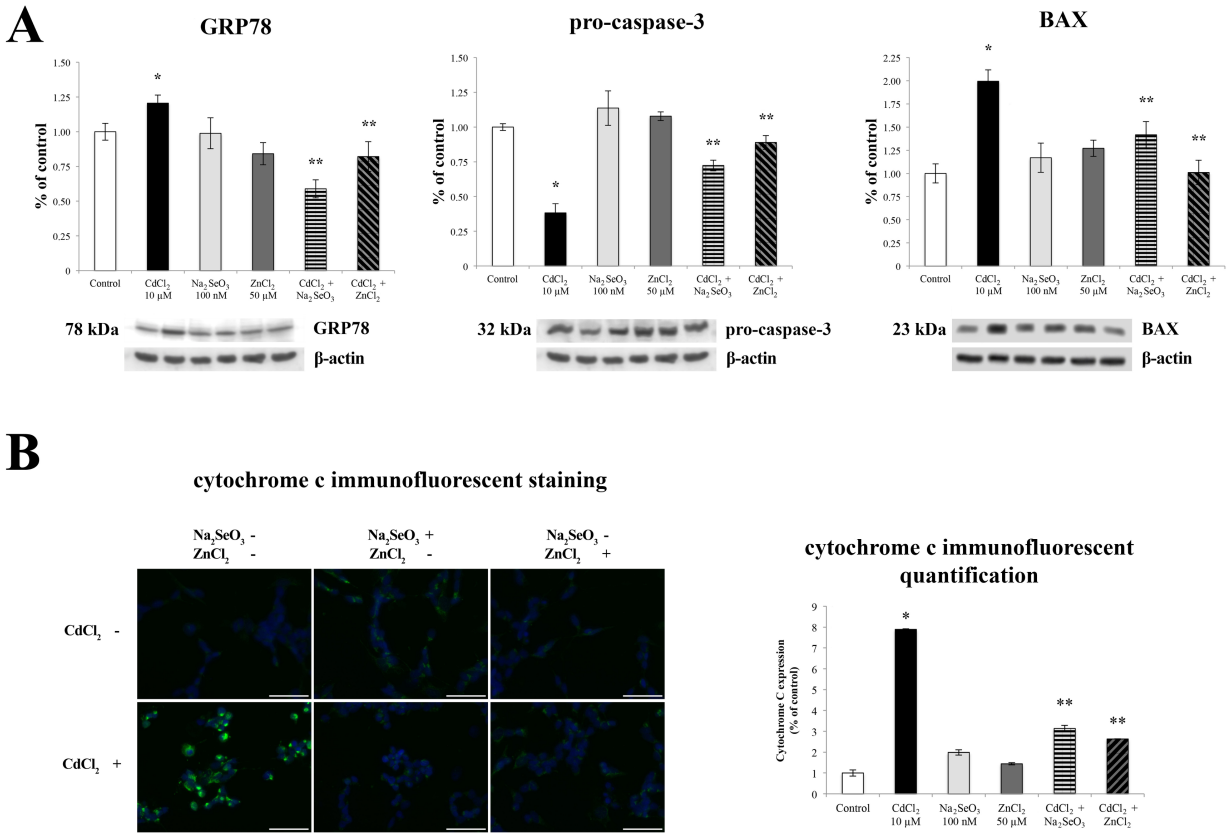


Figure 2. ROS production in SH-SY5Y cells.

SH-SY5Y cells were pre-treated with 50 µM $ZnCl_2$ or 100 nM Na_2SeO_3 in the presence of 10 µM $CdCl_2$ for 24 h and then underwent to evaluation of ROS expression by the 2',7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) probe. ***Panel A***. Representative fluorescent microscope images showing intracellular ROS levels in undifferentiated cells; fluorescence semi-quantitative analysis (histograms in figure 2A) showed that Se and Zn treatments hindered the Cd-dependent ROS formation. ***Panel B***. Representative fluorescent microscope images showing intracellular ROS levels in RA differentiated cells; semi-quantitative fluorescence analysis (histograms in figure 2B) confirmed that only Zn treatment was able to partially prevent ROS formation. Representative images are shown. Total magnification: 400X and scale bar = 50 µm. The intracellular ROS-derived fluorescence is expressed as the percentage of fluorescence compared with untreated cells. Data are expressed as mean ± S.E.M.. ($^{*}p<0.05$ *vs* control; $^{\#}p<0.05$ *vs* $CdCl_2$ treated cells).

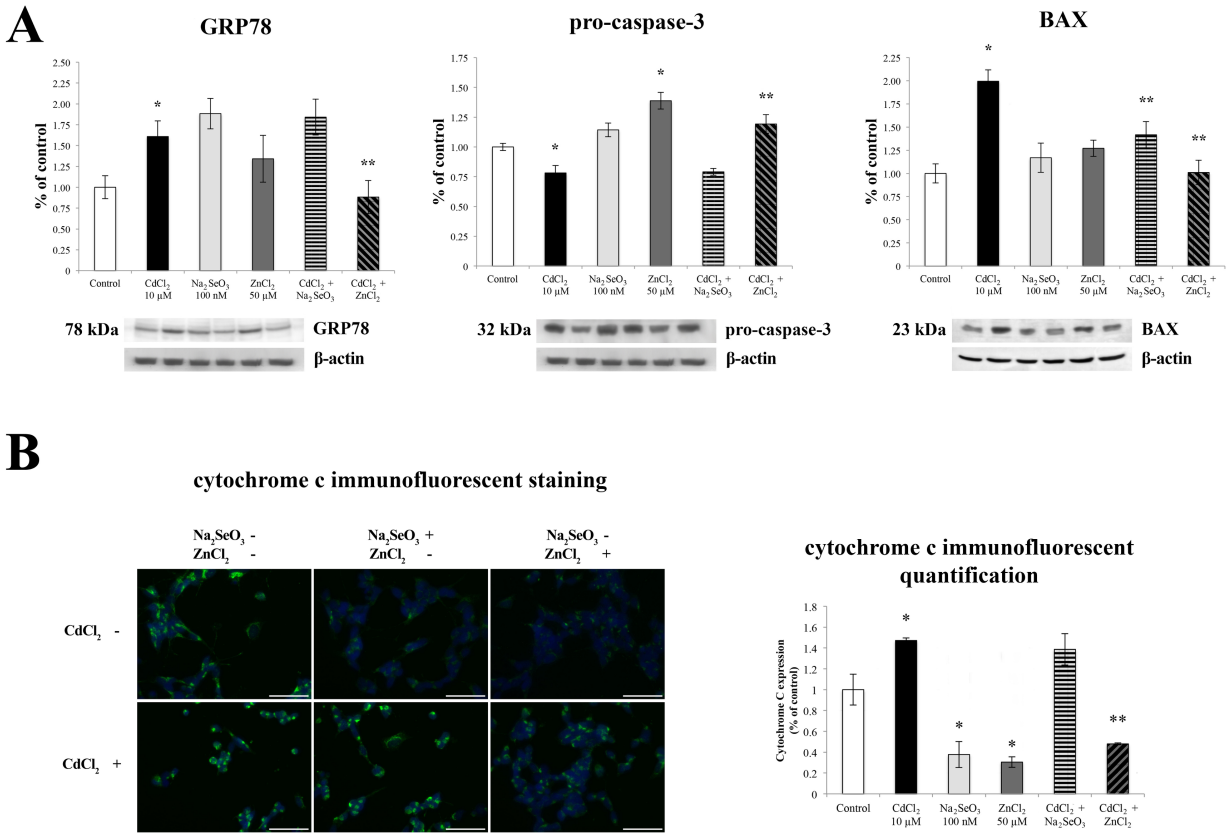


Figure 3. Undifferentiated SH-SY5Y apoptotic pathway: GRP78, caspase-3, BAX, and cytochrome c analysis.

Panel A. Cd-dependent apoptotic pathway in undifferentiated cells. Western blotting analysis showed that $CdCl_2$ significantly ($^{*}p<0.05$ *vs* control) increase SH-SY5Y ER stress, up-regulating GRP78 expression level (left panel). Concurrently with the increase of GRP78, Cd-treated cells showed a significant ($^{*}p<0.05$ *vs* control) decrease of pro-caspase-3 levels (middle panel) paralleled by an increase in its enzymatic activity (***Panel B***), and of BAX protein expression (right panel). The pre-treatment with Zn and Se significantly ($^{\#}p<0.05$ *vs* $CdCl_2$ treated cells) prevented all these cellular stressor markers. β-actin was used as an internal control. Control cells were taken as 100 %. Results are expressed as mean ± S.E.M. The data shown represent the typical data from three independent experiments that yielded similar results. Each experiment was performed in triplicate. ($^{*}p<0.05$ *vs* control; $^{\#}p<0.05$ *vs* $CdCl_2$ treated cells). ***Panel C***. Cytochrome c immunofluorescent staining. The semi-quantitative cytochrome c analysis evidenced a significative ($^{*}p<0.05$ *vs* control) increase in immunofluorescent labelling in $CdCl_2$ treated cells. The presence of $ZnCl_2$ or 100 nM Na_2SeO_3 significantly ($^{\#}p<0.05$ *vs* $CdCl_2$ treated cells) prevented the cytoplasmic increase of cytochrome c in the cell soma. Control cells were taken as 100 %. Results were expressed as mean ± S.E.M. The data shown represent the typical data from three independent experiments that yielded similar results.

Each experiment point was performed in triplicate. (*p<0.05 *vs* control; $^{\#}$p<0.05 *vs* $CdCl_2$ treated cells). Total magnification: 400x and scale bar: 50 µm.

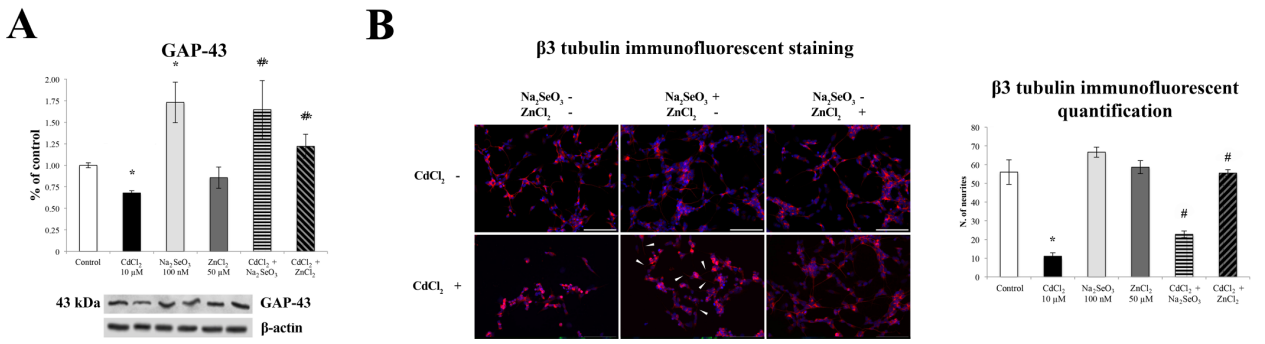


Figure 4. RA differentiated SH-SY5Y apoptotic pathway: GRP78, pro-caspase-3, BAX, and cytochrome c analysis.

Panel A. Effects of Zn and Se on the expression of GRP78, pro-caspase-3, and BAX, as well as on the enzymatic proteolytic activity of caspase-3 (***Panel B***) in RA differentiated SH-SY5Y cells. β-actin was used as an internal control. Quantitative analysis showed that the presence of Zn was able to prevent the Cd-dependent onset of the ER apoptotic pathway ($^{\#}$p<0.05 *vs* $CdCl_2$ treated cells), whereas Se was ineffective to prevent Cd neurotoxicity. Control condition was arbitrarily set as 100 % and results are expressed as mean ± S.E.M. The data shown represent the typical data from three independent experiments that yielded similar results. Each experiment point was performed in triplicate. (*p<0.05 *vs* control; $^{\#}$p<0.05 *vs* $CdCl_2$ treated cells). ***Panel C***. The semi-quantitative cytochrome c analysis evidenced a significative (*p<0.05 *vs* control) increase in immunofluorescent labelling in $CdCl_2$ treated cells. The presence of Zn ($^{\#}$p<0.05 *vs* $CdCl_2$ treated cells) but not Se prevented the cytoplasmic increase of cytochrome c in the cell soma. Five microscopic fields for each experimental point were analysed. Control cells were taken as 100 %. Results are expressed as mean ± S.E.M. The data shown represent the typical data from three independent experiments that yielded similar results. Each experiment was performed in triplicate. (*p<0.05 *vs* control; $^{\#}$p<0.05 *vs* $CdCl_2$ treated cells). Total magnification: 400x and scale bar = 50 µm.

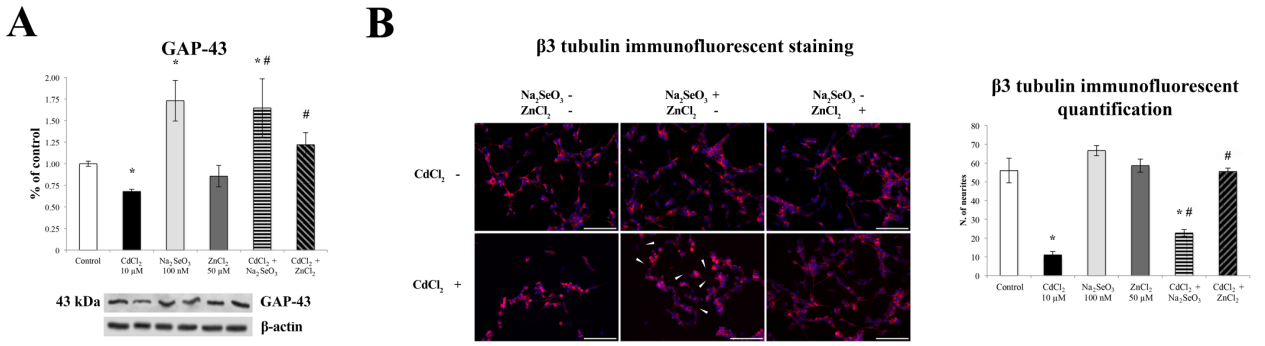


Figure 5. Undifferentiated SH-SY5Y neurite outgrowth.

Panel A. 50 µM $ZnCl_2$ or 100 nM Na_2SeO_3 significantly ($^{\#}p<0.05$ *vs* $CdCl_2$ treated cells) prevented the $CdCl_2$-dependent downregulation of GAP-43 expression levels ($^{*}p<0.05$ *vs* control). Control cells were taken as 100 %. Results are expressed as mean ± S.E.M. ***Panel B***. The quantitative evaluation of β3 tubulin staining evidenced a strong and significative ($^{*}p<0.05$ *vs* control) decrease of cytoplasmic elongations in 10 µM $CdCl_2$ cells, that was partially prevented by the presence of 50 µM $ZnCl_2$ or 100 nM Na_2SeO_3 (white arrowheads). Results are expressed as mean ± S.E.M. All data shown represent the typical data from three independent experiments that yielded similar results. Each experiment was performed in triplicate. ($^{*}p<0.05$ *vs* control; $^{\#}p<0.05$ *vs* $CdCl_2$ treated cells). Total magnification: 200x and scale bar = 100 µm.

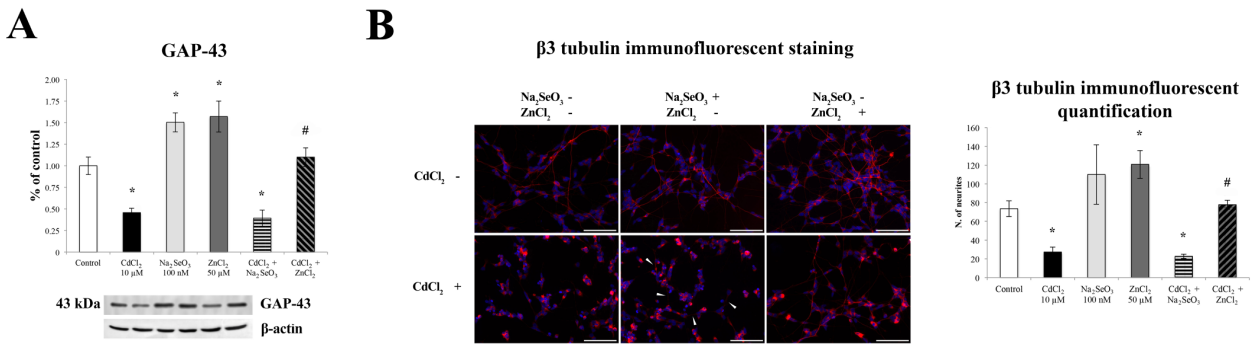
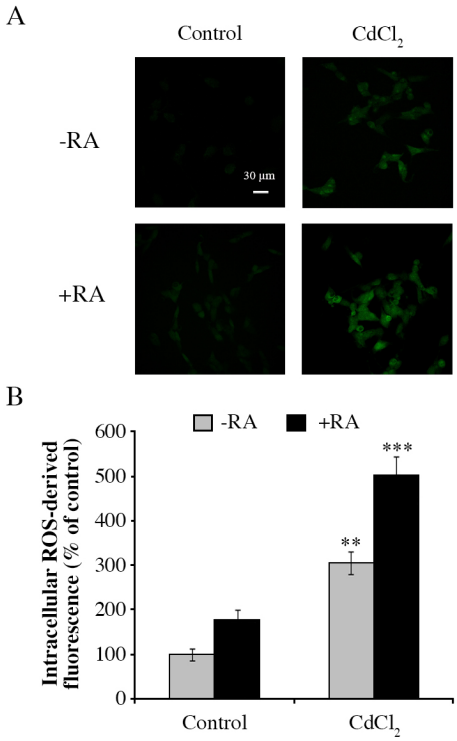
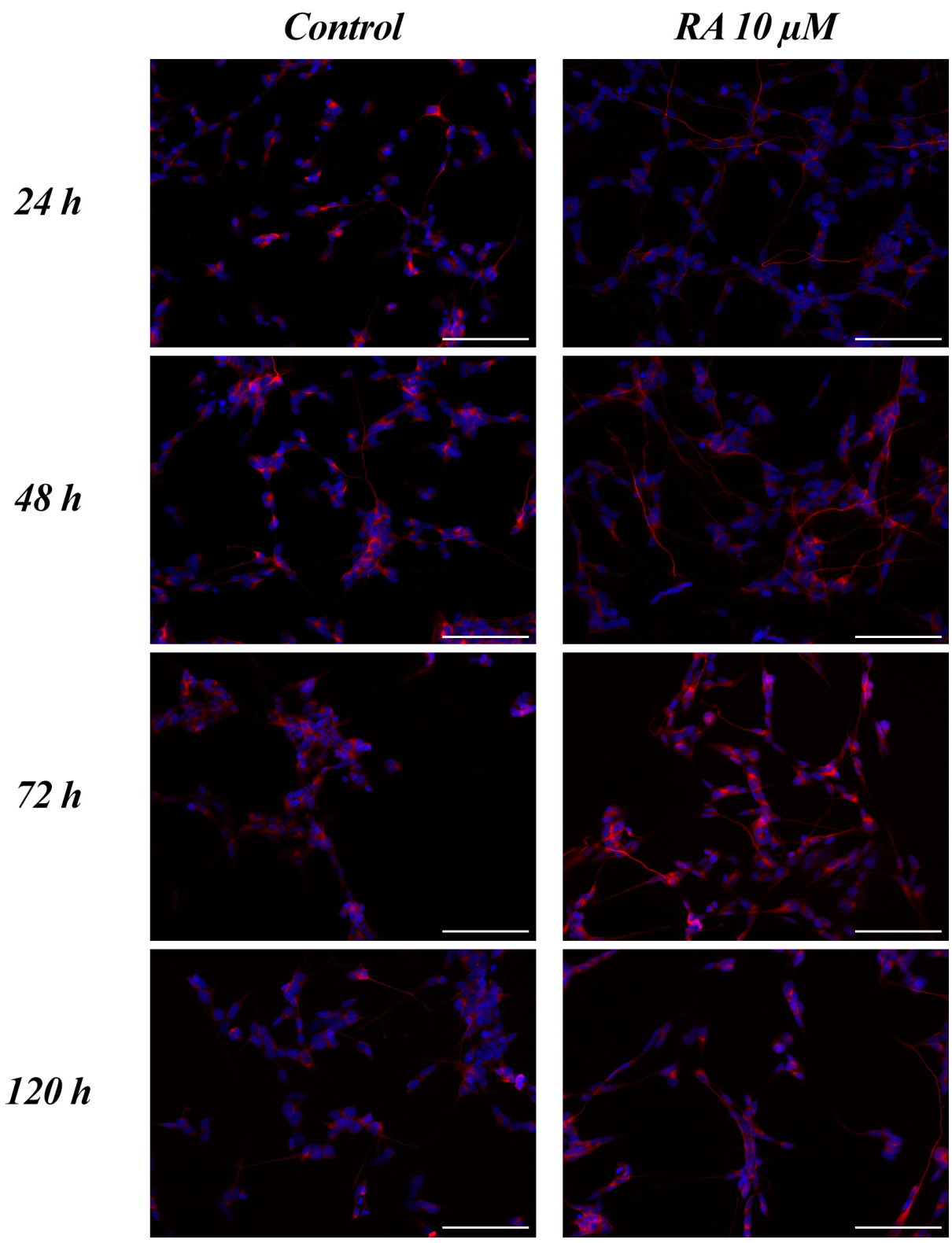


Figure 6. RA differentiated SH-SY5Y neurite outgrowth..

The Cd-dependent decrease of GAP-43 expression levels (***Panel A)*** and the β3 tubulin cytoplasmic elongations, as well as the protective ($^{\#}p<0.05$ *vs* $CdCl_2$ treated cells) effects of Zn treatment reflects the results obtained in undifferentiated cells (see figure 5). Conversely, 100 nM Na_2SeO_3 was not able to hinder the Cd neurotoxicity (***Panel B***). Results are expressed as mean ± S.E.M. Data were analysed as reported in figure 5 legend.



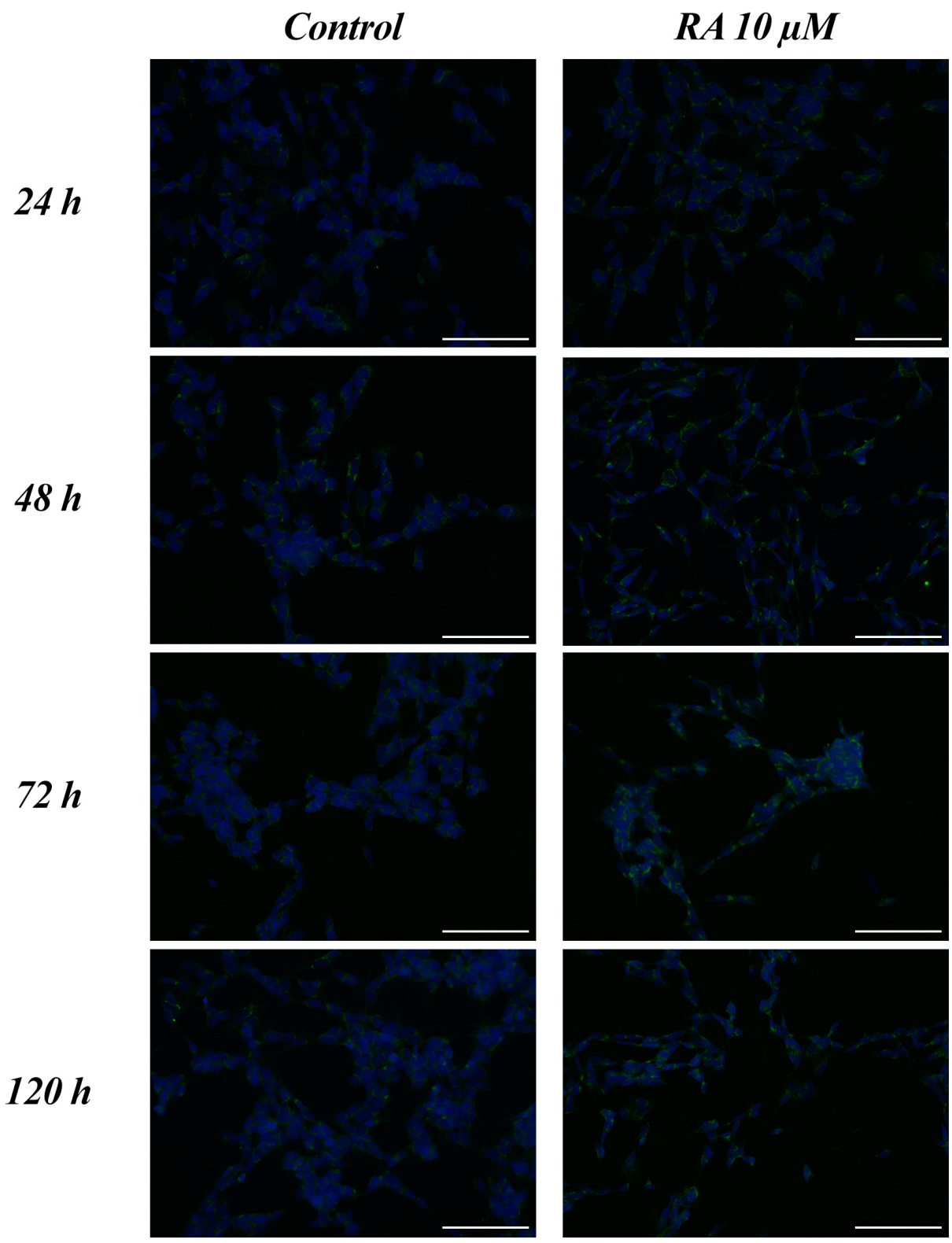
Supplementary Figure S1. Dose-response curves of $CdCl_2$, Na_2SeO_3, and $ZnCl_2$ on SH-SY5Y viability. Viability was measured by MTT assay at different concentration and at 24 h of treatment. Control cells were taken as 100%. Results are expressed as mean ± S.E.M. The data shown represent the typical data from three independent experiments that yielded similar results. Each experiment was performed in quintuplicate. ($^{*}p<0.05$ *vs* control).



Supplementary Figure S2. β3 tubulin immunofluorescent staining during RA differentiation. β3 tubulin labelling was performed to verify SH-SY5Y differentiation during treatment with 10 µM RA at different time points of exposure. As clearly evident, the cytoplasmic elongations increased in

a time-dependent manner. After 48 and 72 h there was a high presence of neurite as well as an increase in their length. To notice that, because of RA intrinsic toxicity, a long time exposure (120 h) induced a significant decrease in the number of branches arising from the cell bodies.

Five microscopic fields for each experimental point were analysed. Representative images were shown. Total magnification: 200x and scale bar = 100 μm.



Supplementary Figure S3. Cytochrome c immunofluorescent staining during RA differentiation.

Cytochrome c labelling was performed to verify if 10 μM RA treatment induce oxidative stress in a time-dependent manner. RA differentiated SH-SY5Y cells increased the expression of cytochrome c, starting from 48 h. No evidence of cytochrome c expression increase was seen after the treatment with 10 μM RA for 24 h.

Five microscopic fields for each experimental point were analysed. Representative images were shown. Total magnification: 400x and scale bar = 50 μm.