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Modulation of chelatable Zn pool in the brain by diethyldithiocarbamate is associated with behavioral impairment in adult zebrafish

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Abstract

The study of the effects of diethyldithiocarbamate (DEDTC) in some diseases is in focus for many years. However, DEDTC is a metal chelator that can present neurotoxicity as side effects. Here we investigate the effect of DEDTC on brain Zinc (Zn) content and behavior. To address this issue we used adult zebrafish exposed to different concentrations of DEDTC. The animal's behavioral parameters were evaluated during the exposure of DEDTC (0.2, 1, 5 mM in home tank water) for 1h. At the end of exposure period, the brain levels of DEDTC were measured. The analysis of reactive Zn content in different regions of the brain and in glutamatergic neurons and radial glial cells were performed using histochemical and immunocytochemical techniques, respectively. We also measured the activity of a Zn-dependent enzyme, δ-aminolevulinate dehydratase (δ-ALA-D). We found that DEDTC exposure at 1 and 5 mM induced seizure-like behavior in the zebrafish and death at 5 mM. DEDTC in the zebrafish brain was detected with exposure of 1 and 5 mM (above 100 $mg.kg^{-1}$ tissue). The reactive Zn was reduced in glutamatergic neurons after 1 and 5 mM DEDTC exposure in radial glial cells after 0.2 and 5 mM. No changes in brain δ-ALA-D activity were detected. Our results showed that DEDTC exposure can accumulate in the brain, leading to impairments in neural behavior and in the homeostasis of reactive Zn in the brain.

Keywords: brain; δ-aminolevulinate-dehydratase; diethyldithiocarbamate; reactive Zn; zebrafish

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Introduction

The use of organosulfur pesticides, like dithiocarbamates,[1-4] is restricted in several countries because of the toxic effets.[5-8] However, experimental studies have indicated that diethyldithiocarbamate (DEDTC) has beneficial properties on restraint stress,[9] ischemia-reperfusion,[10] hypoxic brain injury,[11] ototoxicity caused by cisplatin,[12] arthritis,[13] and cardiac allograft rejection[14] and also presents cytoprotective antioxidant activity[15,16] by triggering apoptosis in human hepatoma cells.[17] For all these reasons, the potential therapeutic use of DEDTC seems to be justified. On the other hand, the side effects induced by DEDTC are quite neglected.

The neurotoxic effect of DEDTC could be attributed to its ability to chelate essential metals, such as zinc (Zn).[18] In the brain, Zn is typically found bound to proteins[19,20] and, thus, Zn-dependent biomolecules are potential targets of DEDTC. One example is the δ-aminolevulinate dehydratase (δ-ALA-D), a key enzyme in the heme biosynthetic pathway that is modulated by Zn.[21,22] Additionally, there is a pool of Zn (chelatable or reactive Zn) in the central nervous system (CNS), susceptible to DEDTC.[23] Reactive Zn is abundantly located in the presynaptic vesicles of hippocampal glutamatergic neurons, is released at the synaptic cleft, and acts as a neuromodulator of excitatory receptors and glial glutamate transporters.[23] Therefore, DEDTC when used can accumulate in the CNS, resulting in brain Zn dyshomeostasis. Here we investigate the relationship between DEDTC, Zn dyshomeostasis, and behavioral impairment. To address this issue we used adult zebrafish (*Danio rerio*) as an animal model.

The zebrafish is an emergent vertebrate model in behavioral neuroscience studies. In addition to its low cost, easy maintenance, and abundant offspring, the species presents attractive features for toxicological research such as the quick absorption of compounds directly added to water and conserved genetic and biochemical

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mechanisms.[24] Particularly, the cellular Zn transporters of zebrafish embryos have been recently characterized, showing a high degree of similarity with their mammalian counterparts.[25] Moreover, a reactive pool of Zn is present throughout the CNS of zebrafish,[26] suggesting that Zn could play a role for brain homeostasis in this species. These characteristics make the zebrafish an appropriate model to investigate the behavioral changes caused by DEDTC via modulation of the Zn pool in the CNS.

Here, we aimed to investigate the acute effects of DEDTC. First, we assessed the influence on behavioral parameters by exposing adult animals to increasing concentrations of DEDTC. Afterwards, we measured the amount of reactive Zn in brain regions by histochemical staining and in glutamatergic neurons and radial glial cells by immunocytochemical analysis. In order to analyze whether DEDTC accumulates in the zebrafish brain and changes the activity of Zn-dependent enzymes, we measured the content of DEDTC and the δ-ALA-D activity in the brain tissue.

Material and Methods

Animals

A total of 220 male and female (50:50 ratio) wild-type adult zebrafish (4–7 months-old) of short-fin phenotype were obtained from commercial distributor (Delphis, RS, Brazil). Animals were kept in an aquarium rack system (Zebtec, Tecniplast, Italy) at 27 ± 1ºC under a light/dark cycle of 14/10 h (lights on at 7:00 am) for at least 2 weeks prior to the experiments. Animals were fed twice a day with commercial flake fish food (alcon BASIC®, Alcon, Brazil). All experiments were performed using reverse osmosis water supplemented with 0.018 mg/L Instant Ocean™ salt. Experimental procedures were conducted in accordance with National Institute of Health Guide for Care and Use

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of Laboratory Animals. The experimental protocols were approved by the Ethics Committee of Universidade Federal do Rio Grande do Sul (number 19780 – CEUA).

DEDTC exposure

Animals were individually placed in beakers containing 400 mL of sodium DEDTC trihydrate (Sigma, St. Louis, MO, USA) solution for 1 h at 28ºC. Experimental groups consisted in (i) Control (home tank water) (n = 55), (ii) 0.2 mM DEDTC (n = 55), (iii) 1 mM DEDTC (n = 55), and (iv) 5 mM DEDTC (n = 55). A stock DEDTC solution was prepared daily, dissolved in home tank water. Experiments with matched controls were simultaneously performed with DEDTC groups.

Behavioral evaluation

Animals were individually tested in beakers and behaviors were recorded using a video camera (Sony Handycam DCR-SX22 SD) during the exposure period. The data were further analyzed by two independent trained blind observers (inter-rater reliability >0.85). The behavioral profiles were determined by the main behavioral phenotype of the animal expressed at each 1 min interval. According to the zebrafish behavioral catalog,[27] each phenotype was determined as follows: 0, normal behavior (regular exploration of the beaker); 1, movements in bursts (rapid changes of swimming direction); 2, seizure-like behavior (or corkscrew-like swimming); 3, loss of posture; 4, death (see **Supplementary video S1**). Afterwards, fish were anesthetized in ice-cold water and euthanatized by decapitation in order to remove the whole brain for the analyses. Throughout the experiments, care was taken to move fish gently between home tanks and beakers and the solutions were replaced after each trial. All fish were equally tested and the behavior was recorded on a stable table in the same room, with

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uniform handling conditions, water quality, and illumination between trials. Fish that died during the exposure period were not used for further biochemical experiments.

Measurement of DEDTC levels in the brain

The levels of DEDTC were measured spectrophotometrically based on previous method described elsewhere.[28] Basically, this method consisted of the addition of saturating concentration of copper (Cu) in the samples. Excess of Cu(II) reacts with either free DEDTC or $Zn(DEDTC)_2$, displacing Zn from this complex. This procedure yields a yellow $Cu(DEDTC)_2$ complex. The calibration curve was prepared by adding 1.2 mM $CuSO_4$ to different concentrations of DEDTC, both dissolved in dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO, USA). The linear calibration range was 6.6 – 99 μg/mL DEDTC with the regression coefficient (r^2) 0.986 (see **Supplementary fig. S1**). We used a pool of 6 weighed whole brains homogenized in DMSO containing 1.2 mM $CuSO_4$ for each independent sample. The samples were centrifuged at 15.000 g at 20°C for 10 min. Then, the yellow supernatants were transferred to 96-well microplates and the absorbance was read at 435 nm. The obtained absorbance was subtracted from the blank (samples from unexposed animals) and the results were expressed in mg of DEDTC per kg of brain tissue.

Histochemical analysis and quantification of reactive Zn

Histochemical staining of reactive Zn was performed using the Neo-Timm method previously described for zebrafish brains.[26] After the exposure period, brains were excised and immersed in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 24 h, and then transferred to sodium sulfide solution (1% Na_2S) in 0.12 M Millonig's buffer for 24 h. Coronal (30-μm-thick) slices were cut in a vibroslicer (VTS-1000;

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Leica), and mounted on slides. For histochemical staining of reactive Zn, the slices were incubated in a solution containing silver nitrate. The reaction was carried out in a dark room, and only ultrapure and metal-free reagents were used. Images were captured by NIS Elements AR 2.30 software (Nikon) using the same parameters with a light microscope (Nikon Eclipse E-600) coupled with a camera (Nikon DXM1200C CCD). Quantification of Neo-Timm was performed by analyzing the total area stained for each CNS region under 10x magnification. Images were converted to 8-bit gray scale and the optic density was quantified using ImageJ 1.37v software.

Flow cytometric analysis of the intracellular content of reactive Zn in neural cells

The analysis of reactive zinc content in neural cells was based in Malavolta et al.[29] Neural cell suspensions (**Fig. 5A**) were obtained by mechanical dissociation of each brain with a glass Pasteur pipette in 0.3 M phosphate-buffered saline (PBS, pH 7.4) containing 1 mM ethylenediamine tetraacetic acid (EDTA). The EDTA was used throughout the experiment in order to avoid possible interferences due to free Zn in the medium and adsorbed to the cell membrane. Cells were filtered through a 40 μm nylon mesh (Cell Filter Strainer-BD Biosciences) and incubated with PBS containing 1% paraformaldehyde at 4°C for 20 min. Samples were centrifuged at 1,000 g at 4°C for 10 min, the supernatants were discarded, and the pellets were further suspended in permeabilization buffer containing 1% bovine serum albumin (BSA) and 0.0001% triton X-100 (Sigma, St. Louis, MO, USA) in PBS for 20 min at room temperature. Cells were processed for specific immunostaining of glutamatergic neurons or radial glial cells using rabbit anti-vesicular glutamate transporter 1/2 (Vglut, 1:100, Synaptic Systems, Germany) and rabbit anti-glial fibrillary acidic protein (GFAP, 1:100, Dako, Denmark A/S), respectively. Antibodies were diluted in permeabilization buffer and

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incubated for 1 h at room temperature. Cells were then washed (1x) with PBS and centrifuged as previously described. The supernatants were removed and the cells were bathed in secondary fluorescent antibody Alexa fluor anti-rabbit 635 (1:200, Invitrogen Life Technologies; Carlsbad, CA) diluted in permeabilization buffer, being further incubated for 1 h at room temperature. The cells were rinsed (1x) with PBS and centrifuged as previously described. The supernatants were removed and the cells were incubated with 20 μM of the membrane permeable Zn fluorescent probe, Zinpyr-1 (ZP1), diluted in 0.02% DMSO and PBS. After 30 min, the cells were rinsed (1x) in PBS and centrifuged as previously described. The cells were suspended in PBS for analysis of the relative fluorescence using a FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA). Controls stained only with secondary antibody were used to set the negative region of the graph. Signals from ZP1 were detected in FL1 (530/30) and Vglut, and GFAP were detected in FL4 (661/16) band pass filters. The events (10,000) were collected using Cell Quest software (Becton-Dickinson, San Jose, CA) and data were analyzed using FlowJo software (Tree Star, Inc).

Measurement of δ-ALA-D activity

The analysis of δ-ALA-D activity was performed based on methods as described previously for other models.[21,22] Specific conditions for experimental measurement of the δ-ALA-D activity in zebrafish brain were previously investigated in order to obtain the protein concentration and time of incubation appropriate to maintain the linearity of reaction and saturating amounts of substrate (data not shown). From this initial analysis, 5 whole brains were pooled for each sample and homogenized in 0.9% NaCl (1:5). The homogenate was centrifuged (4,000 g at 4°C for 10 min) and the supernatant (100 μg per assay) was used for enzyme incubation. The δ-ALA-D activity was measured in a

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medium containing 3 mM δ-aminolevulinic acid (Sigma, St. Louis, MO, USA) and 80 mM sodium phosphate buffer, pH 6.8, at 37 °C for 2 h. The levels of porfobilinogen (PBG) were determined using Ehrlich's reagent at 555 nm, with molar absorption coefficient of 6.1 x 10^4 M^{-1}. Protein content was measured according to Lowry et al.[30] using BSA as standard. Results were expressed in nmol of PBG per mg of protein per h.

Statistical analysis

Non-parametric data of the behavioral phenotypes were expressed as median ± interquartile range and the analysis of variance across time was performed by Friedman test followed by Dunn's Multiple Comparison test. The correlation between the main behavioral phenotype and different DEDTC concentrations were further analyzed by non-parametric Spearman's rank correlation test. All other data were described as means ± S.E.M., and statistical comparisons were performed using one-way ANOVA followed by Tukey's test as post hoc. The relationship between parametric data and different DEDTC concentrations was evaluated by linear regression analysis. Statistical significance was set at $p < 0.05$ level.

Results

DEDTC impairs zebrafish behavior

DEDTC significantly impaired the zebrafish behavior during the exposure period ($p < 0.0001$) in a concentration dependent manner ($r^2 = 0.901$, $p = 0.042$) (**Fig. 1**). The representative analysis shows that control fish exhibited normal behavior with spontaneous short swims during the test (**Fig. 2A**). Fish exposed to 0.2 mM DEDTC had no significant changes on behavior (**Fig. 2B**). In contrast, 1 mM DEDTC (**Fig. 2C**) induced significant behavioral changes after ~4 min of exposure, characterized by

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movements in bursts up to the ~16th min. After this period, the fish reestablished the normal swimming up to ~39 min, when they presented seizure-like behavior alternated with movements in bursts up to 60th min. The behavioral changes were more prominent with 5 mM of DEDTC (**Fig. 2D**). At this concentration, after ~2 min, we observed movements in bursts that last up to 3 min. For about 12 min the animals remained with normal behavior and then restarted with seizure-like behavior episodes along with movements in bursts for 20 min. After ~49 min, the animal exhibited a persistent loss of body posture, leading to death at the end of the trial.

DEDTC accumulates in zebrafish brain

Brain levels of DEDTC were below the detection limit in animals exposed to 0.2 mM DEDTC (**Table 1**). However, animals exposed to 1 and 5 mM presented DEDTC at values of 114 and 960 mg.kg^{-1} tissue, respectively. The increase of DEDTC in the brain was strongly correlated with the exposure concentration ($r^2 = 0.974$, $p < 0.001$).

DEDTC decreases reactive Zn content in the brain

The DEDTC exposure substantially affected the levels of reactive Zn in zebrafish brain (**Fig. 3**). Fish exposed to 0.2 mM DEDTC exhibited small changes in the reactive Zn content in the optic tectum (**Fig. 3B, 3F and 3J**). In contrast, 1 mM (**Fig. 3C, 3G and 3K**) and 5 mM (**Fig. 3D, 3H and 3L**) DEDTC induced a prominent decrease in the amount of reactive Zn throughout the brain. The optic density quantification of the periventricular gray zone (PGz) shown in **Figs. 3I-3L** confirmed these decrement in reactive Zn content after 1 and 5 mM DEDTC exposure (**Fig. 4**). A significant correlation between optic density of PGz and different DEDTC concentrations was confirmed by linear regression ($r^2 = 0.320$, $p = 0.045$).

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Neo-Timm analysis showed a normal staining pattern throughout the brain, excluding the possibility of cytoarchitectonic re-distribution of reactive Zn after DEDTC exposure.

DEDTC decreases intracellular content of reactive Zn in both glutamatergic neurons and radial glial cells

The analysis of the mean fluorescence intensity (MFI) of reactive Zn revealed a significant decrease of Zn content in all neural cells from animals exposed to 1 and 5 mM DEDTC (**Fig. 5B**), with a significant correlation with DEDTC concentration (r^2 = 0.166, p = 0.032). To investigate whether DEDTC affected reactive Zn content in different neural cell types we performed immunostaining for Vglut and GFAP to identify glutamatergic neurons and radial glial cells respectively. Both concentrations of DEDTC, 1 mM and 5 mM were able to decrease Zn content in glutamatergic neurons in 28 and 40%, respectively (**Fig. 5C**); No changes were found with 0.2 mM DEDTC. Flow cytometry analysis also showed a significant correlation between reactive Zn in glutamatergic neurons and different DEDTC concentrations (r^2 = 0.421, $p < 0.001$). The radial glial cells had lower reactive Zn content than glutamatergic neurons (data not shown). Nonetheless exposure to 0.2 and 5 mM DEDTC decreased the reactive Zn levels in radial glial cells (35 and 32%, respectively), with no changes at exposure to 1 mM DEDTC (**Fig. 5D**). We could observe only a trend of association between reactive Zn in radial glial cells and different DEDTC concentrations (r^2 = 0.106, p = 0.085).

DEDTC does not change δ-ALA-D activity

In order to investigate the effect of DEDTC on the fraction of Zn strongly bound to biomolecules, we evaluated the Zn-dependent activity of the δ-ALA-D in zebrafish

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brain. As shown in **Table 2**, DEDTC did not change the enzyme activity ($r^2 = 0.025$, $p = 0.463$).

Discussion

In the current report, we investigated the acute effect of DEDTC on behavior and Zn pool in the brain, by exposing adult zebrafish to different concentrations of DEDTC. Our main findings included behavioral impairment and decreased reactive Zn fraction in glutamatergic neurons in a concentration dependent manner of DEDTC in zebrafish brain (**Fig. 6**). To our knowledge, this is the first study that concomitantly evaluated the toxic effect of DEDTC on behavior and reactive Zn-brain levels in a vertebrate model.

It is quite remarkable the small number of studies addressing the behavioral effects of DEDTC at different concentrations.[18,31-33] Here, we observed that 5 mM DEDTC induced loss of posture and death in about 40% of animals, while 1 mM DEDTC caused movements in bursts and seizure-like behaviors. Studies have reported that DEDTC may decrease locomotor activity[18,34] and induce seizure-like behaviors[31] in rodents, acting as a proconvulsant.[32,34] Interestingly, Haycock et al.[33] have demonstrated an electrographic recording of seizures induced by high concentration of DEDTC in rats. Here we observed that zebrafish acutely exposed to DEDTC displayed seizure-like behavior, similar to that induced by common convulsant agents to the same species.[35-38] Therefore, caution should be taken with the therapeutic use of DEDTC.

Considering that DEDTC may cross the blood-brain barrier,[39] the abnormal behavior is a possible consequence of DEDTC exposure. Accordingly, we showed that the behavioral impairment were associated with the accumulation of DEDTC in the zebrafish brain after acute exposure. Moreover, histochemical and flow cytometry analyses revealed that these behavioral effects were accompanied by a reduction of

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reactive Zn content in the brain. Given the ability of DEDTC to chelate the pool of reactive Zn,[18] our data strongly suggests that the chelation of reactive Zn can be responsible, at least in part, for the harmful effects in zebrafish. Similarly, previous studies have reported that brain clearance of reactive Zn by DEDTC result in a proconvulsant effect in rodents.[31-33] Accordingly, our results showed that seizure-like behavior was observed only in DEDTC at concentrations able to chelate Zn. These results reinforce the idea that the zebrafish could be a useful model to assess the neurotoxical effects of DEDTC in CNS.

Since a subset of glutamatergic neurons have high content of reactive Zn,[23] these cells are most likely to be affected by DEDTC. Indeed, the glutamatergic neurons presented a significant reduction in intracellular content of reactive Zn after exposure to 1 mM and 5 mM DEDTC. Interestingly, the decrease of reactive Zn levels in glutamatergic neurons is associated with seizure susceptibility in Zn-deficient diet-treated rats[40] and in vesicular Zn transporter-knockout mice.[41] Our results also support an important role of the reduced Zn content in glutamatergic neurons by DEDTC on the behavioral impairment in zebrafish. Such effects could have implication on the dyshomeostasis of neural network, since reactive Zn released from glutamatergic neurons can modulate neighboring neural cells, including glial cells.[23] Although glial cells have lower reactive Zn content than the glutamatergic neurons,[42] radial glial cells had a reduction of about 35% in the pool of reactive Zn after exposure to 0.2 mM and 5 mM DEDTC. Similarly, a previous study has shown that DEDTC decreases the intracellular levels of reactive Zn in glial cells.[42] Contrastingly, the content of reactive Zn in radial glial cells of zebrafish exposed to 1 mM DEDTC was similar to control, indicating a possible ability of these cells to take up extracellular Zn, as observed in astrocytes.[43] In fact, the astrocytic uptake of Zn from glutamatergic neurons was

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reported in an animal model of seizures induced by kainic acid.[44] This could be the mechanism of increasing the amount of reactive Zn in glial cells after exposure to 1 mM DEDTC.

Although DEDTC affected the pool of reactive Zn in the brain, it was not able to change the Zn-dependent activity of δ-ALA-D, a highly conserved enzyme containing Zn rigidly associated to the molecular structure and that could be inactivated by Zn chelator.[45] Indeed, previous studies have shown no changes in δ-ALA-D activity in other tissues, such as blood and hepatic tissue in rats exposed to DEDTC.[46] Further studies are necessary to clarify whether DEDTC is unable to mobilize the pool of tightly bound Zn in the CNS. Nevertheless, it is reasonable to affirm that the neurotoxic effects of DEDTC described here could be attributed to the chelation of the reactive Zn pool rather than the Zn content strongly bound to biomolecules.

In summary, we demonstrated that exposure to DEDTC leads to a buildup of DEDTC concentration in the brain with consequent chelation of reactive Zn pool in the brain and behavioral impairment of adult zebrafish. The use of zebrafish may be an interesting strategy to assess the potential effects of DEDTC and unravel novel mechanisms of actions of this compound in CNS. As DEDTC is able to chelate other metals (e.g., copper) or act on other molecular targets and other neural cell types, additional studies must be carried out to better elucidate its toxic effects. In any case, we reinforce the idea that it is crucial to consider the side effects of DEDTC in future pharmacological studies.

Conflict of Interest Statement

The authors report no conflicts of interest.

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Table 1. Level of DEDTC in the brain after acute exposure.

[DEDTC] in the water	[DEDTC] in the brain ($mg.kg^{-1}$)	r*
0.2 mM	< 100	
1 mM	113.8 ± 19.6^{a}	0.987 $p = 0.001$
5 mM	960.2 ± 72.6^{b}	

The experiments were performed at least three times, and a pool of 6 whole brains was used for each independent sample (each group was $n = 3$). Values were expressed as means ± S.E.M. Distinct letters indicate statistical differences between groups at $p < 0.05$.

* Linear regression was performed without 0.2 mM DEDTC group.

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Table 2. δ-ALA-D activity in the zebrafish brain after DEDTC exposure.

[DEDTC]	δ-ALA-D activity (ηmol PBG.mg protein^{-1}.h)	*r**
Control	0.62 ± 0.05[a]	0.157 $p = 0.463$
0.2 mM	0.64 ± 0.04[a]	
1 mM	0.59 ± 0.03[a]	
5 mM	0.66 ± 0.04[a]	

The experiments were performed at least five times, and a pool of 5 whole brains was considered to $n = 1$ (each group was $n = 5$). Values were expressed as means ± SEM. Distinct letters indicate statistical differences between groups at $p < 0.05$.
* Linear regression

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Figure Captions

Fig. 1 Effects of DEDTC on behavioral phenotypes during the 60 min of exposure. Quantitative analysis of behavioral phenotypes for each experimental group (n = 8). Values were expressed as median ± interquartile range of observed phenotypes, which were numbered as 0 (normal behavior), 1 (movements in bursts), 2 (seizure-like behavior), 3 (loss of posture), and 4 (death). Data were analyzed by Friedman test followed by Dunn's Multiple Comparison test. Spearman correlation between the phenotypes and different DEDTC concentrations was obtained and plotted in the graphic

Fig. 2 Scheme of the main behavioral phenotypes for representative profiles of control (A), 0.2 mM (B), 1 mM (C) and 5 mM DEDTC groups (D). Exposure to lower concentration of DEDTC (0.2 mM) did not change the behavior across time (B). Exposure to 1 mM DEDTC induced seizure-like behavior at the end of the test (C). Administration of 5 mM DEDTC caused several seizure-like episodes up to death (D). The x-axis represents the exposure time to DEDTC (in minutes) and the color bars indicate the main behavioral phenotype played by each animal

Fig. 3 Histochemical staining of reactive Zn in the periventricular gray zone after DEDTC exposure. In comparison to the control (A and E) 0.2 mM DEDTC caused small changes on the reactive Zn content (B and F), while 1 mM (C and G) and 5 mM (D and H) DEDTC produced a reduction in the reactive Zn levels. At higher magnification, the abundant content of reactive Zn of the periventricular gray zone (I) was unaltered at 0.2 mM DEDTC (J), and significantly decreased at 1 mM (K) and 5 mM (L). The images were obtained by the analysis of four brains for each group (n = 4).

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OT, optic tectum; PGz, periventricular gray zone. Bars represent 60 μm in A-D panels, 200 μm in E-H panels, and 400 μm in I-L panels

Fig. 4 Optic density quantification of reactive Zn in the periventricular gray zone. Results confirmed the substantial decrease in the reactive Zn levels at 1 mM and 5 mM DEDTC (n = 4). Values were expressed as the mean ± S.E.M of arbitrary units (a.u.). Data were analyzed by one-way ANOVA followed by Tukey's test as post hoc. Different letters indicate statistical differences between groups at $p < 0.05$. The linear regression between optic density and different DEDTC concentrations was obtained and plotted in the graphic

Fig. 5 Flow cytometry measurement of intracellular reactive Zn content from glutamatergic neurons and radial glial cells. Representative plots of side scatter (SSC) and forward scatter (FSC) of neural cells isolated from each zebrafish brain (n = 6 - 8) are shown (A). Percentage of mean fluorescence intensity (MFI) of reactive Zn obtained for all the neural cells of zebrafish brain after exposure to different DEDTC concentrations (B). Percentage of MFI of reactive Zn in glutamatergic neurons at different DEDTC concentrations (C). Percentage of MFI of reactive Zn in radial glial cells caused by exposure to DEDTC (D). Values were expressed as the mean ± S.E.M. in percentage of MFI related to the control. Data were analyzed by one-way ANOVA followed by Tukey's test as post hoc. Different letters indicate statistical differences between groups at $p < 0.05$. The linear regression between cellular reactive Zn and DEDTC concentrations was obtained and plotted in each graphic

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Fig. 6 Schematic representation of the effects caused by DEDTC in zebrafish. Numbered stripes represent each parameter and the colors correspond to specific changes. In general, increased DEDTC concentrations cause most significant changes in the parameters

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Supplementary Figure Caption

Supplementary fig. S1 Calibration curve of absorbance and corresponding concentrations of DEDTC ($mg.mL^{-1}$)

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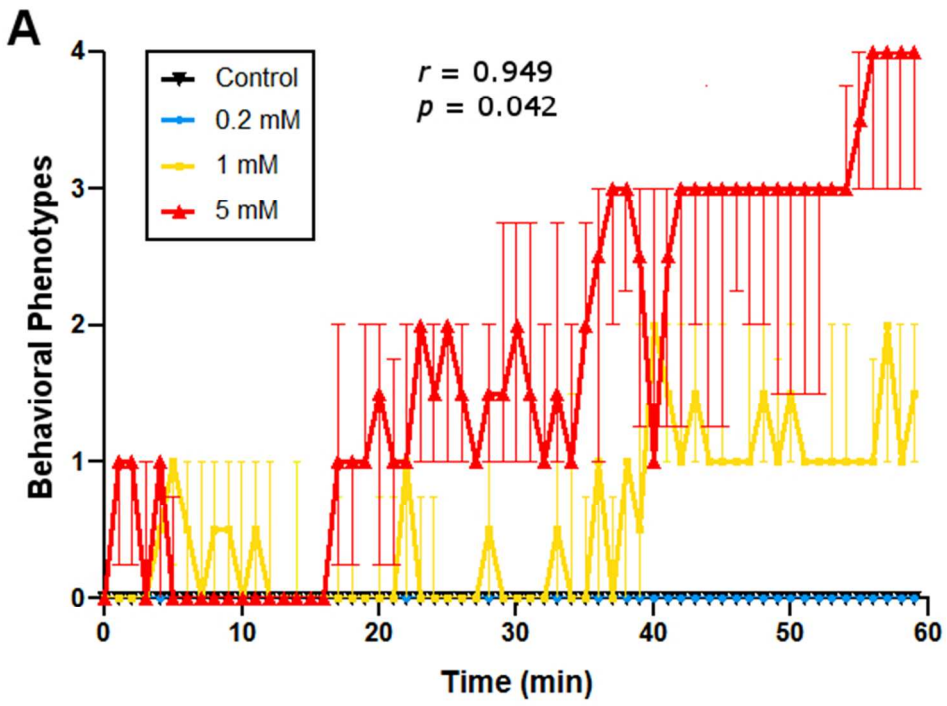

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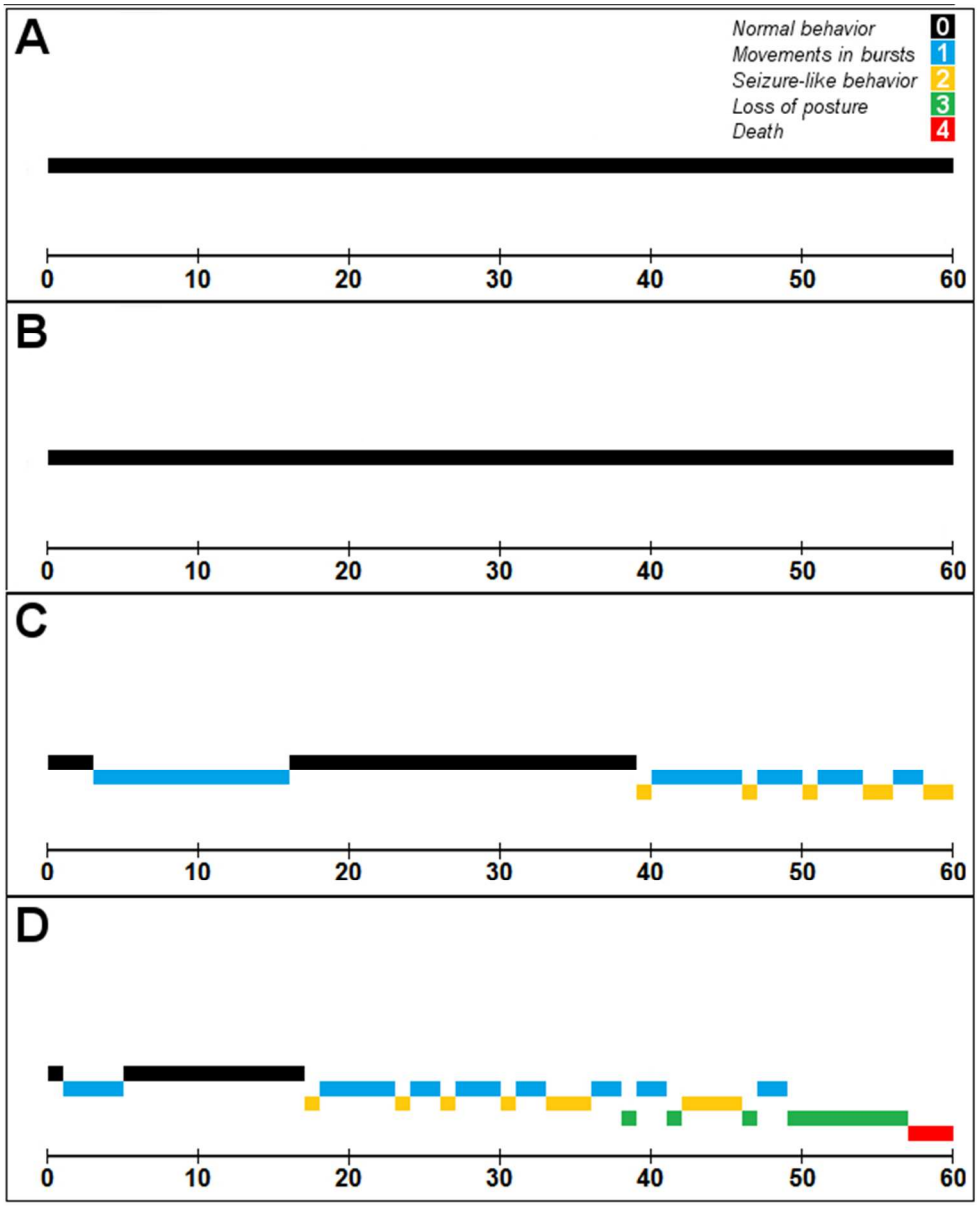

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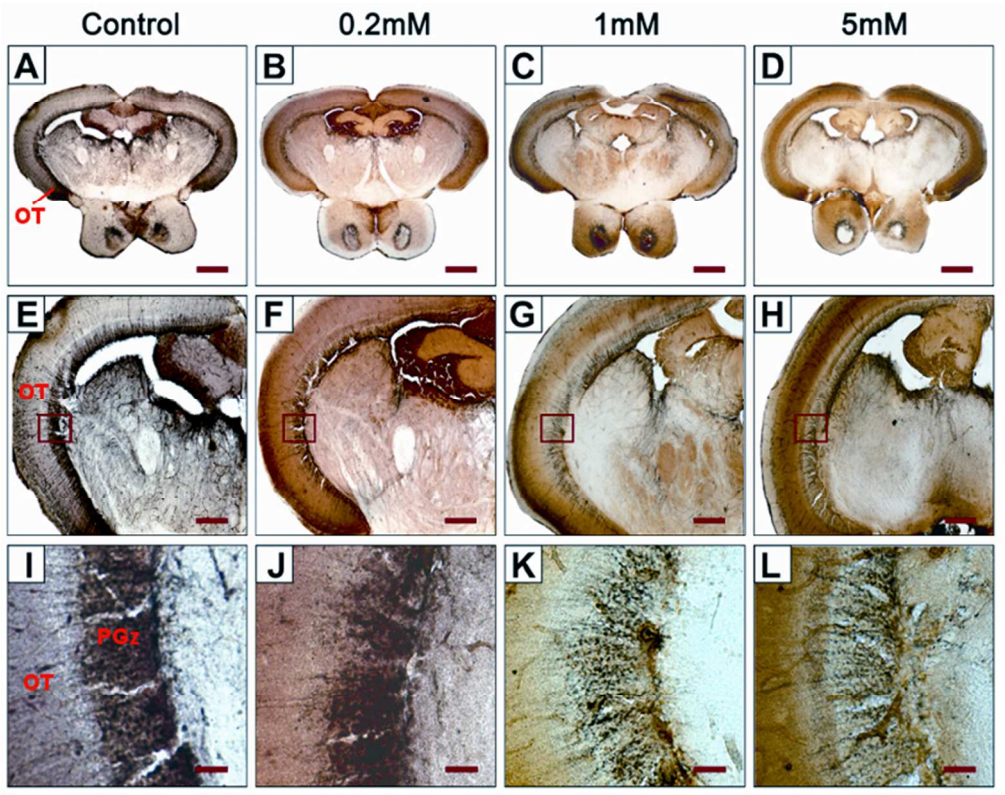

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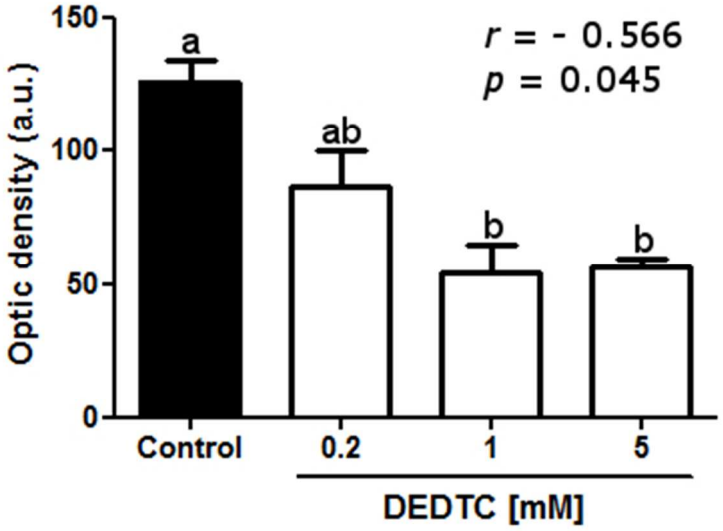

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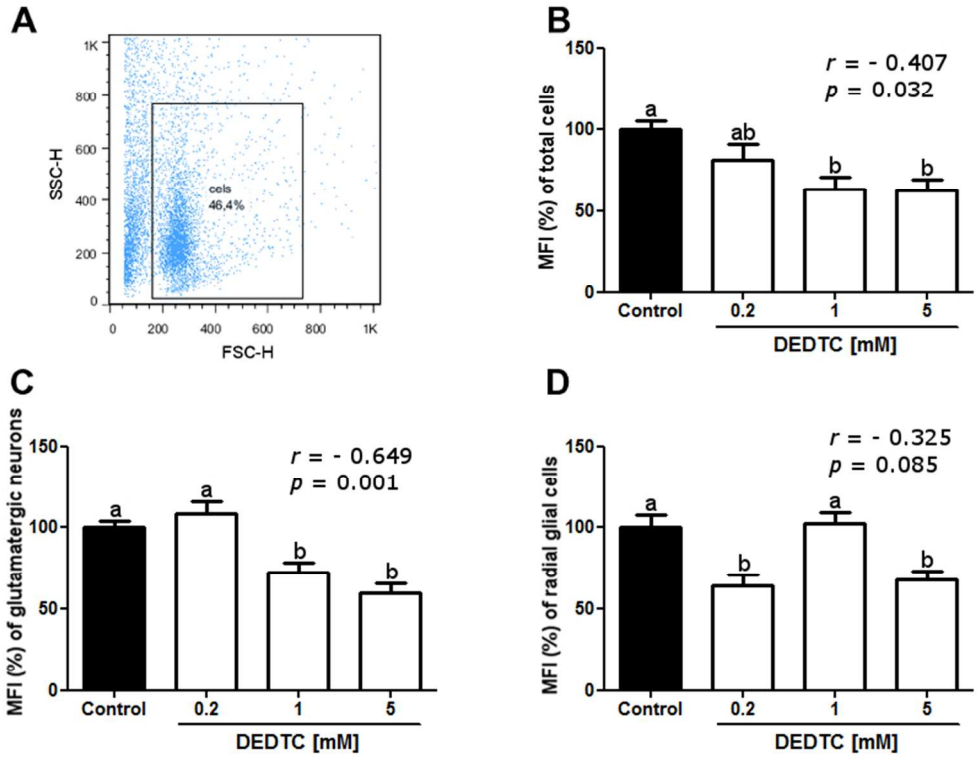

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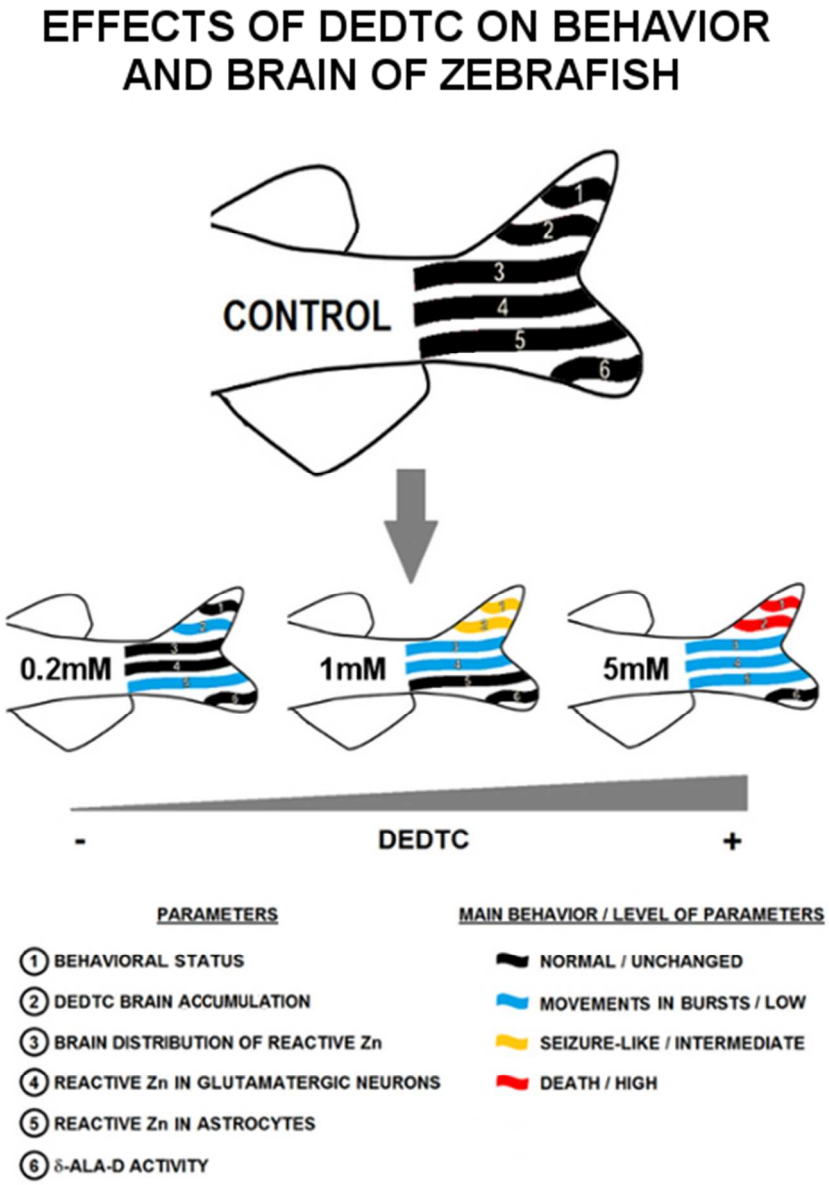

Fig. 6 Schematic representation of the effects caused by DEDTC in zebrafish. Numbered stripes represent each parameter and the colors correspond to specific changes. In general, increased DEDTC concentrations cause most significant changes in the parameters
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DEDTC leads to a buildup of DEDTC in the brain with consequent chelation of reactive Zn and behavioral impairment of zebrafish.