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Methylmercury alters the number and topography of NO-synthase positive neurons in embryonic retina: Protective effect of alpha-tocopherol

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ABSTRACT

Vertebrate retina has been shown to be an important target for mercury toxicity and very studies have shown the effect of mercury on the retinal ontogenesis. The nitrergic system plays an important role in the retinal development. The current work studied the effects of methylmercury (MeHg) exposure on the NO-synthase positive neurons (NADPH-diaphorase neurons or NADPH-d+) of the chick retinal ganglion cell layer at embryonic E15 and postnatal P1 days. Retinal flat mounts were stained for NADPH-diaphorase histochemistry and mosaic properties of NADPH-d + were studied by plotting isodensity maps and employing density recovery profile technique. It was also evaluated the protective effect of alpha-tocopherol treatment on retinal tissues exposed to MeHg. MeHg exposure decreased the density of NADPH-d + neurons and altered cell mosaic properties at E15 but had very little or no effect at P1 retinas. Alpha-tocopherol has a protective effect against MeHg exposure at E15. MeHg alterations and alpha-tocopherol protective effect in embryonic retinas were demonstrated to be at work in experimental conditions. MeHg effect in the early phases of visual system development in natural conditions might use the nitrergic pathway and supplementary diet could have a protective effect. At later stages, this mechanism seems to be naturally protected.

1. Introduction

Methylmercury (MeHg) is an organic form of mercury that causes severe damage to the central nervous system (Hunter et al., 1940; Hunter and Russell, 1954; Mottet et al., 1984; Clarkson, 1987; Philbert et al., 2000; Crespo-López and Herculano, 2005; Silva-Pereira et al., 2005; Nascimento et al., 2008). MeHg neurotoxicity has been described in people living in mercury contaminated environment (Bakir et al., 1973a, 1973b; Harada, 1995; Boischio and Henschel, 1996; Lebel et al., 1996, 1998; Rodrigues et al., 2007; Fillion et al., 2013). Visual function impairment comprises important symptoms associated with MeHg intoxication as reported in controlled experiments with animals (Gunderson and Grant-Webster, 1988) and in selected group of exposed persons (Bakir et al., 1973a,b; Tsubaki, 1979; Harada, 1995; Lebel et al., 1996, 1998; Silveira et al., 2003; Saint-Amour et al., 2006; Rodrigues et al., 2007; Fillion et al., 2013; Freitas et al., 2015). The toxic effects of MeHg on the adult visual system have been evaluated but few studies have described alterations caused by MeHg in retinal tissue during its embryonic development.

It has been shown that the chick retina is a good model to study visual system development (Adelmann, 1966; Fischer and Reh, 2000; Belecky-Adams et al., 2008). Embryonary chick retinas have been largely used to describe molecular and cellular phenomena associated with the development of vertebrate retina (Belecky-Adams et al., 2008). Studies of the embryonic chick retina have showed that orchestrated events such as migration and cellular differentiation are necessary for adequate retinal development (Belecky-Adams et al., 2008). All these cellular events are evolutionary conserved in vertebrates, including humans. Martins and Pearson (2008) described that ganglion cells arise in the early stages of retinal development of human, rat, mouse, rabbit and chicken. It is well documented that at chick E10 embryonic period, which is correspondent to 19 embryonic weeks in humans, the mainly

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cell types that comprise the adult retina are present in chick embryonic retinal tissue. Several neurotransmitters such as acetylcholine, glutamate and GABA are important to assure these events during retinal development in vertebrates. In this context, it has been well documented that nitric oxide (NO) acts as an important modulator for retinal development (Garthwaite, 2008).

NO is a gaseous neuromodulator synthesized by NO-synthase (NOS) activity during the conversion of L-arginine to L-citruline in the presence of NADPH as a co-factor (Dawson et al., 1992). NOS can be expressed as constitutive isoforms (NOS-1 and NOS-3) or inducible enzymatic isoform (NOS-2) (Hope et al., 1991; Bredt and Snyder, 1994; Knowles and Moncada, 1994; de Faria et al., 1995; Garthwaite and Boulton, 1995). Both kinds of NOS isoforms are able to reduce nitroblue tratazolium (NBT) to formazan salts in paraformaldeyde-fixed tissues. This enzymatic property is known as NADPH-diaphorase activity (Hope et al., 1991; de Faria et al., 1995). It has been shown that NADPH-diaphorase histochemistry is a simple technique for localizing NOS positive neurons in the retina and other regions of the central nervous system (Goureau et al., 1997; Nacsa et al., 2015).

NADPH-diaphorase positive cells (NADPH-d+) are mainly localized in the retinal ganglion cell layer (RGCL) at earlier stages of development (Goureau et al., 1993, 1994, 1995; Yamamoto et al., 1993; Koch et al., 1994). NO released from cells of the retinal ganglion cell layer controls guidance of axonal migration, photoreceptor proliferation, cell death, and production of growth factors such as NGF and BDNF (Jung et al., 1997; Lang et al., 2007; Klöcker et al., 2001). The organization of NADPH-d + in the retinal ganglion cell layer is essential for the perfect development of axonal projections to the diencephalon and mesencephalon as well as for the organization of retinal external layers. It has also been demonstrated that the regularity of NADPH-d + mosaics is important for receptive field development. Thus, changes in the organization of NADPH-d + mosaic may cause significant alterations in the perfect functionality of the adult retina.

Toxicological studies regarding the effect of MeHg on the central nervous system have demonstrated that MeHg toxicity is frequently mediated by induction of oxidative stress (Aschner et al., 2007; Farina et al., 2011a, 2011b; Roos et al., 2012). We have showed that inhibition of NO production prevents retinal cell death induced by MeHg exposure (Herculano et al., 2006). Although antioxidant treatment has became a strategy for protection or prevention of mercury toxicity, no protective studies using classical antioxidants have been performed in retinal tissue exposed to MeHg. Alpha-tocopherol is the most abundant vitamin E isoform present in vertebrates. Alpha-tocopherol is an important antioxidant present in the diet and several studies have demonstrated that alpha-tocopherol has a protective effect on the central nervous system (Rodriguez-Martinez et al., 2004; Zaidi and Banu, 2004; Sridevi et al., 2007) but this effect has not yet been evaluated in embryonic or adult retina exposed to xenobiotics such as MeHg. Alpha-tocopherol is highly liposoluble, it is able to cross the blood-brain barrier and to exert its effect in both the extracellular and intracellular milieu (Balazs et al., 2004; Ferri et al., 2015). This work aimed to evaluate the MeHg effect on the spatial density and organization of chick NADPH-d + cells at different stages of the retinal development as well as to test the protective effect of alpha-tocopherol in retinal tissue exposed to MeHg.

2. Materials and methods

2.1. Animals

White Leghorn chick retinas were obtained from animals at the 15th embryonic day (E15) and in the first day post-hatch (P1) as described previously by Hamburger and Hamilton (1992). All animal experiments were carried out in accordance with the National Institutes of Health guide for the care and use of laboratory animals (NIH Publication #8023, revised in 1978) as well as in accordance with the guidelines established by the Association for Research in Vision and Ophtalmology (ARVO). All animal experiments complied with the ARRIVE guidelines. The experiments were approved by the Ethical Research Committee for Animal Experiments of the Institute of Biological Sciences, Federal University of Pará (CEPAE #122-13).

2.2. Tissue preparation and experimental design

Stock solution of MeHg (100 μM) was diluted in phosphate buffer saline (PBS) solution at pH 7.5 under over-night gitation at 37 °C. Stock solution of alpha-tocopherol (100 μM) was previously diluted in 0.025% DMSO solution. These steps were followed by sequential dilution in DMEM without bovine serum. The work solution used in MeHg group, alpha-tocopherol group and MeHg + alpha-tocopherol group were adjusted to pH 7.5. The final concentration of DMSO in the work solutions used to treat the groups exposed to alpha-tocopherol was 0.012%. Right eyes of the chick embryos were enucleated and its eyecups were treated by 3 h at 37 °C as follows: control group (DMEM medium); Control vehicle (DMEM +0.012% DMSO); alpha-tocopherol group (50 μM alpha-tocopherol); MeHg group (5 μM MeHg); and alpha-tocopherol + MeHg group (50 μM tocopherol +5 μM MeHg). After this exposure period, retinal tissue was quickly dissected and fixed by direct immersion in 4% paraformaldehyde solution (PFA) as previously described (Pow, 1997; Pow et al., 1995). All experiments were conducted twice and using double-blind evaluation.

2.3. NADPH-diaphorase assay

NADPH-diaphorase histochemistry was performed as described previously by Goureau et al. (1997). Retinas were incubated during 4 h at 37 °C in solution containing 0.6% malic acid, 0.03% nitroblue tetrazolium (NBT), 1% dimethylsulfoxide (DMSO), 0.03% manganese chloride, 0.5% beta-NADP and 1.5% Triton X-100 in 0.1 M Tris buffer pH 8.0. Chemicals used throughout tissue preparation and NADPH-diaphorase assay were acquired from Sigma-Aldrich (St. Louis, Missouri, USA).

2.4. Analysis of mosaic spatial regularity

After the reaction period, the retinas were mounted on glass slides and oriented according to their dorsal-ventral and temporal-nasal axes. Then, digital images from the retinal ganglion cell layer were obtained at 1 mm intervals using a trinocular BX41 Laboratory Microscope equipped with x40 objective and Olympus E-500 digital camera (Olympus Corporation, Shinjuku, Tokyo, Japan).

NADPH-diaphorase positive cells (NADPH-d+) from the retinal ganglion cell layer were quantified using the ImageJ 1.38× software (Wayne Rasband, National Institutes of Health, Bethesda, Maryland, USA). The values obtained were converted to cells per mm^2 and density topography maps were generated using the Delta Graph 4.0 software (Delta Point, Monterey, California, USA). Using the optic disk as a landmark, NADPH-d + cells mosaics were sampled along the dorsal-ventral axis. Images from 100 fields (1000 × 1000 μm) for each retina were digitized. The centre of each cell was identified in the digitized images and their Cartesian coordinates were determined using the Scion Image software (http://www.scioncorp.com, Scion Corporation, Frederick, Maryland, USA).

The NADPH-d + cells mosaics were analysed using a version of the Rodieck (1991) Density Recovery Profile (DRP) software written by Cézar Akiyoshi Saito (de Lima et al., 2005; Gomes et al., 2005) to compute the Effective Radius (ER) and Packing Factor (PF) for each cell mosaic. The PF is a non dimensional parameter used as an indirect measure of mosaic regularity, ranging from 0 (Poisson distribution such as an ideal random mosaic) to 1 (hexagonal distribution like a crystalline mosaic) (Rodieck, 1991; Kouyama and Marshak, 1997; Martin and Grünert, 1999; Cornish et al., 2004; Gomes et al., 2005; Rocha et al., 2009). The ER is a scalar measure in the cell distribution that

quantify the "dead space" about each cell (Rodieck, 1991; Gomes et al., 2005). Autocorrelograms of 140 µm radius and bin width of 20 µm or 150 µm radius and bin width of 10 µm were chosen to yield a reliability factor K of approximately 3 and 6, respectively, for control retinas, which allowed a direct comparison between mosaics with different areas and densities (Rodieck, 1991; Gomes et al., 2005).

2.5. Nissl stain

After 3 h of incubation with 0, 2.5 µM and 5 µM of MeHg E15 retinal tissues were fixed by immersion in a solution of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2–7.4) for 2 h at room temperature. After fixation period retinas were placed back into PBS and its orientation was defined by the pecten, that occupies the ventral quadrant of the retina. Pigment epithelium tightly adhered to the retina was bleached with a solution of 3% hydrogen peroxide in PBS for 24 h at room temperature. Subsequently, the retina was rinsed with PBS, flattened onto a gelatinized slide, and exposed to formaldehyde vapors at 60 °C for 2 h, then the retina was stained with the following protocol: ethanol 90%, ethanol 70%, etanol 50%, distilled water +5% acetic acid, per 3 min,an aqueous solution of 0.1% cresyl Violet per 7 min, wash in Distilled water, ethanol 70%, ethanol 90%, ethanol 100%, ethanol 100%,ethanol-xilol (1:1),xilol 100% e xilol 100%, and coverslipped using Entellan.

2.6. Statistical analysis

The parameters measured were submitted to statistical analysis using BioEstat 3.0 software (Ayres et al., 2003) and *p*-values < .05 were considered significant. Data were expressed as mean ± standard deviation of two independent experiments. Variations in cell density were evaluated using one-way ANOVA followed by Tukey post hoc test.

3. Results

3.1. Retinal ganglion cell layer NADPH-d + cell density is affected by MeHg treatment

Fig. 1 shows isodensity maps illustrating the topography of the distribution of NADPHd+ neurons in the retinal ganglion cell layer of E15 (Fig. 1A–B) and P1 (Fig. 1C–D) chick retinas. Neuronal density reaches about 400 cells/mm^2 in regions showing the highest density of labelled neurons. In control retinas, at E15, the labelled neurons are distributed mainly in the dorsal and ventral retinal regions, showing higher densities in the dorsal region in comparison with the ventral region, and very low densities in the central region (Fig. 1A). At P1, the labelled neurons were found throughout the retina, except at the borders where densities were very low, showing higher densities in the dorsal region when compared with the ventral region. The effect of 5 µM MeHg on NADPHd+ neurons of the retinal ganglion cell layer is also illustrated in Fig. 1. At E15, there was a generalized decrease in cell density (Fig. 1B) while at P1 no effect was observed (Fig. 1D). No significant difference in NADPH-d + number was observed between DMEM treated group and DMEM +0.012% DMSO group (data not showed). Our data also demonstrated that 5 µM MeHg did not decrease the total number of ganglion cells present in the chick retinal tissue at E15 embryonic period (supplementary figure).

Fig. 1 also illustrates the statistical comparison performed on the data obtained from these two groups of retinas ($n = 7$ for each group). At E15, the mean density of NADPHd+ neurons had significantly decreased after exposure to 5 µM MeHg ($p < 0.05$, *t*-test) (Fig. 1E), while at P1 there was no significant difference ($p > .5$, *t*-test). Areas with very low density in the retinal periphery were not analysed (Fig. 1F).

3.2. Alpha-tocopherol treatment prevents MeHg effect on the cell density and topographic organization of NADPH-d + of the retinal ganglion cell layer

Fig. 2 shows isodensity maps illustrating the topography of the distribution of NADPHd+ neurons in the retinal ganglion cell layer of

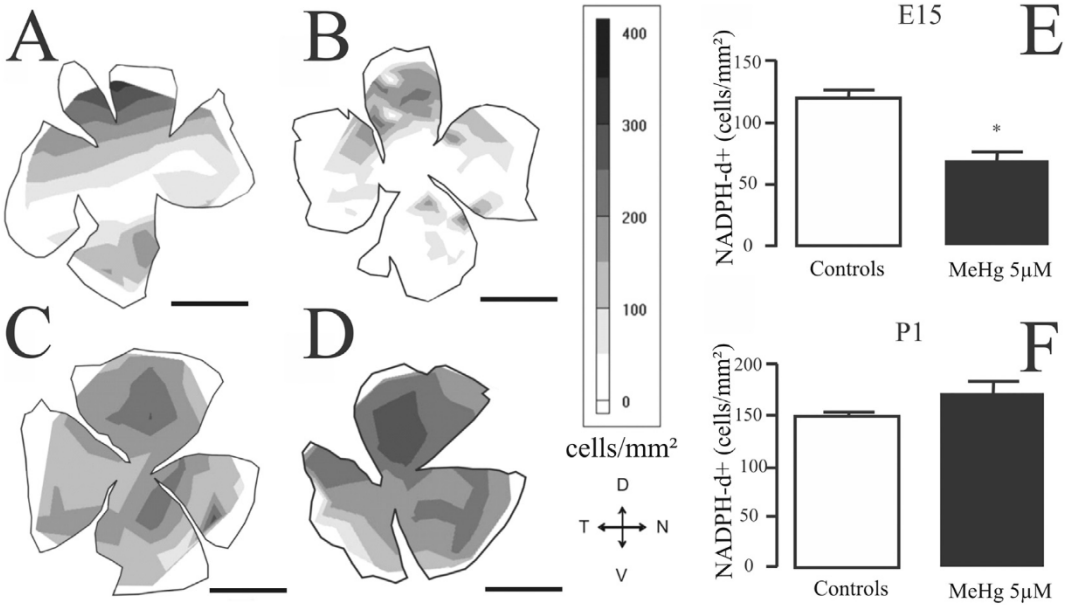

Fig. 1. The effect of MeHg on NADPHd+ neurons of the chick retinal ganglion cell layer. A-D) Isodensity topography retinal maps of NADPHd+ neurons at two different developmental stages: A) E15 embryonic day retina without any treatment; B) E15 embryonic day retina treated with 5 µM MeHg; C) P1 postnatal day retina without any treatment; D) P1 postnatal day retina treated with 5 µM MeHg. E-F) Comparison between mean density of NADPHd+ neurons in control retinas and MeHg-treated retinas at E15 (E) and P1 (F) ($n = 7$ for each group) made from two independent experiments. Areas with very low density in the retinal periphery were not analysed. Difference between control retinas and MeHg-treated retinas was found only at E15: density of NADPHd+ neurons decreased in MeHg-treated retinas in comparison with control retinas (*$p < 0.05$, *t*-test). D: dorsal; N: nasal; V: ventral; T: temporal. Scale bars: 5 mm.

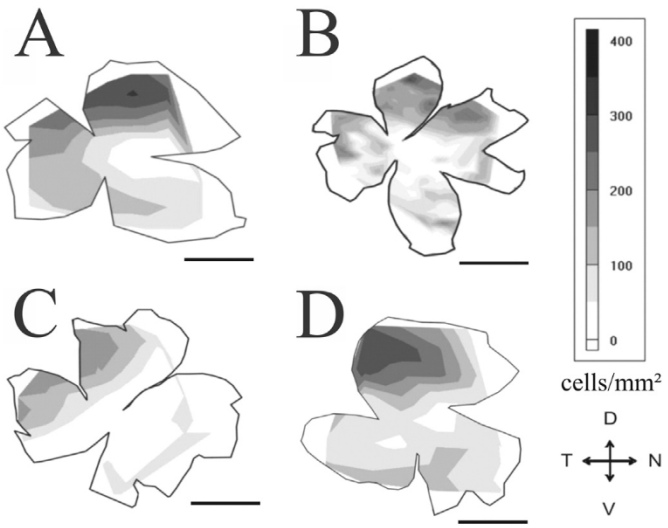

Fig. 2. The protective effect of alpha-tocopherol on MeHg-treated retinas – isodensity maps. Alpha-tocopherol was used in MeHg-treated retinas to protect them from neurodegenerative effects observed in NADPHd+ neurons at E15 (see Fig. 1). A-D) Isodensity topography maps of NADPHd+ neurons in the chick retinal ganglion cell layer at E15 embryonic day: A) Control retina; B) 50 µM alpha-tocopherol treated retina; C) 5 µM MeHg-treated retina; D) 50 µM alpha-tocopherol +5 µM MeHg treated retina. Areas with very low density in the retinal periphery were not analysed. See next figure for statistical comparisons. D: dorsal; N: nasal; V: ventral; T: temporal. Scale bars: 5 mm.

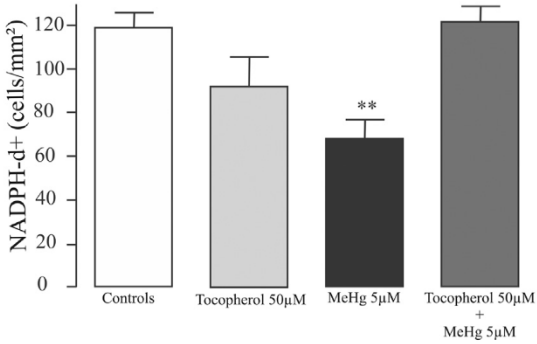

Fig. 3. The protective effect of alpha-tocopherol on MeHg-treated retinas – statistical comparison. Mean densities of NADPHd+ neurons in the ganglion cell layer of chick retinas at E15 embryonic day divided in four groups were compared: control retinas; retinas treated with 50 µM alpha-tocopherol; retinas treated with 5 µM MeHg; retinas treated with 50 µM alpha-tocopherol +5 µM MeHg ($N = 7$ retinas in each group). Mercury-treated retinas had lower NADPHd+ neuron density than the other two groups (**$p < 0.01$, ANOVA/Tukey post hoc test).

E15 (Fig. 2A–D), one example from the four group studied in this comparison: control retinas (Fig. 2A); retinas treated with 50 µM alpha-tocopherol (Fig. 2B); retinas treated with 5 µM MeHg (Fig. 2C); retinas treated with 50 µM alpha-tocopherol +5 µM MeHg (Fig. 2D). In control retinas, retinas treated with alpha-tocopherol and retinas treated with alpha-tocopherol plus MeHg, neuronal density reaches about 400 cells/mm^2 in regions showing the highest density of labelled neurons. In retinas treated with MeHg only, neuronal density reaches only about 200 cells/mm^2. As previously illustrated in Fig. 1B, the effect of 5 µM MeHg on NADPHd+ neurons of the retinal ganglion cell layer at E15 comprises a generalized decrease in cell density (Fig. 2C) when compared with control retinas (Fig. 2A). Retinas treated with alpha-tocopherol (Fig. 2B) are similar to controls (Fig. 2A). In addition, treatment with MeHg associated with alpha-tocopherol (Fig. 2D) prevents the effect of MeHg on the density of NADPHd+ neurons (Fig. 2C).

Fig. 3 shows the statistical comparison performed on the data obtained from these four groups of retinas ($n = 7$ for each group). At E15, the mean density of NADPHd+ neurons had significantly decreased after exposure to 5 µM MeHg in comparison with the other three groups (**$p < 0.01$, ANOVA/Tukey post hoc test). On the other hand, there were no statistical significant differences between control retinas, retinas treated with alpha-tocopherol, and retinas treated with alpha-tocopherol plus MeHg ($F = 10.01$ and $p > 0.05$ ANOVA/Tukey post hoc test).

The significant effect of MeHg on the density of NADPH-d + neurons as well as the protective effect of alpha-tocopherol encouraged us to evaluate whether similar effects would be observable studying the spatial properties of NADPH-d + mosaics in the four groups of retinas at E15: control retinas; retinas treated with 5 µM MeHg; retinas treated with 50 µM alpha-tocopherol; retinas treated with 50 µM alpha-tocopherol +5 µM MeHg. Thus, a series of cell mosaics from the four groups of retinas were digitezed and analysed using the density recovery profile technique. The results of such analysis are illustrated with four representative mosaics depicted in Figs. 4 and 5. Additional numerical results for these mosaics are listed in Table 1. The mosaics shown in Figs. 4–5 were chosen to represent the central tendency of a large range of values observed in retinas treated with MeHg, alpha-tocopherol or alpha-tocopherol + MeHg, while mosaics from control retinas were more homogeneous. The illustration shows that mosaics from control retinas, alpha-tocopherol treated retinas, and retinas treated with alpha-tocopherol + MeHg are generally similar regarding cell density, effective radius, and packing factor, while MeHg-treated retinas largely differ.

Descriptive statistics for the two groups of NADPH-d + mosaics studied at P1 and the four groups of mosaics studied at E15 are shown in Figs. 6 and 7 for effective radius and packing factor, respectively ($n = 7$). Very little difference was found at P1 between MeHg-treated retinas and controls for the effective radius (Fig. 6, upper plot) and packing factor (Fig. 7, upper plot), with some tendency for MeHg-treated retinas to show less variation than controls with slightly lower values for effective radius and slightly higher values for packing factor than control retinas.

More substantial differences were found between MeHg-treated retinas and the other three groups at E15 (Figs. 6 and 7, lower plots). MeHg-treated retinas differed from the others by showing wider range of values associated with very low values of effective radius. Control retinas, alpha-tocopherol and alpha-tocopherol treated retinas did not show very low values as MeHg-treated retinas, but both alpha-tocopherol groups showed wider variation than control retinas towards high values of effective radius. MeHg-treated retinas differed from the others by showing wider range of values of packing factor associated with the interquartile range displaced towards lower values of packing factor than the other three groups. Control retinas, alpha-tocopherol, and alpha-tocopherol treated retinas were similar but did not show the range of low values observed in MeHg-treated retinas.

4. Discussion

Since the discovery that MeHg is one of the more toxic substances affecting central nervous system either by occupational exposure or environmental pollution, high concern has been expressed about the effects of this toxicant during pregnancy and childhood. MeHg exposure increases the rate of abortion during pregnancy and could also result in neural sequels with serious consequences for children that were born. Impairment of the nervous system development could result in a variety of mild and severe sequels depending upon the level and duration of exposure and it is very important to determine threshold levels for neurotoxicity during nervous development as well as to understand the mechanisms underlying such neurotoxicity. Thus, MeHg exposure during pregnancy as well as of women in reproductive age has been monitored in several locations where the presence of the metal raised

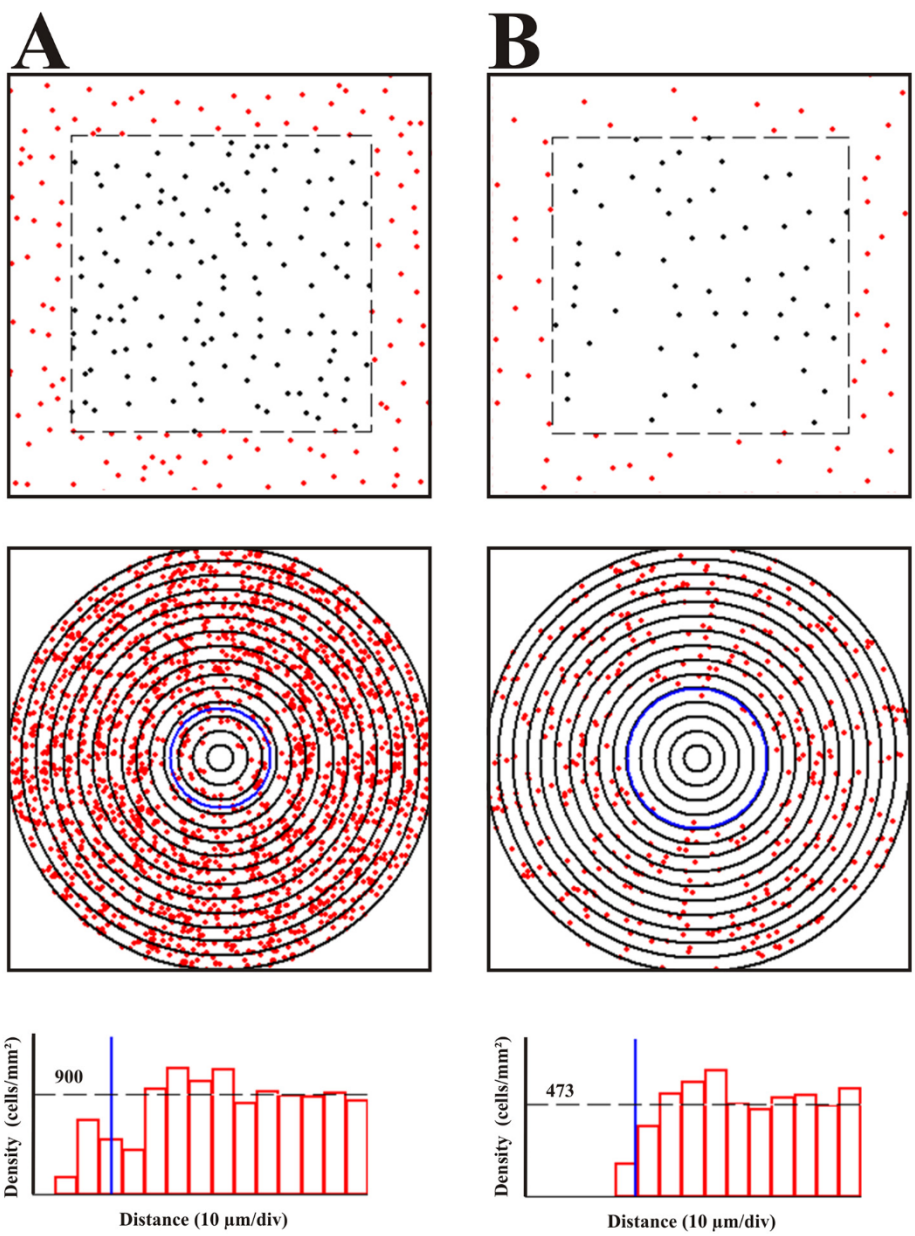

Fig. 4. The effect of methyl mercury (MeHg) on NADPHd+ neurons of the chick retinal ganglion cell layer – spatial statistical comparison. The plots illustrate the results of density recovery profile analysis for NADPHd+ neurons of the chick retinal ganglion cell layer, E15 embryonic day. Two groups of retinas were compared ($n = 7$). A field taken from a representative retina of each group is illustrated: A) control retina; B) retina treated with 5 µM MeHg. Top panels show the position of NADPHd+ neurons in the studied area measuring 1 mm of side. The analysed are measured 0.7 mm of side and the buffer area comprised 0.15 mm at each side of the analysed area. Middle panels show the spatial autocorrelograms, 150 µm of radius, 10 µm of bin size. Each dot represents one cell in relation to each reference cell centered in the autocorrelogram. Blue circles represent the effective radius. Lower panels show the density recovery profile histograms. Bars represent cell density in each autocorrelogram bin, horizontal dashed lines represent cell density estimated from the effective radius, and vertical lines represent the effective radius. These two fields illustrate the main results of the spatial statistical analysis of the two groups of retinas: the mosaics of NADPHd+ neurons in the MeHg-treated retinas had lower density and were less organized than in the control retinas. Additional numerical values obtained with density recovery profile analysis from these two mosaics are listed in Table 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

concerns, including the Brazilian Amazon (Boischio and Henschel, 1996; Barbosa et al., 1998; Hacon et al., 2000; Pinheiro et al., 2005, 2007; Corvelo et al., 2014; Marinho et al., 2014). In addition, children living in such environments have also been monitored to check for possible effects of MeHg exposure on their organisms, including impairment of cognitive, motor, and sensory development (Grandjean et al., 1998, 1999; Tavares et al., 2005; Bose-O'Reilly et al., 2010; Dórea et al., 2012; Freitas et al., 2015).

Previous studies using cells from human brains have described LC50 values of 6.5 µM and 8.1 µM for neurons and glial cell exposed for 24 h to MeHg, respectively. Rush et al. (2012) described MeHg at 3 µM as a subtoxic concentration for brain mixed cell cultures until 6 h of exposure. Our group utilizing chick retinal cell cultures has demonstrated that 10 µM of MeHg for 2 h did not induce significant decrease in the cell viability. Although retina represents a recognized target of MeHg toxicity, there are no reports describing the exact value of IC50 for MeHg in retinal tissue. In the current study, chick retinal tissues were treated with a MeHg concentration which is among IC50 values previously described for other regions of the central nervous system (Rush et al., 2012). Subtoxic concentration used in our study did not change the cell number in retinal ganglion layer (supplementary figure), but it has altered significantly the number and organization of NO-synthase positive cells in that layer. Some reports have suggested that visual system development could be an important target for MeHg toxicity on exposed human populations (Freitas et al., 2015). Yoneyama et al. (1983) described the effect of MeHg on the retinal ontogenesis, by showing in dissociated embryonic chick retinal cells that this toxicant inhibits some enzymes involved in cellular aggregation. In the present study we have observed that MeHg exerts more evident effect in retinas from E15 embryonic chickens than post hatched chickens. These results support previous studies showing that embryonic tissues are more sensible to mercury toxicity than adult tissues (Myren et al., 2007; Huang et al., 2008). Although similar phenomenon have been observed in other animal models as well as in human exposed to environmental MeHg, the present work is the first to demonstrate it in the retinal tissue. In this way, our data contribute with literature by supporting the hypothesis that embryonic exposure to MeHg can induce significant alterations in retinal development and consequently alterations in visual processing.

The current study demonstrated that MeHg is able to alter the cell

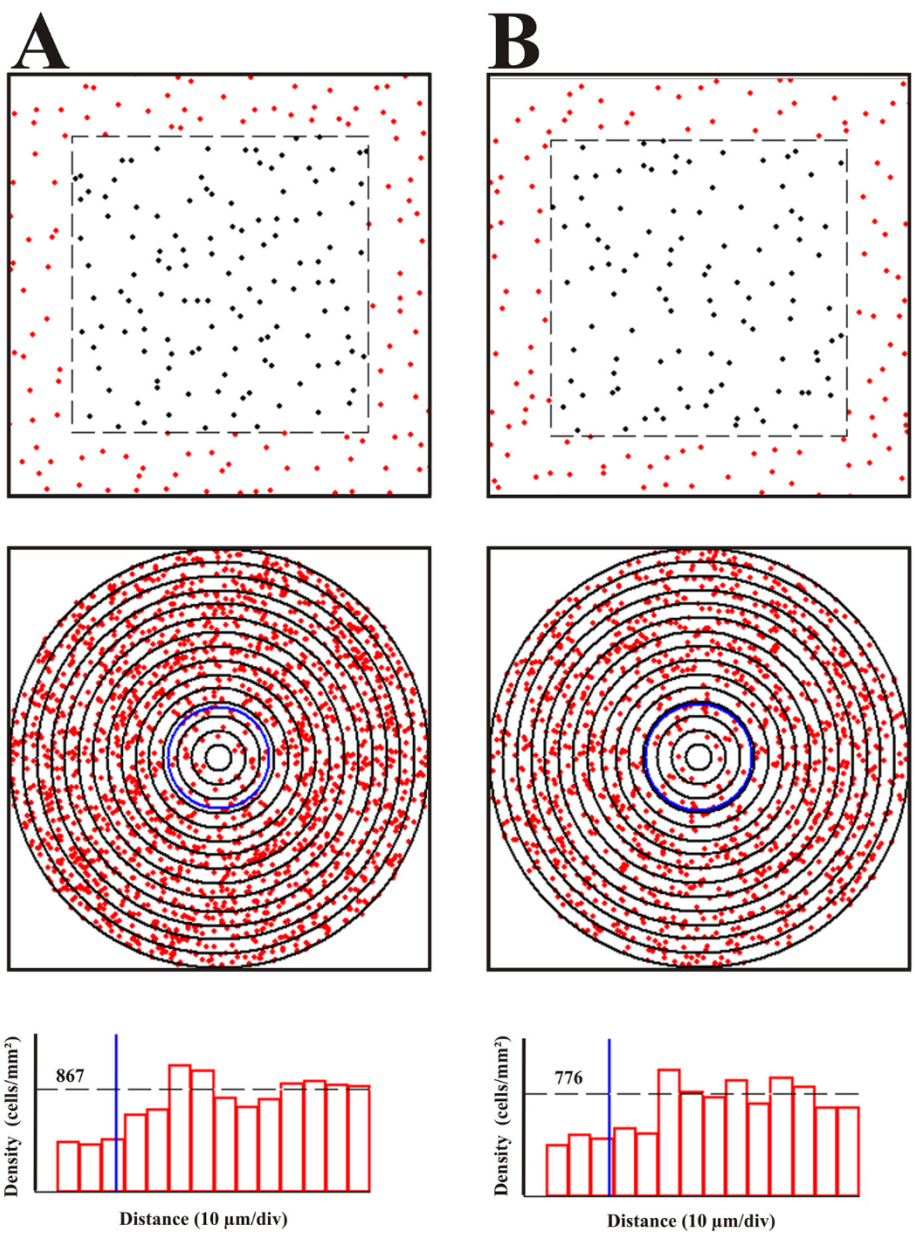

Fig. 5. The protective effect of alpha-tocopherol on MeHg-treated retinas – spatial statistical comparison. The plots illustrate the results of density recovery profile analysis for NADPHd+ neurons of the chick retinal ganglion cell layer, E15 embryonic day. Two groups of retinas were compared ($n = 7$). A field taken from a representative retina of each group is illustrated: A) retinas treated with 50 µM alpha-tocopherol; B) retinas treated with 50 µM alpha-tocopherol + 5 µM MeHg. Panels, labels, and conventions as in the previous Fig. 4. These two fields illustrate the main results of the spatial statistical analysis of the two groups of retinas: the mosaics of NADPHd+ neurons in the alpha-tocopherol + MeHg treated retinas had approximately the same density and the same organization as the alpha-tocopherol treated retinas, similar to the control retinas was well. Additional numerical values obtained with density recovery profile analysis from these two mosaics are listed in Table 1.

Table 1
Spatial statistical analysis of NADPH-d + neurons of the chick retinal ganglion cell layer at E15. Values of the spatial statistics parameters obtained with density recovery profile technique from mosaics showed in Figs. 4–5.

Parameter	Control	MeHg	Tocoph	MeHg + Tocoph
Area analysed (mm^2)	0.49	0.49	0.49	0.49
Number of cells (area analysed)	124	58	123	102
Cell density (cells/mm^2) (area analysed)	253	118	251	208
Number of cells (mosaic)	438	190	424	393
Autocorrelogram radius (µm)	150	150	150	150
Bin size (µm)	10	10	10	10
Effective radius (µm)	36	49	36	39
Packing factor	0.28	0.25	0.29	0.27
Confiability factor	3.14	1.47	3.11	2.58
Density using effective radius (cells/mm^2)	900	473	867 s	777

Control, control retina, no treatment. MeHg, retina treated with 5 µM MeHg; Tocoph, retina treated with 50 µM alpha-tocopherol; MeHg + Tocoph, retinas treated with 50 µM alpha-tocopherol + 5 µM MeHg.

density and spatial organization of the NADPHd+ neurons on the ganglion cell layer of E15 chick retina. These mechanisms could be responsible for the visual impairment observed in the childhood of exposed humans and need further investigation. It is well documented that NADPH-d + cells coordinate specific events during visual system ontogenesis such as axonal migration, cellular proliferation and cell differentiation (Goureau et al., 1993, 1994, 1995, 1997; Belecky-Adams et al., 2008). In addition, NADPH-d + cells are essential for consolidation of the connections along the visual pathways during the development of vertebrate visual system (Belecky-Adams et al., 2008). The results of the present study, showing that MeHg exposure causes significant changes in the number and spatial arrangement of NADPH-d + neurons of the ganglion cell layer of embryonic chick retina also point for a possible impact on the consolidation of the visual pathways (Ventura et al., 2004; Costa et al., 2008; Mela et al., 2012). Although in the present study it was not determined the specific NADPH-d + cell type affected by MeHg in ganglion layer of embryonic chick retina, Cellerino et al. (2000) have showed that NADPH-d + cells in the chick ganglion cell layer are retinal ganglion cells. The idea that eventually some embryonic displaced amacrine cells could have NADPH-

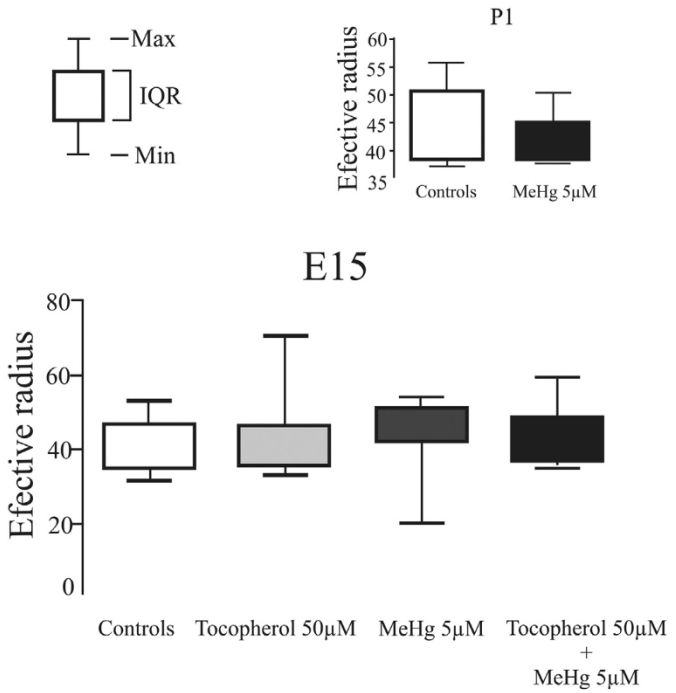

Fig. 6. The effect of methyl mercury (MeHg) on NADPHd+ neurons of the chick retinal ganglion cell layer and the protective effect of alpha-tocopherol – effective radius statistics. Box plots show the upper and lower (maximum and minimum) values and the interquartile range (first and third quartiles) for the effective radius. The plot on the upper right show the comparison for retinas at P1, showing very little difference for control retinas and MeHg-treated retinas (n = 7). The lower plot show the comparison between the four groups for E15 (n = 7): control retinas; 50 µM alpha-tocopherol treated retinas; 5 µM MeHg-treated retinas; 50 µM alpha-tocopherol + MeHg 5 µM treated retinas. MeHg-treated retinas differed from the others by showing wider range of values associated with very low values of effective radius. Control retinas, alpha-tocopherol and alpha-tocopherol treated retinas did not show very low values as MeHg-treated retinas, but both alpha-tocopherol groups showed wider variation than control retinas towards high values of effective radius.

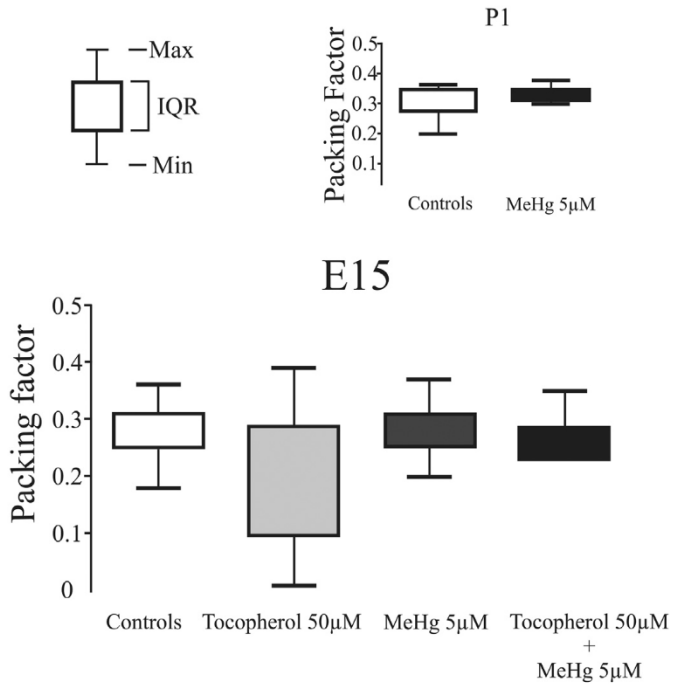

Fig. 7. The effect of methyl mercury (MeHg) on NADPHd+ neurons of the chick retinal ganglion cell layer and the protective effect of alpha-tocopherol – packing factor statistics. Box plots show the upper and lower (maximum and minimum) values and the interquartile range (first and third quartiles) for the packing factor. The plot on the upper right show the comparison for retinas at P1, showing very little difference for control retinas and MeHg-treated retinas (n = 7). The lower plot show the comparison between the four groups for E15 (n = 7): control retinas; 50 µM alpha-tocopherol treated retinas; 5 µM MeHg-treated retinas; 50 µM alpha-tocopherol + MeHg 5 µM treated retinas. MeHg-treated retinas differed from the others by showing wider range of values of packing factor associated with the interquartile range displaced towards lower values of packing factor than the other three groups. Control retinas, alpha-tocopherol, and alpha-tocopherol treated retinas were similar but did not show the range of low values observed in MeHg-treated retinas.

diaphorase activity cannot be discarded, but until now there are no studies supporting this hypothesis. At cellular and molecular levels, it is widely described in the literature that NO-synthase cells exert important role in the retinal physiology as well as the nitric oxide produced in by ganglion cells is an important neurotransmitter regulating the intracellular levels of the GMPc (Lima et al., 2014). GMPc is an important intracellular second messenger acting both in the development of retinal tissue and in the processing of visual information in adult retina. In this way, alterations in the number and distribution of NO-synthase cells in embryonic retinal could implicate in failures in retinal development and impairment in retinal physiology.

Previous works demonstrated that embryonic central nervous system is most sensible to intoxicants than adult central nervous system (Myren et al., 2007; Huang et al., 2008). The current study showed that the retinal tissue, also part of the central nervous system, is equally sensitive in the proper time window of the embryonic development. The possible explanation for this effect of MeHg on embryonic neural tissue can be related with its high propriety to interact with sulfhydryl (-SH)-containing proteins (Aschner, 1996). Mercury association with proteins causes protein inactivation and alteration s in cellular metabolism and it is well documented that protein synthesis represents a fundamental mechanism for development and establishment of embryonic central nervous system (see Steward et al., 1988; Elmer and McAllister, 2012).

It is well documented that oxidative stress represents an important intermediary of MeHg toxicity in the central nervous system (Aschner et al., 2007; Farina et al., 2011a,b; Roos et al., 2012). Different studies have demonstrated that ROS formation and lipid peroxidation mediate retinal injuries associated to MeHg exposure. In the current work, it was shown that treatment with alpha-tocopherol, a well known antioxidant compound, was able to prevent the MeHg effects on cellular density and mosaic arrangement of NADPH-d + neurons. These results are in agreement with those of Kleinschuster et al. (1983), which previously showed the protective effect of alpha-tocopherol against cellular disaggregation induced by MeHg in retinal cell cultures. In fact, protective effect of alpha-tocopherol against MeHg neurotoxicity already has been described in literature at biochemical and cellular levels (see Prasad, 1991; Andersen and Andersen, 1993; Watanabe et al., 2009). However, our study demonstrated for the first time that alpha-tocopherol is able to protect against histological disarrangement induced by a subtoxic concentration of MeHg in embryonic retina.

The concentration of alpha-tocopherol used to protect against toxicity of different agents on the CNS is very variable. Many studies describe concentration range between 200 nM and 100 µM used for example in in vitro experiments. Khanna et al. (2003) show neuroprotective effect of nmol concentration of alpha-tocopherol in hippocampal cultures submitted to toxicity induced by glutamate treatment. Nakajima et al. (1991) demonstrated that 10 µM of alpha-tocopherol is able to support the survival of cultured neurons from different regions of rat brain. Mojon et al. (1994) also describe that treatment with 100 µM of alpha-tocopherol inhibits proliferation and cell death of retinal pigment epithelium cells in model of vitreoritinopaty. In the present study, we have tested an intermediary concentration of alpha-

tocopherol in chick retinal tissue at 15 embryonic period acutely exposed to MeHg. The protective effects described in the current work suggest that enriched diet with vitamin E could be useful to protect retinal development against the toxicity induced by MeHg exposure.

In fact, oxidative stress seems to be a conserved phenomenon associated with metal toxicity in the CNS and the use antioxidant during pregnancy could be a strategy to prevent against these toxic.

An important mechanism described for generation of oxidative stress induced by MeHg intoxication is associated with deregulation of glutamate uptake by glial cells followed by hyper stimulation of ionotropic glutamate receptor in neurons. These events trigger intense calcium influx to neurons evoking ROS formation and cell (see Aschner et al., 2000, 2007). It is well documented that MeHg can be scavenged by molecules or proteins containing sulfhydryl group such as glutathione (GSH) and metallothioneins (see Aschner, 1997). Depletion of GSH content also induces oxidative stress in the CNS since GSH is an important co-substrate for GSH peroxidase (GPx) which converts H_2O_2 to H_2O. ROS production can inhibit or to block the expression of NO-synthase in the CNS. It can be a possible explanation for the protective effect assured by the alpha-tocopherol on the retinas treated with MeHg, but posteriors experiments should be performed to ratify this hypothesis.

Our founds can open new perspectives to understand the toxicological mechanism of MeHg toxicity on the embryonic human visual system since chick retinal development is controlled by homologues molecular events observed in all vertebrates.

5. Conclusions

The current study concludes that adult retinal tissue seems to be less sensible to MeHg toxicity than embryonic retinal tissue. In addition, MeHg is able to alter the normal retinal development by altering the organization of NADPH-d + neurons in ganglion layer of embryonic retina. We also conclude that alpha-tocopherol treatment protects against this tissue alteration induced by MeHg exposure.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tiv.2018.07.018.

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The following are the supplementary data related to this article.

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