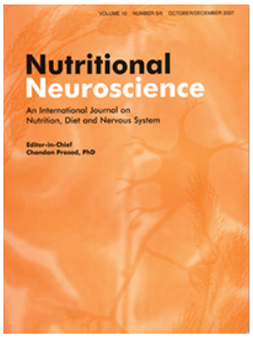

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Diet enriched with the Amazon fruit açaí (*Euterpe oleracea*) prevents electrophysiological deficits and oxidative stress induced by methyl-mercury in the rat retina

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Background: The protective effect of a diet supplemented by the Amazonian fruit Euterpe oleracea (EO) against methylmercury (MeHg) toxicity in rat retina was studied using electroretinography (ERG) and biochemical evaluation of oxidative stress.

Method: Wistar rats were submitted to conventional diet or EO-enriched diet for 28 days. After that, each group received saline solution or 5 mg/kg/day of MeHg for 7 days. Full-field single flash, flash and flicker ERGs were evaluated in the following groups: control, EO, MeHg, and EO+MeHg. The amplitudes of the a-wave, b-wave, photopic negative response from rod and/or cone were measured by ERGs as well as the amplitudes and phases of the fundamental component of the sine-wave flicker ERG. Lipid peroxidation was determined by thiobarbituric acid reactive species.

Results: All ERG components had decreased amplitudes in the MeHg group when compared with controls. EO-enriched food had no effect on the non-intoxicated animals. The intoxicated animals and those that received the supplemented diet presented significant amplitude reductions of the cone b-wave and of the fundamental flicker component when compared with non-intoxicated control. The protective effect of the diet on scotopic conditions was only observed for bright flashes eliciting a mixed rod and cone response. There was a significant increase of lipid peroxidation in the retina from animals exposed to MeHg and EO-supplemented diet was able to prevent MeHg-induced oxidative stress in retinal tissue.

Conclusion: These findings open up perspectives for the use of diets supplemented with EO as a protective strategy against visual damage induced by MeHg.

Keywords: Retina, Electroretinogram, Methyl-mercury, Oxidative stress, *Euterpe oleracea*

Introduction

Methyl-mercury (MeHg) is a biohazard pollutant released into the aquatic environment by gold mining or industrial activity.[1] Several studies have describe that a diet based on contaminated fish is the main source of MeHg intoxication in humans.[1,2] As described previously by our group, riverside populations living close to mining areas present significant visual alterations as a result of MeHg exposure.[3] Visual impairment represents a classical and well-described symptom of MeHg toxicity in humans.[3–5] To date no remedy against MeHg-induced visual damage has been identified.

Diverse nutraceuticals have been shown to protect human health.[6] Diets enriched with natural products have been used to protect the central nervous system (CNS) from injury.[7] Based on this idea, the present work aimed to evaluate the protective effect of a diet enriched with the fruit pulp of the Brazilian

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Amazonia fruit (*Euterpe oleracea*) against MeHg toxicity in rat retina.

Euterpe oleracea Mart. (EO) is an indigene palm tree present in the Amazon region that is commercialized as a juice called 'açai'. Fruit pulp of EO is widely consumed by the inhabitants of the Amazon region and it has high socio-economic value in Brazil.[8] The chemical analysis of EO fruit shows high levels of antioxidants such as anthocyanins and tocopherols.[9] The protective effects of EO fruit pulp on neurons of the CNS and its role as a natural antioxidant or as an anti-inflammatory agent have already been described previously.[10,11] However, it is still not clear whether EO fruit pulp diet can exert any protective effect on retinal tissue and if there are any functional consequences.

Retinal physiology can be studied using an electroretinograph (ERG) that measures electrophysiological responses to light stimulation. Two components of the flash ERG are often evaluated: the *a*-wave (first large negative component) and *b*-wave (positive wave and usually larger in amplitude) amplitudes and the implicit time under photopic (light-adapted ERG response) and scotopic (dark-adapted ERG response) conditions. The *a*- and *b*-waves are thought to originate in the photoreceptors (rod and cone cells) and in the bipolar/Muller cells activity, respectively. Furthermore, other components could be evaluated such as the photopic negative response (PhNR), which reflects the ganglion cells and inner retinal function, and the phase and amplitude of fundamental flicker component which is a sensitive cone pathway-driven response.[12] Studies have already demonstrated that MeHg accumulates in retinal tissue and can significantly decrease ERG responses.[13,14] Toxicological mechanisms associated with ERG alterations induced by MeHg are still unclear, but overproduction of reactive oxygen species (ROS) and oxidative stress as a resultant phenomenon of MeHg toxicity in the brain has been extensively described.[15] The purpose of the present work was to study if a diet enriched with EO fruit pulp could be protective against MeHg-induced retinal dysfunction.

Methods

Animals

Thirty-two male Wistar rats (150–200 g) were used for the ERG measurements. The animals were obtained and kept at a central animal facility of the Federal University of Pará (UFPa) under a light/dark cycle (12 hours on/12 hours off). Thirty-three animals were used to carry out the biochemical assays after the ERG measurements. All experiments were conducted in accordance with the use and care of Animals in Ophthalmic and Vision Research (ARVO) and were approved by the Ethics Committee on Experimental Animals of the Federal University of Pará (UFPA).

Diet

Pulp of EO fruits was obtained from a farm at Castanhal, Pará, Brazil. The plant species was identified by specialists of the UFPa herbarium. EO-enriched food was produced by mixing regular commercial food (PURINA Company Cia, São Paulo, Brazil) and fruit pulp (10:1 g/g) with ultrapure water. Then the mixture was compressed into pellets and dried by warming at 40°C for 2 hours. Finally, the enriched ration was given to the animals after cooling to room temperature. The commercial ration used to feed the control group was the same kind used to prepare EO-enriched food.

The animals were divided into two diet groups: one group was fed regular commercial ration ($n = 16$) and the other group was fed the supplemented diet ($n = 16$) for 28 days before experimentation. Body mass was weighted once a week and no significant differences in animal weight and consumption was observed (data not shown). After this period, two sub-groups of animals with a conventional diet were formed: one subgroup ($n = 8$) was exposed to 5 mg/kg/day methyl-mercury chloride (MeHg) by oral administration for seven consecutive days as described previously by Liu *et al*.[16] The other subgroup received a saline solution by oral administration. Similarly, the group that received the supplemented diet was divided into eight animals that it was exposed to MeHg and eight animals were sham treated with saline. During MeHg treatment all groups were maintained in separate cages at the central animal facility of UFPa at the same conditions described above.

ERG

In the present work, the retinal activity was studied using full-field electroretinography (ffERG). A xenon lamp (PS33-PLUS) was used to deliver white light flashes for 10 ms. Stimulus calibration was performed using a CS-100A Colorimeter (Minota, Osaka, Japan). ERG responses were amplified 50,000X in a Model P511 amplifier (Grass Technologies, Warwick, RI, USA), filtered between 0.3 and 300 Hz and digitalized at 1 kHz sampling rate (National Instruments, Austin, TX, USA). Labview 3.0 (National Instruments, Austin, TX, USA) was used a data acquisition software.

Prior to the ffERG recordings, the animals were adapted to the dark overnight and anesthetized by intraperitoneally injection of ketamine (100 mg/kg)/xylazine (6 mg/kg) and transferred to a Faraday cage. Pupils were dilated by topical administration of a drop of tropicamide 1% (Alcon Surgical, Inc., Fort Worth, TX, USA). (1) An electrode placed in the ear of the animal served as the ground electrode (model F-E6SHC-12, Grass Technologies); (2) a subcutaneous steel needle electrode in the eyelid was used

as the reference electrode (model F-E3-48, Grass instruments, Warwick, USA), and (3) a silver ring electrode placed on the conjunctiva was used as the active electrode. Electrode placement was carried out under deep red room lighting.

Visual stimuli

A visual stimulator (Model PS33-PLUS, Grass Technologies) was used and ffERG was evaluated based on scotopic and photopic responses according to Harazny *et al.*[17]Rod responses were recorded to 0.09 cd.s.m^{-2} flashes. We also measured mixed rod- and cone-driven responses by measuring the responses to 0.378 cd.s.m^{-2} interstimulus intervals (called 'mixed 1' condition) and to 10.215 cd.s.m^{-2} (called 'mixed 2' condition). To obtain photopic responses (cone single flash and flicker), the animals were light-adapted during 10 minutes. Thereafter, the responses to 10.21 cd.s.m^{-2} flashes were recorded. Photopic flicker responses were measured using square-wave stimuli at different temporal frequencies (12, 18, and 24 Hz) presented for 6 seconds. Interflash time intervals were 15 seconds for scotopic conditions and 4 seconds for photopic conditions. For scotopic (rod and mixed response) and photopic single flash responses, the responses among 6 and 12 flashes were averaged. Average of six samples of photopic flicker responses were carried out.[17] To single flash responses, the *a*-wave amplitude was measured from baseline to the first negative peak. The *b*-wave amplitude was defined as the voltage difference between the peak of the a-wave and the first positive peak component of ERG. We also evaluated the PhNR that consists of the amplitude from the baseline to the peak of the second negative component. The *a*-wave, *b*-wave, and PhNR implicit times were measured from the flash onset to the *a*- and *b*-wave peaks and the peak of PhNR, respectively. The flicker amplitudes were measured peak to peak of each response. After electrophysiological evaluations, all groups were conducted to biochemical analysis of oxidative stress in the retinal tissue.

Lipid peroxidation assay

Animals were deeply anesthetized with ketamine (100 mg/kg 90 mg/kg)/xylazine (6 mg/kg 10 mg/kg) by intraperitoneally injection. After that, the animals were quickly killed by decapitation. The eyes were enucleated and whole retina was dissected. The tissue was homogenized in phosphate buffer saline pH 7.4 at 4°C. The homogenates were centrifuged at 3000 rpm for 5 minutes and the supernatant was kept for the biochemical analysis of oxidative stress using the thiobarbituric acid reactive substances method.[18] Analysis of the lipid peroxidation was carried out based on standard curve concentrations of malondialdehyde (MDA), measured by the absorbance at a 535 nm wavelength. MDA concentration was quantified in nmols per milligram of protein; the protein levels were determined by the Bradford method.[19] The values were expressed as a percentage of control.

Statistical analyses

Electrophysiological and biochemical data were expressed as averages±standard deviation. The normality test was performed using the Shapiro–Wilk test and the statistical difference between all groups was evaluated using one-way ANOVA followed by the Tukey *post* test. Statistical analysis was carried out using the BioEstat 5.0 software and *P*-values <0.05 were considered significant.

Results

Retinal impairment induced by MeHg is prevented by EO fruit diet

Our data showed that EO-enriched diet did not alter the electrophysiological response of rat retina. On the other hand, rats submitted to MeHg exposure showed decreased a-wave and b-wave amplitudes for the photopic condition (Fig. 1A, upper and middle). Similar results were observed for PhNRs (Fig. 1A, lower). Values of the b/a-wave ratio were not altered in the MeHg group when compared with control. All these results suggest that both primary- and second-order retinal neurons were affected by MeHg toxicity (Fig. 1B). Photopic flicker responses after retinal stimulation at 12 Hz ($P < 0.05$), 18 Hz ($P < 0.05$), and 24 Hz were decreased in the MeHg group when compared with control ($P < 0.05$) (Fig. 2A–C).

Data from protective experiments showed that diet enriched with EO fruit pulp protects against toxicological effect of MeHg on the *b*-wave and PhNR amplitudes ($P < 0.05$). Nevertheless, EO diet was not able to prevent the MeHg-induced decrease on the *a*-wave amplitude during photopic stimulation (Fig. 1A). Animals submitted to EO diet and treated with MeHg present increased values of the b/a-wave ratio when compared with both the control group and the MeHg group ($P < 0.01$) (Fig. 1B). These results suggest that EO diet seems to exert protection on the second-order neurons of rat retina.

We also observed that supplementation of the enriched diet prevented alterations of photopic flicker responses elicited by different temporal frequencies (12, 18, and 24 Hz) in the animals treated with MeHg ($P < 0.05$) (Fig. 2A–C).

The b-wave amplitude of the scotopic rod response was also altered in the group treated with MeHg, and no protective effect of EO fruit dietary was observed on the decrease MeHg-induced in the b-wave amplitude (Fig. 3). Nevertheless, EO fruit diet prevented the decrease of the scotopic mixed

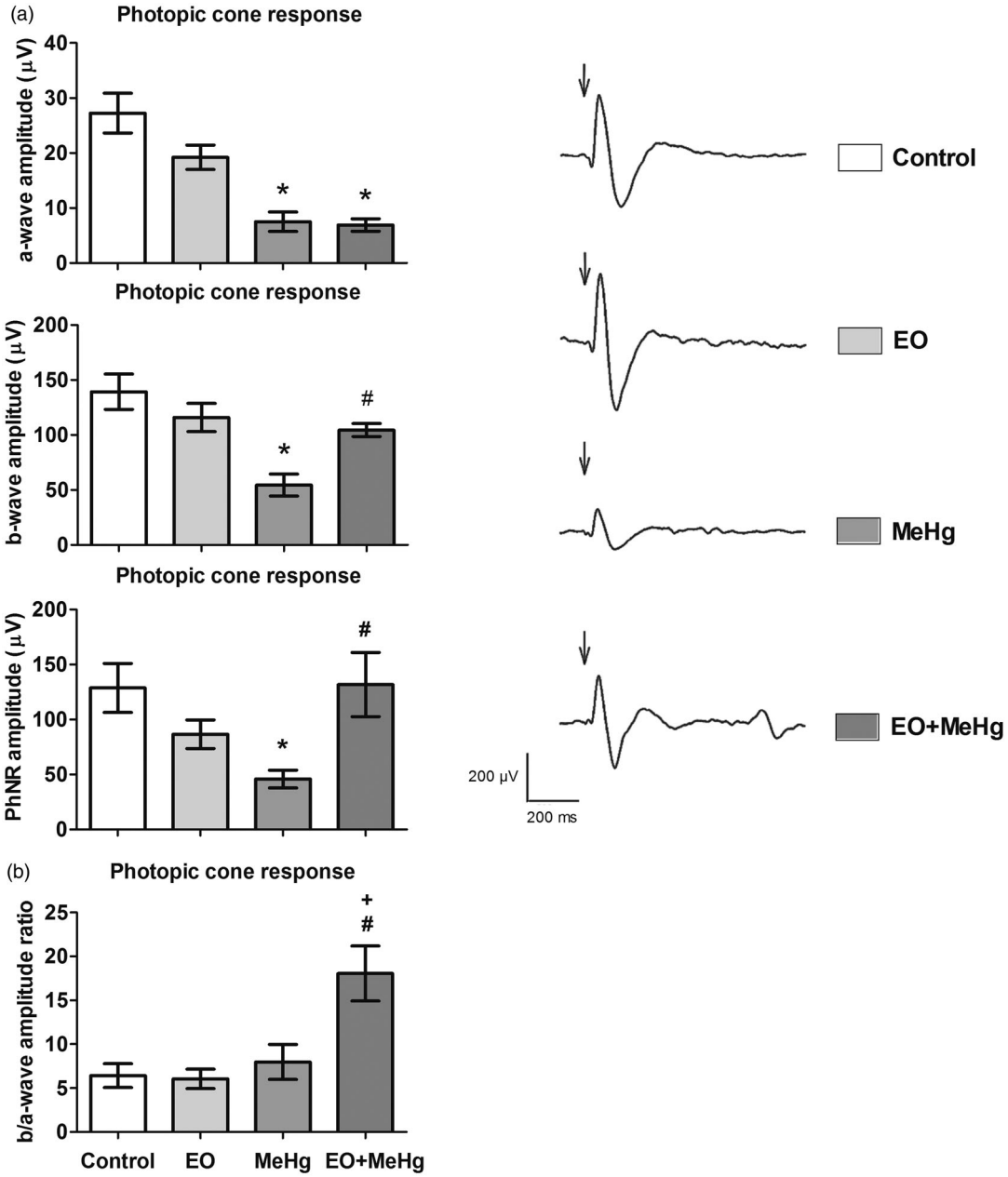

Figure 1 Photopic cone responses from rat retina. (A) a-wave (upper), *b*-wave (middle), and PhNR-wave (lower) amplitude responses of control, EO dietary, MeHg, and EO+MeHg groups. (B) Analysis of the *b*/*a*-wave ratio of the amplitudes in control, EO dietary, MeHg, and EO+MeHg groups. Data represent average and standard error. Statistical analyses were performed by one-way ANOVA followed by the Tukey *post* test $^{*}P < 0.05$ vs. control (A); $^{+}P < 0.01$ vs. control, and $^{\#}P < 0.05$ vs. MeHg (BioStat 5.0 software).

component (rod–cone response) induced by MeHg ($P < 0.01$) (Fig. 4A and B). Implicit time and time of cell response at photopic or scotopic conditions were not altered in neither the groups evaluated in the present study (data not shown).

Oxidative stress induced by MeHg in retinal tissue is prevented by EO fruit diet

Our biochemical analysis demonstrated that EO fruit diet did not alter MDA production in the rat retina; but intoxicated animals showed high MDA levels in retina when compared with the control group (Fig. 5). Rats submitted to EO fruit diet and intoxicated with MeHg did not present increased MDA levels when compared with control (Fig. 5).

Discussion

The use of nutraceuticals to treat different diseases has opened up new perspectives for the use of diets in the prevention of CNS injuries.[6,7] MeHg intoxication evokes severe visual function impairment in populations living close to mining areas.[3–5] Ventura *et al.*[20] and Da Costa *et al.*[21] have described that humans chronically exposed to mercury vapor show decreased photoreceptor responses. Animal models also develop similar visual function impairments as

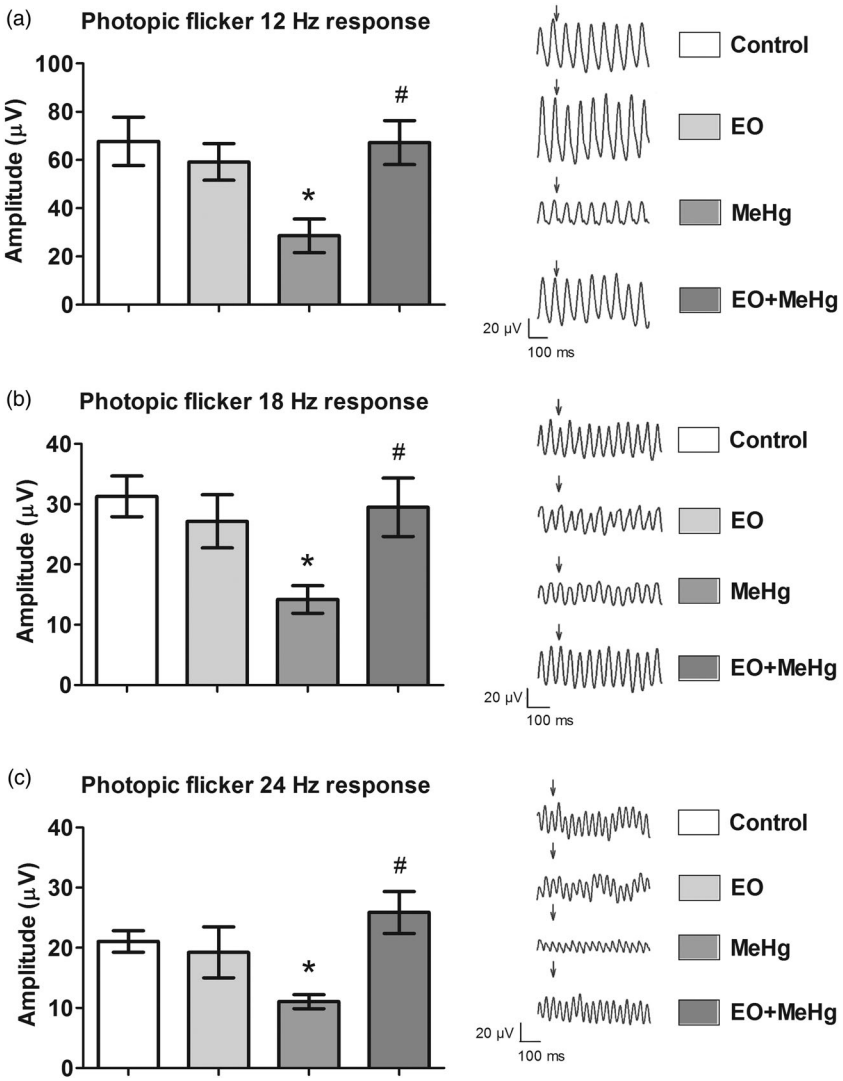

Figure 2 Photopic flicker response after (A) 12 Hz, (B) 18 Hz, and (C) 24 Hz stimulus. Data are expressed as average of wave amplitudes. Statistical analyses were performed by one-way ANOVA followed by the Tukey *post* test $^{*}P < 0.05$ vs. control and $^{\#}P < 0.05$ vs. MeHg (BioStat 5.0 software).

previously demonstrated by Goto *et al.*[14] and Tanan *et al.*[22] and our results are in agreement with these results since we have demonstrated low electrical response from photoreceptor cells of rats exposed to MeHg.

Considering the electrophysiological data from the animals submitted to the diet and exposed to MeHg, our results demonstrated a protective effect on the response elicited by second-order neurons and ganglion cells of the retina but not a protective effect

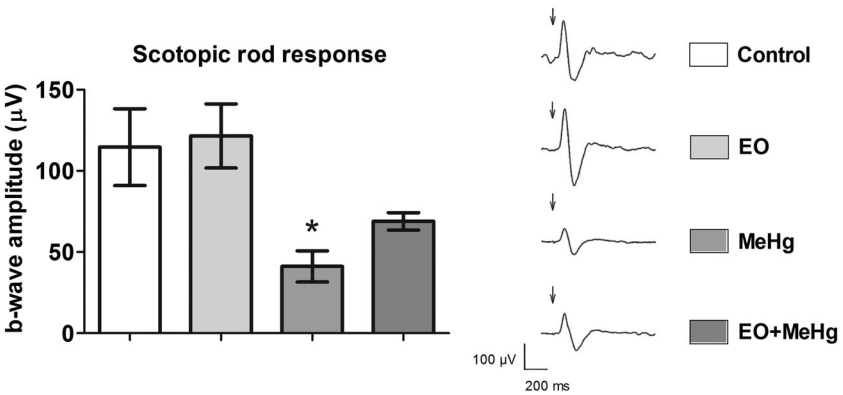

Figure 3 Scotopic rod responses from rat retina. *b*-wave amplitude of control, EO dietary, MeHg, and EO + MeHg groups. Data are expressed as average of *b*-wave amplitudes. Statistical analyses were performed by one-way ANOVA followed by the Tukey *post* test $^{*}P < 0.05$ vs. control (BioStat 5.0 software).

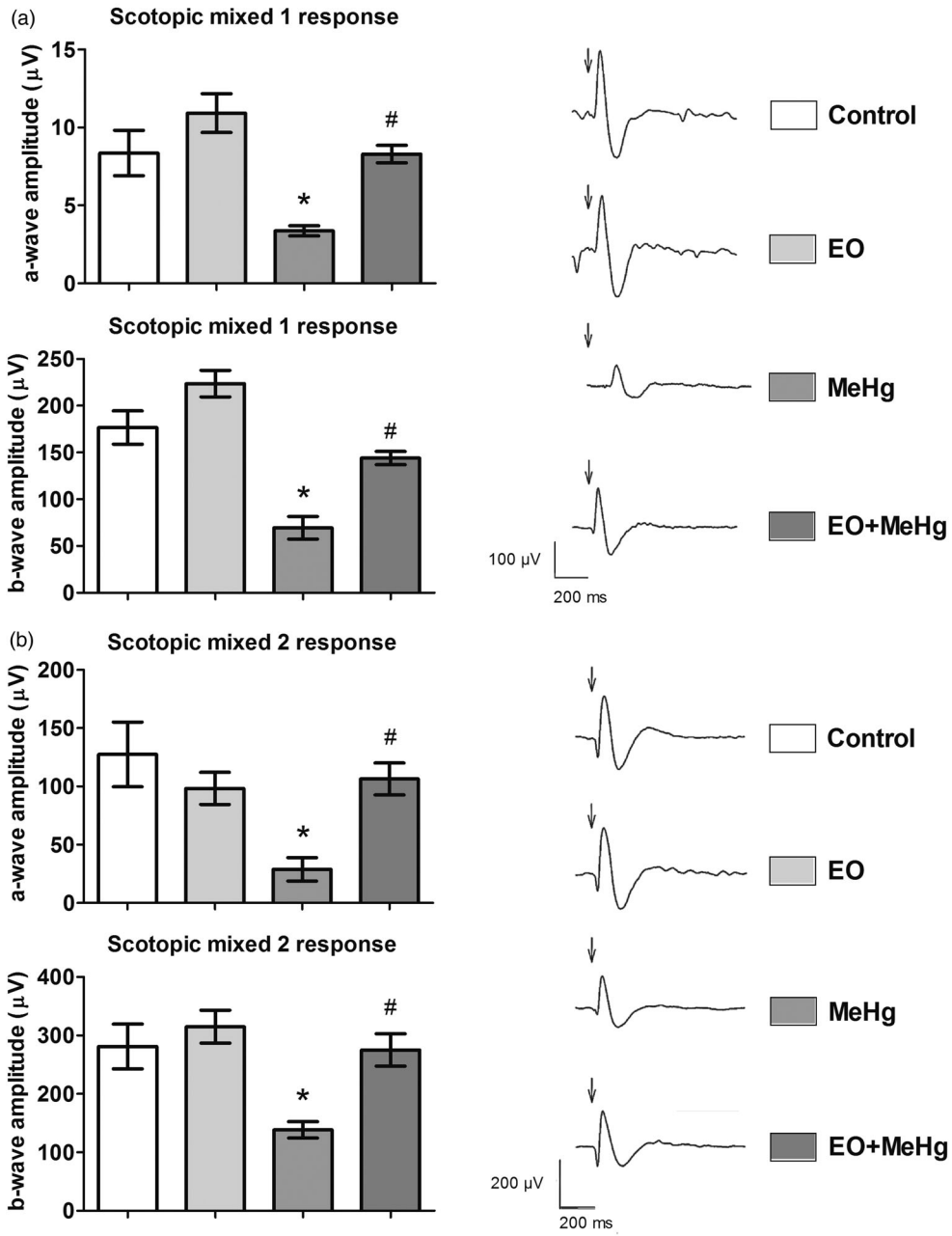

Figure 4 Scotopic mixed responses after experimental treatment. (A) *a*-wave (upper) and *a b*-wave (lower) amplitudes of scotopic mixed 1 stimulus. (B) *a*-wave (upper) and *a b*-wave (lower) amplitudes of scotopic mixed 2 stimulus amplitudes. Responses were measured from the electroretinographic waves of control, EO dietary, MeHg, and EO+MeHg. Data represent average and standard error. Statistical analyses were performed by one-way ANOVA followed by the Tukey *post* test $^{*}P < 0.05$ vs. control and $^{\#}P < 0.05$ vs. MeHg (BioStat 5.0 software).

on the response elicited by cone photoreceptors during photopic conditions (Fig. 1). Also, second-order neurons from rod pathways were not protected by the supplementary fruit diet, but a protection attributed to the rod photoreceptor was identified from the *a*-wave record of scotopic mixed responses (Figs. 4A and B). These results led us to hypothesize that MeHg toxicity could occur via different mechanisms in different cells and in different layers of retinal tissue. The effective action of EO fruit diet on the rod response in our work is in agreement with Matsumoto *et al.*,[23] who describes a rhodopsin turnover in rod photoreceptors stimulated by anthocyanin cynidin-3-glucoside. Thus, our results suggest that the enriched diet with EO fruit is able to protect against injuries affecting the photoreceptor cell physiology but the biochemical mechanism involved in this phenomenon still needs to be clarified.

Oxidative stress is described as an important phenomenon in terms of biochemical alterations induced by MeHg.[15] In fact, the relationship between ROS production and electrophysiological deficits has not yet been described for retinal tissue; however, oxidative stress followed by ERG alterations is well reported in retinal diseases such as glaucoma and retinitis pigmentosa.[24,25] The present work

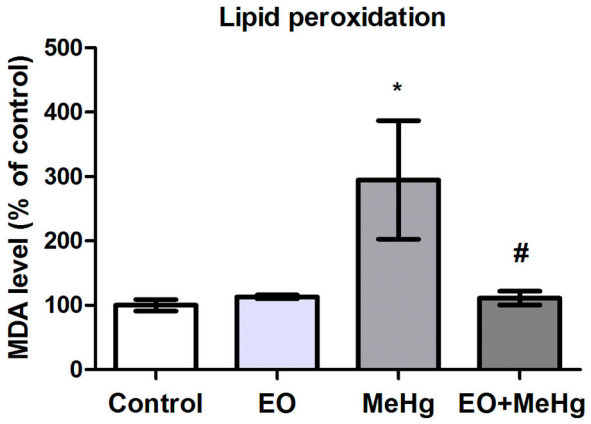

Figure 5 Lipid peroxidation in retinal tissue. MDA levels were determined in different experimental groups and the values were expressed as a percent of control. Data represent average and standard deviation and statistical analyses were performed by one-way ANOVA followed by the Tukey *post* test *$P < 0.05$ vs. control # vs. MeHg (BioStat 5.0 software).

demonstrated for the first time that MeHg intoxication induces oxidative stress in retinal tissue. Although the biochemical mechanisms that lead to ROS production as a response to MeHg toxicity in the retina have not been clarified, Herculano *et al*. [26] reported that treatment with an inhibitor of nitric oxide production decreases retinal cell death induced by MeHg. Here, we demonstrated that diet enriched with EO fruit pulp was able to prevent oxidative stress evoked by MeHg in retinal tissue. The high concentration of antioxidants such as anthocyanins and tocopherols in EO fruit could explain its protective effect, but we did not discard the participation of other compounds present in EO fruit. In fact, the present study only aimed to demonstrate that diets enriched with a natural fruit are able to prevent oxidative stress and consequent the toxicity of MeHg on retinal tissue.

Generally speaking riverside populations live under low socio-economic conditions which mean that it is important to develop and to characterize cheap diets with easy access especially for people living in contaminated areas. The present work is a first step along these lines, since we have demonstrated, for the first time, that diet enriched with EO fruit pulp from Amazon is able to prevent electrophysiological and biochemical impairment induced by MeHg on retinal tissue.

Disclaimer statements

Contributors All authors contributed equally.

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Conflicts of interest The present work do not present any conflicts-of-interest.

Ethics approval The present study was conducted in accordance with the use and care of Animals in Ophthalmic and Vision Research (ARVO) and were approved by the Ethics Committee on Experimental Animals of the Federal University of Pará (UFPA).

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