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Extracellular Zinc Chelator *In Vivo* On System Of Taurine in Retina: Transport, Concentrations and Localization of Transporter

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Abstract

Taurine and zinc are relevant molecules in the retina, both are highly concentrated and have similar functions in this structure. Little is known about the mechanisms of action of this amino acid and the interaction with other molecules such as zinc. The objectives of the present study were to evaluate the consequences of zinc deficiency by the extracellular chelator diethylenetriaminepenta-acetic acid (DTPA) on taurine levels, transport and localization of taurine transporter in rat retina. Various concentrations of the extracellular zinc chelator, DTPA, dissolved in dimethylsulfoxide, was administered intraocular: 10, 100, 250 and 500 μM. Retinas were dissected 3, 5 and 10 days later. Zinc was determined by spectrophotometry. Taurine levels, determined by high performance liquid chromatography with fluorescence detection, DTPA administration, 10 μM, decreased taurine in 56% at 3 days. Capacity of taurine transport, using [^{3}H]taurine, was decreased in 44% without changes in the affinity after DTPA. The treatment produced differences in the distribution of taurine transporter in all layers of retina, the least in the outer plexiform layer. The observations contribute to understanding of taurine-zinc interaction in the retina and could have functional implications. The effects of zinc or decrease on the system of taurine, transport, levels and location of the transporter are indicative of functional relevances. They are aspects crucial to the understanding of taurine-zinc interaction in the retina.

Keywords: Retina; Taurine; Taurine transport; Zinc

Abbreviations BCA: Bicinchoninic Acid; BSA: Bovine Serum Albumin; CNS: Central Nervous System; DAPI: 4',6-Diamidino-2-Phenylindole; DTPA: Diethylenetriaminepentaacetic Acid; FITC: Fluorescein-5-Isothiocynateconjugated; GC: Ganglion Cells; GCL: Ganglion Cell Layer; GliC: Glial Cells; HPLC: High Performance Liquid Chromatography; ICP: Inductively Coupled Plasma Emission Spectrophotometry; io: Intraocular; INL: Inner Nuclear Layer; IOD: Integrated Optical Density; IPL: Inner Plexiform Layer; ONL: Outer Nuclear Layer; OPL: Outer Plexiform Layer Phot photoreceptors; SEM: Standard Error Of The Mean; TAUT taurine transporter: TPEN N,N,N,N-Tetrakis-(2-Pyridylmethyl) Ethylenediamine

Introduction

The high concentration of taurine in the retina has triggered a series of investigations to understand their role in various pathophysiological conditions. For instance, it has a critical role in diseases: cardiovascular, diabetes, in migraine, in cerebral ischemia and epilepsy [1,2]. The majority of eye diseases in humans have been associated with a zinc deficiency, in relation to decreased serum zinc levels or by improvement with metal supplement [3]. Today, researchers use chelating agents as an alternative to restrict access of zinc to the cells because zinc deficient diets have little effect in reducing the metal in the tissues in a relatively short time such as the brain, where 30% of zinc reduction in the hippocampus occurs after 3 months [4]. Chelating agents have the advantage for restrict access of zinc to cells and prevent the effects of systemic administration by performing local injections, which prevents the interpretation of results is less confusing [4,5].

We have previously shown that intermediate concentrations of zinc extracellular chelator, diethylenetriaminepentaacetic acid (DTPA) *ex vivo* significantly decreased [^{3}H]taurine transport [6]. In addition, the reduction of zinc, by zinc intracellular chelator, N,N,N,N-tetrakis-(2-pyridylmethyl) ethylenediamine (TPEN) in the retina, significantly decreased TAUT in all layers, as detected by immunohistochemistry, except in photoreceptors (Phot), indicating that the presence of zinc is necessary for maintenance of the transporter in the retina [7].

Taurine and zinc perform similar functions in the central nervous system (CNS), interacting in development and in various functions of the retina, and both are related to retinal protection and regeneration. Thus, for studying the structural and functional correlations between taurine and zinc, deficiency was produced with DTPA and levels of taurine, taurine transport and taurine transporter were determined in rat retina. Thus, the effect of decreasing extracellular zinc, caused by chelating agents, could affect taurine system in retina.

Materials and Methods

Animals

Male Sprague-Dawley rats (150-200 g) from the animal housing at Instituto Venezolano de Investigaciones Científicas had an adaptation period of at least 48 h in the experimental room of the Laboratory, food and water provided ad libitum. The animals were decapitated

between 8:00 and 10:00 am, and the eyes were extracted from orbit. Handling of animals was conducted following the standards of animal bioethics [8] and was approved by the Bioethics Committee for Animal Research of the Institute.

Treatment with the extracellular zinc chelator *in vivo*

Various concentrations of extracellular zinc chelator DTPA, 10, 100, 250 and 500 μM, were administered for different periods of time 3, 5 and 10 days, for selecting the dose to be used according to zinc levels reduction [9]. Three experimental groups of rats were included:

- Intraocular administration (io) of 10, 100, 250 and 500 μM, 2 μl of saline and dimethyl sulfoxide (DMSO, 0.001%).
- Administration, io, of 2 μl of DMSO 0.001% final concentration (vehicle, control group).
- Needle puncture (sham intervention).

Isolation of rat retinal cells

Retina was dissected and cells were separated with 0.25% trypsin in Locke buffer (500 μl), composed (in mM) of 154 NaCl, 2.7 KCl, 2.1 K_2HPO_4, 0.95 KH_2PO_4, 2.7 sucrose and 2.5 HEPES, at 37°C for 10 mints, followed by mechanical further separation with Pasteur pipette. The cells were washed with phosphate saline buffer Na^+-K^+ (PBS) 0.1 M pH 7.4, centrifuged for 10 mints at 2,000 rpm (300 g), and counted in Neubauer chamber. Integrity of membrane was determined by 50% Trypan blue exclusion (>96%) [6].

Determination of total zinc

The tissue was homogenized with manual teflon homogenizer in 320 μl of deionized distilled water, 20 μl were collected for protein determination by bicinchoninic acid (BCA) assay, and the remainder was used for determination of total zinc [10]. Tissue samples were dried, suspended in 1 ml of concentrated HNO_3, heated for 6 h at 60°C and made up to 10 ml with deionized water. Inductively coupled plasma emission spectrometry (ICP-AES) was performed in a Perkin-Elmer Model Optima 3000 equipped with a U-5000AT ultrasonic nebulizer, a standard demountable-type quartz plasma torch and alumina injection (1.5 mm internal diameter). A ten-roller peristaltic pump was used to supply the gas with the sample solution. The atomic line for zinc was 213.856 nm [9,11]. After verified reduction of zinc levels, 10 μM of DTPA, 3 days after io injection was chosen for all the experiments.

Determination of taurine

Taurine was determined in isolated cells of the retina and after DTPA injection. High Performance Liquid Chromatography (HPLC) with fluorescent detection was employed by a modified method [12]. The system consisted of a Waters 2690 Separation System and a Waters 2475 fluorescent detector. Separation was done with a Supelco LC-18 column 4.6 mm × 15 cm, 5 μm. The samples was resuspended and homogenized in 50 μl of 20% sulfosalycilic acid and 300 μl of 0.4 M potassium borate buffer pH 10.4 and the aliquots were subjected to protein analysis by BCA assay [9]. Centrifugation of samples was carried out at 38,000 g for 20 min at 4°C. Derivatization was performed by addition of 200 μl of the following mix: 25 mg ophthaldehyde, 500 μl methanol, 25 μl β-mercaptoethanol (1g/ml), and 4.5 ml 0.4 M potassium borate buffer of pH 10.4. Aliquots of the derivatized preparation were injected into the chromatographic system. Taurine was quantified by the method of the external standard and expressed in nmol/mg of protein using the program Millenium (Waters, MA, USA) [13].

Saturation of taurine transport and extracellular zinc chelator *in vivo* on transport

According to Márquez et al. hipotaurine and beta-alanine were specific inhibitors, number of cells, temperature and time of pre- and incubation were fixed [6]. For saturation assays, the cell preparation (250,000 cells per tube) was incubated in the presence of various concentrations of [^{3}H] taurine from 15 to 165 nM (488 and 629 GBq). For each concentration, duplicate tubes were prepared in a final volume of 500 μl. The preparation was pre-incubated at 37°C for 5 min in Locke solution. Incubation, 25s, was started by the addition of the substrate. Subsequently, the process was stopped by rapid filtration through fiberglass filters (Watman GF/C), followed by two washed with 5 ml of cold Locke [6]. High and low-affinity components of transport were evaluated, and transport capacity (V_{max}) and affinity constant (Kt) were calculated for control and treated rats with DTPA.

Immunohistochemistry of taurine transporter

As described above, 3 days after injection of 10 μM DTPA, retinas were obtained for immunohistochemistry assays of TAUT. Dissection and fixation of tissue were done. Rats were anesthetized with Equitesin, (1 ml i.p./kg) (Na+-pentobarbital in 95% ethanol chloral hydrate in propylene glycol and magnesium sulfate in water). The animals were decapitated; eyes were removed and fixed in 4% formaldehyde for 24 h. After fixation, the eyes were dehydrated by several washings with 70% ethanol for 4 h, later were cleared in xylene solvent at room temperature. The eyes were placed in xylene, and three changes were made for 1 h each at room temperature, were included in paraffin for 1 h in an oven at 56-58°C. They were allowed to solidify overnight at 4°C to form the histological blocks. Tissues were cut in the microtome into sections of 5-7 μ at room temperature [7]. The sections were mounted on slides coated with egg albumin as previously reported [7,14]. Antigen retrieval was performed with 0.5% trypsin for 15 min, and the slides were washed with PBS. To permeabilize, the sections were incubated with Triton X-100 (0.1%) diluted in PBS for 10 min and the slides were washed with PBS. To block non-specific binding was added 5% BSA diluted in PBS for 1 h. They were then incubated overnight at 4°C with a polyclonal antibody developed in mouse anti-TAUT-1 (Santa Cruz Biotechnology), at a dilution of 1:500 in 5% BSA. After incubation, the sections were washed with PBS and incubated in the dark at room temperature for 1 h with rhodamine conjugated secondary antibody (goat anti-mouse IgG) (Santa Cruz Biotechnology) at a dilution of 1:500 in 5% BSA. For staining the nuclei of cells, the sections were incubated for 5 min with 1 μg/ml of 4',6-diamidino-2-phenylindole (DAPI). After incubation, the sections were washed with PBS. To examine the immunofluorescence of DAPI and rhodamine was used a Nikon microscope (Eclipse E600) with Coolpix 4300 camera attached. For negative control, cells were incubated with secondary antibody only to determine the nonspecific fluorescence [7]. The photographs were combined and analyzed with the program Image J version 1.47, which is a program of digital image processing the programmed public domain in Java and developed at the National Institutes of Health [15]. Fluorescence was measured as integrated optical density (IOD). For the determination of the IOD selection "circle" was used, 100 pixeles2 (25 pixeles2=10-15 μm^2) in four areas for each tissue layer randomly selected for then calculating the average

of each area [16]. In the case of ganglion cell layer (GCL), measurements were made by cells, ten cells were chosen randomly and in each cell IOD was measured in three different areas, using 20 pixeles2 for calculating average [7].

Statistical analysis

Each value represents mean ± standard error of the mean (SEM).The statistical significance of the specific data was determined by analysis of variance (ANOVA) with the program INSTAT followed by Tukey test [17]. Saturation curves were analyzed using the program GraphPad Prisma 2. V_{max} and K_t of taurine transport were calculated either by Lineweaver-Burk plots or curvilinear analysis. For immunohistochemical assays, reported n refers to the number of rats. To establish differences between the presence of transporter in cells or in layers of the retina, ANOVA followed by Tukey test was done Values of $P<0.05$ were considered statistically significant.

Results

Zinc levels after io injection of extracellular zinc chelator

There was no significant difference between the groups receiving the vehicle, DMSO, or the needle puncture, thus control group was that with injection of DMSO. The concentration of zinc in the control group was 105.76 ± 14.12 µg/mg of protein. Zinc levels were significantly decreased to 46.82 ± 6.9, 54.84 ± 10.63, 50.63 ± 2.02 and 56.28 ± 5.63 µg/mg of protein at 3 days after the io administration of DTPA in a concentrations of 10, 100, 250 and 500 µM respectively, compared to DMSO (Figure 1). At 5 and 10 days, DTPA did not produce a significant decrease in zinc levels in the retinas of the eyes treated with different concentrations of the chelator (data not shown). The treatment with DTPA did not modify Ca^{2+}, Mg^{2+}, Fe^{2+}, and Na^{+} levels in the retina.

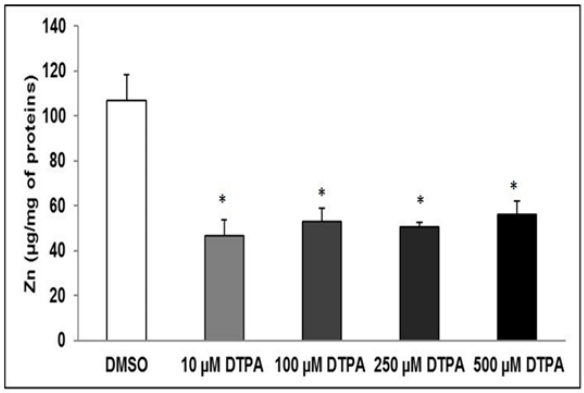

Figure 1: Effect of DTPA *in vivo* on the levels of zinc in the rat retina. Zinc levels were determined at concentrations of the chelator 10,100, 250 and 500 µM and 3 days after injection io. Each value is the mean ± SEM, n=5. *p<0.05 with respect to DMSO.

Taurine levels after io injection of extracellular zinc chelator

The concentration of taurine in isolated cells from controls was 44.11 ± 5.01 nmoles/mg of protein and was significantly reduced in 34% (29.25 ± 4.68 nmoles/mg of protein) at 3 days after the io administration of 10 µM DTPA (Figure 2).

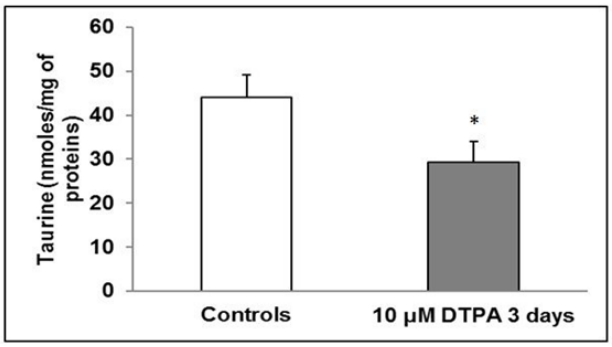

Figure 2: Effect of DTPA *in vivo* on taurine levels in the rat retina. Taurine levels were determined by HPLC to 10 µM of the chelator and 3 days after injection io. Each value is the mean ± SEM, n=4. *p<0.05 with respect to controls (DMSO).

Taurine transport after io injection of extracellular zinc chelator

3 Days after the io administration of 10 µM DTPA, taurine transport decreased respect to control group (54.21 ± 1.57 fmol/106 cells) (Figure 3 and Table 1). There was a significant reduction of 44% (30.16 ± 1.91 fmol/106 cells) in the capacity and no significant changes in affinity for the taurine transport (Table 1).

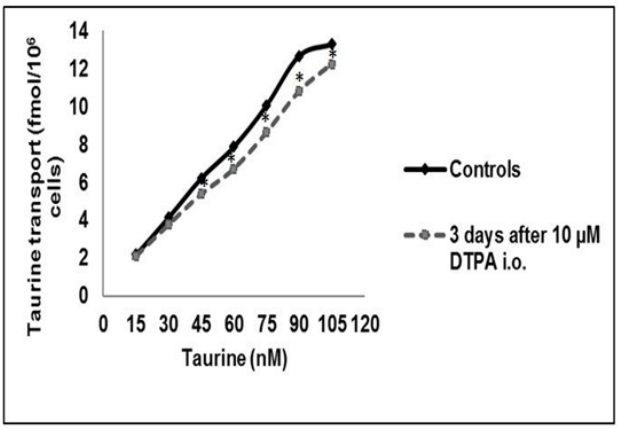

Figure 3: Effect of zinc chelator DTPA *in vivo* on the of [^{3}H] taurine transport. Cells (200,000) were preincubated 5 min in Locke buffer at 37°C. The transport was determined at different concentrations of [^{3}H]taurine of 15-105 nM and incubation time of 25 s. The animals were treated with 10 µM of DTPA and the transport was determined 3 days after the administration io. Each value is the mean ± SEM, n=5. *p<0.05 with respect to control group (DMSO).

Uptake of [^{3}H] taurine		
Days	V_{max} (fmol/10^6 cells)	K_t (μM)
Control DMSO 3rd	54.21 ± 1.57	0.48 ± 0.03
10 μM DPA 3d	30.16 ± 1.91*	0.30 ± 0.06
Each value is the mean ± SEM, n=5. *p<0.05 with respect to control group. $V_{max\ and}$ K_t were determined by the method of double reciprocal of Lineweaver-Burk.		

Table 1: Effect of administration io. 10 μM of DTPA on the kinetics parameters of [^{3}H] taurine transporter in isolated cells from rat retinal after 3 days of injection.

Localization of taurine transporter in layers of rat retina after io injection of extracellular zinc chelator

The IOD values are reported in Table 2. The treatment with DTPA produced significant differences in the distribution of TAUT (Figure 4). Significant decrease of IOD was observed in all layers of retina, the least in the outer plexiform layer (OPL) compared with TAUT control group (Figure 5 and Table 2).

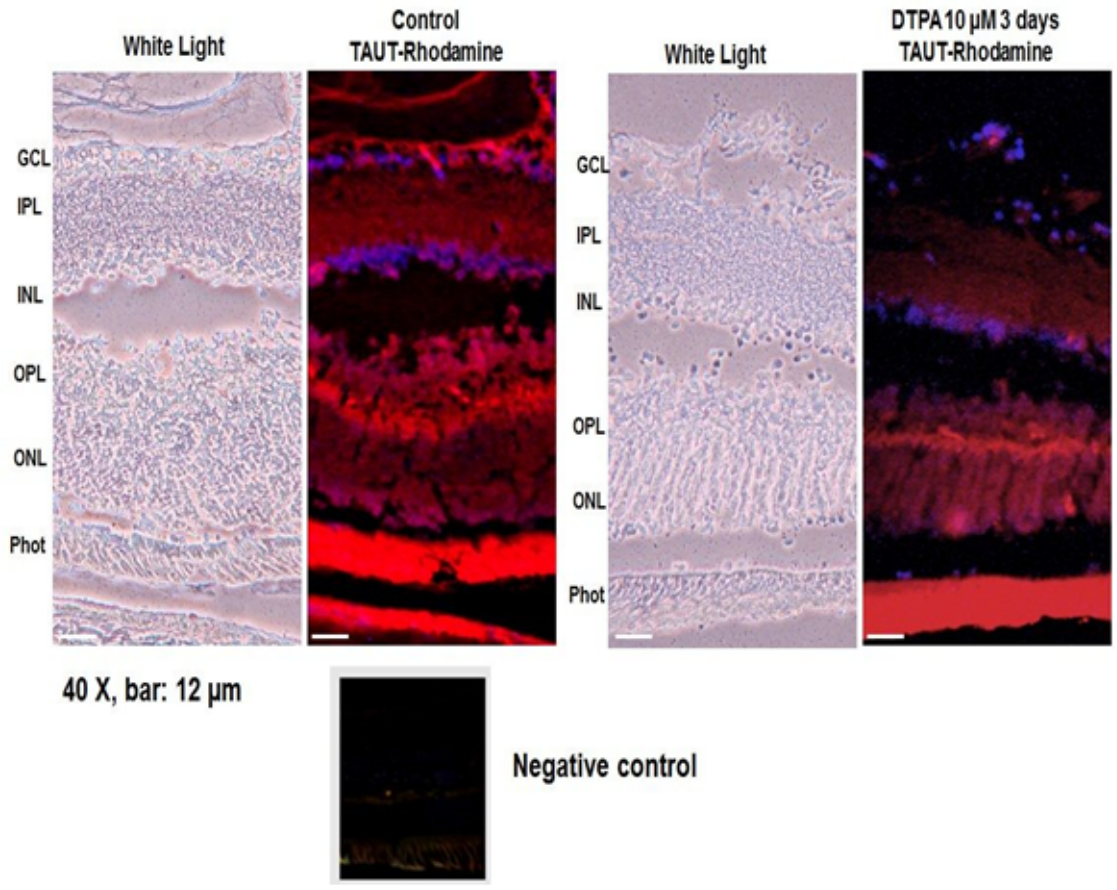

Figure 4: Effect the diethylenetriaminepentaacetic acid on localization of taurine transporter in layers of rat retina. TAUT was labeled with rhodamine (red). The retinal layers are observed GCL: Glial Cells, GliC: Inner Nuclear Layer; INL: Inner Plexiform Layer; IPL: Outer Nuclear Layer; ONL: Outer Plexiform Layer; OPL: Photoreceptor Phot. For each condition, the negative control is shown. Boxes indicate the location of the transporters. n = 6 for each TAUT. The image was taken with a Nikon microscope, x 40. Bar: 12 μm.

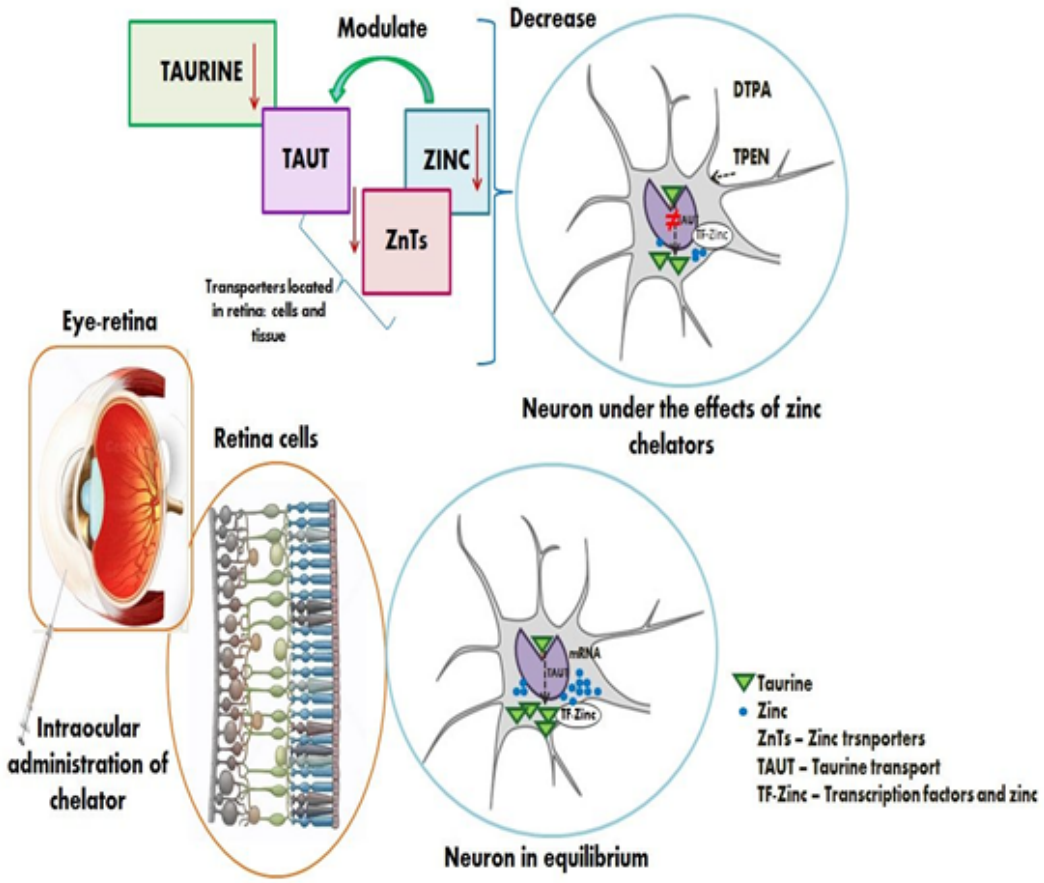

Figure 5: Schematic diagram of the effect of zinc chelators on, taurine transport, the localization of the transporter and taurine levels in the retina. Neuron is shown in equilibrium, observed TAUT, taurine concentrations in equilibrium and TAUT mRNA levels. Zinc levels in equilibrium, zinc could bind to a TAUT site and modify its activity. Neuron under the effects of the zinc chelators TPEN and DTPA. Zinc chelators decrease zinc levels, which affect TAUT activity, which results in a decrease in taurine concentrations and a decrease in TAUT mRNA levels as reported by Márquez et al. when using the zinc chelator TPEN. This might originated by alterations on transcription factors regulated by zinc. The decrease in zinc affects the functions of taurine and zinc in the retina described in the diagram.

Retinal Layers	Control TAUT	TAUT-DTPA
GCL	141.69 ± 1.80	112.06 ± 2.49*
IPL	124.56 ± 6.24	108.85 ± 3.17*
INL	156.35 ± 5.98	112.59 ± 6.77*
OPL	132.45 ± 3.14	119.12 ± 8.06
ONL	141.39 ± 6.30	112.13 ± 1.65*
Phot	156.23 ± 4.72	147.78 ± 2.29*

Taurine transporter TAUT: n=6; integrated optical density was determined in the layers of retina in four areas randomly selected to calculate the average. In GCL, ten cells were randomly chosen in three areas of same size for obtaining average. Each value in the mean ± SEM. *P<0.05 respect to layers retina of control. IOD: Integrated optical density; GCL: Ganglion Cell Layer; IPL: Inner Plexiform Layer; OPL: Outer Plexiform Layer; SEM: Standard Error of the Mean; INL: Inner Nuclear Layer; ONL: Outer Nuclear Layer

Table 2: Integrated optical density of TAUT in rat retinal layers after chelator extracellular zinc.

Discussion

Taurine and zinc are highly concentrated in the retina and have similar properties in this structure, such as neuroprotection, membrane stabilization, regeneration and modulation of development, perhaps by acting in parallel or as interacting agents [11], therefore attention has been paid to the effects of deficiency.

In macular degeneration of the retina, damage or deterioration of the macula are appreciated, which is the area that provides visual acuity, that allows the eye to perceive fine and small details [18,19]. This age-related disease has been associated with decreased levels of taurine, zinc and antioxidant defenses of the retina [20,21].

Zinc deficiency is a pathological state involving abnormalities in the metabolism of metal. This may be due to inadequate dietary intake, to higher requirements or excretion, to conditional deficiency, or genetic causes [22]. Zinc deficiency can cause different clinical manifestations depending on the intensity of this deficiency, but the classic features include anorexia, retarded growth, weight loss, impaired immune function, delayed sexual maturation, testicular atrophy, blindness, and other [22,23]. These features support the crucial role of zinc in the cell metabolism [3,23]. Was found that zinc deficiency during prenatal development of rats caused irreversible disruption of normal brain development [24,25], spinal cord and eye [25,26]. Among the defects in the eye, they are included microphthalmia and anophthalmia, dysplasia in photoreceptors (Phot) [26]. Leure-Dupree and McClain [27] demonstrated, by electron microscopy, which male rats with zinc deficient diet for 7 weeks, showed structural alterations in the retinal pigment epithelium (RPE). In studies in cats and dogs, it has been shown that diet deficient in zinc cause alteration in the electroretinogram [28], malformations in rods [29], conjunctivitis and cataracts [30]. The use of chelating agents has been an alternative to restrict access of zinc to the cells because; zinc deficient diets have little effect in reducing the metal in the tissues or have long-term effects [31]. There is also the advantage of local injections, which prevents the effects of systemic administration, so that the action is limited to the area of interest and interpretation of the results is less confusing [31]. The extracellular zinc chelator, DTPA, is not toxic in the concentration range used and is effective in causing the decrease of metal [31,32]. DTPA is zinc chelating extracellular impermeable membrane [33]. Like the TPEN, it is also a hexadentate ligand. The affinity of DTPA for the zinc is 10 times less than that of TPEN [34]. Lees et al. [32] noted that the extracellular chelators such as DTPA have less effects intracellular chelators like TPEN, can occur as intracellular release of zinc into the extracellular space and therefore, the intracellular chelators are more effective for studies of metal decreased because it requires less concentration to cause the effect, which coincides with our results *ex vivo* [6]. In goldfish retinal, DTPA, 25 to 300 μM, decreased outgrowth from explants after 5 days in culture in the absence of taurine. DTPA, 50 μM, modify the trophic effect of the taurine (decreased), but 4 mM taurine counteracted the inhibitory effect of DTPA [11]. The results indicate that an optimal zinc concentration is necessary for outgrowth of goldfish retinal explants and that, in zinc deficient retina, taurine could stimulate outgrowth [11]. The present study, since low levels of zinc, caused by DTPA treatment *in vivo*, produced significant decrease in taurine levels, transport, and localization of TAUT in retina. As mentioned by Márquez et al. [6] the mechanism by which zinc is affecting taurine transport remains unknown, the zinc might bind some site of TAUT that affect union or translocation of taurine, or possibly the formation of taurine-zinc complexes could be involved in maintaining the functional stability of the retina and resulting in modifications of transporter function. In addition, optimal zinc concentration is necessary for operation of taurine system in retina.

It has not been shown whether taurine transporter has histidine residues (His) in one of its domains, which could be the recognition site of the metal, as has been shown for different transporters from the same family, serotonin [35] and dopamine [36,37]. Zinc has a direct inhibitory effect on glutamate transporter in cells of salamander retina [38]. Reduced the capacity of TAUT without changes in affinity and caused a noncompetitive inhibition of high affinity taurine transport in goldfish retina [39]. In fact, it has been demonstrated that zinc has a biphasic effect on taurine transport ex vivo, stimulating it at low concentrations and inhibiting at high [6]. All these mechanisms are possible when there is zinc deficiency in the retina by DTPA. In the findings of this study, DTPA not only affected the TAUT function, it also caused a significant decrease in taurine concentrations in the retina. Both modifications in the taurine system are related. Significant changes in the levels of taurine and activity of TAUT could be caused by changes in transporter expression. Since it has been suggested that one of the mechanisms regulating transporter taurine is by transcription factors, as described promoter TAUT has several binding sites to different transcription factors that require zinc to maintain its structure. It could be that the decline of zinc caused by DTPA, change taurine levels, activity and localization of the TAUT by its effects on transcription factors regulated by zinc. The effect *in vivo* of chelating zinc on the system of taurine in the retina is crucial for comprehension and understanding the interaction taurine-zinc in this structure.

TAUT is responsible for maintaining levels of taurine in different tissues, and taurine and TAUT in self have been located in retina of goldfish [14] and rat retina [7,40]. We also show in Márquez et al. [7] the localization of TAUT in ganglion cells (GC) and glial cells (GliC), which play relevant roles in the retina and its coexistence with zinc transporters, ZnT1, 3 and 7. Also, there is only one report of the effect of declining zinc caused by TPEN on the localization of TAUT and was demonstrated that treatment with TPEN *in vivo*, intracellular zinc chelator, produced significant differences in the distribution of TAUT. We observed significant decrease IOD in all layers of retina least in the Phot, similar to that observed in the results of this study. Treatment with the chelating zinc *in vivo* extracellular, DTPA, produced significant differences in the distribution of TAUT in all retinal layers least in the OPL. To study the action of zinc effectively, specialized tools are required that probe the changes of zinc ions within live tissue and cells. Intracellular and extracellular levels of zinc are critical for the proper functioning of the nervous system, for that reason the use of the extracellular chelator, DTPA, and intracellular chelator, TPEN, it allows complementing what has been reported about the mechanisms of action of taurine and zinc in this tissue.

Together, these findings on localization by immunohistochemical technique, levels of taurine and activity of taurine transporter, contributes to understand the interaction taurine-zinc in the retina as novel results linking structure and function.

Conflict of Interest

There is no conflict of interest. The authors have non-financial competing interests in an exclusive academic way.

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