1. **Trace-Element Analysis of the Pineal Gland in the Presence of Zn Excess**

8. Abstract

9. The TXRF technique (total reflection X-ray fluorescence) analyzed the concentration
10. of zinc (Zn) and other metals in the pineal of rats submitted to orally administered
11. excess dosages of Zn sulfate. The histochemical localization of Zn was also performed.
12. TXRF results showed a 42.9% increase in Zn concentration, and alterations of
13. homeostasis of other essential elements in rats. It was concluded that TXRF is suitable
14. technique for measuring, for the first time in this work, the concentration of Zn
15. accumulated in the pineal which may be either directly or indirectly related with
16. alteration in the homeostasis of other chemical elements.
17. Keywords: Pineal, Rat, Zinc, TXRF, Hyperzincemia

18. Article highlights:
19. The X-Ray Fluorescence measured for the first time in the pineal the effects of excess
20. zinc on the homeostasis of trace elements.
21. The excess of zinc altered the homeostasis of S, Cl, K, Ca, Ti, Cr, Mn, Fe, increasing
22. these elements in the pineal.
23. Histochemical techniques to zinc showed that after the overdose of zinc occurred
24. changes in the parenchyma of pineal.

Introduction

The pineal (PG, *epiphyse cerebri*) [21, 22, 23,24,49] is a neuroendocrine gland that integrates the circumventricular organs; PG parenchyma is mainly composed of pinealocytes, microglia and astrocytes [9]. The pinealocytes secrete melatonin, a neurohormone that is synthesized and secreted almost entirely at night. Among other important functions, melatonin affects the functioning of other important glands (as the thyroid, adrenal, and gonads) and it can modulate the bioavailability of zinc (Zn) in the plasma [60]. Metals perform many important physiological functions in the human body. The Zn oligo-metallic ion is one of the most common and essential elements that are involved in brain function, and it plays an important role in both physiological and pathophysiological processes [8,13]. Zn is highly concentrated in the synaptic vesicles of subsets of glutamatergic neurons in some brain regions being particularly abundant in the hippocampus, amygdala, cerebral cortex, thalamus and olfactory bulb [6, 29]. After iron (Fe), Zn is one of the most abundant D-block metal [7,33, 61] and is essential for several biochemical processes such as in the control of cell proliferation, myelination and degeneration and serving to structural, catalytic, and regulatory functions, protection against reactive oxygen species (ROS) and it is thought to play a role as a neuromodulator [6, 55, 40, 65] besides that, Zn and Fe play a pivotal role during neurodevelopment and mediate cognitive development [5,33]. Zn is especially important in the immune system because plays a role as a molecular signal to immune cells that are involved in the expression of inflammatory cytokines and yet more, several transcription factors need Zn to bind directly to specific regions of DNA [41].

The multi-elemental composition of the human brain is important to its physiological function, however, the distribution of elements in different tissues is not uniform, and some structures can be the site of accumulation of toxic metals leading to multi-directional intracellular damage principally in the central nervous system (CNS) where these disorders are especially dangerous. In general, with respect to the total metal concentration, the brain should possess efficient homeostatic mechanisms that prevent abnormally high concentrations of metallic ions. Zn concentration in the brains of both rats and humans increased after birth and remained relatively steady throughout adult life [20, 36].

Despite the important physiological role of Zn in modulating several CNS functions,

circumstantial evidence suggests that high concentrations of Zn in the CNS can be neurotoxic [12, 81].

Based on *in vitro* studies, the amount of Zn released during neurotransmission (~300 μM) is more than sufficient to cause fast neurotoxic effects [10]. Results from cultures showed that the survival of neurons is compromised when they are exposed to extracellular Zn at concentrations ranging from 200 to 1000μM [45]. Accordingly, in CNS the alteration of homeostatic mechanisms should lead to neurodegenerative disorders [16,51]. Research on various brain diseases has indicated that trace metals such as Fe, Zn, copper (Cu), manganese (Mn) are key neurochemicals in the neuropathology of diseases [35]. The neurodegenerative disorders in which these metals are implicated are all characterized by a failure to maintain homeostasis, for example, the anomalous accumulation of weakly bound Zn deposits that has been observed in senile plaques in the brains of Alzheimer's patients [1, 11,86]. The association of Zn (and Cu) to amyloid-β in Alzheimer's disease suggests a central role for the abnormal metabolism of these metals in the pathology of this disease [71]. Then, metals dyshomeostasis has been linked to a variety of neurological disorders, and it was found that inappropriate distribution of trace elements, as well as the accumulation of toxic elements in structures of the human brain, is associated with the occurrence of neurodegenerative diseases [12,16,].

Total reflection X-ray fluorescence (TXRF) is a variant of Energy Dispersive X-Ray Fluorescence (EDXRF) and is a multi-element technique. Typically, TXRF only requires a few microliters of a liquid or micrograms of a solid, as a thin film, and the effects of absorption and enhancement can be neglected [46] which simplifies the quantitative analysis.

In general, TXRF quantifications are performed using the internal standardization method [72] which involves the addition of an element that is not present in the sample, for example, gallium (Ga). Internal standardization is useful because the thin film formed on the perspex sample support does not have a regular geometry and the X-ray intensity depends on its position. This geometry effect [47] can be corrected by normalizing each element's X-ray line to the internal standard added to each sample and standard. Therefore, in contrast to conventional X-ray fluorescence (XRF), the concentration in TXRF is simply determined by the relationship between the intensity of the radiation emitted by the sample and the relative sensitivity of the system, which is determined using an internal standard as described elsewhere [47]. This technique

(TXRF) appeared to be suitable for the present study, whose objective was to quantify the amount of zinc in the pineal gland (small samples as the PG of rats) in animals receiving large oral doses of zinc relative to untreated rats of the same age, specifically young adult females which are the object of our studies.

In an earlier study, we demonstrated that adult female rats treated with excess zinc experienced severe changes to their motor behavior, and we considered this dose used in the study as a high dose of zinc capable of also producing hyperzincemia and amyloidosis [24]. In the present study the histology of pineal in young adult females rats was evaluated using histochemical methods for the detection Zn in the animals treated with excess Zn as cited in our study [22]. Meanwhile, in the present study we focus on the potential role of induced hyperzincemia in the disruption of homeostasis of some other metals.

Experimental

Animals and Administration of oral doses

Female Wistar rats (n=48) at postnatal (PN) day 90 (birth was considered PN 0) obtained from different colonies were kept in cages biological under normal laboratory conditions (12/12 h light/dark cycle) with water provided *ad libitum* and controlled food. These cages were doubles (with internal separation) allowing each rat to be isolated in its compartment. The rats were put in these cages a week prior to the postnatal age 90, with the aim to ambient them in these cages. All the rats were weighed before (greater , weight), during and after the experiment (average final body weight = 188 g). The animals were divided into three groups: two controls groups (CG and NCG) and an experimental group (EG). The experimental group (EG) received zinc sulfate ($ZnSO_4$ solution 0.1M, Sigma), one control group (CG) received sterile buffered saline and the other control group (NCG) did not receive any solution. The total dose of hyperzincemia [22] corresponded to 600 mg/kg of solution ZnSO4 and was administered as follow: The dose was divided into 10 sub-doses being administered 1 sub-dose/day of $ZnSO_4$ solution (or the corresponding volume of saline solution); each rat received 10 sub-doses which were administered as daily oral dose over a ten-day period, always administered without previous anesthesia and in the morning (~10:00 h), using a gavage needle for rats (diameter = 1,2 with ball) embedded in glycerin,

accordance with the norms and procedures from Experimental Ethics[1]. Forty-eight hours after the administration of the last dose, the rats were killed by deep anesthesia (ketamine, xylazine, and acepromazine solution) and intracardiac perfusion. All protocols used for the animals were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the appropriate commission[2]. The distribution of animals by technique is seen in Table 1. The NCG used for the TXRF technique served to analyze whether the saline solution administered in the control group would change the concentration of chemical elements in biological samples.

Table 1 Techniques and Number of rats and slides.

Techniques	Animals/ group		
	EG	CG	NCG
Optical and fluorescence analysis and electron microscopy analysis (material embedding in Epon/Araldite)	11	11	4
TXRF	9	9	4
Slides /group and sections/slides			
TSQ	06/rat 4 sections/slide	06/rat 4 sections/slide	06/rat 4 sections/slide
NEO-TIMM	10 /rat 6 sections/slide	10 /rat 6 sections/slide	10 /rat 6 sections/slide

Histological methods and indicators for zinc

Two techniques were used for zinc labeling to observation by optical microscopy: the Neo-Timm histochemical method (NTm) and the TSQ method (6-methoxy-8-quinolyl-*para*toluenesulfonamide) (TSQm) [28]. The Neo-Timm has high selectivity to Zn and is the most used method to detect heavy metal in the mammalian brain [34,75]. The NTm

[2]Riviera, E.A.B. Ética, bem-estar e legislação. In: Manual para Técnicos em Bioterismo. 2nd Ed. São Paulo, EPM, 1996.

[3] Comissão de Ética no Uso de Animais em Experimentação Científica (CEUA) do Centro de Ciências da Saúde da , Universidade Federal do Rio de Janeiro, under protocol number: **DAHEICB094-07/16 (year: 2013)**

is based on the conversion of the metal ion in the tissue into metal sulfide molecules, upon which the metallic silver is deposited. In this way, after the incubation of the sections in the developer solution, black precipitates of silver-metallic precipitates appear and mark the location of the zinc sulfide [18]. The TSQ histochemical method marks by blue fluorescence the zinc free or weakly bound [28]. For NTm was administered during the perfusion of rats a 0.9% saline solution (buffered - PBS 0.1 M) followed by 5 mL of sodium sulfide solution (Na_2S/Sorensen's buffer 0.15M, pH 7.4) for 10 minutes. and then 3% glutaraldehyde (in 0.15 M Sorensen buffer pH 7.4) for 3 minutes. Then the same sodium sulfide solution was passed again for 7 min. The encephalons containing the pineal glands were removed, post-fixed in glutaraldehyde 3% (~ 1h); the pineal glands were removed from the brain, oriented to obtain parasagittal sections and processed for embedding in paraffin. The blocks were cut into 5-µm sections (Rotary Microtome, Lipshaw) and sections (table 1) were mounted on gelatinized slides. The slides were treated using the Neo-Timm method simplified as described in the literature [18,19,35]: histological preparations were deparaffinized and hydrated and were placed in the developer solution (gum Arabic, citrate buffer, hydroquinone and silver nitrate) for 60 min (in the dark) and passed in 5% sodium thiosulfate solution to stop the developer process [19]. The histological preparations were counterstained with hematoxylin [4], dehydrated, clarified in xylene and then mounted with entellan (GTIN8 Entellan/Merck).

To TSQm we used the non-fixed pineal glands; the glands were cryoprotected in sucrose (10%, 20%, and 30%) overnight, soaked in O.C.T. (Tissue Tek) and placed in this same medium. The blocks were sectioned in a cryostat (14 µm thick) (Slee Cryostal) at -14°C, and the sections were collected (Table 1) on gelatinized slides that were then immerse (for 60 s) in the TSQ solution (4.5 µM) buffered (140 mM barbital buffer and 140 mM sodium acetate buffer, pH 10.5 - 11) and after incubation, were washed with saline 0,9%. The material was analyzed using a fluorescence microscope with an ultraviolet filter (Zeiss, excitation at 355-375 nm; dichroic mirror, 380 nm; barrier, 420 nm). The dithizone method was used as a detection control. Dithizone [73] specifically removes zinc from tissues and prevents TSQ detection. The sections were immersed in 10 mM dithizone for 5 min at room temperature. After a 60 s immersion in TSQ buffer solution, the samples were washed with normal saline and examined for TSQ fluorescence. For electron microscopy, the rats deeply anesthetized as explained above were perfused intracardiacally with 0.9% buffered

saline solution (PBS 0.1 M) followed by 4% paraformaldehyde fixative solution and 4% glutaraldehyde buffered. The encephalons were removed and post-fixed for 24h in the same fixative. The pineal glands were removed from and placed in 0.1 M PBS. The material was included in pure Eppon for 72 h in a 60°C (oven) for polymerization and analyzed (Zeiss 900 transmission electron microscope, Laboratory of Protozoan Biology UFRJ).

TXRF analysis: sample and standard preparation

The relative sensitivity of this technique for different elements can be calculated using multi-element standard solutions. These standard solutions were prepared with varying and well-known concentrations and contained Al, Si, K, Ca, Ti, Cr, Fe, Ni, Zn, Ga, Se, Sr, and Mo for the K series (Table 2). Gallium (Ga) was also added as an internal standard for all the multielement standard solutions and samples. The TXRF technique was used according to the protocols cited in the literature [57,58, 70]and summarized here: animals were sacrificed via decapitation and their brains were quickly and carefully removed and frozen with liquid nitrogen. The pineal glands were dissected and maintained at -70°C until the experiments were performed. The feces from each animal were collected before, during and after the administration of the 10 doses (total of 3 samples per animal), were weighed and to chemical digestion (in stove) by adding nitric acid (HNO_3 - 65%.) over 2 h, at 60°C. After the chemical digestion the volume of the samples was adjusted with deionized water and Gallium solution to a final volume (µL). The blood from each animal, collected at the time of the perfusion was centrifuged (2,500 rpm for 15 minutes) and 200 µL of serum was removed from each sample. For blood serum without acid digestion, the volume of 200 µL was adjusted in the same way. The pineal from the EG, CG and NCG were individually weighed (as a single sample for each group) to provide three samples (EG = 3 mg; CG = 3.3 mg; NCG=3.3 mg) and the samples were submitted to chemical digestion adding nitric acid (HNO_3 - 65%.). After dissolution, the samples were mixed with a gallium standard solution (102.5 ppm). All samples (including the blood serum samples) were prepared in duplicate to provide better results. Blank samples (only water, gallium and HNO_3) were prepared to evaluate any source of contamination (without nitric acid for the blood serum). Small amounts (5 µL) of the final solutions were pipetted onto a clean perspex sample support (lucite), and each sample was dried under an infrared light. To create the calibration curve, standard solutions containing

the chemical elements (Table 2, Fig. 1), for the K-lines were prepared in varying, well-known concentrations, with gallium as the internal standard. The TXRF measurements were performed at the D09-B beamline from the Brazil Light Synchrotron Laboratory in Campinas, São Paulo, Brazil. The sample carrier was placed in a horizontal plane relative to the hyper-pure germanium (HPGe) detector (resolution 140 eV at 5.9 KeV), which was positioned perpendicularly to the sample carrier, and excited with a white beam of synchrotron light with a maximum energy of 20 keV and filtered by 0.5 mm of aluminum (with an incidence angle of 1.0 mrad). The sample and standards were excited for 100 s. The X-ray spectra obtained were evaluated using Quantitative X-ray Analysis System (QXAS) software which is distributed by the International Atomic Energy Agency (IAEA), to obtain the X-ray intensities and associated uncertainty for each element. The fluorescence intensities were obtained by fitting the spectra to the QXAS.

TABLE 2 Multielemental standard solution concentrations (mg/L) used for calibration of the system for the K series.

Element	1K	2K	3K	4K	5K	6K
Al	50	40.9	36.36	31.82	27.27	22.73
K	100	81.82	72.73	63.64	54.54	45.45
Ca	10	8.2	7.27	6.36	5.45	4.5
Cr	50	40.9	36.36	31.82	27.27	22.73
Mn	10	8.2	7.27	6.36	5.45	4.5
Fe	10	8.2	7.27	6.36	5.45	4.5
Co	10	8.2	7.27	6.36	5.45	4.5
Ni	50	40.9	36.36	31.82	27.27	22.73
Cu	10	8.2	7.27	6.36	5.45	4.5
Zn	10	8.2	7.27	6.36	5.45	4.5
Sr	10	8.2	7.27	6.36	5.45	4.5
Mo	50	40.9	36.36	31.82	27.27	22.73

Inserte Fig. 1

Statistical analysis

The results were expressed as the mean values ± the standard error, and the means were compared using an analysis of variance (ANOVA) with a 5% significance level. The means were also compared between groups using Tukey's test. All statistical analyses were performed using BioEstat 5.0 computer software (Free Statistics/www.freestatistics.info).

Results

Using the TXRF technique we found that the pineal glands of animals (EG) receiving an excess dose of $ZnSO_4$ showed a 42.9% (150 $\mu g.g^{-1}$) increase in zinc concentration relative to the control groups (CG and NCG) (Table 3). This increase may represent changes in zinc homeostasis. The homeostatic balance of other chemical elements also changed in the PG under hyperzincemia conditions. The concentrations ($\mu g.g^{-1}$) of S, Cl, K, Ca, Ti, Mn and Fe also increased relative to those of the PG of the control animals. However the concentrations of P and Ni decreased ($\mu g.g^{-1}$) (Table 3). It was not possible to analyze the concentration of copper (Cu) in the pineal gland because the values were below the limit of detectability. The excess administered zinc did not significantly alter the zinc concentration in the serum. However, alterations were observed in the serum concentrations of other elements. There was a statistically significant decrease in Fe concentration and a significant increase in the S, Cl, and K concentrations in serum of animals which received zinc.

Table 3 Percentage comparison of the concentrations of the chemical elements of the control group with those of the experimental group. The chemical elements are in ascending order according to their atomic number (Z).

Groups / Element	Pineal Gland Elemental Concentration ($\mu g.g^{-1}$)			Blood Serum Elemental Concentration ($\mu g.ml^{-1}$)		
	CG	EG	(%)	CG	EG	(%)
Si	52±0.67	89±36	-	<LMD	7±2	<LMD

P	8478±109[a]	1404±62[a]	83.44 D	15±6	14±6	-
S	1244±20[a]	2215±23[a]	78.10 I	40±8[a]	62±18[a]	55 I
Cl	864±26[a]	1736±22[a]	101 I	218±91[a]	290±72[a]	33 I
K	2893±8[a]	4040±43[a]	39.66 I	281±54[a]	491±88[a]	74.7 I
Ca	1835±17[a]	4272±56[a]	132.80 I	228±118	221±123	-
Ti	43±4[a]	62±6[a]	44.20 I	7±2	9±3	-
Cr	29±1	28.8±5	-	4±2	4±2	-
Mn	3.5±0.6[a]	6.0±0.85[a]	71.44 I	0.9±0.2	1.5±1	-
Fe	645±11[a]	1953±26[a]	202.8 I	348±173[a]	245±96[a]	29.6 D
Ni	100±0.78[a]	47±0.5[a]	53 D	2±1	4±2	-
Zn	105±0.5[a]	150±1[a]	42.90 I	13±5	13±4	-

Values are the means ± standard error. (a) indicates statistically significant differences among the groups at $P \leq 0.05$; LMD, minimum detectable value; concentrations (%); I, increase; D, decrease.

Analysis of pineal parenchyma using electron microscopy showed disorganized fibrillar depositions and alterations in the vessels walls that appeared thinner and smoother and with imperfections in experimental animals (EG) (Fig. 2B) in relation to the control groups (Fig.2A). The pineal gland's parenchyma in the EG evidenced that the excess zinc disrupted the normal architecture and this it was particularly visible with respect to the wall of the blood vessels and enlarged peripherical spaces (Fig.2B; white stars). In rats' controls the parenchyma was more homogeneous and had well-defined structure vessels with fingerlike projections (Fig. 2A and insert) characteristic of normal pineal vessels. These fingerlike projections were modified in the EG (Fig. 2B; black arrows), where some degree of disorganization was apparent in the periphery of vessel (Fig. 2B; white arrows) and there is a noticeable decrease in the vessel wall thickness in EG rats (Fig. 2B). In rats of CG (Fig. 3 A) the pineal vessels stained by hematoxylin and eosin show a regular organization and the parenchyma is more organized compared to the

Insert Fig. 2

experimental group (Fig. 3C and insert). The Neo-Timm method revealed Zn deposits (zinc aggregates in black) adjacent to blood vessels (Fig. 3B, a) in the pineal gland. These deposits appeared in larger quantities in the experimental group than in the controls (not

showed here) (EG = 161 aggregates; CG = 64 aggregates; NCG = 65 aggregates). In figure 3C, the arrows show the significant disruptions in relation to the blood vessel walls, and it is possible to realize a decrease of thickness in these walls.

The TSQ method produced an intense bright blue fluorescence (Fig. 4A) that was brightest in the pseudorosette (Fig. 4A, demarcated area) cellular arrangements around blood vessels [24], and in the pineal cells scattered throughout the parenchyma. In the control groups (Fig. 4B) the fluorescence was less evident and this weak fluorescence perceived is considered to represent the location of zinc that normally exists in tissue.

Insert FIG. 3 and Fig. 4

Discussion

The present study quantified Zn in the pineal gland by the TXRF technique. This technique [72] was required because the pineal is small and thus provides a very limited sample for analysis. By using TXRF, we were able to obtain significant quantitative information, even with the small amount of tissue available, from animals administered with excess Zn. It is important to note that this type of sensitive quantification of pineal gland samples had never been achieved, particularly under conditions where excess of Zn was administered. Zn is an essential trace element that is normally present in small amounts in the body [36]. However, high concentrations may accumulate in the cerebral cortex and hippocampus and can have toxic effects, as observed in neurons cultured from mice exposed to increased Zn concentrations [17, 29, 45]. In Alzheimer's disease, metals such as Zn and Cu favor the aggregation of β-amyloid peptides and can be histochemically detected in the amyloid deposits of senile plaques [1, 75]. Despite evidence of the toxic effects of Zn the literature provides more information on the consequences of Zn deficiency (hypozincemia) than Zn excess (hyperzincemia) [66]. For example, Zn deficiency, besides Cu overload, has been identified as a risk factor for autism spectrum disorders (ASD) [25,26]. According to the results (Table 3) there was a increase (42.9%) in Zn concentration in the pineal gland after excess Zn administration, implying that the pineal gland is a target for Zn accumulation.

This finding is strengthened by evidence of higher levels of Zn are always found in the pineal gland of some animals (calves, cows and pigs) [83]. Particularly, in the human pineal gland was cited a beneficial association between high zinc content and specific physiological roles [21]. In mammalian cells, approximately 98% of the total Zn concentration in the body is intracellular, and only a small portion accumulates in The extracellular matrix [63,83]. Neurons containing "free ionic zinc" (Zn^{2+}) are found

in various areas of the brain, including the cortex, amygdala, olfactory bulb, and hippocampal neurons, which appear to have the highest concentration of zinc in the brain [6,13]. The intracellular homeostasis of Zn is regulated by membrane importers and exporters, known as zinc carriers and these are divided into two distinct families: the ZIP and ZnT families [17,62]. ZIP transporters mediate the influx of Zn into the cytoplasm, resulting in an increased level of intracellular zinc: the ZnT family acts to reduce intracellular zinc by promoting its efflux from cells or intracellular vesicles [41,42]. Zn homeostasis is also regulated by the intracellular Zn storage protein, metallothionein (MT) [69] that readjust the intracellular stock and maintain ion homeostasis [64]. MT acts as a scavenger when Zn is present in high concentrations, as well as a zinc reservoir to supply Zn when it is deficient. The Zinc homeostasis is essential for cellular events and its dysfunction can lead to several human disorders [9]. In humans the main mechanisms regulating zinc homeostasis are absorption and excretion, and organs such as the small intestine, pancreas and liver play central roles in its maintenance [50]. In these organs the transepithelial transport refers to the transfer of metals across the apical and basolateral membrane to be picked up and distributed by soluble transporters [47]. The transepithelial zinc movement is orchestrated by many mammalian zinc transports specialized for selective capture, and movements of Zn ions across the membrane barrier that depend on an electrochemical gradient [79]; for instance the bacterial zinc acquisition, depends of ZIPB transports zinc in an opposite direction, down a zinc concentration gradient. Zinc fluxes across apical and basolateral membranes need to be balanced and the abundance of ZIP4 and ZnT1 on the respective cell surfaces is tightly regulated according to the Zn availability. The high extracellular Zn levels induce internalization of surfaced ZIP4, as well as promote drastic removal of cellular ZIP4 via proteasomal and lysosomal degradation pathways [82]. In mouse and rat fed a diet with Zn, ZIP4 is hardly detected (74, 94) but is required a detailed analysis of the mouse and rat and human differences in the kinetics of endocytosis and degradation of ZIP4, to gain a more complete understanding of the regulated ZIP4 and their underlying mechanisms endocytosis in mammalian [42].

When Zn is orally administered it is absorbed (20-30% of the ingested content) by the gastrointestinal tract through both active (saturable) and passive (diffusion) transport [52,81]. Zinc uptake takes place on the intestinal brush border membrane of the enterocytes. After ingestion and absorption, there is a vectorial Zn movement from the intestinal lumen to the blood. The excretion of Zn on the basolateral side of the

enterocytes release it into the portal blood, where it is predominantly bound to albumin, which distributes the metal in the body [50]. In general, absorption of Zn is promoted through the presence of small molecular compounds (amino acids and hydroxiacides) and animal proteins [38]. A role of metallothionein (MT) is to bind zinc with high affinity and to serve as an intracellular zinc reservoir. When needed the MT can release free intracellular zinc. MT expression is induced by zinc elevation, and thus, zinc homeostasis is maintained [3]. Notwithstanding, there is a critical period (CP) of 6 to 12 days [59] for the homeostatic regulation of Zn in plasma, and recovery from the effects of either hypozincemia or hyperzincemia. A study using ^{65}Zn showed considerable variation in Zn elimination from different brain regions in rats with half-lives ranging from 16 to 43 days [77]. During the CP, metallothionein (MTs) play a role as metabolic zinc-binding proteins [14] and are capable of regulating Zn bioavailability, preventing alterations in ion concentrations from disturbing homeostasis.

For the pineal gland discussed in this paper, we have no enough information on the transport mechanisms of Zn, and the activity of the metallothioneins to discuss our results about Zn drive after ingested of this ion, although there is data suggesting the presence of a metallothionein-I-II expression system in the pineal gland in bovines. [85]. Although these proteins are involved in metal detoxification, the mechanism of this protection is unclear. Therefore, it is impossible to clearly explain how the high amounts of Zn were retained by the pineal gland of young rats exposed to an excess of administered Zn. In the same way, one study evaluated the effect of zinc overdose (the ingestion of two different doses of zinc chloride, ZnCl2) on the homeostasis of metals (Mn, Cu, Fe and Zn) in the liver of rats and, the results showed that this excess of ZnCl2 causes an accumulation of these metals in the liver compared to controls [67]. Recent studies relate the difficulty of to elucidate the cellular transport systems, for others trace elements when toxics, as cadmium (Cd), mediated by the transporter for manganese (Mn) and by members of the ZIP transporter family (ZIP8 and ZIP14), identified in studies as transporters having high affinities for both Cd and Mn [30,35].

According to the literature, the primary transportation route for Zn to the brain involves the blood-brain barrier and, the choroid plexus may participate in the slow supply of Zn to the brain [78], and the blood-brain barrier maintains the homeostasis of the microenvironment regulating the balance of zinc in the brain [68]. For this reason, the blood-brain barrier is important for Zn homeostasis because disturbance to metal homeostasis is a common characteristic of neurological dysfunction and

neurodegenerative diseases [15, 44]. It has been reported that L-histidine is involved in Zn transport into the brain through the blood-brain barrier via a divalent metal transporter (DMT1) expressed in capillary endothelial cells and choroidal epithelial cells in the brain. DMT1 is also involved in the transportation of other highly toxic cations trace elements such as Pb^{2+}, Cd^{2+}, Co^{2+}, Ni^{2+} and Pt^{2+} Another element for Zn^{2+} transport is ZIP8 a member of the ZIP transporter family [62], principally involved in the transport of this metal at renal tubules [31]. In the case of pineal gland that has a strong blood circulation and there is no true blood-brain barrier [43], DMT1 is a transport not cited in the pineal literature to date, as well as the ZIP8; but the present study is inclined to consider to the accumulation of Zn in the pineal gland might be favored by the absence of a blood-brain barrier in this gland.

Although there is a relationship between melatonin (the primary product of the pineal gland) and the plasma zinc level, the analysis of any relationship between melatonin levels and excess zinc were not the objective of this study, although it is important to evaluate this relationship in the future, but considering that in the present study plasma zinc did not change (Table 3) .

Using the Neo-Timm and TSQ histological techniques [18,19],the present study demonstrated that Zn is primarily accumulated in the perivascular space and appears as dark granules that reveal the location of Zn deposits when tissues are treated with NTm [34]; these aggregates near blood vessels were more evident in rats orally treated with excess zinc (Fig. 3B). Unfortunately in the present study it was not possible to perform a more specific technique to determine the precise location of these aggregates in the pineal vessel wall (Fig 3B), something that should be done. The presence of Zn in the perivascular space was also histochemically reactive to TSQ (diffuse fluorescence) (Fig. 3B) in the rats treated with excess zinc (EG). TSQ is a dye that can penetrate cellular membranes to stain both vesicular Zn and zinc that is only weakly bound to proteins. Therefore, the material detected by TSQ in some cells and in the perivascular space of the pineal gland may represent either of this type of zinc, and a possibility of this abnormal accumulation could be due to changes in the Zn transport system of the endothelium, a saturable system mediated by families of transporters (ZnT and Zip) [42, 53]. As previously mentioned, these transporters are regulated by the Zn concentration. Even though the brain has strict regulatory mechanisms that keep fluctuating concentrations of metals to prevent their shortage or excess, which may be related to various neuropathies [12,85], very little is known about how the potential

system transport of Zn in the pineal responds to the accumulation of this ion. TXRF analysis also indicated that the serum Zn concentration did not change significantly. This result is not surprising because it is known that the concentration of Zn in serum is mostly influenced by the circadian rhythm, with lower values in the morning (when the animals were euthanized) and increased values in the afternoon [22]. To better understand why the Zn concentration in the serum of rats did not change, the results obtained after 10 zinc doses were compared with the Zn concentrations found in serum from the controls (CG and NCG) and serum taken after administration of the first to fifth doses of Zn (D5). No changes occurred in the Zn concentration in the serum. However, after D5, the rats were already experiencing increased sensitivity and weakness, and it was more difficult to continue taking blood samples. Thus, a last blood sample was taken at D10 (before the addition of the fixative solution, during the passage of saline solution) and the blood serum was collected and analyzed. The resulting comparison verified that the Zn concentration in the serum remained virtually constant at all analyzed times (Table 3), the values of CG and EG are very similar, with a very small decrease in serum of EG animal after the 10 doses of zinc. Fecal samples collected from the animals throughout the experiment also showed no significant changes in Zn concentration. It is difficult to explain with certainty why there was such a small decrease in serum zinc in the EG animal, if it was assumed that there was an overdose of Zn in the pineal cells after the 10 zinc sulfate doses. Considering what was said before about the efflux of zinc out of the cell through the membrane, one hypothesis could be some disturbance in the Zn transport system which promotes its efflux. In addition to the increased concentration of Zn in the pineal gland, statistically significant alterations were observed for the concentrations of other elements such as S, Cl, K, Ca, Ti, Mn and Fe (Table 3). The relationship among the concentrations of various chemical elements is essential for the proper functioning of the body, and alterations in the concentration of some metals can affect the bioavailability of other essential metals [39] . This is important because according to current knowledge it turns out that metals such as Na, K, Mg, Ca, Fe, Mn, Co, Cu, Zn and Mo are essential elements for life and our body must have adequate amounts of them [87]. Thus, it is important to quantify the correlation among chemical elemental concentrations as done for some areas in the brains of Wistar rats of different ages by the TXRF method [72]. In the current study, the changes in the concentration of the other chemical elements was also detected by TXRF (Table 3) and such changes could be either directly or indirectly linked to the administration of excess

zinc, but a discussion of these factors is beyond the scope of this study. However, there are some remarkable aspects of our results, such as the >100% increase in the concentrations of certain ions (Table 3) as Fe. In relation to the iron (Fe) which is an essential element for normal body functioning [2] its concentration in the pineal gland increased significantly following the addition of excess zinc (Table 3). In general, a large increase in iron concentration is harmful because it can promote the generation of toxic reactive oxygen species (ROS) that can damage proteins, lipids, and DNA and the irregular deposition of iron is common in some pathologies [7]. Studies of Fe and Zn interactions in neural tissues are scarce but the literature suggests a biological (micronutrient status) interdependence between iron [2] and zinc in the brain. Previous studies demonstrated an antagonism between Fe and Zn, citing absorptive competition between the iron and zinc at the receptor DMT1, however, evidence showed that the DMT1 is not the primary intestinal transporter of zinc [48]. Moreover, there are well known evidence that excess Zn intake through diet or supplements, can affect iron absorption: it has been shown that following the administration of Zn overdose it is possible to see an increase of Fe accumulation in the liver, suggesting a strong disturbance of Zn homeostasis in this organ after overdose of zinc, and interference with iron metabolism. For the authors, these increases and decreases in chemical elements after zinc excess intake, led to the conclusion of a synergic relationship between Fe, Mn and Cu and Zn, but this accumulation of Fe for example, can determine an intensification of cell oxidative reactions and an oxidative stress appearance [67).

Our results showed increased Fe concentration within the pineal gland in parallel to its reduction (~ 30%) in the serum (Table 3); however, it is unclear why the increased Zn concentration triggered an imbalance of iron, likewise that described to the overdose of $ZnCl_2$, for instance [67]. Calcium can appear as free Ca_{2+} both extracellularly and intracellularly [64]. The increased concentration and accumulation of Ca observed in the pineal gland by TXRF method (Table 3) may be reflecting the Ca release from intracellular stores, such as those in the mitochondria or endoplasmic reticulum [27]. There is evidence that Zn can increase the permeability of cell membranes to Ca and thus contributing to homeostatic imbalance [6, 37]. Release of Ca from the mitochondria may also occur because of changes in Mn concentration [65]. Mn may induce cellular damage through mitochondrial dysfunction and can release Ca from intracellular stores under certain conditions [58,79]. In the current study, it was observed a substantial increase in the concentration of Ca and Mn (Table 3). There are reports about an excess

of manganese which can cross the blood-brain barrier (BBB), and accumulate in some regions of the brain (consider that pineal do not have BBB) thereby producing toxicity and neuropathies [70]. Complementary studies are needed to determine whether these observed changes in Ca and Mn homeostasis are interrelated. Speculations regarding these findings are beyond the scope of the present study. An unexpected result was the reduced concentrations of phosphorus (P) and nickel (Ni) observed in the pineal gland (but not in the serum) after excess of Zn, a difficult result to interpret. P in the body is in the form of phosphate, and phosphate has a reciprocal relationship with Ca (a decrease in phosphate content implies an increase in Ca concentration) [80], therefore, the excess of one implies increased excretion of the other. It is possible that the changes in P are more closely related to Ca than to excess of Zn. Among the increased concentrations in the pineal gland of S (sulfur), Cl (chloro) and K (potassium) (Table 3), and in the serum related to excess Zn, it is particularly important to note the increase in K concentration. The high K concentration in the serum could be explained by a sudden release of intracellular K reservoirs into the blood that exceeds the elimination capacity of the kidneys, creating a potentially lethal condition [32]. The reason for the increased K concentration into the blood following the administration of excess zinc requires further investigation. We also find it important to consider that rats subjected to a dosage of ZnSo4 like that used in this study showed decreased motor activity when tested in the open field maze after the 10 doses of zinc sulfate [22]. One hypothesis raised is that this response is due to the breakdown of homeostasis of the various trace elements how analyzed in the present study, and this would be corroborating studies [5] where changes in the homeostasis of Zn and other metals would be implicated in the pathogenesis of certain diseases.

Conclusions

This study used the TXRF technique to demonstrate, for the first time, that the concentrations of Zn and other essential elements (S, Cl, K, Ca, Ti, Mn, and Fe) in the pineal gland of young rats increased considerably following the administration of excess zinc sulfate. Further studies are needed to determine the factors involved in changing (the breakdown) homeostasis of Zn and these other chemical elements. TXRF successfully quantified the elemental changes within this gland, thus proving to be an effective, reliable, and efficient technique for quantifying chemical elements in small samples such as the pineal gland. TXRF can be considered another important analytical tool for the study of the mammalian pineal gland. Although this study clearly showed

alterations in ion homeostasis, further investigations are needed to determine the mechanisms underlying such alterations and clarify the role of the increased concentrations of these ions in the pineal gland, following the administration of excess zinc. However, these results are sufficient to validate our suggested model of animal hyperzincemia [22] where the animals showed a significant change in motor behavior after 10 doses of zinc sulphate: in addition, irregular deposits of zinc appeared adjacent to pineal gland vessels, in the same area occupied by amyloid deposits [22]. The present study contributes to the pineal literature because it shows, also for the first time, the effect of an excess of zinc sulfate on trace elements homeostasis in female rat pineal gland.

References:

1. Adlard PA, Bush, AI (2018) Metals and Alzheimer's disease: How far have we come in the clinic? J. Alzheimers. Dis 62:1369–1379.

2. Atyabi N, Gharagozloo F, Nassiri SM (2006) The necessity of iron supplementation for normal development of commercially reared suckling calves. Comp Clin Pathol 15:165-168.

3. Baltaci AK, Yuce K, Mogulkoc R (2018) Zinc Metabolism and Metallothioneins. Biol Trace Elem Res 183(1):22-3.

4. Bancroft, JD, Gamble, M (2008) Teory and Practice of Histological Techniques, Reino Unido: Churchill Livingstone 121-134.

5. Bitanihirwe, BK, Cunningham, MG. (2009) Zinc: the brain's dark horse. Synapse 63:1029–1049.

6. Blakemore LJ, Trombley, PQ (2017) Zinc as a Neuromodulator in the Central Nervous System with a Focus on the Olfactory Bulb. Frontiers in cellular neuroscience 297:1-20.

516. 7. Bonini-Domingos CR (2007) Iron increases, hereditary hemochromatosis and HFE
517. gene disorders: what do we know of the Brazilian population? Rev Bras Hematol
518. Hemoter 29(4): 341-342.
519. 8. Brito S, Lee MG, Bin BH, Lee JS (2020) Zinc Homeostasis Regulates Epigenetics.
520. Mol. Cells 43(4): 323-330.
521. 9. Bukreeva I, Junemann O, Cedola A, Palermo F, Maugeri L, Provinciali GB, Pieroni
522. N, Sanna A, Otlyga DA, , Buzmakov A, Krivonosov Y, Zolotov D, Chukalina M,
523. Ivanova A, Saveliev S, Asadchikov V, Fratini M (2020) Investigation of the human
524. pineal gland 3D organization by X-ray phase contrast tomography. Journal of Structural
525. Biology 212:1-12.
526. 10. Bush AI (2000) Metals and neuroscience. Curr Opin Chem Biol 4:184-191.
527. 11. Bush AI, Pettingell WH, Multhaup G, Paradis M, Vonsattel JP, Gusella JF,
528. Beyreuther K, Masters CL, Tanzi RE (1994) Rapid induction of Alzheimer A beta
529. amyloid formation by zinc. Science 265:1464-1467.
530. 12. Campbell A, Smith MA, Sayre LM, Bondy SC, Perry G (2001) Mechanisms by which
531. metals promote events connected to neurodegenerative diseases. Brain Resear Bull
532. 55:125-132.
533. 13. Choi S, Hong Dk, Choi B, Suh SW (2020) Zinc in the Brain: Friend or Foe? Int J
534. Mol Sci 21(23) 8941: 1-24.
535. 14. Chung RS, West AK (2004) A role for extracellular metallothioneins in CNS injury
536. and Repair Neurosc 123:595-599.
537. 15. Cicero, CE, Mostile G, Vasta R, Rapisarda V.; Signorelli SS, Ferrante M, Zappia M,
538. Nicoletti A (2017) Metals and neurodegenerative diseases. A systematic review. Environ
539. Res 159: 82–89.
540. 16. Cuajungco MP, Less G (1997) Zinc metabolism in the brain: relevance to human
541. neurodegenerative disorders. Neurobiol Dis 4:137-169.
542. 17. David J, Eide DJ (2006) Zinc transporters and the cellular trafficking of zinc.
543. Biochimica et Biophysica Acta (BBA)Molecular Cell Research 1763(7):711-722.
544. 18. Danscher G (1981) Histochemical demonstration of heavy metais. Histochemi 71:1-
545. 16.
546. 19. Danscher G, Zimmer J (1978) An improved Timm sulphide silver method for light
547. and electron microscopic localization of heavy metals in biological tissue. Histochemi
548. 55:27-40.
549. 20. Demmel U, Höck A, Kasperek K, Feinendegen LE (1982) Trace element

550. Concentration in the human pineal body. Activation analysis of cobalt, iron, rubidium,
551. selenium, zinc, antimony and cesium. Sci. Total Environ 24(2):135-146.
552. 21. Ekstrom P, Meissl H (2003) Evolution of photosensory pineal organs in new light:
553. the fate of neuroendocrine photoreceptors. Phil Trans R Soc Lond B 358:1679-1700.
554. 22. Ferezin-Pinto C (2010) Investigações mineralógicas, histopatológicas e
555. Ultraestruturais usando o modelo experimental de hyperzincemia na glândula pineal de
556. ratas jovens.Tese de Mestrado, Universidade Federal do Rio de Janeiro (UFRJ),
557. Programa de Ciências Morfológicas do Instituto de Ciências Biomédicas, CCS, Rio de
558. Janeiro, Brasil.
559. 23. Ferreira-Medeiros M, Correa-Gillieron EM (2004) Recognition of N-
560. acetylglucosamine and Poly-N-acetyl lactosamine residues in vessels of the rat pineal
561. gland. Int J Morphol 22:285-290.
562. 24. Ferreira-Medeiros, Mandarim-de-Lacerda CA, Correa-Gillieron, EM (2007).Pineal
563. gland pos-natal growth in rat revisited. Anat Histol Embryol 36(4): 284-289.
564. 25. Fluegge K BA. (2017). Zinc and Copper Metabolism and Risk of Autism: a reply to
565. 26. Sayehmiri et al. Iranian Journal of Child Neurology 11(3), 66–69.
566. 27. Formigari A, Gregianin E, Irato P (2013) The effect of zinc and the role of p53 in
567. copper-induced cellular stress responses. J Appl Toxicol 33: 527–536.
568. 28. Frandsen A, Schousboe A (1993) Excitatory amino acid-mediated cytotoxicity and
569. calcium homeostasis in cultured neurons. J Neurochem 60:1202-1211.
570. 29. Frederickson CJ, Kasarkis EJ, Ringo D, Frederickson RE (1987) A quinoline
571. fluorescence method for visualizing and assaying the histochemically reactive zinc
572. (bouton zinc) in the brain. J Neurosci Methods 20:91-103.
573. 30. Frederickson CJ, Suh SW, Silva D, Frederickson CJ, Thompson RB (2000)
574. Importance of zinc in the central nervous system: The zinc-containing neuron. J Nutr
575. 130:1471S–1483S.
576. 31. Fujishiro H, Kambe T (2022) Manganese transport in mammals by zinc transporter
577. family proteins, ZNT and ZIP. J Pharmacol Sci148(1):125-133.
578. 32. Gachot B, Tauc M, Morat L, Poujeol P (1991) Zinc uptake by proximal cells
579. isolated from rabbit kidney: effects of cysteine and histidine. Pflugers Arch 419:583–
580. 587.
581. 33. Gennari FJ (2002) Disorders of potassium homeostasis. Hypokalemia and
582. hyperkalemia. Crit Care Clin 18: 272-288.
583. 34. Georgieff MK (2008) The role of iron in neurodevelopment: Fetal iron deficiency

584. and the developing hippocampus. Biochem Soc Trans 36:1267–1271.
585. 35. Hamani C, de Paulo I, Mello LEAM (2005) Neo-Timm staining in the thalamus of
586. chronically epileptic rats. Braz J Med Biol Res 38:1677-1682.
587. 36. Himeno S, Fujishiro H. (2021) Roles of Zinc Transporters That Control the
588. Essentiality and Toxicity of Manganese and Cadmium. Cited in Yakugaku Zasshi
589. 41(5):695-703. Japanese.
590. 37. Hock A, Demmel U, Schicha H, Kasperek K, Feinendegen LE (1975) Trace element
591. concentration in human brain. Brain 98:49-64.
592. 38. Horning MS, Blakemore LJ, Trombley PQ (2000) Endogenous mechanisms of
593. neuroprotection: role of zinc, copper, and carnosine. Brain Research 852:56-61.
594. 39. Hongfang G, Guanghui C, Khan R, Huanxia J, Jianxin Z, Abbas Raza SH, Ayaz M,
595. Shafiq M, Zan L (2020) Review: Molecular structure and functions of zinc binding
596. metallothionein-1 protein in mammalian body system. Pak J Pharm Sci 33(4):1719-
597. 1726.
598. 40. Janssen CR, De Schamphelaere K, Heijerick D, Muyssen B, Lock K, Bossuyt B,
599. Vangheluwe M, Sprang P (2000) Uncertainties in the environmental risk assessment of
600. metals. Hum Ecol Risk Assess 6:1003-1018.
601. 41. Jarosz M, Olbert M, Wyszogrodzka G, Mlyniec K, Librowski T(2017) Antioxidant
602. and anti-inflammatory effects of zinc. Zinc-dependent NF-kappaB signaling.
603. Inflammopharmacol 25:11–24.
604. 42. Kambe T, Tsuji T, Hashimoto A, Itsumura N (2015). The physiological,
605. biochemical, and molecular roles of zinc transporters in zinc homeostasis and
606. metabolism. Physiol Rev 96:749-784
607. 43. Kambe T, Taylor KM, Fu D (2021). Zinc transporters and their functional
608. integration in mammalian cells. J Biol Chem 296:1-27.
609. 44. Kaur C, Ling EA (2017) The circumventricular organs. Histol
610. Histopathol32(9):879-892.
611. 45. Kawahara, M, Kato-Negishi, M, Tanaka, KI (2020). Amyloids: Regulators of metal
612. homeostasis in the synapse. Molecules, 25(6):1441-1460
613. 46. Koh JY, Suh SW, Gwag BJ, He YY, Hsu CY, Choi DW (1996). The role of zinc in
614. selective neuronal death after transient global cerebral ischemia. Sci. 272: 1013-1016.
615. 47. Klockenkamper R. (1996) Total-Reflection X-Ray Fluorescence Analysis.Institut
616. fur Spectrochemie und Angewandte Spektroskopie. v. 140. Dourtmund, Germany.
617. 48. Klockemkãmper R, Von Bohlen A (1996) Elemental analysis of environmental

618. Samples by total reflection fluorescence: a review. X-Ray Spectrom 25: 156-162.
619. 49. Kordas K, Stoltzfus RJ (2004) New Evidence of Iron and Zinc Interplay at the
620. Enterocyte and Neural Tissues. J Nutr 134: 1295–1298.
621. 50. Korf HW (1994) The pineal organ as a component of the biological clock.
622. Phylogenetic and ontogenetic considerations. Ann. NY Acad Sci 719:13-42.
623. 51. Krebs NF (2000) Overview of zinc absorption and excretion in the human
624. gastrointestinal tract.J Nutr 130:1374S–1377S.
625. 52. Leal MFC, Catarino RIL, Pimenta Am, Souto MRF (2020) Roles of Metal M
626. Microelementos in neurodegenerative Diseases. Neurophysiol 52, 80:88.
627. 53. Lee HH (1989) Zinc absorption in human small intestine. American J Phy 256:87-
628. 91.
629. 54. Liuzzi JP, Cousins RJ (2004) Mammalian zinc transporters. Ann Rev Nutr 24:151-
630. 172
631. 55. López-García C, Varea E, Palop JJ, Nacher J, Ramirez C, Ponsoda X, Molowny A
632. (2002) Cytochemical techniques for zinc and heavy metals localization in nerve cells.
633. Microsc Res Tech 1,56(5):318-31.
634. 56. Macdonald RS (2000) The role of zinc in growth and cell proliferation. J of
635. Nutri130:1500S -1508S.
636. 57. Matsushima S, Reites RJ (1975) Ultrastructural observations of pineal gland
637. Capillaries in four rodent species. Am J Anat 143:265-282.
638. 58. McCormick N, Velasquez V, Finney L, Vogt S, Kelleher SL (2010) X-Ray
639. Fluorescence Microscopy Reveals Accumulation and Secretion of Discrete Intracellular
640. Zinc Pools in the Lactating Mouse Mammary Gland. PLOS ONE 5(6):e11078.
641. 59. Milatovic D, Zaja-Milatovic S, Gupta RC, Yu Y, Aschner M (2009) Oxidative
642. damage and neurodegeneration in manganese-induced neurotoxicity. Toxicol Appl
643. Pharmacol 240:219-225.
644. 60. Opresko DM (1992) Toxicity summary for zinc and zinc compounds, Chemical
645. Hazard Evaluation and Communication Group Biomedical and Environmental
646. Information Analysis Section, Health and Safety Research Division – Prepared for Oak
647. Rdge Reservation Environmental Restoration Program, Oak Ridge, Tennessee.
648. 61. Ozturk G, Akbulut KG, Afrasyap L (2008) Age-related changes in tissue and
649. Plasma Zinc levels: kodulation by exogenously administered melatonin. Exp Aging Res
650. 34:453-462.
651. 62. Pratibha P, Ting JM, Agarwal S, Ichikawa T, Jain A (2021) The Catalytic Role of

652. D-block Elements and Their Compounds for Improving Sorption Kinetics of Hydride
653. Materials: A Review. Reactions, 2, 333–364.
654. 63. Palmiter RD, Cole TB, Quaife CJ (1996) ZnT-3 putative transporter zinc into
655. synaptic vesicles. Proc Natl Acad Sci USA 93:14934-14939.
656. 64. Person OC, Botti AS, Péres MCLC (2006) Clinical repercussions of zinc deficiency
657. in human beings. Arq Med ABC 31:46-52.
658. 65. Poweel SRA (2000) The antioxidant properties of zinc. J. Nutr. 130:1447-1454.
659. Pozzan T, Rizzuto R (2000) The renaissance of mitochondrial calcium transport. Eur J
660. Biochem 267:5269-5273.
661. 66. Prasad AA, Beck FW, Snell DC, Kucuk O. Zinc in cancer prevention. *Nutr Cancer*.
662. 2009; 61:879-887.
663. 67. Pup M, Ahmadi-Vincu M , Velciov, AB , Gârban Z, Dronca, D. (2006)The effect
664. of zinc chloride administration on some trace metals in Wistar rats liver. J Agroaliment
665. Proc and Technol XII(2):521-528.
666. 68. Qi Z, Liu KJ (2019) The interaction of zinc and the blood-brain barrier under
667. physiological and ischemic conditions. Toxicol Appl Pharmacol 364:114-119.
668. Ruttkay-Nedecky B, Nejdl L, Gumulec J, Zitka O, Masarik M, Eckschlager T,
669. Stiborova M,Adam V, Kizek R (2013) The role of metallothionein in oxidative stress.
670. Int J Mol Sci 15;14(3):6044-66.
671. 69. Santos APM, Milatovic D, Au C, Yin Z, Batoreu MC, Aschner M (2010) Rat brain
672. endothelial cells are a target of manganese toxicity. Brain Res. 1326:152-16.
673. Sastre M, Ritchie CW, Hajji N (2015) Metal Ions in Alzheimer's Disease Brain. JSM
674. Alzheimer's Dis Related Dementia 2(1): 1014.
675. 70. Serpa RFB, Jesus EFO, Anjos MJ, Carmo MGT, Moreira S, Rocha MS, Martinez
676. AMP, Lopes RT (2006) Elemental concentration analyze in brain structures from
677. young, adult and old Wistar rats by total reflection X-ray fluorescence with synchrotron
678. radiation. Spectrochim Acta Part B 61:1205-1209.
679. 71. Shaw E, Dean LA (1952) use of Dithizone as an extractant to estimate the zinc
680. nutrient Status of soils. Soil Sci 73(5):341-348.
681. 72. Shen H, Zhang Y, Xu J, Long J, Qin H, Liu F, GuoJ (2007). Zinc distribution and
682. Expression pattern of ZnT3 in mouse brain. Biol Trace Elem Res119:166-174.
683. 73.Slovitter R (1982) A simplified Timm stain procedure compatible with
684. formaldehyde fixation and routine paraffin embedding of rat brain. Brain Res Bull
685. 8:771-774.

686. 74. Suh SW, Jensen KB, Jensen MS, Silva DS, Kesslak PJ, Danscher G, Frederickson
687. CJ (2000). Histochemically-reactive zinc in amyloid plaques, angiopathy, and
688. degenerating neurons of Alzheimer's disease brains. Brain Res 852:274-278.
689. 75. Takeda A (2001). Zinc homeostasis and functions of zinc in the brain. BioMetals
690. 14:343-351.
691. 76. Takeda A (2004) Analysis of Brain Function and Preventions of Brain Diseases: the
692. actions of trace metals. J Health Sci 50(5), 429-44.
693. 77. Takeda A, Sotogaku N, Oku N (2003) Influence of manganese on the release of
694. neurotransmitters in rat striatum. Brain Res 965:279-282.
695. 78. Taylor JG, Bushinsky DA (2009) Calcium and phosphorus homeostasis. Blood
696. Purif 27:387-394.
697. 79. Tubek S, Grzanka P, Tubek I (2008) Role of zinc in hemostasis: a review. Biol
698. Trace Elem Res 121:1-8.
699. 80. Weaver BP, Dufner-Beattie, J, Kambe, T, Andrews, GK (2007). Novel zinc-
700. responsive post-transcriptional mechanisms reciprocally regulate expression of the
701. mouse Slc39a4 and Slc39a5 zinc transporters (Zip4 and Zip5). Biol Chem 388, 1301–
702. 1312.
703. 81. Wong PY, Fritze K (1969) Determination by neutron activation of copper,
704. manganese and zinc in the pineal body and other areas of brain tissue. J Neurichem
705. 16:1231-1234.
706. 82. Yokoyama M, Koh J, Choi DW (1986) Brief exposure to zinc is toxic to cortical
707. neurons. NeurosciLett 71:351-355.
708. 83.Zatta P, Raso M, Zambenedetti P, Rocco P, Petretto A, Mauri P, Cozzi B (2006)
709. Metallothionein-I-II expression in young and adult bovine pineal gland. J Chem
710. Neuroanat 31(2):124-129.
711. 84. Zatta P, Drago D, Bolognin S, Sensi SL (2009) Alzheimer's disease, metal ions and
712. metal homeostatic therapy. Trends Pharm Scien 30: 346-355.
713. 85. Zoroddu MA, Aaseth J, Crisponi G, Medici S, Peana M, Nurchi VM (2019) The
714. essential metals for humans: a brief overview. J Inorg Biochem 195:120-129.

Supplementary information.

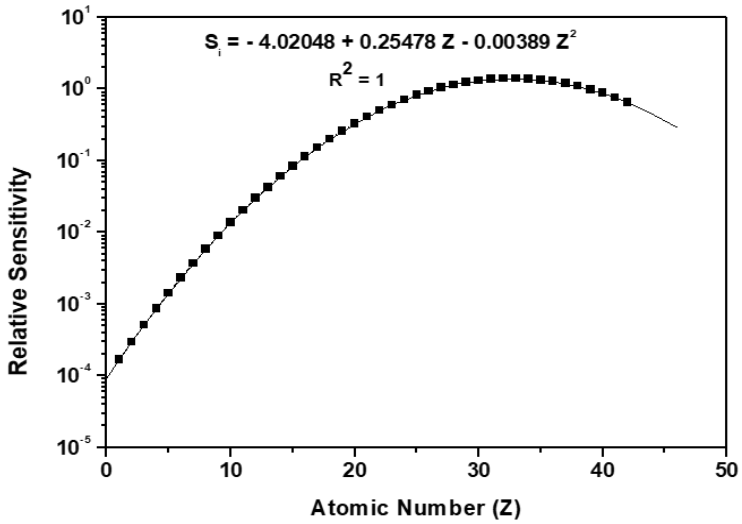

Fig. 1 Calibration curve for the K-line elements using TXRF

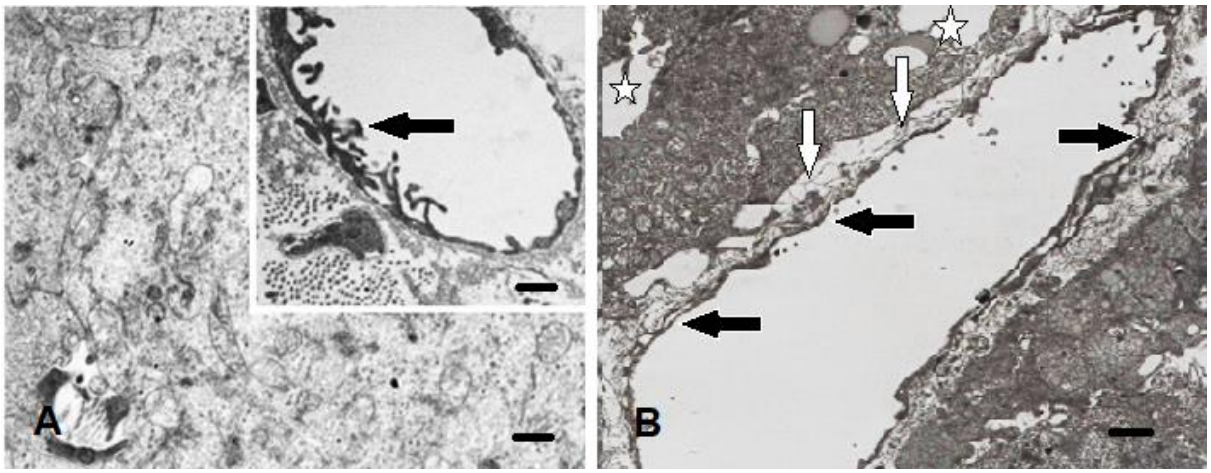

Fig. 2. Electron micrograph of the pineal. Group CG (A) group EG (B). (A) The pineal parenchyma is homogeneous, and the intercellular spaces are much smaller. The insert in A represents a blood vessel where it is possible to observe the various fingers projections (black

arrows) from the vessel wall to the lumen of this vessel. In the parenchyma (B) in the rat with excess Zn, the white arrows indicate areas where the architecture is not uniforms, and appear to be extended with some arrangements fibrillar disorganized or absent fibrils. Many intercellular spaces seem enlarged. Changes can also be observed in the wall of blood vessels (black arrows in B) where a noticeable the decrease in finger like projections, characteristic of normal pineal vessels can be observed and also the appearance of the walls is thinner and smoother and contain imperfections. Scale bar: 0.45 µm.

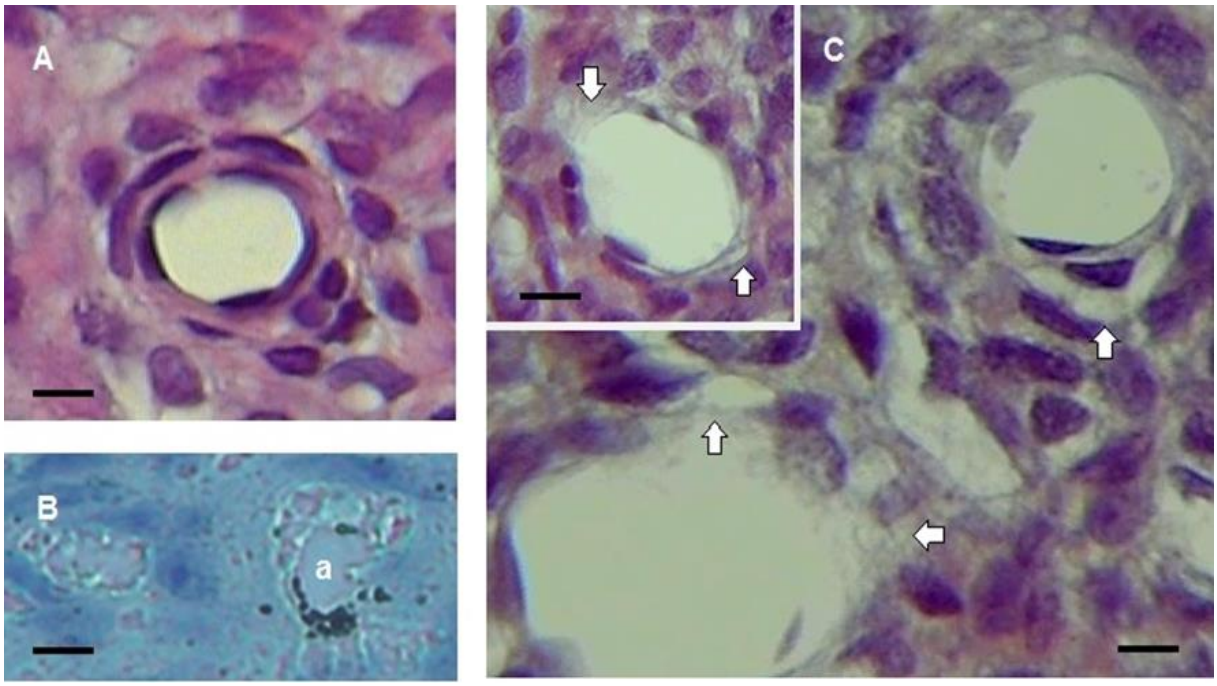

Fig. 3. Micrograph of parasagittal sections of the pineal glands. (A) in the control group (CG) showing a blood vessel stained by hematoxylin and eosin (HE) [4] with well-preserved wall. The peripherical parenchyma seem well organized. (B) Pineal gland from rats in the experimental group (EG). The Neo-Timm method revealed Zn aggregates (granulations in black) adjacent to blood vessels (a) seemingly in the perivascular space. Some pineal parenchymal cells (darker blue) had low visibility because of the need for more adjustments in the granulations of the over-focus peripheral area. (C) Experimental group (EG) pineal parenchymal (stained with HE) was not intact (white arrows in the insert) in contrast to the CG. This disruption (white arrows in C) was significant in relation to the blood vessel walls, sometimes in own wall the vessel other times were in the disorganized cytoarchitecture of the gland in various regions of the pineal parenchymal. Scale Bars: A,C, insert = 5.0 μm; B = 6.0 μm.

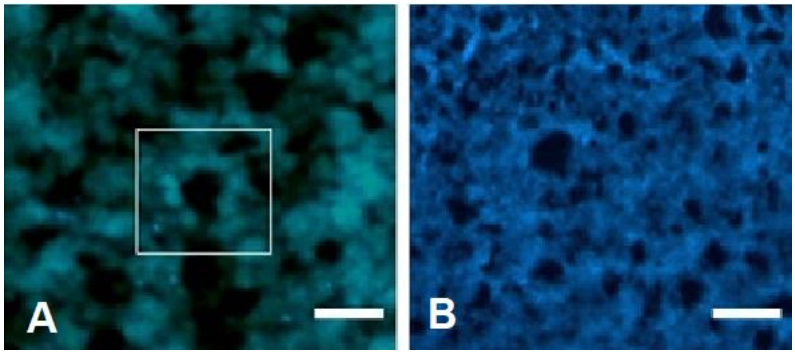

Fig. 4: A and B: Pineal gland treated by TSQ method. A: Sagittal sections of the pineal gland in animals treated with excess zinc (EG). Intense fluorescence of TSQ was observed in the pineal parenchyma and in the pseudo-rosette cells (demarcated area); B: Showing less evident fluorescence in the pineal parenchyma including the pseudo-rosette, with a lower degree of fluorescent labeling in the pinealocytes, and in these controls fluorescence is considered to represent the location of zinc that normally exists in tissue; and Scale bar: 3,5 μm.