

**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** [\(meaning not conveyed rewrite the sentence\).](#)

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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.

## 25. Introduction

26. The pineal (PG, *epiphyse cerebri*) [21, 22, 23,24,49] is a neuroendocrine gland  
 27. that integrates the circumventricular organs; PG parenchyma is mainly composed of  
 28. pinealocytes, microglia and astrocytes [9]. The pinealocytes secrete melatonin, a  
 29. neurohormone that is synthesized and secreted almost entirely at night. Among other  
 30. important functions, melatonin affects the functioning of other important glands (as the  
 31. thyroid, adrenal, and gonads) and it can modulate the bioavailability of zinc (Zn) in the  
 32. plasma [60]. Metals perform many important physiological functions in the human  
 33. body. The Zn oligo-metallic ion is one of the most common and essential elements  
 34. that are involved in brain function, and it plays an important role in both physiological  
 35. and pathophysiological processes [8,13]. Zn is highly concentrated in the synaptic  
 36. vesicles of subsets of glutamatergic neurons in some brain regions being particularly  
 37. abundant in the hippocampus, amygdala, cerebral cortex, thalamus and olfactory bulb  
 38. [6, 29]. After iron (Fe), Zn is one of the most abundant D-block metal [7,33, 61] and is  
 39. essential for several biochemical processes such as in the control of cell proliferation,  
 40. myelination and degeneration and serving to structural, catalytic, and regulatory  
 41. functions, protection against reactive oxygen species (ROS) and it is thought to play a  
 42. role as a neuromodulator [6, 55, 40, 65] besides that, Zn and Fe play a pivotal role  
 43. during neurodevelopment and mediate cognitive development [5,33]. Zn is especially  
 44. important in the immune system because plays a role as a molecular signal to  
 45. immune cells that are involved in the expression of inflammatory cytokines and yet  
 46. more, several transcription factors need Zn to bind directly to specific regions of DNA  
 47. [41].  
 48. The multi-elemental composition of the human brain is important to its physiological  
 49. function, however, the distribution of elements in different tissues is not uniform, and  
 50. some structures can be the site of accumulation of toxic metals leading to multi-  
 51. directional intracellular damage principally in the central nervous system (CNS) where  
 52. these disorders are especially dangerous. In general, with respect to the total metal  
 53. concentration, the brain should possess efficient homeostatic mechanisms that prevent  
 54. abnormally high concentrations of metallic ions. Zn concentration in the brains of  
 55. both rats and humans increased after birth and remained relatively steady throughout  
 56. adult life [20, 36].  
 57. Despite the important physiological role of Zn in modulating several CNS functions,

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58. circumstantial evidence suggests that high concentrations of Zn in the CNS can be  
59. neurotoxic [12, 81].  
60. Based on *in vitro* studies, the amount of Zn released during neurotransmission (~300  $\mu\text{M}$ )  
61. is more than sufficient to cause fast neurotoxic effects [10]. Results from cultures showed  
62. that the survival of neurons is compromised when they are exposed to extracellular Zn at  
63. concentrations ranging from 200 to 1000 $\mu\text{M}$  [45]. Accordingly, in CNS the alteration of  
64. homeostatic mechanisms should lead to neurodegenerative disorders [16,51]. Research  
65. on various brain diseases has indicated that trace metals such as Fe, Zn, copper (Cu),  
66. manganese (Mn) are key neurochemicals in the neuropathology of diseases [35].  
67. The neurodegenerative disorders in which these metals are implicated are all  
68. characterized by a failure to maintain homeostasis, for example, the anomalous  
69. accumulation of weakly bound Zn deposits that has been observed in senile plaques in  
70. the brains of Alzheimer's patients [1, 11,86]. The association of Zn (and Cu) to  
71. amyloid- $\beta$  in Alzheimer's disease suggests a central role for the abnormal metabolism  
72. of these metals in the pathology of this disease [71]. Then, metals dyshomeostasis has  
73. been linked to a variety of neurological disorders, and it was found that inappropriate  
74. distribution of trace elements, as well as the accumulation of toxic elements in  
75. structures of the human brain, is associated with the occurrence of neurodegenerative  
76. diseases [12,16,].  
77. Total reflection X-ray fluorescence (TXRF) is a variant of Energy Dispersive X-Ray  
78. Fluorescence (EDXRF) and is a multi-element technique. Typically, TXRF only  
79. requires a few microliters of a liquid or micrograms of a solid, as a thin film, and the  
80. effects of absorption and enhancement can be neglected [46] which simplifies the  
81. quantitative analysis.  
82. In general, TXRF quantifications are performed using the internal standardization  
83. method [ 72] which involves the addition of an element that is not present in the sample,  
84. for example, gallium (Ga). Internal standardization is useful because the thin film  
85. formed on the **perspex** sample support does not have a regular geometry and the X-  
ray  
86. intensity depends on its position. This geometry effect [47] can be corrected by  
87. normalizing each element's X-ray line to the internal standard added to each sample  
88. and standard. Therefore, in contrast to conventional X-ray fluorescence (XRF), the  
89. concentration in TXRF is simply determined by the relationship between the intensity  
90. of the radiation emitted by the sample and the relative sensitivity of the system, which

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91. is determined using an internal standard as described elsewhere [47]. This technique

92. (TXRF) appeared to be suitable for the present study, whose objective was to quantify  
93. the amount of zinc in the pineal gland (small samples as the PG of rats) in animals  
94. receiving large oral doses of zinc relative to untreated rats of the same age, specifically  
95. young adult females which are the object of our studies.  
96. In an earlier study, we demonstrated that adult female rats treated with excess zinc  
97. experienced severe changes to their motor behavior, and we considered this dose  
98. used in the study as a high dose of zinc capable of also producing hyperzincemia and  
99. amyloidosis [24]. In the present study the histology of pineal in young adult females rats  
100. was evaluated using histochemical methods for the detection Zn in the animals treated  
101. with excess Zn as cited in our study [22]. Meanwhile, in the present study we focus on  
102. the potential role of induced hyperzincemia in the disruption of homeostasis of some other  
103. metals.

#### 104. Experimental

105. Animals and Administration of oral doses  
106. Female Wistar rats (n=48) at postnatal (PN) day 90 (birth was considered PN 0)  
107. obtained from different colonies were kept in cages biological under normal laboratory  
108. conditions (12/12 h light/dark cycle) with water provided *ad libitum* and controlled food.  
109. These cages were doubles (with internal separation) allowing each rat to be isolated in its  
110. compartment. The rats were put in these cages a week prior to the postnatal age 90,  
111. with the aim to ambient them in these cages. All the rats were weighed before (greater ,  
112. weight), during and after the experiment (average final body weight = 188 g).  
113. The animals were divided into three groups: two controls groups (CG and NCG)  
114. and an experimental group (EG). The experimental group (EG) received zinc  
115. sulfate ( $ZnSO_4$  solution 0.1M, Sigma), one control group (CG) received  
116. sterile buffered saline and the other control group (NCG) did not receive any solution.  
117. The total dose of hyperzincemia [22] corresponded to 600 mg/kg of solution  $ZnSO_4$  and  
118. was administered as follow: The dose was divided into 10 sub-doses being administered  
119. 1 sub-dose/day of  $ZnSO_4$  solution (or the corresponding volume of saline solution); each  
120. rat received 10 sub-doses which were administered as daily oral dose over a ten-day  
121. period, always administered without previous anesthesia and in the morning (~10:00 h),  
122. using a gavage needle for rats (diameter = 1,2 with ball) embedded in glycerin,

123. accordance with the norms and procedures from Experimental Ethics<sup>1</sup>. Forty-eight hours  
 124. after the administration of the last dose, the rats were killed by deep anesthesia (ketamine,  
 125. xylazine, and acepromazine solution) and intracardiac perfusion. All protocols used for  
 126. the animals were conducted in accordance with the Guide for the Care and Use of  
 127. Laboratory Animals and were approved by the appropriate commission<sup>2</sup>. The distribution  
 128. of animals by technique is seen in Table 1. The NCG used for the TXRF technique served  
 129. to analyze whether the saline solution administered in the control group would change  
 130. 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

**131. Histological methods and indicators for zinc**

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

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

<sup>3</sup> 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)**

136. is based on the conversion of the metal ion in the tissue into metal sulfide molecules,  
137. upon which the metallic silver is deposited. In this way, after the incubation of the  
138. sections in the developer solution, black precipitates of silver-metallic precipitates  
139. appear and mark the location of the zinc sulfide [18]. **The TSQ histochemical method**  
140. **marks by blue fluorescence the zinc free or weakly bound [28].** {Rewrite the sentence as  
The TSQ histochemical method exhibits blue fluorescence for free zinc or weakly  
bound one.}-For NTm was  
141. administered during the perfusion of rats a 0.9% saline solution (buffered - PBS 0.1 M)  
142. followed by 5 mL of sodium sulfide solution (Na<sub>2</sub>S/Sorensen's buffer 0.15M, pH 7.4)  
143. for 10 minutes. and then 3% glutaraldehyde (in 0.15 M Sorensen buffer pH 7.4) for 3  
144. minutes. Then the same sodium sulfide solution was passed again for 7 min. The  
145. encephalons containing the pineal glands were removed, post-fixed in glutaraldehyde  
146. 3% (~ 1h); the pineal glands were removed from the brain, oriented to obtain  
147. parasagittal sections and processed for embedding in paraffin. The blocks were cut into  
148. 5- $\mu$ m sections (Rotary Microtome, Lipshaw) and sections (table 1) were mounted on  
149. gelatinized slides. The slides were treated using the Neo-Timm method simplified as  
150. described in the literature [18,19,35]: histological preparations were deparaffinized and  
151. hydrated and were placed in the developer solution (gum Arabic, citrate buffer,  
152. hydroquinone and silver nitrate) for 60 min (in the dark) and passed in 5% sodium  
153. thiosulfate solution to stop the developer process [19]. The histological preparations  
154. were counterstained with hematoxylin [4], dehydrated, clarified in xylene and then  
155. mounted with entellan (GTIN8 Entellan/Merck).  
156. To TSQm we used the non-fixed pineal glands; the glands were cryoprotected in  
157. sucrose (10%, 20%, and 30%) overnight, soaked in **O.C.T.** (full form to be mentioned)  
(Tissue Tek) and placed in  
158. this same medium. The blocks were sectioned in a cryostat (14  $\mu$ m thick) (Slee  
159. Cryostal) at -14°C, and the sections were collected (Table 1) on gelatinized slides  
160. that were then immersed (for 60 s) in the TSQ solution (4.5  $\mu$ M) buffered (140 mM  
161. barbital buffer and 140 mM sodium acetate buffer, pH 10.5 – 11) and after incubation,  
162. were washed with saline 0,9%. The material was analyzed using a fluorescence  
163. microscope with an ultraviolet filter (Zeiss, excitation at 355-375 nm; dichroic mirror,  
164. 380 nm; barrier, 420 nm). The dithizone method was used as a detection control.  
165. Dithizone [73] specifically removes zinc from tissues and prevents TSQ detection.  
166. The sections were immersed in 10 mM dithizone for 5 min at room temperature. After  
167. a 60 s immersion in TSQ buffer solution, the samples were washed with normal saline

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168. and examined for TSQ fluorescence. For electron microscopy, the rats deeply
169. anesthetized as explained above were perfused intracardially with 0.9% buffered

170. saline solution (PBS 0.1 M) followed by 4% paraformaldehyde fixative solution and  
 171. 4% glutaraldehyde buffered. The encephalons were removed and post-fixed for 24h  
 172. in the same fixative. The pineal glands were removed from and placed in 0.1 M  
 PBS. (full form to be written)  
 173. The material was included in pure Epon for 72 h in a 60°C (oven) for polymerization  
 174. and analyzed (Zeiss 900 transmission electron microscope, Laboratory of Protozoan  
 175. Biology UFRJ).

#### 176. TXRF analysis: sample and standard preparation

177. The relative sensitivity of this technique for different elements can be calculated using  
 178. multi-element standard solutions. These standard solutions were prepared with varying  
 179. and well-known concentrations and contained Al, Si, K, Ca, Ti, Cr, Fe, Ni, Zn, Ga, Se,  
 180. Sr, and Mo for the K series (Table 2). Gallium (Ga) was also added as an internal  
 181. standard for all the multielement standard solutions and samples. The TXRF technique  
 182. was used according to the protocols cited in the literature [57,58, 70] and summarized  
 183. here: animals were sacrificed via decapitation and their brains were quickly and  
 184. carefully removed and frozen with liquid nitrogen. The pineal glands were dissected  
 185. and maintained at -70°C until the experiments were performed. The feces from each  
 186. animal were collected before, during and after the administration of the 10 doses  
 187. (total of 3 samples per animal), were weighed and to chemical digestion (in stove) by  
 188. adding nitric acid (HNO<sub>3</sub> - 65%) over 2 h, at 60°C. After the chemical digestion  
 189. the volume of the samples was adjusted with deionized water and Gallium solution to a  
 190. final volume (μL). The blood from each animal, collected at the time of the  
 191. perfusion was centrifuged (2,500 rpm for 15 minutes) and 200 μL of serum was  
 192. removed from each sample. For blood serum without acid digestion, the volume of 200  
 193. μL was adjusted in the same way. The pineal from the EG, CG and NCG were  
 194. individually weighed (as a single sample for each group) to provide three samples (EG  
 195. = 3 mg; CG = 3.3 mg; NCG=3.3 mg) and the samples were submitted to chemical  
 196. digestion adding nitric acid (HNO<sub>3</sub> - 65%). After dissolution, the samples were mixed  
 197. with a gallium standard solution (102.5 ppm). All samples (including the blood serum  
 198. samples) were prepared in duplicate to provide better results. Blank samples (only  
 199. water, gallium and HNO<sub>3</sub>) were prepared to evaluate any source of contamination  
 200. (without nitric acid for the blood serum). Small amounts (5 μL) of the final solutions

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201. were pipetted onto a clean perspex sample support (lucite), and each sample was dried
202. under an infrared light. To create the calibration curve, standard solutions containing

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

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

**222. Results**

223. Using the TXRF technique we found that the pineal glands of animals (EG) receiving an  
 224. excess dose of  $\text{ZnSO}_4$  showed a 42.9% ( $150 \mu\text{g}\cdot\text{g}^{-1}$ ) increase in zinc concentration relative  
 225. to the control groups (CG and NCG) (Table 3). This increase may represent changes in  
 226. zinc homeostasis. The homeostatic balance of other chemical elements also changed in  
 227. the PG under hyperzincemia conditions. The concentrations ( $\mu\text{g}\cdot\text{g}^{-1}$ ) of S, Cl, K, Ca, Ti,  
 228. Mn and Fe also increased relative to those of the PG of the control animals.  
 229. However the concentrations of P and Ni decreased ( $\mu\text{g}\cdot\text{g}^{-1}$ ) (Table 3).  
 230. It was not possible to analyze the concentration of copper (Cu) in the pineal gland because  
 231. the values were below the limit of detectability. The excess administered zinc did not  
 232. significantly alter the zinc concentration in the serum. However, alterations were  
 233. observed in the serum concentrations of other elements. There was a statistically  
 234. 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 ( $\mu\text{g}\cdot\text{g}^{-1}$ )			Blood Serum Elemental Concentration ( $\mu\text{g}\cdot\text{ml}^{-1}$ )		
	CG	EG	(%)	CG	EG	(%)
Si	52 $\pm$ 0.67	89 $\pm$ 36	-	<LMD	7 $\pm$ 2	<LMD

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

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

### Insert Fig. 2

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

251. showed here) (EG = 161 aggregates; CG = 64 aggregates; NCG = 65 aggregates). In  
252. figure 3C, the arrows show the significant disruptions in relation to the blood vessel  
253. walls, and it is possible to realize a decrease of thickness in these walls.  
254. The TSQ method produced an intense bright blue fluorescence (Fig. 4A) that was  
255. brightest in the pseudorosette (Fig. 4A, demarcated area) cellular arrangements around  
256. blood vessels [24], and in the pineal cells scattered throughout the parenchyma. In the  
257. control groups (Fig. 4B) the fluorescence was less evident and this weak fluorescence  
258. perceived is considered to represent the location of zinc that normally exists in tissue.

#### Insert FIG. 3 and Fig. 4

#### 259. Discussion

260. The present study quantified Zn in the pineal gland by the TXRF technique. This  
261. technique [72] was required because the pineal is small and thus provides a very limited  
262. sample for analysis. By using TXRF, we were able to obtain significant quantitative  
263. information, even with the small amount of tissue available, from animals administered  
264. with excess Zn. It is important to note that this type of sensitive quantification of pineal  
265. gland samples had never been achieved, particularly under conditions where excess of  
266. Zn was administered. Zn is an essential trace element that is normally present in small  
267. amounts in the body [36]. However, high concentrations may accumulate in the cerebral  
268. cortex and hippocampus and can have toxic effects, as observed in neurons cultured  
269. from mice exposed to increased Zn concentrations [17, 29, 45]. In Alzheimer's disease,  
270. metals such as Zn and Cu favor the aggregation of  $\beta$ -amyloid peptides and can be  
271. histochemically detected in the amyloid deposits of senile plaques [1, 75]. Despite  
272. evidence of the toxic effects of Zn the literature provides more information on the  
273. consequences of Zn deficiency (hypozincemia) than Zn excess (hyperzincemia) [66].  
274. For example, Zn deficiency, besides Cu overload, has been identified as a risk factor for  
275. autism spectrum disorders (ASD) [25,26]. According to the results (Table 3) there was a  
276. increase (42.9%) in Zn concentration in the pineal gland after excess Zn administration,  
277. implying that the pineal gland is a target for Zn accumulation.  
278. This finding is strengthened by evidence of higher levels of Zn are always found  
279. in the pineal gland of some animals (calves, cows and pigs ) [83]. Particularly, in the  
280. human pineal gland was cited a beneficial association between high zinc content and  
281. specific physiological roles [21]. In mammalian cells, approximately 98% of the total  
282. Zn concentration in the body is intracellular, and only a small portion accumulates in  
283. ~~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

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317. 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 <sup>65</sup>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 not 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, ZnCl<sub>2</sub>) on the homeostasis of metals (Mn, Cu, Fe and Zn) in the liver of rats and, the results showed that this excess of ZnCl<sub>2</sub> 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

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349. blood-brain barrier is important for Zn homeostasis because disturbance to metal
350. homeostasis is a common characteristic of neurological dysfunction and

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

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

367. Using the Neo-Timm and TSQ histological techniques [18,19],the present study  
368. demonstrated that Zn is primarily accumulated in the perivascular space and appears  
369. as dark granules that reveal the location of Zn deposits when tissues are treated with  
370. NTm [34]; these aggregates near blood vessels were more evident in rats orally treated  
371. with excess zinc (Fig. 3B). Unfortunately in the present study it was not possible to  
372. perform a more specific technique to determine the precise location of these aggregates  
373. in the pineal vessel wall (Fig 3B), something that should be done. The presence of Zn in  
374. the perivascular space was also histochemically reactive to TSQ (diffuse fluorescence)  
375. (Fig. 3B) in the rats treated with excess zinc (EG). TSQ is a dye that can penetrate cellular  
376. membranes to stain both vesicular Zn and zinc that is only weakly bound to proteins.  
377. Therefore, the material detected by TSQ in some cells and in the perivascular space of  
378. the pineal gland may represent either of this type of zinc, and a possibility of this  
379. abnormal accumulation could be due to changes in the Zn transport system of the  
380. endothelium, a saturable system mediated by families of transporters (ZnT and Zip) [42,  
381. 53]. As previously mentioned, these transporters are regulated by the Zn concentration.  
382. Even though the brain has strict regulatory mechanisms that keep fluctuating  
383. concentrations of metals to prevent their shortage or excess, which may be  
384. related to various neuropathies [12,85], very little is known about how the potential

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

419. zinc, but a discussion of these factors is beyond the scope of this study. However, there  
420. are some remarkable aspects of our results, such as the >100% increase in the  
421. concentrations of certain ions (Table 3) as Fe. In relation to the iron (Fe) which is an  
422. essential element for normal body functioning [2] its concentration in the pineal gland  
423. increased significantly following the addition of excess zinc (Table 3). In general,  
424. a large increase in iron concentration is harmful because it can promote the generation of  
425. toxic reactive oxygen species (ROS) that can damage proteins, lipids, and DNA and the  
426. irregular deposition of iron is common in some pathologies [7]. Studies of Fe and Zn  
427. interactions in neural tissues are scarce but the literature suggests a biological  
428. (micronutrient status) interdependence between iron [2] and zinc in the brain. Previous  
429. studies demonstrated an antagonism between Fe and Zn, citing absorptive competition  
430. between the iron and zinc at the receptor DMT1, however, evidence showed that the  
431. DMT1 is not the primary intestinal transporter of zinc [48]. Moreover, there are well  
432. known evidence that excess Zn intake through diet or supplements, can affect iron  
433. absorption: it has been shown that following the administration of Zn overdose it is  
434. possible to see an increase of Fe accumulation in the liver, suggesting a strong disturbance  
435. of Zn homeostasis in this organ after overdose of zinc, and interference with iron  
436. metabolism. For the authors, these increases and decreases in chemical elements after  
437. zinc excess intake, led to the conclusion of a synergic relationship between Fe, Mn and  
438. Cu and Zn, but this accumulation of Fe for example, can determine an intensification of  
439. cell oxidative reactions and an oxidative stress appearance [67].  
440. Our results showed increased Fe concentration within the pineal gland in parallel to its  
441. reduction (~ 30%) in the serum (Table 3); however, it is unclear why the increased Zn  
442. concentration triggered an imbalance of iron, likewise that described to the overdose of  
443. ZnCl<sub>2</sub>, for instance [67]. Calcium can appear as free Ca<sub>2+</sub> both extracellularly and  
444. intracellularly [64]. The increased concentration and accumulation of Ca observed in the  
445. pineal gland by TXRF method (Table 3) may be reflecting the Ca release from  
446. intracellular stores, such as those in the mitochondria or endoplasmic reticulum [27].  
447. There is evidence that Zn can increase the permeability of cell membranes to Ca and  
448. thus contributing to homeostatic imbalance [6, 37]. Release of Ca from the mitochondria  
449. may also occur because of changes in Mn concentration [65]. Mn may induce cellular  
450. damage through mitochondrial dysfunction and can release Ca from intracellular stores  
451. under certain conditions [58,79]. In the current study, it was observed a substantial  
452. increase in the concentration of Ca and Mn (Table 3). There are reports about an excess

453. of manganese which can cross the blood-brain barrier (BBB), and accumulate in some  
454. regions of the brain (consider that pineal do not have BBB) thereby producing toxicity  
455. and neuropathies [70]. Complementary studies are needed to determine whether these  
456. observed changes in Ca and Mn homeostasis are interrelated. Speculations regarding  
457. these findings are beyond the scope of the present study. An unexpected result was the  
458. reduced concentrations of phosphorus (P) and nickel (Ni) observed in the pineal gland  
459. (but not in the serum) after excess of Zn, a difficult result to interpret. P in the body is in  
460. the form of phosphate, and phosphate has a reciprocal relationship with Ca (a decrease in  
461. phosphate content implies an increase in Ca concentration) [80], therefore, the excess of  
462. one implies increased excretion of the other. It is possible that the changes in P are more  
463. closely related to Ca than to excess of Zn. Among the increased concentrations in the  
464. pineal gland of S (sulfur), Cl (chloro) and K (potassium) (Table 3), and in the serum  
465. related to excess Zn, it is particularly important to note the increase in K concentration.  
466. The high K concentration in the serum could be explained by a sudden release of  
467. intracellular K reservoirs into the blood that exceeds the elimination capacity of the  
468. kidneys, creating a potentially lethal condition [32]. The reason for the increased K  
469. concentration into the blood following the administration of excess zinc requires further  
470. investigation. We also find it important to consider that rats subjected to a dosage of  
471. ZnSo<sub>4</sub> like that used in this study showed decreased motor activity when tested in the  
472. open field maze after the 10 doses of zinc sulfate [22]. One hypothesis raised is that this  
473. response is due to the breakdown of homeostasis of the various trace elements how  
474. analyzed in the present study, and this would be corroborating studies [5] where changes  
475. in the homeostasis of Zn and other metals would be implicated in the pathogenesis of  
476. certain diseases.

#### 477. **Conclusions**

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

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

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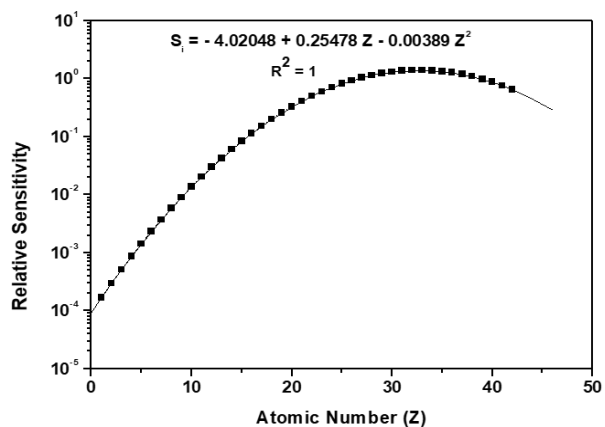
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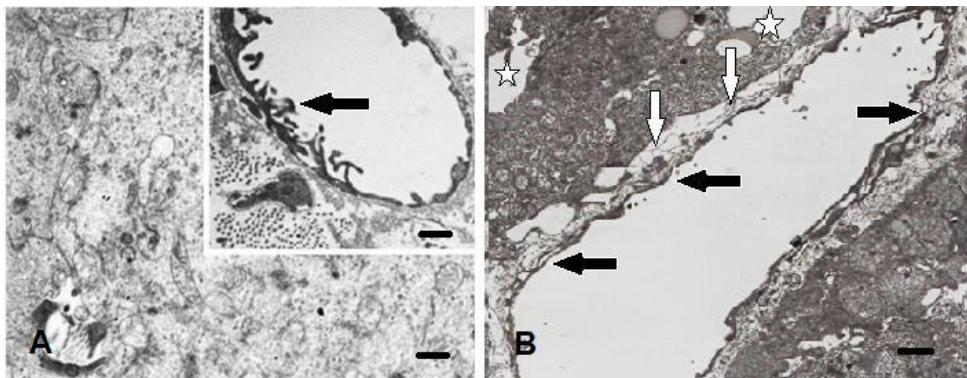
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Supplementary information.

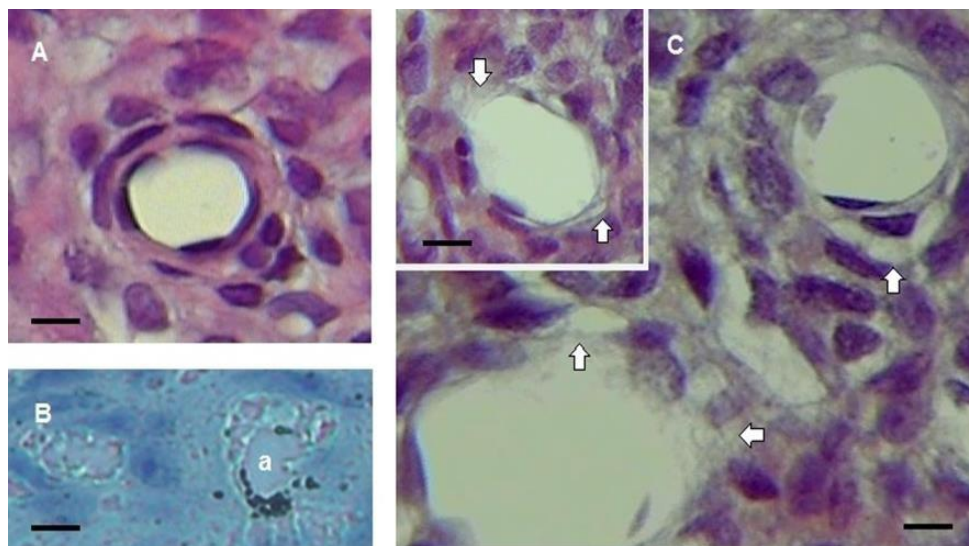


**Fig. 1** Calibration curve for the K-line elements using TXRF



**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 uniform, 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 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  $\mu\text{m}$ .



**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 peripheral 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  $\mu\text{m}$ ; B = 6.0  $\mu\text{m}$ .

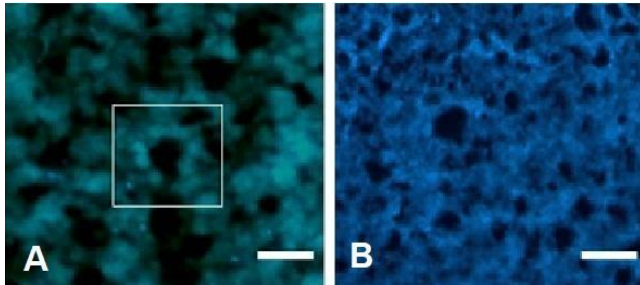


Fig. 4: A and B: Pineal gland treated by TSQ method. A: Sagittal sections of the pineal gland in animals treated with excess zinc (EG). Intense fluorescence of TSQ was observed in the pineal parenchyma and in the pseudo-rosette cells (demarcated area); B: Showing less evident fluorescence in the pineal 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  $\mu\text{m}$ .