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Analysis of the Influence of Zn Excess in the Pineal Gland by Total Reflection X-ray Fluorescence

Correa-Gillieron, Elenice Maria [a,b*], Barbosa, Renata Faria [c,d], Ferezin-Pinto, Caroline [e,f] and Martinez, Ana Maria Blanco [g,h]

[a] Federal University of Rio de Janeiro, Institute of Medical Biochemistry Leopoldo de Meis (IBqM), Center of Health Science, Rio de Janeiro, RJ, Brazil.
[b] Institute of Medical Biochemistry Leopoldo de Meis, Center of Health Science, University City, Av. Carlos Chagas, 373, Zip Code 21-941-590, Ilha do Fundão, Rio de Janeiro, RJ, Brazil.
[c] Multidisciplinary Department, Federal University of Sao Paulo, Osasco, SP, Brazil.
[d] Multidisciplinary Department, Paulista School of Politics, Economics and Business, R. Oleska Winogradow 100, Jardim das Flores, Osasco, zip code 06110295, São Paulo, Brazil.
[e] Oswaldo Cruz Foundation-Fiocruz, Bio-Manguinhos, Rio de Janeiro, RJ, Brazil.
[f] Department of Marked Relations, Institute of Technology in Immunobiologycals, Commercial Division, Bio-Manguinhos, Fundação Oswaldo Cruz – Fiocruz Av, 4365, Manguinhos, Rio de Janeiro, RJ, zip code 21040-900, Brazil.
[g] Department of Pathology, Faculty of Medicine, Federal University of Rio de Janeiro, Neurodegeneration and Repair Laboratory, University Hospital Clementino Fraga Filho, Center of Health Science, Brazil.
[h] Department of Pathology, Faculty of Medicine, University Hospital Clementino Fraga Filho, University City, R. Prof. Rodolpho Rocco 255, zip code 21941-913, Ilha do Fundão, Rio de Janeiro, RJ, Brazil.

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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**Corresponding author: E-mail: corgillieron@ufrj.br;*

ABSTRACT

The TXRF technique (total reflection X-ray fluorescence) was employed to analyze the concentration of zinc (Zn) and other metals in the pineal gland of rats submitted to orally administered excess dose of Zn sulfate. The histochemical localization of Zn was also performed. TXRF results showed a 42.9% increase in Zn concentration, and alterations on the homeostasis of other essential elements in rats. It was concluded that TXRF is a suitable technique for measuring, for the first time in this work, the concentration of Zn accumulated in the pineal gland after administration of an excess dose of Zn, and this result may be either directly or indirectly related to alteration in the homeostasis of other chemical elements, such as S, Cl, K, Ca, Ti, Cr, Mn and Fe.

Keywords: Pineal; rat; Zinc; TXRF; hyperzincemia.

1. INTRODUCTION

The pineal gland (PG, *epiphyse cerebri*) [1-5] is a neuroendocrine gland that integrates the circumventricular organs; PG parenchyma is mainly composed of pinealocytes, microglia and astrocytes [6]. 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 (such as the thyroid, adrenal, and gonads) and it can modulate the bioavailability of zinc (Zn) in the plasma [1,7].

Metals perform many important physiological functions in the human body. The Zn oligo-metallic ion is one of the most common and essential elements involved in brain function, and it plays an important role in physiological and pathophysiological processes [8-9].

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 [10]. After iron (Fe), Zn is one of the most abundant D-block metal [11-14] and is essential for several biochemical processes such as 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 [14-17] besides that, Zn and Fe play a pivotal role during neurodevelopment and mediate cognitive development [18]. Zn is especially important in the immune system because it plays a role as a molecular signal to immune cells that are involved in the expression of inflammatory cytokines and, yet several transcription factors need Zn to bind directly to specific regions of DNA [19].

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 [20-21]. Zn concentration in the brains of both rats and humans increase after birth and remain relatively steady throughout adult life [20]. In general, with respect to the total metal concentration, the brain should possess efficient homeostatic mechanisms that prevent abnormally high concentrations of metallic ions. Despite the important physiological role of Zn in modulating several CNS functions, substantial evidence suggests that high concentrations of Zn in the CNS can be neurotoxic [21-23]. Based on *in vitro* studies (in cortical cell culture) the amount of Zn released during neurotransmission (~225-600 μM) depending of time exposition is more than sufficient to cause fast neurotoxic effects [23-24].

Research on various brain diseases has indicated that trace metals such as Fe, Zn, copper (Cu), and manganese (Mn) are key neurochemicals in the neuropathology of diseases and the alteration of homeostatic mechanisms in CNS should lead to neurodegenerative disorders [25-26]. The neurodegenerative disorders in which these metals [27-30] are implicated are all characterized by a failure to maintain homeostasis, for example, the anomalous accumulation of weakly bound Zn deposits that have been observed in senile plaques in the brains of Alzheimer's patients [27-30]. The

association of Zn (and Cu) to amyloid-β in Alzheimer's disease suggests a central role in the abnormal metabolism of these metals in the pathology of this disease. 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 [28-30].

Total reflection X-ray fluorescence (TXRF) is a variant of Energy Dispersive X-Ray Fluorescence (EDXRF) and is a multi-element technique. One of the major advantages of this technique is the small sample amount required, a few microliters of a liquid or micrograms of a solid. In the case of solid samples, after the chemical digestion, a thin film is formed in the sample carrier, and the effects of absorption and enhancement can be neglected which simplifies the quantitative analysis [31]. In general, TXRF quantifications are performed using the internal standardization method 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 [32]. This geometry effect 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 [32]. This technique (TXRF) appeared to be suitable for the present study because the pineal gland of rats young adult females are very small samples.

In an earlier study [2] we demonstrated that adult female rats treated with excess zinc experienced severe changes in their motor behavior; this dose used was considered as a high dose of zinc and inclusive capable of producing hyperzincemia and amyloidosis. In the present study we focus on the potential role of induced hyperzincemia in female rats that received this large oral doses of zinc relative to female rats of the same age and, untreated with Zn. The main objective would be to evaluate the role of excess Zn in pineal histology, including revealing the zinc in pineal tissue by means of specific methods for this ion [33-38]. The technique of TXRF seemed quite adequate for the present study to quantify the amount of Zn in the pineal of the female rats that received the oral doses of excess zinc, as well as, evaluate the potential role of induced hyperzincemia in a possible one disruption of homeostasis of few other metals [31] which are also quantified by TXRF.

2. METHODOLOGY

Animals and experimental groups:
Female Wistar rats (n=48) at postnatal (PN) day 90 (birth was considered PN 0) obtained from different colonies were kept in biological cages 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 rats were weighed before (increased weight), during, and after the experiment (mean final weight = 188 g).

The animals were divided into three groups: two control groups (CG and NCG) and an experimental group (EG). The experimental group (EG) received zinc sulfate (ZnSO4 solution 0.1M, Sigma), one control group (CG) received sterile buffered saline, and the other control group (NCG) did not receive any solution.

Experimental Dose and oral administration:
The total dose of hyperzincemia] given [2] corresponded to 600 mg/kg of ZnSO4 solution and was administered as follows: The dose was divided into 10 sub-doses being administered 1 sub-dose/day of ZnSO4 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, in 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

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

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

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.

2.1 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*toluene-sulfonamide) (TSQm) [34-38]. The Neo-Timm has high selectivity to Zn and is the most used method to detect heavy metals in the mammalian brain [38]. The NTm 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 [36,38]. The TSQ histochemical method exhibits blue fluorescence for free zinc or weakly bound one [37].

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 (Na2S/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 brains 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 paraffin embedding.

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 [36,38]. 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. The histological preparations were counterstained with hematoxylin [39] dehydrated, clarified in xylene, and then mounted with Entellan (GTIN8 /EtellanMerck).

As for TSQm, [37] we used the non-fixed pineal glands; the glands were cryoprotected in sucrose (10%, 20%, and 30%) overnight, soaked in OCT (optimum cutting temperature 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 immersed (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 0,9% saline. 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 [33] 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 were deeply anesthetized, as explained above, perfused intracardially with 0.9% saline solution in 0,1 M phosphate buffer (PBS 0.1 M) followed by 4% paraformaldehyde fixative solution and 4% glutaraldehyde solution in 0,1 M phosphate buffer. The brains were removed and post- fixed for 24h in the same fixative. The pineal glands were removed and placed in 0.1 M phosphate buffer PBS. The material was embedded in pure Epon for 72 h in a 60°C oven for polymerization and analyzed by electron microscopy (Zeiss 900 transmission electron microscope, Laboratory of Protozoan Biology UFRJ).

2.2 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

[2]*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).*

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 [31,40,41] 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 subjected to chemical digestion (in a stove) by adding nitric acid (HNO3 - 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 (HNO3 - 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 HNO3) 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 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) were used for the 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

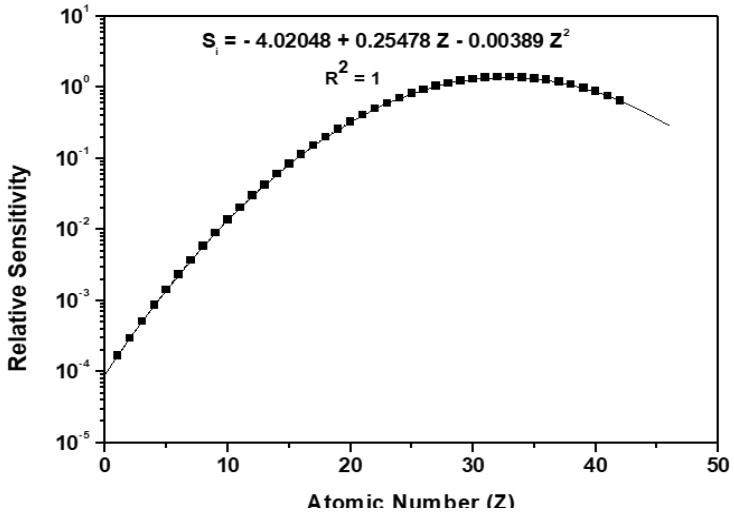

Fig. 1. Calibration curve for the K-line elements using TXRF

2.3 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).

3. RESULTS

Using the TXRF technique we found that the pineal glands (EG) of animals receiving an excess dose of ZnSO4 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 the serum of animals that received zinc.

Analysis of pineal parenchyma using electron microscopy showed disorganized fibrillar depositions and alterations in the blood 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 was particularly visible with respect to the wall of the blood vessels and enlarged peripherical spaces (Fig.2B; white stars). In group control rats, the parenchyma was more homogeneous and had a well-defined structure of blood 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 the blood vessel (Fig. 2B; white arrows), and there was a noticeable decrease in the vessel wall thickness (in EG rats) (Fig. 2B). In rats of CG (Fig. 3 A) the pineal blood vessels stained by hematoxylin and eosin showed a regular organization and the parenchyma was more organized as compared to the 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 (in group EG). These deposits appeared in larger quantities in the experimental group than in the controls (not shown here) (EG 161 aggregates; CG = 64 aggregates; NCG = 65 aggregates). In Fig. 3C, the arrows show the significant disruptions in relation to the blood

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 ≤ 0.05; LMD, minimum detectable value; concentrations (%); I, increase; D, decrease

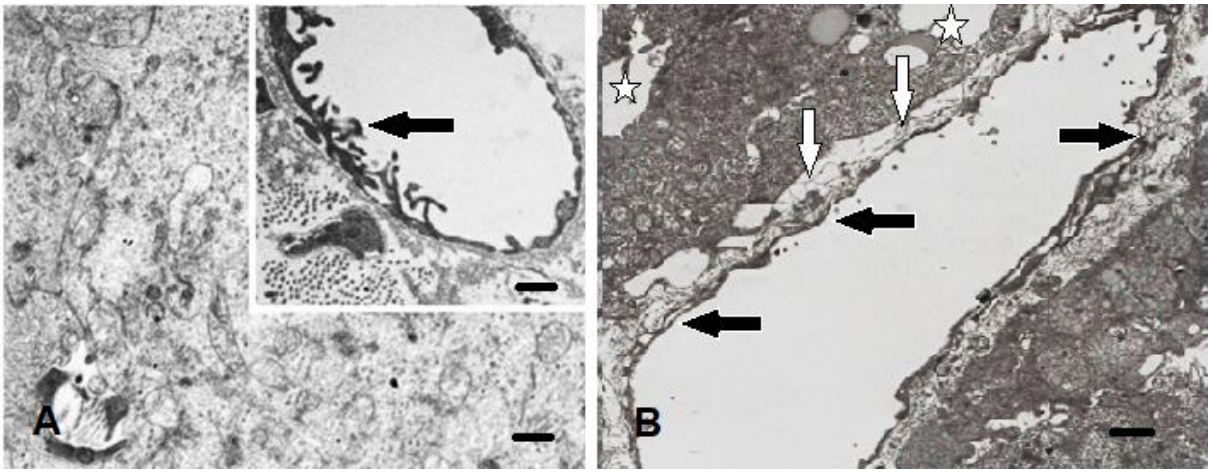

Fig. 2. Electron micrograph of the pineal. Group CG (A) group EG (B). (A) The pineal parenchyma is homogeneous, and the intercellular spaces are much smaller. The insert in A represents a blood vessel where it is possible to observe the various fingers projections (black arrows) from the vessel wall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

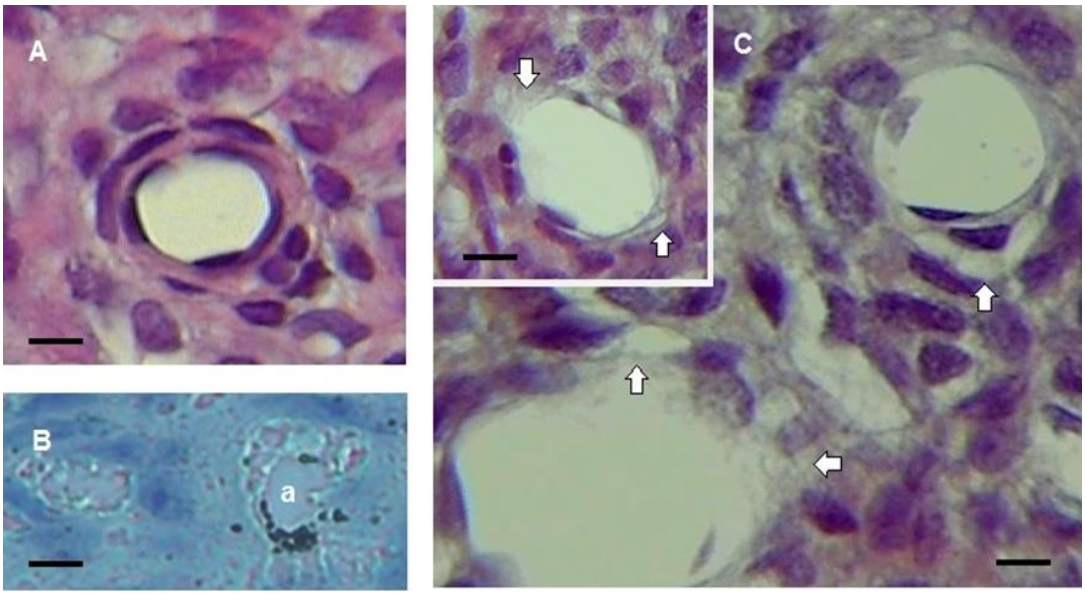

Fig. 3. Micrograph of parasagittal sections of the pineal glands. (A) in the control group (CG) showing a blood vessel stained by hematoxylin and eosin (HE) [39] with well-preserved wall. The peripherical parenchyma seem well organized. (B) Pineal gland from rats in the experimental group (EG). The Neo-Timm 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

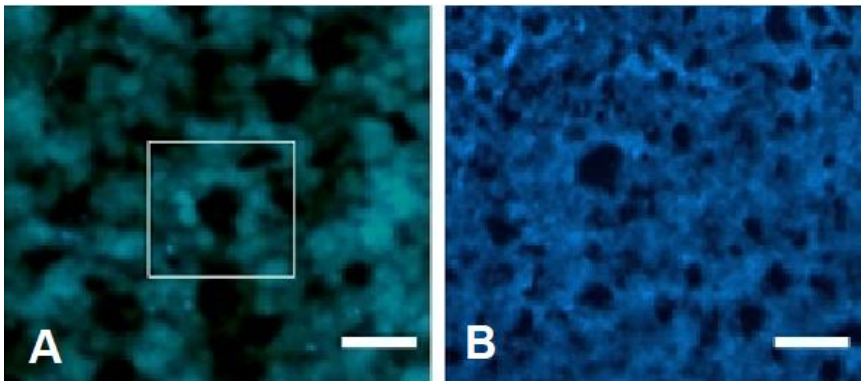

Fig. 4. A and B: Pineal gland treated by TSQ method. A: Sagittal sections of the pineal gland in animals treated with excess zinc (EG). Intense fluorescence of TSQ was observed in the pineal parenchyma and in the pseudo-rosette cells (demarcated area); B: Showing less evident fluorescence in the pineal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

vessel walls, and it is possible to observe a decrease in 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 [4] 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.

4. DISCUSSION

The present study quantified Zn in the pineal gland by the TXRF technique [41]. This technique was required because the pineal gland 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 an excess of Zn was administered. Zn is an essential trace element that is normally present in small amounts in the body [42]. 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 [43,44]. In Alzheimer's disease [45] 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. Despite evidence of the toxic effects of Zn, the literature provides more information on the consequences of Zn deficiency (hypozincemia) than that of Zn excess (hyperzincemia) [46]. For example, Zn deficiency, besides Cu overload, has been identified as a risk factor for autism spectrum disorders (ASD) [47].

According to our results (Table 3) by TXRF, there was an increase (42.9%) in Zn concentration in the pineal gland after excess Zn administration, which allows us to infer that the pineal gland is a target for Zn accumulation. This finding is strengthened by evidence that higher levels of Zn were found in the pineal gland [48] of some animals (calves, cows and pigs) [49]. Particularly, in the human pineal gland and in some brain tumors it has been reported a beneficial association between high zinc content and specific physiological roles [6,49,50,51,52]. 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. Neurons containing "free ionic zinc" (Zn^{2+}) are found in various areas of the brain, including the cortex, amygdala, olfactory bulb, hippocampal neurons, and pineal, which appear to have the highest concentration of zinc in the brain [9,10,51,52]. 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 [42,52,53,54,55,56]. 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 [57-59].

Zn homeostasis is also regulated by the intracellular Zn storage protein, metallothionein (MT) which readjusts the intracellular stock and maintains ion homeostasis [49,60,61,62,63,64]. 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. Zinc homeostasis is essential for cellular events and its dysfunction can lead to several human disorders [65,66]. 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 [60,65]. In these organs, transepithelial transport refers to the transfer of metals across the apical and basolateral membranes to be picked up and distributed by soluble transporters [69] The transepithelial zinc movement is orchestrated by many mammalian zinc transporters specialized for selective capture, and movements of Zn ions across the membrane barrier that depend on an electrochemical gradient, for instance, the bacterial zinc acquisition depends on ZIPB transporter zinc in an opposite direction, down a zinc concentration gradient [58,66]. 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 the drastic removal of cellular ZIP4 via proteasomal and lysosomal degradation pathways [67-71]. In mice and rats fed with a diet containing Zn, ZIP4 is hardly detected but a detailed analysis of the mouse and rat and human differences in the kinetics of endocytosis and degradation of ZIP4 is required, to gain a more complete understanding of the regulated ZIP4 and their underlying mechanisms of endocytosis in mammals [70]. 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). Zinc uptake takes place on the intestinal brush border

membrane of the enterocytes transport. 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 releases it into the portal blood, where it is predominantly bound to albumin, which distributes the metal in the body. In general, absorption of Zn is promoted through the presence of small molecular compounds (amino acids and hydroxy acids and animal proteins [68-72].

The role of metallothionein (MT) [73,74,75] 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. Notwithstanding, there is a critical period (CP) of 6 to 12 days [7] 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 [64]. During the CP, metallothionein (MTs) play a role as metabolic zinc-binding proteins and are capable of regulating Zn bioavailability, preventing alterations in ion concentrations from disturbing homeostasis [7]. For the pineal gland discussed in this paper, we do have not enough information on the transport mechanisms of Zn, and the activity of the metallothioneins to discuss our results about Zn drive after ingestion of this ion, although there is data suggesting the presence of a metallothionein-I-II expression system in the pineal gland of bovines [49]. 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 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 when compared to controls [76]. Recent studies relate the difficulty to elucidating the cellular transport systems for other trace elements when toxics, such 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 [77].

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 and, the blood-brain barrier maintains the homeostasis of the microenvironment regulating the balance of zinc in the brain [78]. 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 [79,80]. 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+} [81]. Another element for Zn^{2+} transport is ZIP8, a member of the ZIP transporter family [82] principally involved in the transport of this metal at renal tubules [82,83].

In the case of the pineal gland [84] that has strong blood circulation, there is no true blood-brain barrier [2]. DMT1 is a transporter not cited in the pineal literature to date, as well as the ZIP8; but the present study is inclined to consider that the accumulation of Zn in the pineal gland might be favored by the absence of a blood-brain barrier in this gland. The fact that the pineal has no blood-brain barrier and is highly vascularized by a capillary network organized by the parenchyma, with its capillaries presenting fenestrations is a factor that implies in exchanges of substances between tissues and vessels faster and easier [85,86]. The intrapineal vascular architecture varies with specific features concerning the peripheral part, poorly vascularized by small and fine blood vessels and, the central part of the gland highly vascularized by large sinusoid capillaries [84-86].

Although there is a relationship between melatonin (the primary product of the pineal gland) [87] and the plasma zinc level, the analysis of any relationship between melatonin levels and excess zinc was 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, 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. 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 blood 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. 4) 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 types 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). These transporters are regulated by the amount of 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 [30,45,54] 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 [2]. 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 weakness, and it was more difficult to continue taking blood samples [2]. Thus, the 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 [67]. 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 the proper functioning and growth of brain [67] and our body must have adequate amounts of them. 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 [31].

In the current study, the changes in the concentration of the other chemical elements were 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) such as Fe. In relation to iron (Fe), which is an essential element for normal body functioning [80] 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 a common feature in some diseases. Studies of Fe and Zn interactions in

neural tissues are scarce but the literature suggests a biological (micronutrient status) interdependence between iron and zinc in the brain [79]. Previous studies demonstrated an antagonism between Fe and Zn, citing absorptive competition between iron and zinc at the receptor DMT1, however, evidence showed that the DMT1 is not the primary intestinal transporter of zinc. 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 detect 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 some 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 [88].

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 in the pineal, likewise that described to the overdose of ZnCl2, for instance [67].

Calcium can appear as free Ca2+ both extracellularly and intracellularly [89].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 [90]. There is evidence that Zn can increase the permeability of cell membranes to Ca and thus contributing to homeostatic imbalance. Release of Ca from the mitochondria may also occur because of changes in Mn concentration [90].

Mn may induce cellular damage through mitochondrial dysfunction and can release Ca from intracellular stores under certain conditions [91]. 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 (considering that pineal do not have BBB) thereby producing toxicity and neuropathies [91]. Complementary studies are needed to determine whether these observed changes in Ca and Mn homeostasis are interrelated.

However, 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 the 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) 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. 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 [2] One hypothesis raised is that this response is due to the breakdown of homeostasis of the various trace elements as analyzed in the present study, and this would be corroborate previous studies [18] where changes in the homeostasis of Zn and other metals would be implicated in the pathogenesis of certain diseases.

5. 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 exactly determine the factors involved in changing (the breakdown) the 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 [2] where the animals showed a significant change in motor behavior after 10 doses of excess of zinc sulphate; in addition, irregular deposits of zinc appeared adjacent to pineal gland vessels, in the same area occupied by amyloid deposits [2].

The present study contributes to the pineal literature, because it shows, for the first time, that a dose with excess zinc sulfate (corresponding to 600 mg/kg ZnSO4 solution) may cause hyperzincemia and to affect the homeostasis of some trace elements in the pineal gland of female rats

ARTICLE HIGHLIGHTS

The X-Ray Fluorescence measured for the first time in the pineal the effects of excess zinc on the homeostasis of trace elements.

The excess of zinc altered the homeostasis of S, Cl, K, Ca, Ti, Cr, Mn, Fe, increasing these elements in the pineal.

Zinc histochemical techniques showed that after the overdose occurred important changes in the pineal parenchyma.

ETHICAL APPROVAL

Animal Ethic committee approval has been collected and preserved by the author(s)

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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