Multielement analysis of swiss mice brains with Alzheimer's disease induced by beta amyloid oligomers using a portable total reflection X-ray fluorescence system

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ABSTRACT

Alzheimer's disease (AD) is a progressive dementia that, in early stages, manifests as a profound inability to form new memories. The pathological features of AD include β-amyloid (Aβ) plaques, intracellular neurofibrillary tangles, loss of neurons and synapses, and activation of glia cells. Recently, several groups have raised the "metal hypothesis" of AD. Metal ions, such as Cu and Zn, have been demonstrated to modulate amyloid aggregation along different pathways. Extensive research has been conducted on the effects of metals on Aβ aggregation and all of them have shown that both Cu and Zn accelerate the aggregation by shortening, or eliminating, the lag phase associated with the amyloid fibrillation process. The metal ions mentioned previously may have an important impact on the protein misfolding and the progression of the neurodegenerative process. The TXRF technique is very important, because can be used to identify and quantify trace elements present in the sample at very low concentrations ($\mu g.g^{-1}$). In this work, three groups of females were studied: control, AD10 and AD100. The groups AD10 and AD100 were given a single intracerebroventricular injection of 10 pmol and 100 pmol of oligomers of β-amyloid peptide respectively to be induced AD. The TXRF measurements were performed using a portable total reflection X-ray fluorescence system developed in the Laboratory of Nuclear Instrumentation (LIN/UFRJ) that uses an X-ray tube with a molybdenum anode operating at 40 kV and 500 mA used for the excitation and a detector Si-PIN with energy resolution of 145 eV at 200 eV. It was possible to determine the concentrations of the following elements: P, S, K, Fe, Cu, Zn and Rubidium. Results showed differences in the elemental concentration in some brain regions between the AD groups and the control group.

1. INTRODUCTION

Metals play important roles in the human body, for example, maintaining cell structure and regulating the gene expression, the neurotransmission and the antioxidant response. However, elevated levels of metals can induce various detrimental intracellular events, including the oxidative stress, the mitochondrial dysfunction, the DNA fragmentation, the protein misfolding, the endoplasmic reticulum (ER) stress, the autophagy dysregulation and the activation of apoptosis [1-3]. Excessive metal accumulation in the nervous system can be toxic inducing oxidative stress and impairing the activity of numerous enzymes. These effects can change neurotransmission and lead to neurodegeneration, which can manifest as cognitive problems, movement disorders, and learning and memory dysfunction. Damage caused by metal accumulation can result in permanent injuries, including severe neurological disorders like Alzheimer's disease (AD) [4].

Alzheimer's disease is a progressive dementia that, in early stages, manifests as a profound inability to form new memories. Its incidence is higher in people aged over sixty-five. The basis for this specificity is unknown, but some evidences point to the involvement of neurotoxins derived from the self-associating β-amyloid (Aβ). β-amyloid generates the fibrils of the hallmark amyloid plaques of AD. However, the "fibril hypothesis" fails to explain crucial clinical and pathological aspects of this degenerative disease. In AD, it has been pointed out that there is poor or even the absence of correlation between plaque burden and dementia [5]. Alzheimer's disease is increasingly recognized to be linked to the function and status of metal ions and, recently, the amyloid hypothesis has been strongly intertwined with the metal ion hypothesis. In fact, these two hypotheses fit well together and are not mutually contradictory.

β-amyloid oligomers (Aβos) are found at elevated levels and in association with synapses in the brains of demented AD individuals. The build-up of Aβos in the affected brains has been recognized as an additional neuropathological hallmark of AD. In this study, AD is induced by a single intracerebroventricular injection of Aβos.

This work proposes to analyze the distribution and the cerebral multielemental concentration using an experimental model of the AD. For this purpose, six different structures of the brain were analyzed by the Total Reflection X-ray Fluorescence (TXRF). The TXRF technique, in this analysis, is very important, because the detection and quantification of the metals ions in mice brains with AD is fundamental to understanding this disease and the application of neuroprotective and therapeutic measures. The main elements of interest in this proposal are P, S, K, Fe, Zn and Rb [6]. Besides that, in mice brains, these elements concentrations occur in order of $\mu g.g^{-1}$ and TXRF is a powerful and relatively simple analytical technique that can be used to identify and quantify trace elements. This technique has a high sensitivity, needs small sampling amounts [7,8] and it is capable of detecting, simultaneously, several elements present in the sample at very low concentrations.

2. MATERIALS AND METHODS

2.1. **Animals and intracerebroventricular injections**

Three-month-old female Swiss mice were used. The animals were housed in groups of five in each cage, with free access to food and water, under a 12 hours light/dark cycle,

with controlled room temperature and humidity. All procedures followed the Principles of Laboratory Animal Care from the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee of the Federal University of Rio de Janeiro (protocol #IBqM 129/2015). For intracerebroventricular injection of Aβos (or vehicle), the animals were anesthetized for 7 minutes, with 2.5 % isoflurane (Cristália), using a vaporizer system and were gently restrained only during the injection procedure. A 2.5 mm long needle was unilaterally inserted 1 mm to the right of the midline point equidistant from each eye and 1 mm posterior to a line drawn through the anterior base of the eyes [9,10]. Injections of 10 pmol of Aβos (AD10 group), 100 pmol of Aβos (AD100 group) and an equivalent volume of vehicle (control group) were made with a final volume of 3 μL.

2.2 **Sample and standard preparations**

Seven days after the injection of Aβos (or vehicle) were carried out. The animals were sacrificed by cervical dislocation and their brains were quickly and carefully removed. Then, the brains were immersed in cold Phosphate-buffered saline (PBS) solution to lower the temperature of the brain tissue and makes it firm to facilitate dissection. The PSB solution was completely dried with paper towels after a thermal shock to the brain. Subsequently, six structures (temporal cortex, frontal cortex, hippocampus, hypothalamus, cerebellum and substantia nigra) were dissected for trace and major elements analyses. All samples were submitted to acid digestion by adding 0.2 mL of nitric acid (HNO_3 -65%). After 3 hours, at 80 ºC, 0.04 mL of hydrogen peroxide (H_2O_2) were added and the digestion continued for 2 hours, at 80 ºC. After that, they were diluted with 0.1 mL of ultrapure water (Milli Q water). After the dissolution, the samples were mixed with 40 μL of Ga solution, which was used as an internal standard. That solution was homogenized by shaking and a small aliquot of 10 μL was pipetted on a precleaned quartz carrier. After the deposition, the samples were left to dry very slowly under an infrared lamp. All samples were processed in triplicates. The methodology was validated through the analysis of samples of standard reference material (NIST 1577bbovine liver), prepared under the same conditions as the brain compartments samples.

2.3 Experimental setup

TXRF measurements were performed using a portable total reflection X-ray fluorescence system developed in the Laboratory of Nuclear Instrumentation (LIN/UFRJ) in Rio de Janeiro that uses an X-ray tube with a molybdenum anode operating at 40 kV and 500 mA used for the excitation and a detector Si-PIN with energy resolution of 145 eV at 200 eV.

Spectra were analyzed by the AXIL software of the Quantitative X-ray Analysis package of the International Atomic Energy Agency. Concentrations were calculated taking into consideration the Ga standard, the experiment blanks and the weight corrections. In order to investigate the presence of extreme values, we used the Grubb's test and the outliers were excluded. Dunnett's test, for a confidence level of 95% ($\alpha = 0.05$), was used to identify the differences in concentrations between Aβos and control groups.

2.4 Results and discussion

2.4.1 Results

Using the portable total reflection X-ray fluorescence system, it was possible to detect the presence of the following elements of interest: P, S, Cl, K, Ca, Fe, Cu, Ni and Zn, which are present in brain compartments, and Ga, the internal standard. Figure 1 shows a typical X-ray fluorescence spectrum of a sample of brain tissue.

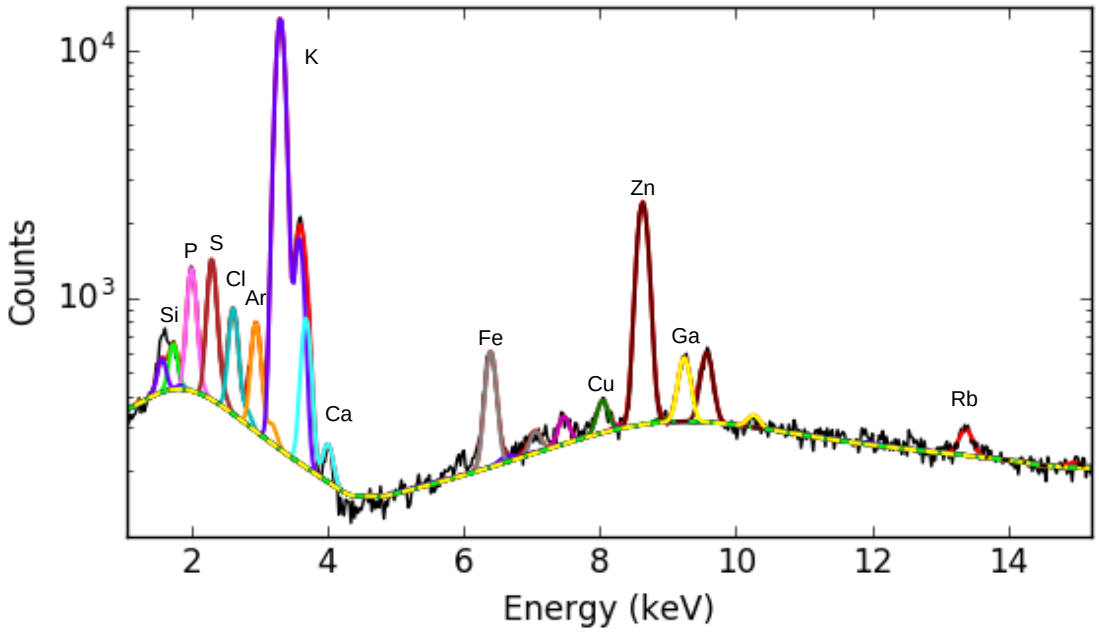

Figure 1. Typical X-ray fluorescence spectrum of a sample of brain tissue.

The accuracy, precision and experimental validations were carried out with elemental analysis of a reference material: NIST Standard Reference Material 1577b (Bovine liver). Table 1 shows the mean concentrations, the certified values and relative errors.

Table 1. Comparison between the experimental results and NIST Standard Reference Material 1577b (Bovine liver).

Element	**TXRF* ($\mu g.g^{-1}$)**	**Certificate value ($\mu g.g^{-1}$)**	**|Relative error| (%)**
P	7900 ± 300	11000	28
S	6000 ± 260	7850	23
K	7000 ± 880	9940	29
Ca	106 ± 28	116	8
Fe	142 ± 50	184	22
Cu	147 ± 7	160	8
Zn	126 ± 3	127	0.01

* Mean ± standard error of the mean.

The values found for Ca and Ni were not considered because their concentrations were lower than detection limits. Chlorine element also was not considered due to losses in the acid digestion (nitric acid and hydrogen peroxide).

Tables 2 and 3 present the elemental concentration determined in female brain compartments of AD10, AD100 and control groups in $\mu g.g^{-1}$.

Table 2. Elemental concentration ($\mu g.g^{-1}$) in the temporal cortex, frontal cortex and hippocampus from the brain of female Swiss mice with experimental Alzheimer's disease.

El.	Temporal Cortex			Frontal Cortex			Hippocampus		
	Control	AD10	AD100	Control	AD10	AD100	Control	AD10	AD100
P	6186 ±414	8559 ±608*	8614 ±512**	8019 ±488	10985 ±428*	11330 ±445**	8064 ±454	11620 ±718*	10914 ±489**
S	1299 ±157	1638 ±181	1456 ±128**	1244 ±65	2055 ±149*	2200 ±196**	1842 ±119	2367 ±235	2230 ±174
K	11195 ±685	15590 ±724*	18620 ±1366**	16075 ±1066*	22474 ±1445*	21729 ±1563	16465 ±1000	24148 ±1673*	23952 ±1056**
Fe	48±2.67	101±14*	118±14	56±3	110±12*	75±6	69±6	121±10*	74±7
Cu	12.6±0.7	20.7 ±2.5*	19±2	15.3±1.4	21±1*	18±2	16±	32±3	19±1
Zn	54.9±4.8	78.6±7.5	96±7	73±4	95±5	71±7	108±9	116±10	122±7**
Rb	21.7±0.12	29±2*	27±2**	23.5±0.9	33.1±1.7*	29±2**	27±1	33±2*	28±1

Values represent means ± standard error of the mean.
* Statistically significant differences ($P < 0.05$) between the AD10 and control groups.
** Statistically significant differences ($P < 0.05$) between the AD100 and control groups.

Table 3. Elemental concentration ($\mu g.g^{-1}$) in the hypothalamus, substantia nigra and cerebellum from the brain of female Swiss mice with experimental Alzheimer's disease.

El.	Hypothalamus			Substantia Nigra			Cerebellum		
	Control	AD10	AD100	Control	AD10	AD100	Control	AD10	AD100
P	11616 ±198	14185 ±665*	14292 ±667**	9433 ±126	9776 ±646	12653 ±269**	7765 ±551	9761 ±690*	8503 ±408
S	2309 ±185	2724 ±144	2764 ±202	2363 ±33	2639 ±75*	6805 ±27**	677 ±69	1146 ±116*	777 ±46
K	20259 ±815	26433 ±1306*	26962 ±1629**	13280 ±186	18254 ±968*	17570 ±424**	1321 ±971	13050 ±1628	14718 ±816
Fe	73±	150±28*	81±8**	98.7±0.4	132±7*	166±3**	54±4	70±5*	60±3
Cu	23±3	35±4	20±2	11±1	37±4*	19±1	17±1	22±3	18±1
Zn	58±5	62±4	127±10*	129±3	172±10*	65±2**	71±7	68±5	61±5
Rb	24±1	32±1*	28±1	22±1	25±2	28±1	20±1	24±2	21±1

Values represent means ± standard error of the mean.
* Statistically significant differences ($P < 0.05$) between the AD10 and control groups.
** Statistically significant differences ($P < 0.05$) between the AD100 and control groups.

As shown in tables above, the P levels are statistically different (SD) in the: temporal and frontal cortex, hippocampus and hypothalamus for both groups (AD10 and AD100), but in the substantia nigra they are SD only for the AD100 group while in the cerebellum, only for AD10 group. Sulphur levels are SD for both groups only in the substantia nigra, being yet SD in the cerebellum for the AD10 group and in the frontal cortex for the

AD100 group. Potassium levels are SD for both groups in all compartments exception in the cerebellum where they are not SD for any group. Iron levels are SD in all compartments for AD10 group, but they are SD only in the frontal cortex and in the substantia nigra for the AD100 group. Copper levels are SD for the AD10 group in the: frontal and temporal cortex, hippocampus and substantia nigra while for the AD100 group they are SD only in the temporal cortex. Zinc levels are SD for both groups in the substantia nigra being yet SD in the temporal cortex and in the hypothalamus for the AD100 group. Finally, Rb levels are SD for both groups in the frontal and temporal cortex and, also, for the AD10 group, in the hypothalamus and in the hippocampus.

2.4.2 Discussion

The findings for cortex are in agreement with a recent study [11] which shows that the Fe content in the cortex was increased at the earliest detectable signs of cognitive decline on the PSAPP mouse model of AD. Another studies also have shown elevated Fe levels in the cortex [12].

The hippocampus is also of neuropathological interest because it is important for long-term memory storage and neurogenesis and it is one of the first and most severely affected brain regions in AD [13] It has shown itself more sensitive to metal perturbation than other brain regions and has unique regulatory demands for Fe, Cu and Zn, since they are involved in synaptic plasticity [14]. Recent studies have shown changes in Fe, Cu, and Zn levels in the hippocampus of animals and humans with AD [13,14]. Findings in this study showed differences for P, K, Fe, Cu and Rb levels.

The most important function of the hypothalamus is to link the nervous system to the endocrine system [14]. Besides that, Type 2 diabetes (T2D), a metabolic disorder, has been associated to AD [15]. Insulin signalling regulates cell metabolism in multiple tissues, including liver, muscle and hypothalamus [16-20]. Brain insulin signalling, particularly in the hypothalamus, controls several metabolic pathways [18]. Animal models have established that the experimental induction of brain insulin resistance can induce whole-body insulin resistance, which is a pre-diabetic state, illustrating the importance of hypothalamic insulin action in metabolic homeostasis [19]. Findings in this study showed differences for P, K, Fe, Cu, Zn and Rb levels. Furthermore, no studies relating the levels of trace elements in the hypothalamus and AD patients were found in literature.

In the normal adult brain, substantia nigra contains the highest Fe concentration, which explains why, in neurodegeneration movement disorders, this region is vulnerable to Fe imbalance [21]. In addition, Cu concentrations in the normal adult brain are higher in the substantia nigra and in the cerebellum [20-23]. Findings presented in this work showed different concentrations of P, S, K, Fe, Cu and Zn.

Although the cerebellum is the major repository of metals, where large amounts of Fe, Cu and Zn are located [24], the findings show statistically significant differences between the groups only for P, S and Fe in this tissue, differently than believed.

3. CONCLUSIONS

Total reflection X-ray fluorescence is a powerful tool to determine elemental concentrations in brain tissue samples. Elements were identified and their concentrations were determined.

Changes in the elemental concentrations in the hippocampus in this study might be associated with the AD induced by β-amyloid oligomers in this experimental model.

The significant differences in concentrations of P, k, Fe, Zn and Rb in the hypothalamus suggest an association between AD and changes in the concentration of these elements.

ACKNOWLEDGMENTS

This work was supported by the National Council for Scientific and Technological Development (Conselho Nacional de Desenvolvimento Cientifico e Tecnológico - CNPq), Foundation for Research Support of the State of Rio de Janeiro (Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro - FAPERJ)

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