A post-mortem study of the anatomical region differences and age-related changes on Ca and Mg levels in the human brain

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Abstract

Calcium and magnesium levels in 14 different areas of the human brain [frontal cortex, superior and middle temporal gyri, caudate nucleus, putamen, globus pallidus, cingulated gyrus, hippocampus, inferior parietal lobule, visual cortex of the occipital lobe, midbrain, pons-locus coeruleus, medulla and cerebellum-dentate nucleus] of adult individuals (n=42; 71±12, range: 50-103 years old) without a known history of neurodegenerative, neurological or psychiatric disorder were studied.

Considering the mean values for the 14 regions, Mg was present at ca. 2.3-fold higher levels than Ca (mean ± sd: 527±34 μg/g *versus* 226±53 μg/g). Calcium distribution within the brain showed to be quite heterogeneous: highest levels were found in the occipital (306±156 μg/g) and frontal cortex (287±78 μg/g), while lowest levels were found in the medulla (186±70 μg/g) and cerebellum (145±42 μg/g). Higher Ca levels were found in women than in men (248±59 μg/g *versus* 213±46 μg/g; $p<0.05$). A tendency for a Ca levels increasing with age in all studied brain regions and in both genders was also observed. On the contrary, Mg presented a highly homogeneous distribution and seems to remain quite unchanged irrespective of ageing.

Keywords: human brain; calcium; magnesium; ageing; post-mortem analysis.

1. Introduction

As a result of longer life expectancy, neurodegenerative diseases (ND) will become leading diseases worldwide during the next decades [1]. Besides "natural" ageing, genetic predisposition and environmental factors [2], imbalances on major and trace element (both essential and toxic) homeostasis in particular areas of the brain have been identified as potentially responsible for the cognitive decline associated with normal ageing and the development of some ND [3,4], but the evidence is still fragmentary and its definite role remains unclear.

Calcium (Ca) plays a central role in neuronal physiology. As an ubiquitous second messenger, Ca regulates energy production, gene expression, membrane excitability, dendrite development and synaptogenesis [5]. In particular, intracellular Ca ions play a major role in neurons as the trigger for neurotransmitter release [6], regulate neuronal plasticity underlying learning and memory and neuronal survival [7].

Besides being a critical cofactor in numerous enzymatic reactions, magnesium (Mg) is involved in several cellular processes, including ATP production, oxygen uptake, electrolyte balance, glucose metabolism, DNA transcription and protein synthesis [8]. Regarding central nervous system, Mg has a neuroprotective role as a blocker of the N-methyl-D-aspartate (NMDA) receptor ion channel, which regulates Ca^{2+} transport through the plasma membrane and prevents ischemic damage to neurons. Since neural activity depends on the Mg concentration, when glutamate is released under anoxia conditions, it acts as a neuromodulator and protect cells from excitotoxicity [9].

Disturbances in the homeostasis of cytosolic Ca have been implicated in the pathogenesis of several acute and chronic ND and in brain ageing [10-12]. Magnesium brain levels seem to be decreased in elderly people and some authors believe that such depletion, particularly in the hippocampus, may represent an important pathogenic factor of some ND [13,14].

Most of the current information about the relationship between metals and human brain functioning is based on animal studies [15,16] or relies on determinations in cerebrospinal fluid, whole blood or blood serum or plasma [15,17-21]. Studies on major metals (such as Ca and Mg) and trace elements levels in normal and pathological human brains are scarce, limited to a few brain areas [22-24] and/or involve a small number of subjects [25,26]. Additionally, and regarding the specific topic of the distribution of metals in brain, most of the published post-mortem [22,26,27] and *in vivo* [28,29] studies are focused solely on iron. Therefore, more extensive elemental

mappings are needed in order to understand the specific roles of major biometals and trace elements in the human brain and to interpret the data obtained from ND patients.

Based on this background, the main goal of the present study was to directly quantify Ca and Mg levels in 14 different areas of the human brain in order to evaluate a) the regional anatomic differences and b) age-related changes in Ca and Mg levels. The results found in one Parkinson's disease and two Alzheimer's disease patients are also presented.

2. Materials and methods

2.1. Subjects

Brain samples were collected from men (n=27; 67±11 years old) and women (n=15; 77±12 years old) not registered in the Portuguese National Registry of Refusal to Organ Donation database and complying with all the current regulations regarding human tissue collection for scientific research purposes.

Samples were obtained from individuals submitted to forensic autopsy exams during the first semester of 2012 at the North Branch (Porto) of the Portuguese National Institute of Legal Medicine (INML). Individuals from each of the following age groups were studied: 50-59 (n=10), 60-69 (n=10), 70-79 (n=10), 80-89 (n=9) and ≥90 (n=3) years old. Inclusion criteria were a) the absence of a history of known neurodegenerative, neurological or psychiatric disorders, b) the absence of injuries involving the central nervous system (CNS), and c) macroscopically normal tissues.

Samples from two individuals with documented Alzheimer's disease (women, 73 and 85 years old) and one with Parkinson's disease (woman, 91 years old) were also collected.

2.2. Sample collection

Samples were collected by the pathologists at the INML following a standard protocol.

To prevent sample contamination, all materials in contact with the samples, including the stainless steel tools used by the pathologists, were previously decontaminated with a 5% (v/v) nitric acid solution prepared from concentrated (≥69%) HNO_3 (Sigma-Aldrich, Germany) and thoroughly rinsed with ultrapure water (resistivity 18.2 MΩ.cm at 25 °C) produced by a Milli-Q water purification system (Millipore, USA).

After removing the brain from the cranium, the contaminating blood was washed away with ultrapure water. The meninges were removed with plastic tweezers, and the brain was washed again with ultrapure water to minimise sample contamination with blood or cerebrospinal fluid. Since each brain hemisphere is divided in four neuroanatomically and functionally lobes, the function of the affected neurons determines the clinical features,

which can be grouped into two main categories: cognitive impairment (e.g. Alzheimer's disease) and movement disorders (e.g. Parkinson's disease).

In order to establish an accurate diagnosis and study the relationship between the disease process and either the clinical features seen in life or the cause of death, Paine and Lowe [30] have recently proposed a post-mortem approach where 14 key areas are suggested to be studied (Fig. 1). In this study, samples from those key areas were obtained.

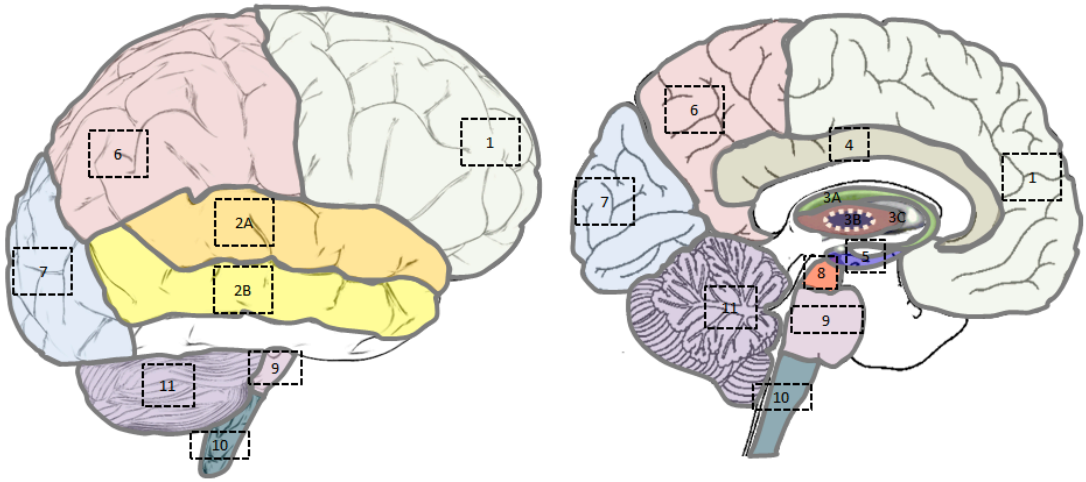

Fig. 1. Sampled brain regions, as suggested by Paine and Lowe [30]: frontal cortex (1), superior (2A) and middle (2B) temporal gyri, basal ganglia including the caudate nucleus (3A), pu- tamen (3B) and globus pallidus (3C), cingulated gyrus (4), hippocampus (5), inferior parietal lobule (6), visual cortex of the occipital lobe (7), midbrain (including the substantia nigra at the level of the third nerve) (8), pons-locus coeruleus (9), medulla (10) and cerebellum-dentate nucleus (11).

Using decontaminated plastic knives, tissue fragments (approximately 1 cm^3) were collected from the following brain areas: frontal cortex (1); superior (2A) and middle (2B) temporal gyri; basal ganglia, including the caudate nucleus (3A), putamen (3B) and globus pallidus (3C); cingulated gyrus (4); hippocampus (5); inferior parietal lobule (6); visual cortex of the occipital lobe (7); midbrain, including the *substantia nigra* at the level of the third nerve (8); pons (locus coeruleus) (9); medulla (10); and cerebellum (dentate nucleus) (11). Samples were stored in decontaminated polypropylene tubes (Sarstedt, Germany) at -4 ºC until analysis.

2.3. Sample pre-treatment

After defrosting, the brain samples were thoroughly washed with ultrapure water and placed in a dry oven (Raypa, Spain) at 110 ºC until constant weight (ca. 24 hours). Dried samples (ca. 100-500 mg) were weighed directly in the microwave digestion vessels, previously decontaminated with 10% (v/v) HNO_3 and thoroughly rinsed with ultrapure water. Samples were digested with 2.5 mL of concentrated (≥65% m/m) HNO_3 (TraceSELECT®, Fluka, France) and 1.0 mL of ≥30% (v/v) H_2O_2 solution (TraceSELECT®, Fluka, Germany) in an MLS 1200 Mega microwave oven (Milestone, Italy), equipped with an HPR 1000/10 rotor, and using the following power (W)/time (min) program: 250/1, 0/2, 250/5, 400/5 and 600/5. After cooling, sample solutions were made up to 50 mL with ultrapure water and stored in closed propylene tubes at 4 ºC until analysis.

2.4. Calcium and magnesium determination

The determination of Ca and Mg was performed by flame atomic absorption spectrometry (FAAS), using a PerkinElmer (Germany) Model 3100 instrument, with an oxidizing air-acetylene flame and Intensitron™ (PerkinElmer) hollow cathode lamps (operated at 10-mA for Ca and 6-mA for Mg) as light source (λ Ca=422.7 nm; λ Mg=285.2 nm; slit width=0.7 nm).

Calibration standards were prepared by diluting a commercial standard solution (1000 mg/L) of Ca (CertiPUR®, Merck, Germany) and Mg (SpectrosoL®, BDH, England) with acidified (0.2% v/v HNO_3) ultrapure water. Calibration curves were obtained with five Ca and Mg calibration standards with concentrations ranging from 1 to 5 mg/L and 0.1 to 0.5 mg/L, respectively.

For analysis, the sample digests were previously diluted (20-fold for Mg and 2-fold for Ca) with ultra-pure water. A 10% (w/v) La solution, prepared by dissolution of La_2O_3 (Sigma-Aldrich, China) in concentrated (30%) HCl (Fluka, Switzerland), was added to all samples and standards (final concentration: 0.5%) in order to suppress interference from elements which give rise to stable oxysalts.

2.5. Analytical quality control

Because human brain tissue is not available as a certified reference material (CRM) for Ca and Mg determination, human serum (Seronorm™ L-1 and L-2, Sero As, Norway) and dogfish liver (DOLT-4, from National Research Council, Canada) were used for analytical quality control purposes. CRMs were subjected to the same sample pre-treatment. The values obtained proved the accuracy of the analytical procedure (Table 1).

One sample blank was performed in each microwave-assisted acid digestion series (10 samples). In total, 77 sample blanks were run, and the mean value (Ca=0.00 μg/L; Mg=1.86 μg/L) was subtracted from the sample values.

Table 1
Results obtained in the determination of Ca and Mg in certified reference materials.

	Ca		Mg	
	Acceptable range[#]	Experimental value (mean±sd;n=3)	Acceptable range[#]	Experimental value (mean±sd;n=3)
Serum L1 (mg/L)	90.2-101.8	94±2.2	19.1-22.1	20.5±0.4
Serum L2 (mg/L)	131-147	138±2	37.9-44.1	40.8±0.5
DOLT-4 (μg/g)	680*	692±4	1500*	1510±12

*Information value (i.e., value which could not be certified because of insufficient information to accurately assess uncertainties).# Acceptable range for ICP-AES determination (no data available for FAAS).

2.6. Statistical analysis

The descriptive statistical parameters and correlations were calculated using Microsoft Office Excel 2010 (Microsoft Co., USA). The means were compared by unpaired Student's t-test at α=0.05 significance level using GraphPad Prism 5 (GraphPad Software Inc., USA).

3. Results and discussion

3.1. Anatomical region differences on Ca and Mg levels

The studies regarding elemental levels in human brain are rather scarce. Our results for Ca and Mg (226±53 μg/g and 527±34 μg/g, respectively) are in good agreement with previously reported data [22,31].

The results (μg/g dry weight) for Ca and Mg in each of the 14 different brain regions studied are summarised in Table 2 and Table 3, respectively.

Table 2. Ca levels (mean ± sd, μg/g dry weight) in 14 different regions of human brain (see footnote) of non-diseased individuals (n = 42; 71 ± 12 years old) according to age group.

Brain regions*	All individuals	**Age groups**				
		[50-59] (*n*=10)	[60-69] (*n*=10)	[70-79] (*n*=10)	[80-89] (*n*=9)	≥90 (*n*=3)
1	287±78	228±42	269±44	291±72	317±96	375±109
2A	254±91	186±63	253±85	242±38	325±128	335±47
2B	221±68	188±51	258±76	266±62	241±47	434±165
3A	250±108	224±76	194±100	258±98	287±190	236±25
3B	202±70	161±64	194±90	210±43	225±59	274±76
3C	256±193	162±53	228±145	230±137	321±193	578±468
4	202±61	157±82	183±40	244±48	196±27	267±61
5	255±105	219±118	233±76	259±84	262±67	416±217
6	276±112	205±41	347±142	242±65	327±146	251±27
7	306±156	265±76	229±68	273±120	392±224	509±195
8	173±53	134±47	173±47	203±64	182±43	172±9
9	184±80	178±59	135±50	196±73	195±80	296±162
10	164±70	183±162	157±75	211±67	148±63	178±68
11	145±42	131±29	157±61	157±36	139±61	180±70
Mean Ca level in the 14 regions	226±53	193±19	233±35	246±54	256±60	321±48

*1-Frontal cortex; 2A-Superior temporal gyrus; 2B-Middle temporal gyrus; 3A-Caudate nucleus, 3B-Putamen; 3C-Globus pallidus; 4-Cingulated gyrus; 5-Hippocampus; 6-Inferior parietal lobule; 7-Visual cortex of the occipital lobe; 8-Midbrain, 9-Pons 10-Medulla; 11-Cerebellum.

The Mg/Ca ratio found in the present study, ranging from 2.0 to 3.3, compares with Andrási et al. [22] data, who reported Mg/Ca ratios between 1.4 and 2.4. However, there are several studies reporting much lower ratios, with Mg and Ca present at similar levels. In an old study by Ward and Manson [32] significantly lower Mg levels were found in cerebral cortex and hippocampus from East Canada and United Kingdom populations, which led to a Mg/Ca ratio in the 1.0 - 1.2 range. In a study by Rajan et al. [25] Mg levels were even lower than Ca in 9 of

the 12 human brain regions studied, resulting in Mg/Ca ratios between 0.94 and 0.57. In a recent study by Tohno et al. [33] a much higher (ca. 18-fold) Ca level was found in basal ganglia (mean value: 4025 μg/g). Subjects in this study (n=45) were older (mean age: 83.3±7.5 years old *versus* 70.5± 12.2 years old in our study (n=42)), nevertheless the difference has to be considered significant. On the contrary, Mg level (429 μg/g) was quite similar.

Table 3. Mg levels (mean ± sd, μg/g dry weight) in 14 different regions of human brain (see footnote) of non-diseased individuals (n = 42; 71 ± 12 years old) according to age group.

Brain regions*	All individuals	**Age groups**				
		[50-59] (*n*=10)	[60-69] (*n*=10)	[70-79] (*n*=10)	[80-89] (*n*=9)	≥90 (*n*=3)
1	579±74	577±96	598±36	589±54	558±52	563±191
2A	576±70	591±42	573±41	575±66	584±107	526±118
2B	609±83	613±39	602±29	596±64	593±39	700±297
3A	567±71	583±73	569±62	579±61	552±76	512±121
3B	577±71	590±79	569±53	591±56	568±91	544±97
3C	476±54	450±43	490±52	480±47	469±69	524±39
4	535±87	522±123	508±115	563±47	528±31	586±67
5	588±87	645±43	551±99	590±65	567±51	662±27
6	557±57	570±43	527±44	582±70	538±58	578±48
7	545±70	558±85	562±75	535±65	521±59	548±56
8	448±45	440±72	446±35	452±23	450±50	465±20
9	434±45	451±67	427±40	436±31	415±36	446±30
10	437±78	440±44	423±35	507±97	385±77	396±39
11	421±67	437±22	446±42	425±64	420±33	439±43
Mean Ca level in the 14 regions	527±34	533±32	521±29	537±31	513±40	535±27

*1-Frontal cortex; 2A-Superior temporal gyrus; 2B-Middle temporal gyrus; 3A-Caudate nucleus, 3B-Putamen; 3C-Globus pallidus; 4-Cingulated gyrus; 5-Hippocampus; 6-Inferior parietal lobule; 7-Visual cortex of the occipital lobe; 8-Midbrain, 9-Pons 10-Medulla; 11-Cerebellum.

The results also showed that the Ca distribution is quite heterogeneous (Table 2). Regardless of age group, the highest levels were found in the frontal cortex (163-518 μg/g) and occipital cortex (109-762 μg/g) and the lowest in medulla (26-295 μg/g) and cerebellum (71-258 μg/g). On the contrary, results showed that brain Mg has a highly homogeneous distribution in the "non-diseased" adult human brain (Table 3).

It has been shown that major metals and some trace element are heterogeneously distributed in the brain [24,27,31] and this pattern is probably related to their specific physiological functions on specific brain areas [34]. Concordant with our results, Duflou et al. [31] also found cerebellum as the brain region with lower Ca

content (90-310 μg/g) and the superior frontal gyrus (an area from frontal cortex) as the Ca richer region (140-900 μg/g).

Regarding Mg, Andrási et al. [35] reported a non-homogeneous distribution of Mg in normal human brain of 20 individuals (mean age 70 years), but our data showed a highly constant Mg level in the 14 regions studied. Rajan et al. [25] also reported relatively constant Mg levels (483-872 μg/g dry weight)[1], as well as for Ca (592-863 μg/g dry weight), in different brain areas.

3.2. Age-related changes on Ca and Mg levels

Ageing is associated with a general decline in physiological functions. Amongst the different aspects of body deterioration, cognitive impairments, and particularly defects in learning and memory, represent one of the most frequent features in the elderly [14]. Neuronal properties and synaptic plasticity closely depend on ion exchanges between intra- and extracellular compartments. Changes in ion regulation during ageing may therefore participate in altering functional properties of neuronal networks [14].

Considering the mean value of the results obtained for each of the 14 regions, a tendency for an age-related increase in brain levels was found for Ca but not for Mg (Fig. 2a and 3, respectively).

This tendency for a direct correlation between Ca brain levels and age was observed in all the 14 regions studied, the most significant being found for the occipital cortex ($r=0.453$; $p=0.003$), globus pallidus ($r=0.349$; $p=0.014$) and middle temporal gyrus ($r=0.616$; $p< 0.0001$) (Fig. 2b-d).

Hebbrecht et al. [24] also found a mild increase on Ca levels in the brainstem (medulla, pons and midbrain) and cerebral white matter (n=18 subjects; 7 to 79 years old). In the basal ganglia, the authors found that Ca levels remained fairly constant until the fifth decade, but thereafter a strong but variable increase was observed ($p<0.01$). These authors also reported a significant ($p<0.05$) Ca increase in cerebellum, but this increase was not so noticeable in our study ($p=0.089$). Another study, by Tohno et al. [33], regarding minerals in the basal

[1] The authors reported their values as wet weight. We converted them into dry weight by using a dry/wet ratio of 0.211, as suggested by the authors.

ganglia, reported a significant (p=0.026) age-related increase of Ca levels in the putamen but not in the caudate nucleus or globus pallidus of non-diseased elderly individuals (n= 45; 70 to 101 years old).

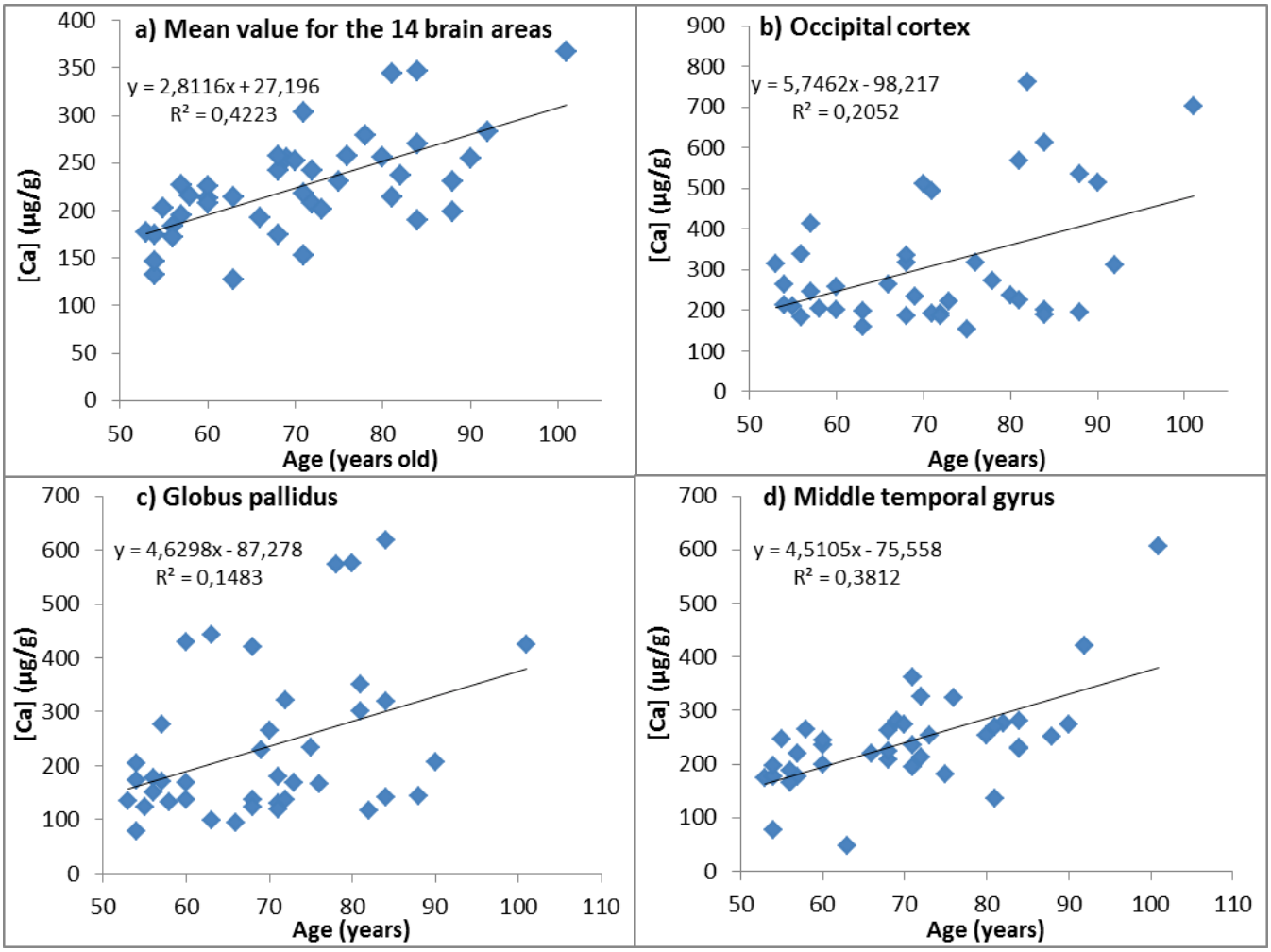

Fig. 2. Relationship between Ca levels [µg/g; mean value for the 14 brain areas of each individual (n = 42)] and age (years) (a) and Ca levels in the occipital cortex (b), globus pallidus (c) and middle temporal gyrus (d).

Calcium ions fluxes across the plasma membrane and between intracellular compartments play critical roles in fundamental functions of neurons, including the regulation of neurite outgrowth and synaptogenesis, synaptic transmission, neuronal plasticity underlying learning and memory, and cell survival [7]. Products of energy

metabolism accumulating with age together with oxidative stress gradually impair Ca homeostasis, resulting in synaptic dysfunction, impaired plasticity and neuronal degeneration [36,37].

It has been hypothesized [24] that increased Ca levels in advanced age may be related to Ca-ATPase malfunction, as a consequence of altered membrane fluidity. Increased levels of free Ca presumably activate DNA fragmentation, characteristic of apoptosis.

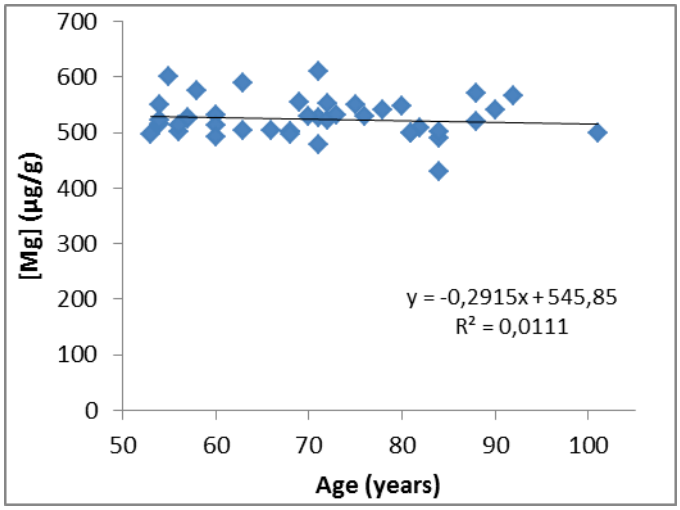

Fig. 3. Relationship between Mg levels [µg/g; mean value for the 14 brain areas of each in- dividual (n = 42)] and age (years).

In our study, Mg showed quite unchanged levels (or a very slight decrease; see Fig. 3) with ageing in all studied regions. Available data are conflicting and either a positive [33,38] or negative [13] correlation between Mg brain levels and age have been reported.

Magnesium dysregulation in brain ageing has received less attention than Ca, though ageing constitutes a risk factor for Mg deficit [14]. Magnesium reduces transmitter release at presynaptic fiber terminals and controls the activation of the N-methyl-D-aspartate receptor, which is critical for the expression of long-term changes in synaptic transmission. In addition, Mg is a cofactor of many enzymes localized either in neurons and glial cells that control neuronal properties and synaptic plasticity [14]. It is therefore likely that a change in Mg brain levels would significantly impair synaptic functions in the aged hippocampus. The experimental studies regarding this question remain too scarce but the data indicate that Mg is involved in age-related deficits in transmitter release,

neuronal excitability and in some forms of synaptic plasticity such as long-term depression of synaptic transmission and suggest that Mg deficit is a relevant factor for ageing-associated susceptibility to hippocampus decline [14].

3.3. Gender-related differences on Ca and Mg levels and smoking habits

A few studies exists regarding gender-related differences on some trace element levels, namely Fe, in human brain [27,39,40] but studies concerning Ca and Mg are rather scarce. Some authors [41] consider two different type of gender-related differences in the elements levels of brain regions. The first one corresponds to a difference that was already present in the adulthood and persists up to the old age. In the second type, although the gender difference in the element levels was absent in the adulthood, it appears in the old age. In particular, the age-related changes on the element levels after adulthood may be different between men and women.

In our study, the correlation between Mg (virtually constant) or Ca levels (a tendency for a steady increase) and age were similar for both men (slope: 2.3; r=0.546; p=0.003) and women (slope: 3.1; r=0.648; p=0.009) (Fig. 4).

The mean value for Ca in the 14 regions were found significantly higher in women than men (248±59 μg/g *versus* 213±46 μg/g; p=0.0463) (Fig. 4a). This difference was particularly significant in the middle temporal (p=0.013), occipital cortex (p<0.0001) and cerebellum (p=0.039).

No gender-related differences were observed for either total Mg levels (527±41 μg/g for women and 527±29 μg/g for men; p=0.970) or Mg levels at any individual brain region studied. Also, no correlation was found between Mg levels and age in both genders (Fig. 4b).

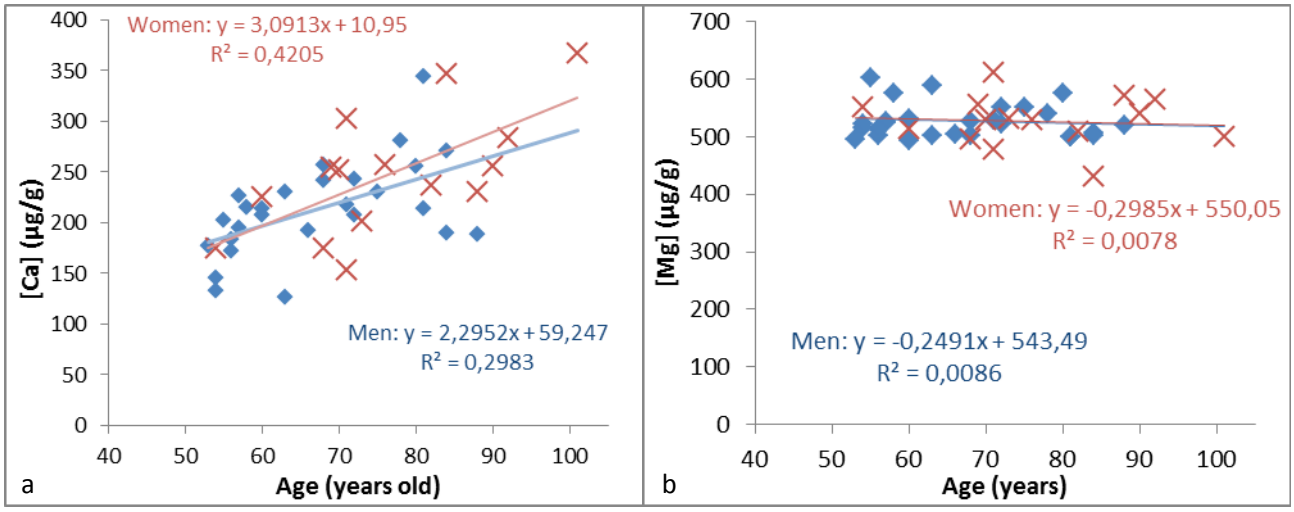

Fig. 4. Relationship between the mean value for Ca (a) and Mg (b) (µg/g) in the 14 brain areas studied and age (years) in men (◆) and women (X).

In a recent study, Tohno et al. [33] found a significantly higher Ca content in the caudate nucleus of women (1.12-fold; p=0.028) but no differences in Mg levels. In our study, higher Ca levels were found in several brain regions, namely in the putamen (p=0.0043), occipital cortex (p<0.0001) and cerebellum (p=0.0217) of women. The cause and physiological significance of these gender-related differences in brain Ca levels remains to be elucidated.

No significant differences were found in Mg levels between smokers and non-smokers (529±30 µg/g vs. 526±42, p=0.8134). Regarding Ca, reduced levels were found in smokers (213±45 µg/g vs. 240±53, p=0.0456), which could be explained by a decreased intestinal Ca absorption in individuals with smoking habits [42,43].

3.4. Neurodegenerative patients

Changes on major and trace element levels have been correlated with neurological degenerative disorders such as Alzheimer's disease (AD) and Parkinson's disease (PD) [3,44,45]. In ND, cellular Ca^{2+}-regulating systems are usually disturbed. Oxidative stress, perturbed energy metabolism, and alterations of disease-related proteins result in Ca^{2+}-dependent synaptic dysfunction, impaired plasticity, and neuronal death [7]. A role for Mg in both physiologic and pathological states of CNS has also become increasingly evident, supporting the

view that the intracellular free Mg^{2+} concentration is critical in normal cell function and ion concentration can change in response to various stimuli [9].

During the sample collection period (first semester of 2012), brain samples from 2 AD and 1 PD patients were also obtained. Although no definite conclusions can be drawn, the results obtained (as the ratio between Ca and Mg levels in the ND patients and matched control subjects) are shown in Fig. 5.

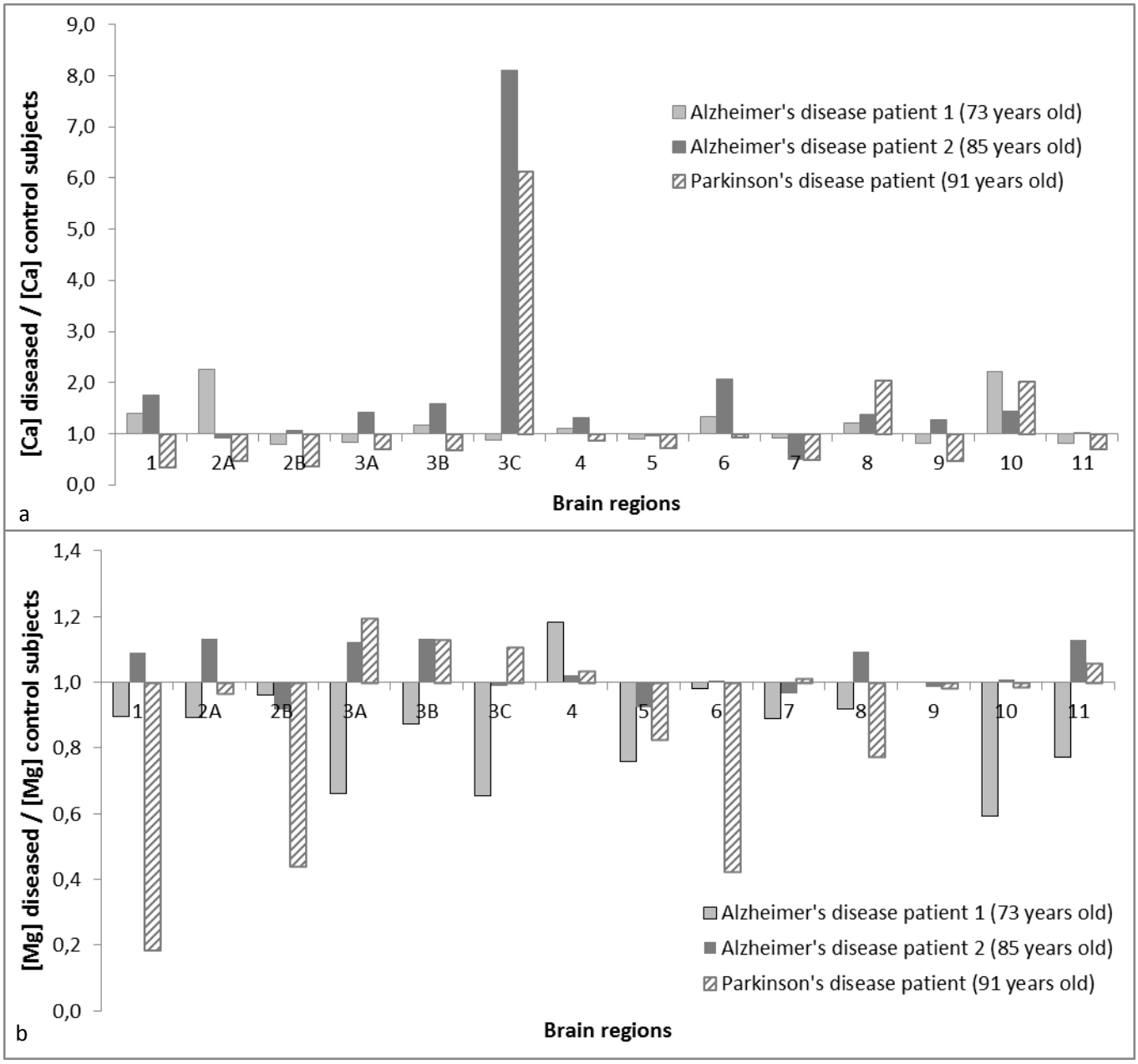

Fig. 5. Ratio between Ca (a) and Mg (b) levels in ND patients and matched control subjects.

Because of the above mentioned gender-related differences in Ca levels, only values for women in the "normal" (non-diseased) group were considered for comparison.

Compared with control group, increased Ca levels were found in frontal cortex, superior temporal and inferior parietal in both AD patients. Although limited and not consistent in the two individuals, our results showed increased Ca levels in some brain regions of AD patients (Fig. 5a).

Previously published studies have already described specific anatomical region changes on Ca levels. Leskovjan et al. [46] found increased levels of Ca in the amyloid plaques (one of the neuropathological hallmarks of the disease) of AD patients . Ward and Mason [32] also found increased Ca levels (1.6-fold) in hippocampus of AD patients (n=58). The exact meaning and implications of these imbalances remains unclear.

Calcium dyshomeostasis has also been recently proposed as the primary age-related condition driving neurodegeneration in the sporadic form of PD supported by the demonstration that dopaminergic neurons expressing higher levels of protein buffers calbindin D28k, calretinin and parvalbumin seem to be resistant to degeneration in this disease [37]. Alfa-synuclein aggregates, neuropathological hallmarks of PD, potentiate neuronal Ca^{2+} dyshomeostasis and overload, which can be linked to glutamate excitotoxicity [36]. Compared to normal subjects of the same age sub-group, Ca was found decreased in all the studied regions of the PD patient studied, except in the globus pallidus, midbrain and medulla, where increased levels were found.

In AD patient #1, it was observed a Mg decrease in the caudate nucleus, globus pallidus, hippocampus, medulla and cerebellum, while in patient #2 no differences were found. Since Mg levels were found decreased in several tissues of AD patients in clinical, experimental and autopsy studies [47], Mg depletion, particularly in the hippocampus, has been considered as an important pathogenic factor in AD [9].

Compared with the respective control group, no differences were found in Mg levels in the PD patient, excluding the frontal cortex, middle temporal, hippocampus, inferior parietal and midbrain, where decreased levels were found.

4. Conclusions

This study, involving the direct determination of Ca and Mg in 14 different brain regions of 42 non-diseased individuals (70.5± 12.2 years old) provides update data on the actual levels, regional distribution and age-related changes of these two important biometals in the human brain, contributing for defining the "normal" levels, a fundamental step in order to allow future comparisons with data obtained from individuals affected by ND.

Globally (i.e., considering the mean value for the 14 regions of the 42 individuals), Mg levels were found ca. 2.3-fold higher than Ca (527±34 μg/g *versus* 226±53 μg/g).

Calcium showed significant heterogeneous distribution, with the highest levels at the frontal (163-518 μg/g) and occipital cortexes (109-762 μg/g) and the lowest at the medulla (26-295 μg/g) and cerebellum (71-258 μg/g). On the contrary, Mg showed highly stable levels in all the studied regions.

A significant age-related tendency for increased Ca levels was found, supporting that it may be involved in age-related neurodegenerative processes. The globus pallidus, occipital cortex and middle temporal gyrus were the brain regions where this direct correlation reached higher significance. On the contrary, Mg showed remarkably stable levels along the age range studied.

Also considering the whole brain mean values, slightly higher Ca levels were found in the female subgroup. Once again, such difference was absent for Mg.

Together, these findings show a different behaviour for Ca and Mg in the normal (non-diseased) human brain, with Mg levels being much more stable, regardless of gender, age and brain region.

Differences in Mg and Ca levels in some brain regions of one PD and two AD patients were found, suggesting that dyshomeostasis of these metals may play a role in ND, but further investigation in necessary.

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References

[1] National Institute on Aging (US National Institutes of Health) and WHO. Global Health and Aging Report. NIH Publication no 11-7737. 2011. [Available from: http://www.nia.nih.gov].
[2] R.J. Castellani, R.K. Rolston, M.A. Smith. Alzheimer disease. Dis Mon. 2010;56(9):484-546.
[3] K. Jomova, D. Vondrakova, M. Lawson, M. Valko. Metals, oxidative stress and neurodegenerative disorders. Mol Cell Biochem. 2010;345(1-2):91-104.
[4] S. Rivera-Mancia, I. Perez-Neri, C. Rios, L. Tristan-Lopez, L. Rivera-Espinosa, S. Montes. The transition metals copper and iron in neurodegenerative diseases. Chem Biol Interact. 2010;186(2):184-99.
[5] E.M. Kawamoto, C. Vivar, S. Camandola. Physiology and pathology of calcium signaling in the brain. Front Pharmacol. 2012;3:61.
[6] J.W. Barclay, A. Morgan, R.D. Burgoyne. Calcium-dependent regulation of exocytosis. Cell Calcium. 2005;38(3–4):343-53.
[7] G. Zundorf, G. Reiser. Calcium dysregulation and homeostasis of neural calcium in the molecular mechanisms of neurodegenerative diseases provide multiple targets for neuroprotection. Antioxid Redox Signal. 2011;14(7):1275-88.
[8] C. Feillet-Coudray, Y. Rayssiguier. Magnesium. In: Caballero B, editor. Encyclopedia of Human Nutrition (Second Edition). Oxford: Elsevier; 2005. p. 191-5.
[9] Y. Nishizawa, H. Morii, J. Durlachs. New Perspectives in Magnesium Research. London: Springer-Verlag London Limited; 2007.
[10] G. Biessels, W.H. Gispen. The calcium hypothesis of brain aging and neurodegenerative disorders: significance in diabetic neuropathy. Life Sci. 1996;59(5-6):379-87.
[11] F. Celsi, P. Pizzo, M. Brini, S. Leo, C. Fotino, P. Pinton, R. Rizzuto. Mitochondria, calcium and cell death: a deadly triad in neurodegeneration. Biochim Biophys Acta. 2009;1787(5):335-44.
[12] A. Palotas, B. Penke, L. Kemeny, Z. Janka, J. Kalman. A chapter in the unity of variety-calcium is the sole author? Brain Res. 2004;1000(1-2):57-9.
[13] J. Durlach. Magnesium depletion and pathogenesis of Alzheimer's disease. Magnes Res. 1990;3(3):217-8.
[14] J.M. Billard. Ageing, hippocampal synaptic activity and magnesium. Magnes Res. 2006;19(3):199-215.
[15] M.C. Paul, C.H. Parsons, M.B. Calford, E.I. von Nagy-Felsobuki. Multi-elemental analysis of brain tissue from healthy Wistar rats using sector field inductively coupled plasma mass spectrometry. Spectrochim Acta B. 2004;59(9):1485-90.
[16] T. Saito, T. Itoh, M. Fujimura, K. Saito. Age-dependent and region-specific differences in the distribution of trace elements in 7 brain regions of Long-Evans Cinnamon (LEC) rats with hereditary abnormal copper metabolism. Brain Res. 1995;695(2):240-4.
[17] I. Hozumi, T. Hasegawa, A. Honda, K. Ozawa, Y. Hayashi, K. Hashimoto, M. Yamada, A. Koumura, T. Sakurai, A. Kimura, Y. Tanaka, M. Satoh, T. Inuzuka. Patterns of levels of biological metals in CSF differ among neurodegenerative diseases. J Neurol Sci. 2011;303(1-2):95-9.
[18] A. Alimonti, B. Bocca, A. Pino, F. Ruggieri, G. Forte, G. Sancesario. Elemental profile of cerebrospinal fluid in patients with Parkinson's disease. J Trace Elem Med Biol. 2007;21(4):234-41.
[19] G. Forte, B. Bocca, O. Senofonte, F. Petrucci, L. Brusa, P. Stanzione, S. Zannino, N. Violante, A. Alimonti, G. Sancesario. Trace and major elements in whole blood, serum, cerebrospinal fluid and urine of patients with Parkinson's disease. J Neural Transm. 2004;111(8):1031-40.
[20] B. Michalke, V. Nischwitz. Review on metal speciation analysis in cerebrospinal fluid-current methods and results: a review. Anal Chim Acta. 2010;682(1-2):23-36.
[21] K. Gellein, J.H. Skogholt, J. Aaseth, G.B. Thoresen, S. Lierhagen, E. Steinnes, T. Syversen, T.P. Flaten. Trace elements in cerebrospinal fluid and blood from patients with a rare progressive central and peripheral demyelinating disease. J Neurol Sci. 2008;266(1-2):70-8.
[22] E. Andrási, L. Orosz, L. Bezur, L. Ernyei, Z. Molnar. Normal human brain analysis. Microchem J. 1995;51(1–2):99-105.
[23] M.A. Deibel, W.D. Ehmann, W.R. Markesbery. Copper, iron, and zinc imbalances in severely degenerated brain regions in Alzheimer's disease: possible relation to oxidative stress. J Neurol Sci. 1996;143(1-2):137-42.
[24] G. Hebbrecht, W. Maenhaut, J.D. Reuck. Brain trace elements and aging. Nucl Instrum Meth B. 1999;150(1–4):208-13.

[25] M.T. Rajan, K.S. Jagannatha Rao, B.M. Mamatha, R.V. Rao, P. Shanmugavelu, R.B. Menon, M.V. Pavithran. Quantification of trace elements in normal human brain by inductively coupled plasma atomic emission spectrometry. J Neurol Sci. 1997;146(2):153-66.
[26] E. Andrási, S. Igaz, N. Szoboszlai, É. Farkas, Z. Ajtony. Several methods to determine heavy metals in the human brain. Spectrochim Acta B. 1999;54(5):819-25.
[27] P. Ramos, A. Santos, N.R. Pinto, R. Mendes, T. Magalhães, A. Almeida. Iron levels in the human brain: A post-mortem study of anatomical region differences and age-related changes. J Trace Elem Med Biol. 2013;http://dx.doi.org/10.1016/j.jtemb.2013.08.001 (in press).
[28] B. Bilgic, A. Pfefferbaum, T. Rohlfing, E.V. Sullivan, E. Adalsteinsson. MRI estimates of brain iron concentration in normal aging using quantitative susceptibility mapping. Neuroimage. 2012;59(3):2625-35.
[29] J.M. Gorell, R.J. Ordidge, G.G. Brown, J.C. Deniau, N.M. Buderer, J.A. Helpern. Increased iron-related MRI contrast in the substantia nigra in Parkinson's disease. Neurology. 1995;45(6):1138-43.
[30] S.M.L. Paine, J.S. Lowe. Approach to the post-mortem investigation of neurodegenerative diseases: from diagnosis to research. Diagn Histopathol. 2011;17(5):211-6.
[31] H. Duflou, W. Maenhaut, J. De Reuck. Regional distribution of potassium, calcium, and six trace elements in normal human brain. Neurochem Res. 1989;14(11):1099-112.
[32] N.I. Ward, J.A. Mason. Neutron activation analysis techniques for identifying elemental status in Alzheimer's disease. J Radioanal Nucl Chem. 1986;113(2):11.
[33] Y. Tohno, S. Tohno, C. Azuma, T. Minami, L. Ke, N. Ongkana, A. Sinthubua, P. Mahakkanukrauh. Mineral composition of and the relationships between them of human basal ganglia in very old age. Biol Trace Elem Res. 2013;151(1):18-29.
[34] W. Maenhaut, G. Hebbrecht, J. De Reuck. Examination of the regional distribution of minor and trace elements in normal human brain by PIXE and chemometric techniques. Nucl Instrum Meth B. 1993;75(1–4):180-7.
[35] E. Andrási, S. Igaz, Z. Molnar, S. Mako. Disturbances of magnesium concentrations in various brain areas in Alzheimer's disease. Magnes Res. 2000;13(3):189-96.
[36] M.P. Mattson. Calcium and neurodegeneration. Aging Cell. 2007;6(3):337-50.
[37] U. Wojda, E. Salinska, J. Kuznicki. Calcium ions in neuronal degeneration. IUBMB Life. 2008;60(9):575-90.
[38] J.D. Stedman, N.M. Spyrou. Elemental analysis of the frontal lobe of "normal" brain tissue and that affected by Alzheimer's disease. J Radioanal Nucl Chem. 1996;217(2):4.
[39] G. Bartzokis, T.A. Tishler, P.H. Lu, P. Villablanca, L.L. Altshuler, M. Carter, D. Huang, N. Edwards, J. Mintz. Brain ferritin iron may influence age- and gender-related risks of neurodegeneration. Neurobiol Aging. 2007;28(3):414-23.
[40] X. Xu, Q. Wang, M. Zhang. Age, gender, and hemispheric differences in iron deposition in the human brain: an in vivo MRI study. Neuroimage. 2008;40(1):35-42.
[41] S. Tohno, N. Ongkana, L. Ke, P. Mahakkanukrauh, T. Minami, P. Suwannahoy, A. Sinthubua, Y. Tohno. Gender differences in elements of human anterior commissure and olfactory bulb and tract. Biol Trace Elem Res. 2010;137(1):40-8.
[42] E.A. Krall, B. Dawson-Hughes. Smoking increases bone loss and decreases intestinal calcium absorption. J Bone Miner Res. 1999;14(2):215-20.
[43] A.G. Need, A. Kemp, N. Giles, H.A. Morris, M. Horowitz, B.E. Nordin. Relationships between intestinal calcium absorption, serum vitamin D metabolites and smoking in postmenopausal women. Osteoporos Int. 2002;13(1):83-8.
[44] K.J. Barnham, A.I. Bush. Metals in Alzheimer's and Parkinson's diseases. Curr Opin Chem Biol. 2008;12(2):222-8.
[45] R.R. Crichton, D.T. Dexter, R.J. Ward. Brain iron metabolism and its perturbation in neurological diseases. J Neural Transm. 2011;118(3):301-14.
[46] A.C. Leskovjan, A. Lanzirotti, L.M. Miller. Amyloid plaques in PSAPP mice bind less metal than plaques in human Alzheimer's disease. Neuroimage. 2009;47(4):1215-20.
[47] S. Ozturk, A.E. Cillier. Magnesium supplementation in the treatment of dementia patients. Med Hypotheses. 2006;67(5):1223-5.