COMPARATIVE STUDIES REGARDING HEAVY ELEMENTS CONCENTRATION IN HUMAN CORTICAL BONE

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The heavy metal metabolism in the human hard tissues biostructures can be assessed by individual studies made on targeted groups from the same living area. During lifetime the exposure to the heavy metals due to environment and professional sources leads to their concentration increment at calcified tissues level, their study reflecting thaw the integrated or cumulative exposure. This study is conducted on 16 samples from human hard tissue in order to assess the metallic elemental concentration in bone and to find the influence of the human subject age on the results. Due to the fact that previous studies showed high differences in bones metals concentrations relative to the sex, living area and bone type we used in this study only male human bones, from the same area (Bucharest) and the same bone type (cortical). We supposed that this selection would allow us to assess the average value of the bone metal concentration function of age factor and similar with the natural concentration. Scanning electron microscopy coupled with energy dispersion spectrometry method was chosen to assess the morphology of the studied bones and Ca/P ratio. X-ray fluorescence spectrometric method is proposed for the heavy elements analysis and its accuracy is proved using atomic absorption spectrometry, a well known precise method.

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1. Introduction

Environment pollution can lead to the heavy metal human bone concentration increase by many ways [1]. Human exposure to the pollutants it is basically produced by air, dust, food and water. In the polluted areas the vegetables and cereals cultures and afterward for example milk includes a higher level of heavy metals, which finally will induce such metals type human consumption [2]. In Romania very few reports on the heavy metals exposure level exists [3, 4], but in more advanced countries a high request on this food exposure evaluation is present. An important aspect should be underlined in the case of children living in polluted areas, because the childhood metabolism makes heavy metals to be more easily absorbed than in the case of adults being easily first accumulated in the soft tissues biostructures type and in time finally in bones. During lifetime the exposure to these elements due to environment and professional sources lead to their concentration increment at calcified tissues level, their study reflecting how the integrated or cumulative exposure. Taking into account the data and facts previously presented, in this paper we've made a study regarding Pb, Cd, Hg, Zn, Fe, Ni and Cu presence in bones preleved from recently exposed persons from the same area, which can lead to the estimation of the existing pollution danger in the same living area. The nocive effects of the heavy elements on the human health are well known, but the low level elements concentration effects observation are of growing

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importance subjects lately. Recent researches underlined the possible dangers with important categories of population are exposing by body burned heavy metal mobilization within the body (eg. pregnant women, children) [5]. A lot of chemical elements are essential for the human body functions but many others are toxic. Due to this toxicity the need of controlling their level in the human tissues and organs is of growing importance, the knowledge on the relationship between their observable toxic effects and their concentration in the human body and environment being essential. The monitoring and environment research/exposure is based on direct measurements on human subjects, on samples retrieved from either human tissues or environment. The same method can be used for determination either of a pure element or a component of a molecule [6]. Some authors [7-9] tried to find the natural level of metals in human bones trough antic human bones, although sometimes their level was higher than the level found in contemporary bones. These results can be assessed to the fact that bone mineral composition depends not only by lifetime conditions but to the processes that took place during bearing. Chemical and physical interactions between bones and soil can lead to enrichment or deficiencies in natural components in bone tissues. On the other hand finding the natural metal concentration in bones of the so called , clean areas" inhabitants proves to be impossible due to large scale and long distance pollution. These facts led us to conduct our research on contemporary person's bones [8]. Recent research [10] in the medical technology field was focused on adapting X-ray fluorescence (XRF) to measure the remaining heavy metal concentration in bones. The physiological basis for this evaluation it is based on the fact that the skeleton serves as a major reservoir for the ingested heavy metals, integrating those in the bone matrix during calcification and where they remain until the bone is remodelled or resorbed [11]. Function of the bone type (trabecullar or cortical) their remanence at the bone level is from 2 to 30 years, while in blood is around one month. The heavy metals toxicity farmacokinetics assumes that beginning with the age of two, the blood existing heavy metals start to get fixed in the bone cells and after age of four the remanent quantity from bones it's over passing the one that returns in blood. After this age the measurable quantity from blood indicates a short time exposure while those measured at teeth and bones level indicates long time exposure [12]. Until the adolescence almost 75% from the heavy metals is deposited in bones while at maturity it grows up to 90-95% at persons being professionally exposed [10].

In order to completely define the role and association of the principal heavy elements, next we will shortly present the ones that can accumulate in the hard tissue human biostructures, and have a important effect to the health. Chronically exposure to cadmium, one of the most toxic environment pollutants, along with kidney affecting, can lead to bone system affections. The destructive impact of cadmium on bones is known by several decades, but only recently scientific papers indicates that even a small dose exposure at this metal can present a high risk for the bones leading through osteopeny, osteoporosis and fractures, although the epidemiological data is rare and refers only at critical levels of exposure [13].

Lead accumulates in bones, the human bone system containing more than 90% from the lead deposits from which 70% in cortical bone (the major part of the human skeleton). The lead concentration in trabecular bone increases by the age of 50, after that his level decreasing or levelling. A reference level of lead in femoral head is of approximately $1\mu g/g$ while at highly exposed children $40\mu g/g$ [11, 13]. Mercury is very poisonous; it damages the central nervous system. It is absorbed easily by the body, but cannot be excreted easily, is volatile and it is possible to breath mercury vapours without ever touching the metal. There are no known benefits for life processes in plants and animals and has no known biological use [9, 13]. Zinc has been recognized as an essential trace element for plants, animals and humans for more than 70 years [8]. Though the average adult body only contains between 2-3g of zinc, this element has some very important functions. This extremely important element is used to form connective tissue like ligaments and tendons. Teeth, bones, nails, skin and hair could not grow without zinc. Zinc is widely considered by doctors to be one of the most important elements to a healthy immune system [13].

Nickel is known to be an essential trace element for several species of animals. Experimental research shows that when chickens and rats are fed a diet that lacks nickel, they develop liver problems. Though many scientists suspect that nickel is necessary for good human health, it has not been proven. Excess nickel in the body is associated with a high incidence of heart disease, thyroid disease and cancer. Whatever the case, nickel certainly appears to affect

human health, even though we do not know exactly how [12, 13]. Iron has many functions in the body. Certain chemicals in brain are controlled by the presence or absence of iron. It is also important for maintaining a healthy immune system and for digesting certain things in the food that we eat. In fact, plays a vitally important part of how our body obtains energy from our food [6]. Copper is an element that is very important for a good health, being critically important for dozens of body functions, a major component of the oxygen carrying part of blood cells and, along with vitamin C, is important for keeping blood vessels and skin elastic and flexible. This important element is also required by the brain to form chemicals that keep us awake and alert. Copper also helps the body to produce chemicals that regulate blood pressure, pulse, and healing [12].

2. Materials and methods

Due to the fact that previous studies [14, 15] showed high differences in bones metals concentrations relative to the sex, living area and bone type we used in this study only male human bones, from the same area and the same bone type. Samples were preleved after total hip artroplasty surgical procedures appertaining to persons living in Bucharest area. The medium age of the persons was 43.8 ± 14.5 years (age interval: 29-68). The group consisted from 16 male which was divided in two groups based on age criteria (<35years and >65years). We supposed that this selection would allow us to assess the average value of the bone metal concentration function of age factor and similar with the natural concentration. After the surgical procedure the femoral heads were mechanically divided using surgical instruments in order to divide them in the three types (cortical, spongious and cartilage surface), and than aseptically kept at -20°C until the following analysis. SEM/EDS method was chosen to assess the morphology of the studied bones and Ca/P ratio. XRF method is proposed for the heavy elements analysis and its accuracy is proved using atomic absorption spectrometry (AAS), a well known precise method.

For this study, we have proposed the preparation of human compact bone samples (taken following a coxofemoral prosthetic surgery involving resection of femoral head and part of the upper femur in most cases) by heat treatment in controlled atmosphere furnace at 600°C and further analysis using imagistic (SEM), compositional (EDS) in order to assess the Ca/P ratio, and morphological changes that occur.

XRF method can be used due to their quantitative advantages compared with other analytical methods. The method has advantages related to the rapid and simple sample preparation, the simultaneous determination of elements from Na to U, the analite concentration in large scale from 1ppm to 100% and very low equipment cost. Ideally XRF measurements should give reproducible results well correlated with the heavy metal human body intoxication [16, 17]. The wave length dispersion X-ray fluorescence spectroscopy (WDXRF) seems to be the most adequate method for heavy metal dozation due to X-ray source and good resolution, but newly the energy dispersion polarized X-ray fluorescence spectroscopy (EDPXRF) method obtained comparable performances at a greatly lower price of the equipment. So the EDPXRF can be considered as one of the most useful non-destructive analytical techniques to estimate heavy metal pollution, and to obtain a good relation between the soil pollution, food products and heavy metal concentration in human bones. As a matter of precaution and in order to validate the EDPXRF obtained results, we made supplementary AAS analysis on the same samples previously investigated by X-ray fluorescence.

The EDPXRF instrumentation used for bone elemental analysis was a SPECTRO equipped with a 50W Rh and a Si-drifted detector with a resolution of 148eV (1000cps Mn K_{α}) (from University Politehnica from Bucharest). The XEPOS 3D geometry is designed for exciting sample X-ray fluorescence with polarized radiation aimed to improve analytical sensitivities [18]. This design leads to a better peak to background ratio and therefore better sensitivity. The sample chamber was purged with helium during data acquisition to lower the radiation absorption and scattering. The obtained spectra were evaluated with TURBOQUANT software package matrix effects which will occur are taken into account. The higher analysis exactness of this method is based on three ways of fluorescence excitation e.g. the light elements Na-V are excited using a HOPG target (intense monochromatic polarized X-rays), the elements Cr-Zr and Pr-U are excited

using a Mo secondary target (intense monochromatic non polarized X-rays) and the high-energy

elements Y-Ce are excited using a Barkla Al_2O_3 target (intense polychromatic polarized X-rays). After heat treatment bone samples were previously powdered and pelletized as disks of 40mm diameter and about 6mm height. There was no need for disk sample ligation because bone plasticity provides an adequate rigidity to the disk.

To validate the results we have made AAS tests, due to the method high precision and very good limit of determination. The AAS spectrometric analysis of lead, cooper, nickel, and iron from a solid extract in acid solvents depends on the quantity of the interest element that might be present in the sample and on the necessity to comprise all the elements in the same sample [19]. The method is applicable when the extractable element content is greater that the limit of detection specific quantity after acid solvents extraction. The limit of detection (mg/kg) in the case of our used AAS method are Zn>2,Cd >2, Pb >15, Cu>5, Ni>12 and Fe>2 ppm. After samples grinding the obligatory operation (when a mixture of organic and inorganic substances or just metal content analysis is assessed) is the mineralization. This step was made in a SpeedwaveTM MWS-3 microwave digestion non-contact temperature measurement device, equipped with a temperature sensor and integrated controller. The samples were mineralized by treatment with 10ml of aqua regia (HNO₃:HCl = 1:3) in two steps working program: heating temperature 180°C, 25 minutes followed by heat at 100°C, 10 minutes. The mineralized sample quantity was 0,5g. After mineralization the samples were filtered in order to eliminate any traces of carbon or undiluted silica (silica elimination is made using fluorhidric acid which favours its vaporing as SiF_4) and than introduced in 100ml glass recipients.

Unlike in the XRF method using AAS the Hg analysis was not possible. The used AAS equipment was a GBC 932 AB PLUS type (from University Politehnica from Bucharest), 185-900nm spectral domain. Due to the fact that only wavelength lower than 350nm were analyzed, the background correction was made with a deuterium lamp. The wavelengths of the analyzed elements were: Zn 213,9nm, Cd 228,8nm, Pb 217,0nm, Cu 324,8nm, Fe 248,3nm and Ni 232,0nm. The principal possible interferences in the flame atomic absorption are in our case: Cd – Fe, Fe – Cd, Ni, respectively Ni – Fe. The instrument calibration was made before each determination series by preparing from the element etalon solution five calibration solutions covering the estimated concentration interval.

3. Results and discussion

Due to significant amounts of water and collagen, the heat treatment applied for their removal, in order to conduct the composition tests, may change trabecular bone structure. With the application of heat treatment is a proven fact that the heat treated bones crystalline phase composition is similar to natural bones [20].

The data presented in literature are confirmed by X-ray diffraction analysis, which reveals that the untreated or heated bones in the oven, the heat treatment does not significantly affect inorganic phase, peaks are almost identical in width, being possible to observe only a slight variation related to the relative height of peak probably due to a difference in orientation [21, 22].



Fig. 1. ESEM morphological aspect of untreated bone after explantation (left); bone sample heat treated for subsequent XRF analysis (right).

Macroscopically, there have been observed color changes, delaminating, fracturing and distortion processes. Significant changes have occurred at structural and morphological level. At high magnification (5000x) collagen microfilaments can be distinguished. After heat treatment at 600°C, pores formation can be observed the morphology being different than for untreated bone. Surfaces are not prepondelently smooth, but rough and branched. High temperatures heat treatment implies the appearance of apatite crystals [23]. Degeneration of the organic matrix under the influence of temperature has a great impact on the structure of bone [24, 25].



Fig. 2. EDS spectra obtained on a) untreated bone after explantation; b) bone sample heat treated at 600 °C for subsequent XRF analysis.

EDS spectral results presented in Figure 2, confirm the stoichiometric composition of the standard samples and provide a ratio to the Ca/P of the studied bone sample close to the ideal value, with the presence of other chemical elements characteristic for a normal bone chemical composition (Na, Mg, Si, O, C). In order to assess the heavy elements concentration there were analysed 16 samples of bones preleved from males by EDPXRF using the same procedure

Each sample was analysed three times in repetitive condition to assess the precision of the analytical results. Each analytical data for a bone sample is provided on the base of the three spectra that are normalized before estimating the elemental concentrations. The typical spectra obtained from one of the subjects bone samples, is shown in Figure 3.



Fig. 3. The typical spectra of a sample used for analyte dozation. (blue-Mo spectrum; cyan-HOPG spectrum, green-Al2O3 spectrum)

The Turboqunt program takes into account the fundamental parameters of fluorescence excitation and the coincidence of the characteristics lines in all the above spectra for a higher exactness assessing of elemental concentrations. From these spectra, it is clear that femoral bone sample contains the well known major elements as Ca, K, Si, Cl, S, Na, Mg, but minor ones as Fe, Ni, Cu, Zn, Pb, Cd, Hg, etc. Figure 4 shows EDPXRF spectra obtained on a sample from first group (<35) compared with one obtained from the second group (>65).



Fig. 4. Example of EDPXRF spectra obtained on a sample from first group (<35) left compared with one obtained from the second group (>65) right.

The average elemental concentrations of the samples together with their relative standard deviations (RSD) give a significant picture of the heavy metal burden of the population in the region but more important suggest metabolic differences among younger and older population regarding heavy metal storage in bones. Thus the Hg, Pb but Fe, Ni burden of older people samples is greater than that of younger ones. The RSDs of older people concentrations are frequently greater than the younger correspondent ones. It means that the variability of the metabolism of hard tissues of older people sample is greater than that of younger ones. The data in Table 1 could bring useful information if they are analyzed by a physician. The error is the statistical error with 1 sigma confidence interval, being presented in Table 2.

The EDPXRF average elemental concentration and their relative standard deviation of the two group samples is presented in Table 1. On the first group (<35years) in the case of heavy elements concentration we obtained a mean value of 1.4ppm in the Cd case, 4.4ppm Pb, the presence of Hg being not detected for this group. Regarding the second group (>65) we found a mean value of 2.7ppm for Cd, 0.9ppm Hg and 7.4ppm in the case of Pb. The comparison of the values obtained for these two selected groups for the Cd, Hg and Pb concentration in the analyzed samples is presented in Figure 5.a). Through the same method we analyzed also the P, and Ca concentration, the mean value comparison for the two groups being presented in Figure 5,b).

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Elem.	UM	Average	RSD[%]	Average	RSD[%]				
		<35		>65					
Р	%	9.67	15	11.11	28				
Ca	%	21.36	15	25.42	27				
Fe	ppm	55	42	98	56				
Ni	ppm	2	-	3.4	151				
Cu	ppm	2.4	11	4.2	24				
Zn	%	0.146	49	0.632	267				
Cd	ppm	1.4	28	2.7	23				
Hg	ppm	ND	-	0.9	45				
Pb	ppm	4.4	56	7.4	55				

Table 1. The EDPXRF average elemental concentration and the relative standard deviation of the two group samples

Table 2. EDPXRF absolute error by element.

Z	Symb.	Norm. Int.	Conc.%	Abs. Err. %
15	Р	41591,32	9.678	0,006
20	Ca	11939,68	21.367	0,02
26	Fe	91,23	0.000551	0,000023
28	Ni	7,14	< 0.00001	(0,0)
29	Cu	29,10	0.000243	0,000005
30	Zn	3476,48	0.1465	0,0003
48	Cd	16,79	0.000145	0,000012
80	Hg	0,45	< 0.0 0001	(0,0)
82	Pb	28,70	0.000448	0,000003

The other detected elements comparison between the mean values for each group is presented in Figure 5,c). Cadmium is one of the most popular elements assayed in bones. Its average concentration taken from our results is higher than the ones determined in the bones by other authors [7, 26]. Lead is one of the elements that is very frequently assayed in bones. The lead concentration in the femur head assayed in our investigations varies much for the two groups but its concentrations reported by different authors also range widely. For example, the average lead concentrations presented in other paper varies from 5 to 65ppm [11, 26, 27]. The average concentration of iron in the femur head was similar to that assayed by other authors [11] and several times higher than the one found in country side population [26]. The concentration of copper in the femur head ranged from $0.3\mu g/g$ to $5.5\mu g/g$, reaching 2.4 $\mu g/g$ on average, and was much lower than the values assayed by other authors [26] of $3.6\mu g/g$. The average Ni content in bones of the analyzed population was close to that observed in other investigations [26].

The EDPXRF obtained results show that there is a significant difference between the two selected groups on one hand in the case of heavy metals concentration and also on the other detected elements. In the case of heavy elements we can notice higher values for the second group meaning that the bone elements accumulation in time is bigger than the elements elimination.



Fig. 5. EDPXRF comparative elemental concentration results of the two groups: a) P; Ca; b) Cd, Hg and Pb; c) Fe, Ni, Cu, Zn.

Elem.	UM	Average	RSD[%]	Average	RSD[%]
		<35		>65	
Р	%	ND	-	ND	-
Ca	%	ND	-	ND	-
Fe	ppm	52	49	94	56
Ni	ppm	2.1	-	3.6	155
Cu	ppm	2.8	16	4.5	21
Zn	%	0.0163	88	0.7758	52
Cd	ppm	1.6	113	2.8	24
Hg	ppm	ND	-	ND	-
Pb	ppm	3.9	40	7.5	49

 Table 3. The AAS average elemental concentration and the relative standard deviation of the two group samples.

By AAS method, the analites concentration determination was made by separate aspiration in flame of the obtained solutions for the whiteness sample and analyzed samples through interest elements absorbance measurement. The solutions were three time measured the final result being the mean value. In the case that the analite concentration in the sample overpasses the calibration interval, the analyzed solutions were diluted with witness calibration solution. From the calibration curve, the element concentration corresponding to the analyzed sample and witness sample absorbance was determined. The X element concentration (w) is determined by $W_{(X)} = (\rho_1 - \rho_0) \cdot f \cdot$ V/W equation, were $W_{(X)}$ is the quantity of the X element in the sample (mg/kg), ρ_1 is the element concentration, corresponding to the absorbance from the analyzed sample (mg/l), ρ_0 is the element concentration, corresponding to the absorbance from the witness sample (mg/l), f is the dilution factor, V the sample volume (l), and m is the sample mass (kg).

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The AAS average elemental concentration and their relative standard deviation of the two group samples is presented in Table 3 (ND-not detected). On the first group (<35years) in the case of heavy elements concentration we obtained a mean value of 1.6ppm in the Cd case, 3.9ppm Pb, the presence of Hg couldn't be detected by AAS. Regarding the second group (>65) we found a mean value of 2.8ppm in the Cd case, and 7.5ppm in the case of Pb.



Fig. 6. AAS comparative elemental concentration results of the two groups.

The comparison of the values obtained by AAS for these two selected groups for the detected elements concentration in the analyzed samples is presented in Figure 6.a) and b). The AAS obtained results show the same difference between the two selected groups on all detected elements and the same higher values for the second group compared with the first group.



Fig. 7: EDPXRF vs. AAS obtained values in the case of first group (left), and the second group (right).

Two comparative histograms for the two selected methods in order to assess the EDPXRF accuracy for this type of analyses are presented in the Figure 7 separately for each group. It can be noticed that for every analyzed elements EDPXRF methods obtained values are very close to the AAS method ones, which theoretically is more accurate.

The available literature data on metal concentrations in bones are fragmentary. Therefore, the possibilities of discussing this issue are limited, a brief comparison with the results previously obtained found in literature being presented before.

4. Conclusions

The heavy metal metabolism in the human hard tissues can be assessed by individual studies made on targeted groups from the same living area. The interpretation of the data in order to assess a conclusion related to the influence of the living area of the subject on the metallic level, induced the need of a data base creation that should contain pre-operative data recording such as: age, sex, weight, previously diseases, diagnosis, smoker or not. The choose of the two well defined groups helped us to conclude there is a certain influence of the age of the subject on the quantity of heavy metal in particularly as this represented the goal of this paper but also of the rest of metallic elements detected in the bone structure. The obtained results show that there is a significant difference between the two selected groups on one hand in the case of heavy metals concentration and also on the other detected elements. In the case of heavy elements we can notice higher values for the second group meaning that the bone elements accumulation in time is bigger than the elements elimination.

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