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Preliminary determination of calcium, phosphorus, and the calcium/phosphorus ratio in cortical bone of Chinstrap penguin using synchrotron X-ray fluorescence analysis

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Abstract Synchrotron radiation X-ray fluorescence (SR-XRF) approach was applied to analyzing of Chinstrap penguin (Pygoscelis Antarctica) cortical bone. The method enabled the in situ determination of Ca and P concentrations and the Ca/P ratio in cortical bone. The preliminary results show that (1) there is the bone site-related difference for Ca and P concentrations. The mean values for the investigated parameters (on a dry-weight basis) are: 30.7% (Ca) and 14.9% (P) for the femoral cortical bone; 21.4% (Ca) and 11.5% (P) for wing cortical bone. (2) The variation for the Ca/P ratio in cortical bone is lower than those for Ca and P separately. This is in agreement with the previous report that the specificity of the Ca/P ratio is better than that of Ca and P concentrations and is more reliable for the diagnosis of bone disorders. The authors suggest that further studies be conducted to establish normal values of Ca, P and Ca/P ratio for polar animals and provide a basis for the diagnosis of bone disorders.

Key words synchrontron radiation X-ray fluorescence (SR-XRF); penguin; bone; Ca, P and Ca/P ratio

1 Introduction

The role of elements in animal health and disease has been extensively studied in recent years using all kinds of analysis methods (Saltzman *et al.* 1990). In comparison with the tissues such as hair, urine and blood etc. (White and Sabbioni 1998), there have been few studies about elements with regard to bone (Kuo *et al.* 2000). Bone tissue can store a wide variety of elements, the levels of which are found to correlate with many physiochemical and enzymic reactions (Saltzman *et al.* 1990). These elements are classified into two categories: essential and trace elements. For bone tissue, Ca and P are two important essential elements, which dominate the bone mineral composition.

It is well known that normal bone mineral generally consists of a poorly crystalline ana-

log of the naturally occurring mineral hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] with 39.9% (Ca), 18.5% (P) and 2.16 (Ca/P, weight ratio) (Zaichick and Tzaphlidou 2002). Changes in the concentration of Ca and P are linked to the variation of bone mineral density and will pose significant impact on the bone disorders and mechanical strength. It is reported that the Ca/P ratio may be a reliable index for the diagnosis of the dynamic and static strength of the skeleton (Zaichick and Tzaphlidou 2002, 2003; Tzaphlidou and Zaichick 2002). Theorectically for stoichiometry, the Ca/P ratio in normal bones must be kept constant, but may follow some changes in diseased bone (e.g. osteoporosis). For example, Osteogenesis Imperfecta (OI) is a heterogeneous heritable disorder affecting both mineralized and non-mineralized connective tissues, resulting low Ca/P ratios (Sarathchandra *et al.* 1999). Determination of the Ca/P ratio thus may add to our understanding of the changes in bone disease (Zaichick and Tzaphlidou 2003).

For polar animals, there are very few data on elements in bone. In a previous preliminary study, qualitative measurements of elements such as Ca, P and the other trace elements were performed in cross section of the intact wing bone of Adelie penguin in Antarctica using synchrotron X-ray fluorescence method (SR-XRF) (Xie *et al.* 2003).

In this note, SR-XRF method was used for quantitative analysis of the cortical bone of wing and femoral bone of Chinstrap penguin. The objectives are (1) applying microscopic method (SR-XRF) to in situ study the cortical bone, which mainly regulated the mechanical strength of bone; (2) determining the levels of Ca, P, and Ca/P in the cortical bone of Chinstrap penguin as a basis for further studies on bone health.

2 Materials and Methods

A fresh skeleton of Chinstrap penguin (*Pygoscelis Antarctica*) was collected on the Ardley Island near the Chinese Great Wall Station during the Chinese Antarctic Research Expedition (December 2001 to February 2002). The sample was placed and sealed in clean plastic bags, and kept at 4 °C until laboratory analysis. A femoral bone and a wing bone, namely CP03 and CP05, were randomly selected for the analysis. A tool made of stainless steel and plastic was used to clean samples of soft tissues and blood. Fat and marrow in the samples were removed using chloroform (99.5%) and ethanol (99.5%) mixture (v/v, 1∶1) for 12 hours, then the samples were rinsed with deionized water in an ultrasonic cleaner several times and air-dried before analysis so that the results were given on a dry-weight basis.

SR-XRF measure was carried out at the synchrotron radiation microprobe XRF experimental station of Beijing Synchrotron Radiation Facility (BSRF) in March, 2004. Electron energy is 2.2 GeV, intensity is 40–80 mA, the energy of radiation is 4.0–30 keV, the exciting radiation is white light, and the beam line is 4W1B. Reflector is not used. The X-ray irradiation area touching the sample surface was set by adjustable slits, which were fixed on $20 \times 20\ \mu m^2$. The Si(Li) detector was working under liquid nitrogen and was placed 4 cm away from the samples with energy resolution about 150–350 eV HWFM. The dead time rate of the detector was between 20–25%. A 2048 multichannel analyzer (MCA) was used to record and analyze the XRF spectrums. The sketch map of the experimental equipment was shown in several previous references (Shen *et al.* 2001; Xie *et al.*

2003).

Samples were immobilized on the sample platform about 1 meter away from the adjustable slits. The angles between the incident X-ray beam, the sample plane and the detector were 45° and 90°, respectively. An optical microscope was used to adjust the position of the samples. For the femoral bone, 4 sites along a line from upside to downside were randomly chosen for analysis. For the wing bone, 2 sites, one is near the tip and the other near the root of wing bone, were randomly selected to be measured. The effective time of X-ray irradiation was 100 secon at room temperature. The qualitative experiment data was processed by software AXIL.

The power material of NIST SRM 1486 was used as standard reference material, which was pressed to be a homogeneous circle slice with 1mm-thickness and 10mm-diameter. The relationship between intensity and the known concentrations of Ca and P in standard material was established. Based upon this relationship, the quantitative data for Ca and P in bone samples were obtained from the measured intensity. The minimum detection limits (MDLs) for Ca and P were approximately 248ppm and 345ppm, respectively.

3 Results and Discussion

3.1 The total concentrations of Ca and P and the Ca/P ratios

There is no standard methodology for analysis of elements in bone to date (Robert *et al.* 1996). In most cases, bone samples are pretreated with solvents such as acetone and alcohol in order to remove collagen, fat, marrow and are then ashed and acid digested. Three methods (dry ash, wet digest and microwave digest) of bio-specimen digestion are widely used (Turker and Yusel 1997). After that, ICP-MS or AAS etc method are commonly used to determine the elements. Recent years, there are some reports using neutron activation analysis to measure intact bone such as rid, trabecular bone and cortical bone of human being (Zaichick and Tzaphlidou 2002, 2003; Tzaphlidou and Zaichick 2002). In comparison with chemical methods, synchrotron radiation X-ray fluorescence method required minimum pretreating and caused non-damage of sample (Wu *et al.* 1995; Chen *et al.* 2000; Shen *et al.* 2001; Xie *et al.* 2003). It is a microscopic approach for measuring a small area of sample or few amount of sample with low MDLs and high accuracy. Its merit makes it favorable to in situ measure the cortical bone sample without pretreating.

The values vary between 3.68% and 45.39% for Ca (mean value, 27.58%) and 1.79% – 24.01% for P (mean value, 13.79%). The highest values of Ca and P are approximately 13 times of the lowest values. The Ca/P ratios range from 1.73 to 2.31 (mean value, 2.02). In spite of the wide variations in the Ca and P concentration values, the mean values lie close to some of the published data such as the values in the intact human femoral cortical bone (23.0% for Ca, 10.7% for P, table 2) and are higher than there in the trabecular bone from human femoral neck (Zaichick and Tzaphlidou 2002). The Ca/P ratio is relatively lower in comparison with that of human cortical bone (2.17) and comparable to that of the trabecular bone (2.07).

Table 1 presents data for the Ca and P concentrations and Ca/P ratio in the cortical bone from femoral bone and wing bone of Chinstrap penguin

Bone Sample		Ca(%)	P(%)	Ca/P
Femoral bone	CP03-1	3.68	1.79	2.05
	CP03-2	45.39	20.11	2.26
	CP03-3	41.53	24.01	1.73
	CP03-4	32.03	13.86	2.31
	Mean	30.7	14.9	2.05
	CV%	61	65	13
Wing bone	CP05-1	22.43	12.41	1.81
	CP05-2	20.39	10.54	1.94
	Mean	21.4	11.5	1.87
	CV%	7	12	5
Total	Mean	27.58	13.79	2.02
	CV%	56	56	12

Table 2 Compared the Ca and P concentrations and Ca/P ratios in the bone of Chinstrap penguin with the previous data

		Ca		P		Ca/P		References
		Mean (%)	CV (%)	Mean (%)	CV (%)	Mean (%)	CV (%)	
Chinstrap Penguin	Femoral Cortical Bone	30.7	61	14.9	65	2.05	13	This study
	Wing Cortical Bone	21.4	7	11.5	12	1.87	5	This study
Human	Trabecular bone from the femoral neck	11.4	25	5.6	27	2.07	12	Zaichick *et al.* (2003)
	Cortical bone from the femoral neck	23.0	17	10.7	22	2.17	14	Zaichick *et al.* (2002)
	Intact rib bone	/	/	/	/	2.33	15	Tzaphlidou *et al.* (2002)

The variations for Ca and P in cortical bone of Chinstrap penguin are both 56% (CV), approximately 5-time higher than the CV for the Ca/P ratio (12%). The high CVs for Ca and P concentrations in the cortical bone indicate that mineral composition, namely hydroxyapatite, heterogeneously distributed in cortical bone. The CV obtained for the Ca/P ratio is obviously lower than that for Ca and P. This finding is in agreement with the previous report about those in the human cortical bone (Zaichick and Tzaphlidou 2002, 2003; Tzaphlidou and Zaichick, 2002), in which the lower CV of Ca/P was ascribed to the strong correlation between Ca and P in bone and the Ca/P ratio was suggested to be a more reliable index for the diagnosis of bone disorders than Ca and P concentrations.

3.2 Bone site-related difference

The bone site-related difference is obviously observed. Both of Ca and P concentrations

peak in the middle part of femoral cortical bone. Ca level is detected only 3.68% in upside surface while it increases to 45.39% in the middle part surface in the femoral cortical bone, indicating a very heterogeneous distribution of Ca and P. This is in agreement with the well-known reason that calcification in the bone varies and the middle part of femoral cortical bone is commonly calcified more significantly than the edge. For wing cortical bone, it shows that Ca level is somewhat lower in the site near the tip than that in the site near the root. The mean values for Ca and P are 30.7% and 14.9% in femoral cortical bone and 21.4% and 11.5% in wing cortical bone, respectively. The Ca and P concentrations in wing cortical bone are approximately 70% of those in the femoral cortical bone, indicating that mineral density seems to be higher in the femur cortical bone than in the wing cortical bone. For Ca/P ratio, it is higher in the femoral cortical bone (mean value, 2.05) than in the wing cortical bone (mean value, 1.87) and lower than the stoichiometric value (2.16). Due to the bone site-related difference for Ca and P concentrations, the authors suggest that more samples should be collected and measured to obtain the normal values of Ca and P levels in the cortical bone.

3.3 The potential causes for the lower values of Ca/P ratio

The Ca/P ratios for the cortical bone of Chinstrap penguin are lower than the stoichiometric value. There may be three possible reasons accounting for this phenomenon. One is a possible loss of Ca and P in the cortical bone during the fat and marrow being removed using organic solvents (Tzaphlidou 2004, personal communication). The organic solvents may incorporate with the Ca and destroy the structure of apatite causing bone erosion. In this case, it will reduce the Ca and P concentrations in the bone and may alter the Ca/P ratios. To evaluate this effect of factor, more experiment should be done.

The second possible reason is the influence of organic matrix. In some samples, the fresh bone may be composed of 90% organic part, most of which is red marrow (Brandt 1977). The P content in red marrow is about 20 times higher than that in the yellow marrow (Woodard and White 1982), thus, the whole cortical bone contains much more P than the inorganic part of the bone and reduces the Ca/P ratio (Zaichick and Tzaphlidou 2003). In this study, although the fat and marrow were removed by chloroform and ethanol, collagen was still remained in the cortical bone, which will contribute to the variations of Ca and P in the cortical bone. Changes in the amounts of Ca and P in collagen do not go hand-in-hand, if Ca/P ratios in the collagen were lower than 2.16, it will naturally reduce the cortical bone Ca/P ratios.

The third possible reason can be ascribed to the bone mineral altered from normal mineral either by lattice substitutions of ions such as CO_3^{2-}, HPO_4^{2-}, Na^+, Sr^{2+}, and Mg^{2+} or by surface adsorption of foreign ions (Sarathchandra *et al.* 1999). Recent studies reveal that the acid phosphate-containing species in bone mineral could lower the Ca/P ratio (Paschalis *et al.* 1996). Especially, it is known that HPO_4^{2-} ion concentrations are the highest in the newly deposited bone and that the concentration decreases as the mineral matures (Rey *et al.* 1991). Commonly, the biological apatite mainly composed by hydroxyapate. If bone diseases or bone disorder occur, Ca and P will exit as other acid phosphate-containing

species such as $CaHPO_4 \cdot 2H_2O$, $Ca_8H_2(PO_4)_6 \cdot 5H_2O$, $Ca_3(PO_4)_2$, etc (Li 2004). For example, there are abnormal mineral compositions in Osteogenesis Imperfecta (OI) bone (Sarathchandra *et al.* 1999). The defective collagen fibrils in OI can hinder the growth of the crystals, resulting in smaller crystals, which would adsorb more HPO_4^{2-} and CO_3^{2-} ions and reduce the ratio (Sarathchandra *et al.* 1999). The status of health of the penguins from which the bone samples collected is not clear. It could not conclude that the low Ca/P ratios reveal bone diseases of Chinstrap penguin.

4 Summary and Outlook

In this study, we firstly applied synchrotron X-ray fluorescence method (SR-XRF) to analysis of the cortical bone of penguins. The preliminary concentrations of Ca, P and Ca/P ratios in cortical bone were obtained. The concentrations of Ca and P varied considerably, while the Ca/P ratios had relative lower CV, revealed that the specificity of Ca/P is better than that of Ca and P concentrations and may be more reliable for the diagnosis of bone disorders for polar animal. The Ca/P ratios in this study were almost lower than the stoichiometric value (2.16), which might be owned to the pretreatment method, organic matrix and substitutions of ions. Since the Ca, P and Ca/P in the bone are generally accepted as an appropriate estimate of bone strength and bone disease, this note represents a first step to establish normal values of Ca, P and Ca/P ratio for polar animals using SR-XRF method.

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