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Calcium Analysis in Bones During Aging Process

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Abstract: The resorption process in bone organ culture can be measured and evaluated by only measuring the calcium concentration in the medium with calcium ion-selective electrode. Reliable and consistent calcium resorption from bone using 500 ng/ml prostaglandine E2 or 250 ng/ml human parathyroid hormone (1-34) have occurred. The results, thus, indicate that calcium can be considered as an independent index of bone resorption. Our preliminary measurements by atomic absorption spectrophotometry (AAS), although statisticaly unrepresentative the sample – group being 9 babies and 9 adults, point to such conclusion. Bones were taken postmortem or post operationem. Calcium concentration measured by AAS was at range of about 260 mg Ca/ mg ash in the human baby bones (costae) and of about 430 mg Ca/mg ash in the human adults bones (femur). Calcium amount measured also in the calvaria of five-day old mice ICR strain by AAS. Values were at range of about 45 mg Ca/mg ash. AAS is a reference method for calcium determination in human bones, however for simplification it is more appropriate to use calcium ion-selective electrode.

INTRODUCTION

Bone is a specialized connective tissue that together with cartilage makes up the skeletal system. These tissues serve three functions: a) mechanical support and site of muscle attachment for locomotion; b) protective: for vital organs and bone marrow, and c) metabolic: reserve of ions for the entire organism, especially calcium and phosphate. It is also a composite structure, consisting of inorganic mineral crystals an extracellular organic matrix, cells, lipids and waters. The mineral crystals are analogous to the geologic mineral hydroxyapatite (Boskey and Coleman, 2010). The cells which produce, nurture and remodel the mineralized extracellular matrix, also respond to mechanical and other signals, which determine the properties (morphology and function) of the bone (Boskey and Coleman, 2010).

Bone remodelling is a complex process which involves a number of cellular functions directed toward the coordinated resorption and formation of new bone (Ronchetti et al., 1996; Goldhaber, 1997; Jin et al., 2000).

Catabolic agents (prostaglandine E_2 (PGE_2) and human parathyroid hormone (h-PTH) fraction 1-34) or anabolic agents (ascorbic acid (AA) and bone morphogenetic proteine 4 (BMP4) could stimulate bone resorption or bone formation directly in a bone organ-culture (Dempster et al., 1993; Goldhaber, 1997; Sampath Kuber, 1999).

The main objective of the current research proposal is to establish calcium as an independent index for observing bone resorption and bone formation processes, which are age dependant.

EXPERIMENTAL

Bone organ-culture system

Calvaria of five-day old mice ICR strain, were dissected aseptically to encompass part of the frontal bone and most of both parietal bones. Dulbecco's Modified Eagle's Medium containing glucose, glutamine, bovine serum albumin, fraction V, penicillin and streptomycin, were added to each bone culture tube. This medium was serum free. Catabolic or anabolic agents were included in the medium. The bone culture tubes were incubated in a roller aparatus for 7 or 14 days at 37^0 C and oxygenated with 50% O_2, 5% CO_2 and 45% N_2. The media were changed every 2-3 days, and after each change of media the used medium from each bone culture tube was analyzed individually for calcium release from the bone into the medium. Bones were

fixed with formalin and processed for histological examination when the experiment was terminated.

Calcium determination

A Varian spektra AA-10 Atomic Absorption Spectrophotometer for calcium determination in human bones was used. The calcium determination was carried out at the 422 nm line; the light source was a hollow cathode lamp. Weighed bone ash samples were hydrolyzed in 6 M HCl. Used media from the bone organ – cultures were analyzed individually for calcium content using Nova Biomedical Calcium Analyzer, Model 7- Ca^{2+} ion selective electrode (Nova Biochemical, Waltham, MA) according to the instructions of the manufacturer (Yoon et al., 2000). The amount of calcium measured from the bone organ-culture medium after PGE_2 or h-PTH fraction 1-34 was included in the medium.

Analysis of hydroxyproline

Bones without fixing with formalin were hydrolyzed and analyzed for hydroxyproline, using HPLC with fluoroscence detection -Pico Tag method -Waters Division of Millipore (Feste, 1992).

Statistical analysis of data

All data were subjected to a one-way analysis of variance (ANOVA) using Fisher's LSD test.

RESULTS AND DISCUSSION

This work is performed to show that calcium analysis is useful for observation and definition of complex biochemical and morphological processes of bone resorption and bone formation. The biological processes of human growth and ageing were reflected in the amounts of calcium in the bones (Boskey and Coleman, 2010)..

Our preliminary measurements by atomic absorption spectrophotometry (AAS), although statistically unrepresentative the sample-group being 9 babies and 9 adults, points to such conclusion. Calcium concentration measured by AAS was in a range of approximately 260 μg Ca / mg ash in the human baby bones and of approximately 430 μg Ca / mg ash in the human adults bones. Amounts of calcium were also measured in the calvaria of five-day old mice ICR strain by AAS. The values were in the range of aapproximately 45 μg Ca / mg calvaria.

Before proceeding with experiments designed to test the agents in our "remodelling system" (Goldhaber, 1997) it was necessary to establish a bone organ culture system that would respond reliably and consistently to bone resorption stimulating agents, such PGE_2 or h-PTH fraction 1-34, so that differences in the amount of resorption could be determined more quantitatively by measuring the amount of calcium released into the medium when the "used" medium was replaced with fresh medium and at the time when the experiment was terminated. Therefore, during the 7-day culture period, therefore, calcium analysis of "used" media was done three times, on day 2 or 3, day 5 and day 7.

From Figure 1. it may be seen that control bones (lacking PGE_2 in the medium) showed little, if any, resorption on gross examination after 7 days in culture. On the other hand, when PGE_2 (500 ng/ml) was added to the culture medium, gross resorption, distortion, and collapse of the calvaria occured (Figure 2.). Similar experiments with different concentrations of PTH revealed that 250 ng/ml of h-PTH gave an adequate bone resorption response.

Figure 1. Control group. Picture of calvaria after 7 days of culture. Note intact calvaria

Figure 2. Experimental group. PGE_2 was added as a bone resorption stimulating agent. Note bone resorption

Addition of 500 ng/ml PGE_2 into the medium resulted in increased calcium release and was statistically significant ($P<0.01$) Figure 3. We observed that calcium release did not occur in the control bones group and group with addition of AA 150μg/ml. Inhibition of increased calcium release occurred when AA was added into the medium with PGE_2.

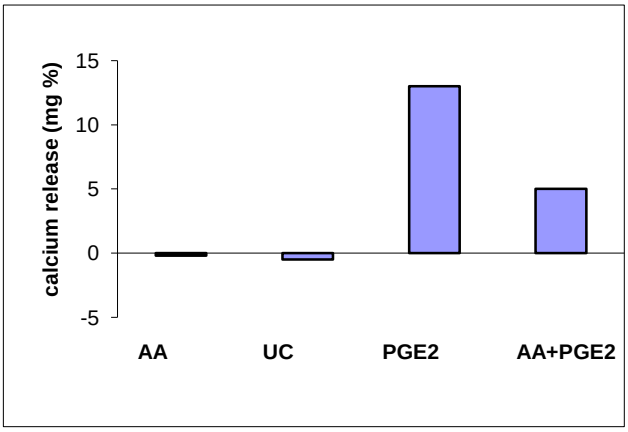

Figure 3. Effect of AA, PGE_2 and combination on calcium release from the bone into the medium, during 7 day culture period. UC -untreated controls; AA -group treated with AA 150μg/ml; PGE_2-group treated with PGE_2 500 ng/ml; AA + PGE_2 – group treated with combination of AA 150μg/ml and PGE_2 500 ng/ml; ** $P<0.01$ (ANOVA)

The addition of AA leads to good new osteoid formation during the culture period.
Biogravimetry of calvaria treated with AA 150µg/ml showed significant increase of calvarial weight (about 50 %) Figure 4.

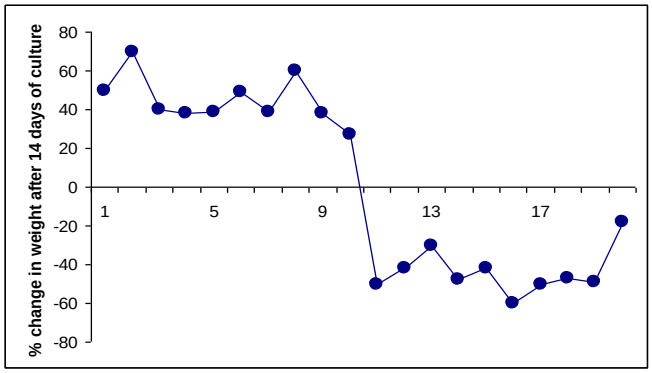

Figure 4. Effect of AA on the weight change of calvaria after 14 day culture period.

150µg/ml AA was added into the medium to stimulate bone formation and biosynthesis of collagen. HPLC analysis of hydroxyproline ("biomarker" for collagen synthesis) approved process of accelerated collagen synthesis and significant increase of hydroxyproline amount compared with untreated control bones (Figure 5.).

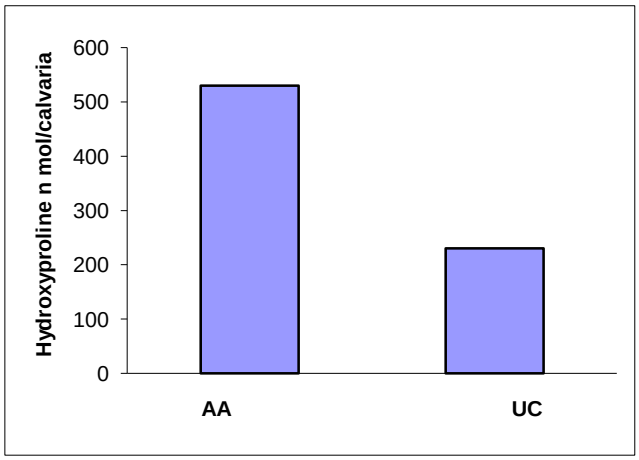

Figure 5. Effect of AA on hydroxyproline amount.
AA – calvaria treated with AA 150µg/ml; UC –untreated controls after 7 day culture period.

The completed experiments confirmed calcium as an independent index of bone resorption and also, as an important parameter in the estimation of bone formation.

CONCLUSION

1. Only with a large number of bone samples would it be possible to draw extrapolation curves reflecting the relationship between the presentation bones calcium amount and human age using AAS.
2. Reliable and consistent calcium resorption from bone using 500 ng /ml PGE_2 or 250 ng /ml h-PTH (1-34) have occured. The results, thus, indicate that calcium can be considered as an independent index of bone resorption.
3. Reliable and consistent bone formation in bone organ culture using ascorbic acid 150 µg /ml or after addition 50 ng /ml of bone morphogenetic protein 4 have been stimulated. Both supstances stimulate osteogenesis. In this case, increased calcium release into medium did not occur.
4. The resorption process in bone organ-culture can be measured and evaluated only by measuring the calcium concentration amount in the medium by calcium ion-selective electrode. Further analysis (for example, histological) is not required.
5. Measuring of calcium release from the bone into the medium using a calcium ion-selective electrode is insufficient for evaluation of bone formation process. Analysis of hydroxyproline by HPLC with fluoroscence detection and histological examination of osteoides are necessary.
6. AAS is a reference method for calcium determination in human bones, however for simplification it is more appropriate to use calcium ion-selective electrode.

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Summary/Sažetak

Resorpcijski proces u organ kulturi kosti može se mjeriti i evaluirati mjerenjem koncentracije kalcija u mediju kalcijevom jon selektivnom elektrodom. Upotreba 500 ng/ml prostaglandina E2 ili 250 ng/ml humanog paratiroidnog hormona (1-34) ostvaruje pouzdan i konzistentan resorpcijski process. Stoga rezultati indiciraju da kalcij služi kao neovisni indicator koštane resorpcije. Naša preliminarna mjerenja atomskom apsorpcionom spektrofotometrijom (AAS), statistički nereprezentativnih uzoraka od 9 beba i 9 odraslih poentiraju takav zaključak. Kosti su uzete postmortem ili post operationem. Koncentracija kalcija mjerena AAS kretala se od 260 mg Ca/ mg pepela u humanim bebi kostima (costae) do 430 mg Ca/mg pepela u humanism kostima odraslih (femur). Količina kalcija mjerena je AAS također i u kalvariji 5 dana starog miša ICR vrste. Vrijednosti su se kretale oko 45 mg Ca/mg pepela. AAS je referentna metoda za mjerenje koncentracije kalcija u humanim kostima, iako je radi pojednostavljenja prikladnija upotreba kalcijeve jon selektivne elektrode.