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by

Submission date: 23-May-2023 09:18AM (UTC+0700)

Submission ID: 2099711360

File name: 8._Increased_bone_calcium.pdf (49.1K)

Word count: 3531

Character count: 18505

1 Increased bone calcium dissociation in lead-exposed rats

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ABSTRACT

BACKGROUND

Lead is still a major environmental and occupational health hazard, since it is extensively used in the production of paints, gasoline and cosmetics. This causes the metal to be ubiquitous in the environment, being found in the air, soil, and water, from which it can enter the human body by inhalation⁴ or ingestion. Absorbed lead is capable of altering the calcium levels in bone. The aim of this study was to demonstrate the effect of lead on bone calcium levels by measuring the reaction constant, Gibbs free energy, and enthalpy.

METHODS

This study was of pure experimental design using 100 male albino rats (*Rattus norvegicus*). The experimental animals were assigned by simple randomization to two groups, one group receiving lead acetate orally at a dosage of 100 mg/kgBW, while the other group did not receive lead acetate. The intervention was given for 4 weeks and the rats were observed weekly for measurement of bone calcium levels by the permanganometric method.

RESULTS

This study found that k_1 (hydroxyapatite dissociation rate constant) was $0.90 \times 10^{-3} \text{ dt}^{-1}$ and that k_2 (hydroxyapatite association rate constant) was $6.16 \times 10^{-3} \text{ dt}^{-1}$ for the control group, whereas for the intervention group $k_1 = 26.20 \times 10^{-3} \text{ dt}^{-1}$ and $k_2 = 16.75 \times 10^{-3} \text{ dt}^{-1}$. Thermodynamically, the overall reaction was endergonic and endothermic ($\Delta G > 0$ and $\Delta H > 0$).

CONCLUSIONS

Lead exposure results in increased dissociation rate of bone in comparison with its association rate. Overall, the reaction was endergonic and endothermic ($\Delta G > 0$ and $\Delta H > 0$).

Key words: Rate constant, hydroxyapatite, Gibbs free energy, enthalpy, rats

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Univ Med 2012;31:151-8

Penguraian kalsium dalam tulang lebih cepat pada tikus terpajan timbal**ABSTRAK****1. LATAR BELAKANG**

Timbal masih tetap merupakan risiko kesehatan asal lingkungan atau akibat kerja, karena digunakan secara luas dalam pembuatan cat, keramik, bensin, dan kosmetik. Dengan demikian logam ini terdapat di mana-mana dalam lingkungan, karena ditemukan di dalam udara, tanah, air, dan tumbuhan. Kemudian logam tersebut dapat memasuki tubuh manusia melalui inhalasi atau melalui makanan dan minuman. Timbal yang diabsorpsi dapat mempengaruhi kadar kalsium pada tulang. Penelitian ini bertujuan untuk menilai pengaruh timbal terhadap kadar kalsium tulang dengan mengukur konstanta laju reaksi, energi Gibbs, dan entalpi.

METODE

Sebuah rancangan eksperimental murni dengan prospektif desain mengikutsertakan 100 ekor tikus putih jantan (*Rattus norvegicus*). Hewan coba secara acak sederhana dibagi dalam 2 kelompok, yaitu kelompok tikus dengan pemberian larutan timbal asetat per oral 100 mg/kg BB dan kelompok tikus tanpa pemberian larutan timbal asetat. Pemberian perlakuan dilakukan selama 4 minggu dan tikus diamati setiap minggu untuk diukur kadar kalsium. Kadar kalsium tulang diukur dengan metode permanganometri.

HASIL

Hasil penelitian diperoleh k_1 (tetapan laju penguraian hidroksiapatit) $0,90 \times 10^{-3} \text{ dr}^{-1}$ dan k_2 (tetapan laju pembentukan hidroksiapatit) $6,16 \times 10^{-3} \text{ dr}^{-1}$ untuk kontrol sedangkan untuk perlakuan $k_1 = 26,20 \times 10^{-3} \text{ dr}^{-1}$ dan $k_2 = 16,75 \times 10^{-3} \text{ dr}^{-1}$. Secara termodinamika, keseluruhan reaksi tergolong reaksi endergonik dan endotermis ($\Delta G > 0$ dan $\Delta H > 0$).

KESIMPULAN

Pajanan timbal menyebabkan penguraian kalsium pada tulang lebih cepat dibandingkan dengan pembentukannya. Secara keseluruhan reaksi tergolong reaksi endergonik dan endotermis ($\Delta G > 0$ dan $\Delta H > 0$).

Kata kunci: Konstanta laju, hidroksiapatit, energi Gibbs, entalpi, tikus

INTRODUCTION

Lead (Pb) is a heavy metal belonging to period IV of the periodic table. This element is grey in color, malleable, soft, and can be extracted from the mineral galena (PbS). Lead and its compounds is widely utilized by man, e.g. in lead-acid batteries and lead solder, for waterproofing (as roofing material and in lead sheath cables), for shielding against radioactive material and X rays. Lead is also used as an additive in gasoline, although the increased production of lead-free gasoline is steadily reducing the use of lead-based additives.^(1,2)

The increasing use of lead makes that this element may become an environmental pollutant and pose a health hazard to man. Lead may enter the body through the digestive and respiratory tracts and the skin. Ingested lead is distributed to bones (60%), liver (25%), kidneys (4%), reticuloendothelial system (3%), intestinal wall (3%), and other tissues.^(3,4)

Accumulation of lead in abovementioned organs may cause organ disorders that are mediated by several mechanisms, such as inactivation of enzymes and other macromolecules through binding of sulfhydryl, phosphate, and carboxyl groups.⁽⁵⁾ In addition,

lead may interact with various cations, particularly calcium, zinc, and iron. The pathological process may be located within the cell membrane and mitochondria, in neurotransmitter synthesis and function, heme synthesis, cellular redox status, and nucleotide metabolism. Lead toxicity causes physiological, biochemical and structural changes in the skeletal, nervous, urinary, hemopoietic and cardiovascular systems. It may also exert carcinogenic and mutagenic effects on the gastrointestinal and reproductive systems.⁽⁶⁻¹¹⁾ The adverse effects of lead have been amply demonstrated in previous studies. The study by Vahedian et al.⁽¹²⁾ demonstrated the occurrence of lead-induced inflammatory changes in the rat proventriculus. A study in Iran found an increased risk of aplastic anemia associated with lead exposure in children.⁽¹³⁾ Furthermore, the study conducted by Todorovic et al.⁽¹⁴⁾ revealed that rats exposed to 100 mg Pb²⁺/kg body weight for 30 days may have a 16% decrease in bone calcium. This is due to the greater affinity of Pb²⁺ for bone as compared with that of Ca²⁺, causing Pb²⁺ to substitute for Ca²⁺ in the bone mineral matrix, thus lowering the Ca²⁺ content of the bone. However, the above study did not cover the kinetic aspects and energy changes accompanying the biochemical or bioenergetic reactions.

There have been numerous previous studies on bioenergetics. The study by Martin et al.⁽¹⁵⁾ has demonstrated the kinetic and thermodynamic aspects of inhibition of serotonin transporter binding. Studies on the effects of lead on bone are also numerous, but no studies have focused on the kinetics and thermodynamics of decreased calcium in bone caused by lead exposure. There is therefore a need to conduct a study on the pathomechanism of the lead-induced decrease in calcium content of bone, by measurement of the reaction rate constant, Gibbs free energy (ΔG) and enthalpy (ΔH).

METHODS

Study design

This study was of pure experimental prospective design, conducted for 4 weeks and observed at weekly intervals from October to December 2011.

Study subjects

The subjects were male albino rats (*Rattus norvegicus*) assigned to an intervention group receiving lead acetate orally at a dosage of 100 mg/kgBW, and a control group receiving no lead acetate. Selection of the animals was by simple random sampling, while the sample size was calculated by means of Federer's formula, yielding a sample size of 50 rats per group.

Experimental animals

This study used male albino rats (*Rattus norvegicus*) aged 2-3 months, weighing 80-200 grams, obtained from Airlangga University, Surabaya. Before intervention, the rats were acclimatized for 7 days, and kept in separate cages according to their assigned group. During the acclimatization period the animals were given exactly the same animal feed pellets and tap water as were to be used during the intervention. Immediately before the intervention, the rats were fasted for 1-2 hours to ensure that their proventriculus was empty. During the 4 weeks of intervention, the rats were given oral lead acetate daily for one week at a dosage of 100 mg/kg, after which 5 of the rats were killed with ether and their bones taken for determination of calcium content by the permanganometric method, as performed in a previous study.⁽⁹⁾ This procedure was repeated in the following 3 weeks.

The collected bones were cleaned and washed, then left to dry in an oven. The dried bones were then pulverized and passed through a 40 mesh sieve. The bone powder was dissolved in HCl (1:4), the resulting solution

was concentrated by evaporation and then placed in a water bath for 1 hour. The dry residue was redissolved in 5 ml concentrated HCl and 50 ml distilled water, then again placed in the water bath for several minutes, and filtered through #52 Whatman filter paper.

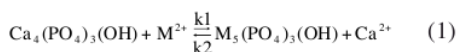
The filtrate was collected in a 200 ml volumetric flask. The precipitate still remaining on the filter paper was washed in distilled water and the wash water was added to the collected filtrate. Distilled water was added to the mixture of filtrate and wash water to yield a volume of 200 ml. This solution was labeled aliquot A.

A volume of aliquot A equivalent to 2 g bone ash was pipetted into a 300 ml beaker and diluted to 200 ml with distilled water. The solution was made slightly alkaline by the addition of NH_4OH (1:4) using methyl orange as indicator. HCl (1:4) was then added to slightly acidify the solution, after which 10 ml 0.5 N HCl and 10 ml 2.5 % oxalic acid solutions were added, and the mixture heated to boil. Under continuous stirring, 15 ml saturated ammonium-oxalate solution was added. The mixture was further heated until a granular precipitate was formed, then the mixture was left to cool. Eight mL of 20% sodium acetate was added and the mixture left for 12 hours. Next the mixture was filtered and washed with hot water until free of chloride [washed with hot water and then with a small amount of HCl (1:4) and finally with hot water until free of chloride]. The residue was then put into a beaker by perforating the tip of the filter paper cone with a glass stirrer, then sprinkled with just sufficient hot water until all precipitate had fallen into the beaker. Next, 10 ml H_2SO_4 (1:4) was added and the dissolved precipitate heated to near-boiling, left to cool, then titrated with 0.1 N KMnO_4 . The calcium content was calculated using the formula:

$$\text{Ca}/100 \text{ mg of sample} = \frac{\text{ml titration} \times 2 \times \text{total volume of dissolved ash}}{\text{volume of dissolved ash} \times \text{weight of sample}} \times 100\%$$

Determination of equilibrium constant and reaction rate constants

Bone contains calcium in the form of hydroxyapatite, which has the formula $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$. Therefore, in the presence of metal ions of equal charge (valence), the reaction may be simplified as follows (equation 1):



where M is a bivalent metal ion (M^{2+}), while k_1 is the hydroxyapatite dissociation constant and k_2 the hydroxyapatite association constant.

The equilibrium constant is calculated using equation 2:

$$K = \frac{[\text{M}_3(\text{PO}_4)_3(\text{OH})]}{[\text{Ca}_4(\text{PO}_4)_3(\text{OH})]} = \frac{k_1}{k_2} \quad (2)$$

The total reaction rate constant is determined with a first-order reversible kinetics model as in equation 3:

$$k = k_1 + k_2 = \frac{1}{t} \ln \frac{[\text{Ca}]_t}{[\text{Ca}]_t - [\text{Ca}]_e} = \frac{1}{t} \ln X \quad (3)$$

where $[\text{Ca}]_t$ is the calcium concentration at time t and $[\text{Ca}]_e$ is the the calcium concentration at equilibrium.

Determination of thermodynamic parameters ΔG and ΔH

Energy changes as well as heat of reaction are expressed as the thermodynamic parameters Gibbs free energy (endergonic energy) (ΔG) and enthalpy (endothermic internal energy) (ΔH). These parameters can be determined using Eyring's equation as follows (equation 4):⁽¹⁶⁾

$$k = \frac{k_B T}{h} e^{-\frac{\Delta G^\ddagger}{RT}} \quad (4)$$

The amount of enthalpy (ΔH) is expressed using the van't Hoff equation (equation 5):

$$\Delta H = -RT \ln K \quad (5)$$

where k =reaction rate constant, k_B =Boltzman constant ($1.381 \times 10^{-23} \text{ J K}^{-1}$), T =absolute temperature (310 K), R =ideal gas constant ($8.314 \text{ J}\cdot\text{K}^{-1}\cdot\text{mol}^{-1}$) and h =Planck's constant ($6.626 \times 10^{-34} \text{ J s}$).

Ethical clearance

The present study was approved by the Research Ethics Commission of the Faculty of Medicine, Lambung Mangkurat University.

Data analysis

The obtained data were converted into a linear graph of $\frac{h}{k_B T} \ln \frac{A}{A_0}$ as a function of time, using Microsoft Exel 2007. The slope of the curve indicates the ΔG value.

RESULTS

The results of bone calcium concentration as a function of time may be seen in Figure 1. Figure 1 shows that at equilibrium the bone calcium content of the albino rats in the control group was 85.25%, whereas that in the intervention group was 39%. The reaction rate constant may be determined by means of a graph as illustrated in Figure 2.

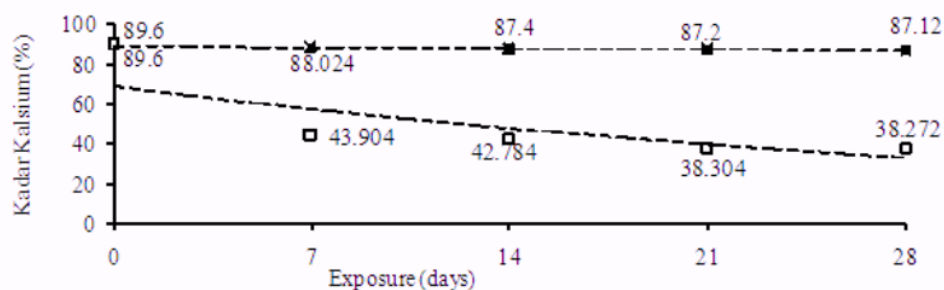


Figure 1. Bone calcium concentration as a function of time (□ = calcium concentrations of intervention group; ■ = calcium concentrations of control group)

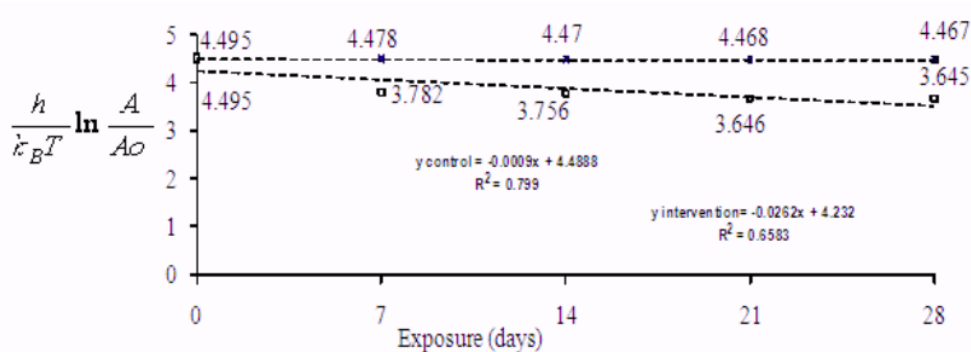


Figure 2. Bone calcium dissociation rate as a function of time (□ = bone calcium in intervention group; * = bone calcium in control group)

Table 1. Kinetic parameters and equilibrium constants

Group	K	k1 (10 ⁻³ dt ⁻¹)	k2 (10 ⁻³ dt ⁻¹)
Control	0.146	0.90	6.16
Intervention	1.564	26.20	16.75

Figure 2 shows that the bone calcium dissociation rate in the intervention group had a steeper slope than that in the control group. Using the expressions in equations (2) and (3), the values of K, k1, and k2 can be calculated, as presented in Table 1.

Table 1 shows that in the control group $k_2 > k_1$, signifying that the rate of association of hydroxyapatite in the control group was greater than its dissociation rate. This is in contrast with the intervention group, where $k_1 > k_2$, signifying that the presence of lead may increase the dissociation rate of hydroxyapatite in comparison with its rate of association. Using the data in Table 1 and equations (4) and (5), the thermodynamic parameters shown in Table 2 may be calculated.

Thermodynamically, the values of ΔG_1 and ΔG_2 in the control group were larger than those in the intervention group (Table 2), indicating that the presence of lead may increase the frequency of spontaneous association and dissociation reactions in hydroxyapatite.

DISCUSSION

The present study shows that the reaction rate constant is a kinetic parameter that can be used to evaluate the reaction rate. The rate of dissociation of calcium in the intervention group was greater than that in the control group. Moreover, the association rate of hydroxyapatite in the control group was greater than its

dissociation rate, while the reverse was true for the intervention group.

Kinetically, these findings may be explained by the fact that lead and calcium have similar properties. However, lead has a larger electron density than calcium, such that it can be precipitated in the metabolically active parts of bone.^(17,18) At present, two compartments may be identified in bone, namely labile trabecular bone and stable cortical bone. Lead accumulation in bone continues throughout life, as shown by the occurrence of intrauterine lead deposition in bone. This lead accumulation increases with each exposure, indicating that there is no threshold for lead absorption in bone. Approximately 95% of the lead content is stored in bone. A high lead content of bone is associated with bone turnover rate or its demineralization rate, such as occurs in hyperthyroidism and immobilization osteoporosis.^(17,18)

The ability to deposit lead in bone is thought to be a defense mechanism of the body, since the deposited lead cannot react with sulfhydryl groups, thus preventing it from inactivating enzymes and other proteins. Nevertheless, the minerals present in the skeletal system are in dynamic equilibrium with minerals from other parts of the body. With each occurrence of bone calcium mobilization, lead is also mobilized. The present study also shows that the free energy changes were lower in the intervention group than in the control group, whereas the enthalpy changes were larger in the intervention group in

Table 2. Thermodynamic parameters of bone calcium dissociation in albino rats

Group	ΔG_1 (kJ/mol)	ΔG_2 (kJ/mol)	ΔH (kJ/mol)
Control	61.510	56.553	0.377
Intervention	52.581	53.974	4.031

comparison with the control group. However, in general the reactions could be categorized as being endergonic and endothermic ($\Delta G > 0$ and $\Delta H > 0$), signifying that the calcium dissociation reaction did not proceed spontaneously or at most very slowly.

The inspontaneity of the calcium dissociation reaction is presumably due to various factors, one of them being diet, especially dietary intake of calcium. Calcium contained in foods is thought to interact with lead in several ways, such as (a) binding of lead in the intestines, causing the lead to become unabsorbable by the small intestine (b) modulating the avidity of intestinal cells for lead, and (c) modulating the affinity of target tissues for lead. These changes in avidity and affinity are regulated by the cholecalciferol endocrine system via 1,25-hydroxycalciferol and calcium-binding proteins.^(19,20)

The results of this study support the report of Todorovic et al.⁽¹⁴⁾ that the decrease in bone Ca^{2+} may be caused by the presence of lead. This is indicated by the greater value of the hydroxyapatite dissociation rate constant (k_2) in comparison with its association constant (k_1), which had not been revealed by previous studies. Therefore, the results of the present study may be used in formulating a model for the development of new drugs that act by decreasing the hydroxyapatite dissociation rate.

CONCLUSIONS

Lead exposure results in a greater rate of dissociation of calcium as compared with its association rate. Overall the reaction can be categorized as being endergonic and endothermic ($\Delta G > 0$ and $\Delta H > 0$). In view of this, further studies are indicated to evaluate the effect of lead on bone calcium in men.

ACKNOWLEDGEMENTS

We thank the Dean of the Faculty of Medicine, Lambung Mangkurat University for

the funding of this study through the DIPA PNBP for the year 2012 of the Faculty of Medicine, Lambung Mangkurat University. ✚

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