Revista Argentina de Clínica Psicológica
2020, Vol. XXIX, N°4, 365-370
DOI: 10.24205/03276716.2020.835

Serum 25-hydroxyvitamin D, Bone Alkaline Phosphatase, Bone Mineral Content and Bone Mineral Density as Important Biochemical Markers for Diagnosis of Nutritional Rickets

Xiaoqian Lv[a, *], Na Liu[a], Zhanru Yin[a]

ABSTRACT

Purpose: To investigate the effectiveness of serum 25-hydroxyvitamin D [25-(OH)D], bone alkaline phosphatase (BAP), bone mineral content (BMC) and bone mineral density (BMD) in diagnosis of nutritional rickets.

Methods: Three-week-old male Sprague Dawley rats (n = 36) weighing 45 - 55 g (mean weight = 50 ± 5 g) were randomly assigned to two groups (18 rats/group): control and rickets groups. Rickets group rats were kept in a dark room and fed vitamin D-deficient feed for 35 days. Serum levels of calcium (Ca^{2+}) and phosphorus (P) and activity of alkaline phosphatase (ALP) were assayed using automated biochemical analyzer. Serum levels of 25-(OH)D and BAP activity were determined using their respective enzyme-linked immunosorbent assay (ELISA) kits. Bone densitometer was used to assay BMC and BMD.

Results: Serum P and 25-(OH) D levels, and bone mineral densities of proximal, middle and distal femur were significantly lower in rickets group than in control group, but the corresponding activities of ALP and BAP were increased significantly in rickets group, relative to the control group ($p < 0.05$). However, there was no significant difference in serum Ca^{2+} level between the two groups ($p > 0.05$). Similarly, the levels of Ca^{2+}, P and Mg^{2+} in the femurs of ricket rats were significantly lower than those of control group ($p < 0.05$).

Conclusion: These results indicate that serum 25-(OH)D, BAP, BMC and BMD are important biochemical markers for diagnosis of nutritional rickets.

Keywords: Bone alkaline phosphatase, Bone mineral content, Bone mineral density, 25-hydroxyvitamin D, Rickets.

INTRODUCTION

Rickets is a disorder of calcium and phosphorus metabolism caused by extreme and prolonged vitamin D deficiency. It is a systemic nutritional disease characterized by skeletal lesions [1]. Rickets, one of the four major diseases of children, is highly prevalent in China. Some symptoms of the disease are delayed/retarded growth, bow legs, as well as weakness and pain in the spine, pelvis and legs [1]. The main clinical feature of the disease is incomplete calcification of the metaphysis and bone tissue. It may be prevented clinically via vitamin D supplementation [1]. Young babies with rickets appear fussy with soft skulls, while older children may have bone pain, bowed legs, and enlarged limbs and joints. Over the last few decades, the incidence of rickets has increased considerably, seriously affecting tissue and organ functions, as well as immunity of children [2]. Early diagnosis and treatment are crucial for effective management of rickets [2].

Serum 25-hydroxyvitamin D [25-(OH) D] amount is known as the strongest predictor of the status of vitamin D, rendering it a powerful ricket biomarker [3]. Many children with calcium deficiency rackets have average serum levels of 25-(OH) D and elevated serum levels of 1, 25-dihydroxyvitamin D [1, 25-(OH)2D]. This child may have raised vitamin D needs as assessed by their

[a]Hengshui People's Hospital, Hengshui, PR China
*Corresponding Author: Xiaoqing Lv,
Address：180 Renmin East Road, Taocheng District, Hengshui City, Hebei Province, China, e-mail: qiaorangpai5533172@163.com

reaction to vitamin D substitution.Bone alkaline phosphatase (BAP), the bone-specific isoform of ALP, is a glycoprotein located on the surface of osteoblasts. It is a crucial enzyme for osteoblast synthesis in bone formation. Studies have shown that the output of BAP is greatly enhanced by rickets [4, 5].

Bone mineral deficits have been implicated in rickets. Bone mineral content (BMC) and bone mineral density (BMD) are two important parameters for assessment of bone mineral deficits. The present study investigated the effectiveness of serum 25-(OH)D, BAP, BMC and BMD as parameters for diagnosis of nutritional rickets.

MATERIALS AND METHODS

Materials

Bone alkaline phosphatase (BAP) ELISA kit was purchased from Wuhan Fine Biotech. Co. Ltd. Calcium, phosphorus and ALP assay kits were obtained from Wuhan Yipu Biotechnology Co. Ltd. Absolute ethanol was a product of Shanghai Hengfei Biotechnology Co. Ltd. Serum 25-(OH)D assay kit was purchased from Shanghai Xinyu Biological Technology Co. Ltd. Automated biochemical analyzer was purchased from Shanghai Yuyan Instruments Co. Ltd. Electric thermostatic water bath was obtained from Beijing Taize Jiaye Technology Development Co. Ltd. Dual-energy X-ray bone densitometer was purchased from Shanghai Hanfei Medical Equipment Co. Ltd. Coupled plasma emission spectrometer was a product of Nanjing Kejie Analytical Instrument Co. Ltd. Magnetic heating agitator was obtained from Sangon Biological Engineering Co. Ltd. (Shanghai).

Experimental rats

Three-week-old male Sprague Dawley rats (n = 36) weighing 45 - 55 g (mean weight = 50 ± 5 g) were obtained from Guangzhou Fuerbo Biotechnology Co. Ltd. The rats were housed in metal cages under standard conditions and allowed free access to standard feed and water. Prior to commencement of the study, the rats were acclimatized to the laboratory environment for 3 days. They were then subject to a 12-h light/12-h dark period and held at an overall temperature of 24 ± 2 ° C and 50 – 60 per cent humidity. The procedure for the research was approved by the Committee on Institutional Animal Treatment and Usage. The methods used have been followed by the standards of the Association for the Evaluation and Accreditation of Laboratory Animal Treatment International (AAALAC). This study has been approved by the Hengshui People's Hospital Animal Ethics Committee according to the Laboratory Animal Treatment Standards, approval number 20198232[6].

Establishment of rat model of nutritional rickets

Rats were randomly allocated to two categories (18 rats / group): control and ricket classes. Rats in the rickets community were housed in a dark environment and given vitamin D-deficient food for 35 days. The diet composed of corn flour (30 per cent), methionine (0.1 per cent), wheat bran (35 per cent), bone meal (1 per cent), yeast powder (5 per cent), bean cake (20 per cent), AD3 (0.1 per cent), multivitamin (0.2 per cent) and calcium (1.18 per cent).

Blood sample collection and serum preparation

At the end of the 35 days treatment period, abdominal aortic blood was collected from each rat after a 4-h fast. The blood was centrifuged at 3,500 rpm for 10 min to obtain serum. The rats were thereafter euthanized and their right tibias and femurs were excised and kept frozen at -80 °C prior to use.

Determination of levels of calcium and phosphorus, and ALP activity

Serum levels of calcium and phosphorus, and activity of ALP were assayed using

automated biochemical analyzer(Nisha et al., Prabhakaran et al., 2019; Ramesh et al., 2019; Muzaffar et al., 2019; Yan et al., 2019).

Enzyme-linked immunosorbent assay (ELISA)

Serum amounts of 25-(OH)D and BAP behaviours have been calculated using their respective industrial ELISA kits. Exactly 50 µL each of the diluent and serum tests is applied to the reaction and the sample wells. The biotin-labelled antibody was then transferred to 50 µL and incubated at 37oC for 1 h. 80 µL of streptomycin-HRP was then applied to each well and incubated at 37oC for 30 min. The wells were washed three times with phosphate-buffered saline-Tween (PBS-T) in order to eliminate unbound molecules. Substrates A and B, each with a volume of 50 µL, were applied to the wells, accompanied by incubation at 37oC in the dark for 10 minutes. The reaction was prevented by inserting 50 µL of terminating solvent, and the absorption of each well was read at 450 nm using a microplate reader.

Determination of BMD

The BMD of the left femur of each rat was measured using a bone densitometer.

Determination of BMC

The left tibia of each rat was digested using the acidic digestion process. Exactly 0.1 g of bone tissue
was inserted in a 50 mL volumetric flask and 10 mL of digestion fluid (a combination of nitric acid and perchloric acid with a 4:1 volume ratio) was applied. The tubes were heated at 210oC for 1 h before the solution became transparent with the cessation of release of brown smoke. The tubes had been allowed to cool down at room temperature. The digested samples were moved quantitatively to 50 mL of volumetric flasks. Transfers were produced using ash-free quantitative philtre paper (Whatman No. 41). The amount of each solution was rendered up to 50 ml of deionized water. Aliquots of the solvent were moved to polyethylene flasks and cooled to 4 ° C. Solutions were further diluted with 1% nitric acid at a volume ratio of 1:5. The Ca2 +, P and Mg2 + amounts were then measured using the coupled plasma emission spectrometer. The operating conditions were: specifications establishing the frequency: 27.12 MHz, output: 1.00 KW, atomizer pressure: 1.0 kPa, plasma gas flow: AR 15 L / min, auxiliary gas flow: AR 1.5 L / min and pump speed: 15 rpm.

Statistical analysis

The data is represented as mean ± SEM. Statistical research has been conducted using SPSS (21.0). Groups have been evaluated using the Student T-Test. Statistical meaning was believed to be p < 0.05.

RESULTS

Levels of Ca^{2+} and P, and serum activity of ALP in rats

The amount of P was significantly lower in the ricket community than in the control group, but the related behaviour of the ALP was significantly higher in the ricket group than in the control group (p < 0.05). However, there was no substantial gap between the two classes in the Ca2 + category (p > 0.05). These findings are summarised in Table 1.

Table 1. **Levels of Ca^{2+} and P, and serum activity of ALP in the rats**

Group	n	Ca^{2+} (mmol/L)	P (mmol/L)	ALP (µmol/L/sec)
Rickets	18	2.37 ± 0.10	1.93 ± 0.17	3.40 ± 0.67
Control	18	2.21 ± 0.09	2.53 ± 0.29	2.24 ± 0.55
t		5.046	7.573	5.678
p		< 0.001	< 0.001	< 0.001

Level of 25-(OH)D and serum BAP activity in the two rat groups

The amount of 25-(OH) D was slightly lower in the ricket community than in the control group, but the corresponding activity of BAP was significantly improved in the ricket group compared to the control group (p < 0.05; Table 2).

Table 2. **Level of 25-(OH)D and serum BAP activity in the two groups of rats**

Group	n	25-(OH)D (ng/mL)	BAP (U/L)
Rickets	18	14.57 ± 5.26	32.45 ± 4.08
Control	18	28.27 ± 5.41	23.06 ± 4.89
t		7.703	6.256
p		< 0.001	< 0.001

Bone mineral density of rat femur

As shown in Table 3, the bone mineral densities of proximal, middle and distal femur were significantly lower in rickets group than in control group ($p < 0.05$).

Table 3. **Bone mineral density of rat femur**

Group	n	Proximal femur (g/cm^2)	Middle femur (g/cm^2)	Distal femur (g/cm^2)
Rickets	18	0.12 ± 0.01	0.09 ± 0.02	0.12 ± 0.01
Control	18	0.19 ± 0.02	0.16 ± 0.03	0.22 ± 0.02
t		13.282	8.237	18.974

p	< 0.001	< 0.001	< 0.001

Bone mineral content of rat femur

The levels of Ca^{2+}, P and Mg^{2+} in the femurs of rats in the rickets group were significantly lower than the corresponding values in control group ($p < 0.05$; Table 4).

Table 4. **Bone mineral content of rat femur**

Group	n	Ca^{2+} (mg/kg)	P (mg/kg)	Mg^{2+} (mg/kg)
Rickets	18	226.32 ± 12.56	141.62 ± 10.52	2.89 ± 0.22
Control	18	236.02 ± 10.74	157.66 ± 7.80	3.20 ± 0.05
t		2.490	5.196	5.830
p		0.018	< 0.001	< 0.001

DISCUSSION

Rickets refers to the softening and weakening of bones in children, and it is highly prevalent in China. However, in recent years, the incidence of rickets has reduced in China due to emphasis on preventive measures. Vitamin D deficiency renders it impossible for the body to retain acceptable amounts of calcium and phosphate. Under this situation, the body releases hormones that allow calcium and phosphate to be extracted from the bone into the systemic circulation, a mechanism known as bone resorption. Rickets adversely affect children's growth and development [7]. Initially, it is characterized radiographically by epiphyseal widening, followed by cupping, splaying, fraying of long bone metaphysis (especially in distal ulna, distal femur, and proximal tibia); deformity, stress fracture lines, diffuse demineralization, and fractures [7].

Rickets are divided into various pathogenesis types: congenital rickets, vitamin D deficient rickets (nutritional rickets) and late-onset rickets. Vitamin D deficiency rickets are the most prevalent type [8]. It is a persistent dietary deficiency induced by calcium and phosphorus deficiency disorders. In rackets, the mineralization defect refers to an aggregation of osteoid in bone tissue below the growth plate (metaphysis). This adds to fragile bones and a time of bowing. Studies have indicated that environmental factors can play a role in racket pathogenesis [9].

The Vitamin D Receptor (VDR) gene polymorphism was hypothesised to lead to rickets. The potential function of VDR gene polymorphism and changes in bone density in rakes has received considerable attention recently [10]. The main circulatory source of vitamin D is 25-(OH)D. Thus, the overall serum amount of 25-(OH)D is generally known to be the strongest measure of the availability of vitamin D to the body from skin synthesis and dietary intake. Its level is substantially decreased in the serum of children with rakes, but the amounts rise after vitamin D supplementation [11]. Serum 25-(OH) D may also be used as a diagnostic index for rakes in children without vitamin D supplementation [12].

Bone alkaline phosphatase (BAP) is an ectoenzyme that is bound to the outer surface of the osteoblast cell membrane through glycosylphosphatidylinositol. It's partially published into circulation. Bone alkaline phosphatase (BAP) and liver ALP account for around 95 percent of the overall human serum alkaline phosphatase production. They are isoforms of non-specific alkaline phosphatase (TNAP) tissue encoded by the chromosome 1 gene and vary mainly by post-translational improvements (N-and O-glycosylation and sialic acid content). Bone alkaline phosphatase (BAP) is a primary surface marker for mature osteoblasts. It is commonly found in human skeleton, kidneys, intestines, placenta and other tissues. It is overexpression is a sign of active osteoblast proliferation. Under normal physiological conditions, osteoblasts are rich in BAP. However, in calcium malnutrition, the inability of osteoblasts to calcify in time and accumulate calcium stimulates the kidneys to synthesize large amounts of 25-(OH)D and BAP, and it also results in activation of stationary osteoblasts. The increased BAP activity is indicative of weakened bone mineralization and failure of bone tissue to proceed beyond the osteoblast stage [13]. In children, the activity of BAP can be used for early diagnosis of rickets since it is more sensitive than Ca^{2+}, P or ALP. Early intervention and treatment are recommended when BAP ≥ 200 U/L [14]. Since BAP activity is positively correlated with the severity of rickets, it can be used as an economical and practical index for the diagnosis of the disease [15]. Decreased BMC and bone decalcification are the main pathological features of rickets.

About 99 % of calcium in the human body is present in the bone, and the measurement of BMC directly reflects calcium nutritional status. Vitamin D deficiency not only affects the metabolism of

calcium and phosphorus, it also plays an important role in maintaining the growth and development of

cells. Low calcium intake affects the height, peak bone mass and bone density of children, thereby increasing the risk of rickets. At present, X-ray and blood biochemical examinations are routinely used tests for clinical screening of nutritional rickets. However, these parameters are not sensitive, since changes in blood Ca^{2+} and P levels are not obvious in the early stage of rickets. Moreover, changes in BMD can only be identified by X-ray examination when reduction in BMC exceeds 30 % [16].

The results of this study showed that BMC and BMD of rickety rats were significantly lower than those of control group. However, the corresponding BAP activity was increased significantly in the rickets group, relative to the control group.

CONCLUSION

The results of this study indicate that serum 25-(OH)D, BAP, BMC and BMD are important biochemical markers for diagnosis of nutritional rickets.

Acknowledgement

This paper was supported by Hebei Hengshui Science and Technology Plan Project (No. 2017014046Z).

Conflict of interest

No conflict of interest associated with this work.

Authors' contributions

This research was carried out by the authors mentioned in this paper, and the authors acknowledge any liability arising from allegations related to this report and its contents. The analysis was planned and planned by Xiaoqing Lv; Xiaoqing Lv, Na Liu, Zhanru Yin, collected and analysed the data; Xiaoqing Lv wrote the text. The manuscript for publication was read and accepted by both contributors.

REFERENCES

[1] Ng YM, Wong SMY, Lee AKH. Very prolonged breastfeeding causing nutritional rickets in a 4-year-old local Hong Kong boy. Hong Kong J Pediatr 2017; 22(2): 103-106.

[2] Uday S, Sakka S, Davies JH, Randell T, Arya V, Brain C, Tighe M, Allgrove J, Arundel P, Pryce R, et al. Elemental formula associated hypophosphataemic rickets. Clin Nutr 2019; 38(5): 2246-2250.

[3] Aparna P, Muthathal S, Nongkynrih B, Gupta SK. Vitamin D deficiency in India. J Family Med Prim Care 2018; 7(2): 324-330.

[4] Liu M, Zeng LF, Yang YJ, Hu LM, Lai WH. Fluorescent microsphere immunochromatographic assays for detecting bone alkaline phosphatase based on biolayer interferometry-selected antibody. RSC Adv 2017; 7(52): 32952-32959.

[5] Diemar SS, Møllehave LT, Quardon N, Lylloff L, Thuesen BH, Linneberg A, Jørgensen NR. Effects of age and sex on osteocalcin and bone-specific alkaline phosphatase—reference intervals andconfounders for two bone formation markers. Arch Osteoporos 2020; 15(1): 1-10.

[6] World Health Organization. Principles of laboratory animal care. WHO Chron 1985; 39: 51-56.

[7] Georges GT, Nájera O, Sowers K, Sowers JR. Fibroblast Growth Factor 23 and Hypophosphatemia: A Case of Hypophosphatemia along the Rickets-Osteomalacia Spectrum. Cardiorenal Med 2017; 7(1): 60-65.

[8] Rao M, Shetty D, Goyal KA, Budania LS. Breath-holding in Vitamin D deficiency rickets: A dilemma for the anesthetist. J Anaesthesiol Clin Pharmacol 2017; 33(4): 562-563.

[9] Zauche HL. Rickets: Not Just a Disease Caused by Vitamin D Deficiency. J Pediatr Health Care 2017; 31(2): 235-240.

[10] Kubota T. Rickets/Osteomalacia. Symptomatic vitamin D deficiency in children and its prevention and treatment. Clin Calcium 2018; 28(10): 1381-1386.

[11] Roizen JD, Li D, O'Lear L, Javaid MK, Shaw NJ, Ebeling PR, Nguyen HH, Rodda CP, Thummel KE, Thacher TD, et al. CYP3A4 mutation causes vitamin D–dependent rickets type 3. J Clin Invest 2018; 128(5): 1913-1918.

[12] Vierucci F, Del Pistoia M, Randazzo E, Massart F, Federico G. The Spectrum of Vitamin D Deficiency: Description of a Family. Exp Clin Endocrinol Diabetes 2017; 125(7): 478-484.

[13] Bergman A, Qureshi AR, Haarhaus M, Lindholm B, Barany P, Heimburger O, Stenvinkel P, Anderstam B. Total and bone-specific alkaline phosphatase are associated with bone mineral density over time in end-stage renal disease patients starting dialysis. J Nephrol 2017; 30(2): 255-262.

[14] Witkowska-Sędek E, Stelmaszczyk-Emmel A, Majcher A, Demkow U, Pyrżak B. The relationship between alkaline phosphatase and bone alkaline phosphatase activity and the growth hormone/insulin-like growth factor-1

axis and vitamin D status in children with growth hormone deficiency. Acta Biochim Pol

2018; 65(1): 269-275.

[15] Aranha RMC, Adriana VCA, Guerra PC, Chaves DP, Melo SA. Bone alkaline phosphatase in donkeys obtained by the thermal inactivation method. Acta Vet Brasilica 2018; 12(1): 1-4.

[16] Nisha, K. J., Janam, P., & Harshakumar, K. (2019). Identifying salivary transcriptome signatures for periodontal diagnosis. Journal of Natural Science, Biology and Medicine, 10(2), 114.

[17] Prabhakaran, G., Bhore, S. J., & Ravichandran, M. (2019). Development of a bait carrier material for apple snail (Pomacea maculata) based on its feed preferences using snail attractant tracking device. Journal of Natural Science, Biology and Medicine, 10(1), 8.

[18] Penoni DC, Fidalgo TK, Torres SR, Varela VM, Masterson D, Leão AT, Maia LC. Bone Density and Clinical Periodontal Attachment in Postmenopausal Women. J Dent Res 2017; 96(3): 261-269.

[19] Ramesh, V., Rao, G. P. C., Ramachandran, D., & Kalyan, A. (2019). One-Pot Syntheses Of 2-(1h-Benzo [D] Oxazole-2-Yl)-N-Arylbenzamides By Self-Catalysis. European Chemical Bulletin, 8(9), 318-321.

[20] Muzaffar, H., Nikolaus, C. J., Ogolsky, B. G., Lane, A., Liguori, C., & Nickols-Richardson, S. M. (2019). Promoting cooking, nutrition, and physical activity in afterschool settings. American journal of health behavior, 43(6), 1050-1063.

[21] Yan, Y., Wang, S., Zhou, W., Wang, S., & Gong, S. (2019). Does immigrant generation matter? Re-examining the ethnic density effects on mental health of ethnic minorities in Britain. American journal of health behavior, 43(5), 924-936.