Effects of porcine bone protein on serum phosphorus level and bone mineral density in a rat model of osteoporosis*★

An Yu-hui, He Si-yu, Song Dan, Li Feng-chun, Zhang Chao, Mao Hong-li, Zhang Qian

Abstract

BACKGROUND: Several studies have demonstrated that many American women who are at high risk of developing osteoporosis have higher levels of serum phosphorus. This indicates that some substances which can lower the serum level of phosphorus will supply a new and effective method to prevent and treat osteoporosis.

OBJECTIVE: To observe the influences of porcine bone protein on bone mineral density (BMD) and serum levels of calcium and phosphorus in a rat model of osteoporosis.

METHODS: Wistar rat models of osteoporosis were established by intramuscular injection of dexamethasone. Rat models were randomly divided into physiological saline, *Jiegu Qili* tablet, 50, 100, 200 mg/kg porcine bone protein groups. Rats that did not receive any treatments served as normal controls. After 12 weeks of treatment, serum was collected and serum levels of phosphorus and calcium were determined by biochemistry method. At the same time, tibia sections were made to determine tibial DMD by QDR-400 dual energy X-ray absorptiometry and to observe tibia marrow cavity by hematoxylin-eosin staining.

RESULTS AND CONCLUSION: There was no significant difference in serum level of calcium among groups ($P > 0.05$). Compared with the physiological saline group, serum level of phosphorus in the 50, 100, 200 mg/kg porcine bone protein groups was significantly decreased ($P < 0.05$). BMD was significantly higher in the 50, 100, 200 mg/kg porcine bone protein, *Jiegu Qili* tablet groups than in the physiological saline group ($P < 0.05$). The tibia marrow cavity was smallest in the normal control group and largest in the physiological saline group. The tibia marrow cavity was larger in the 50, 100, 200 mg/kg porcine bone protein, *Jiegu Qili* tablet groups than in the physiological saline group. These results indicate that porcine bone protein cannot change the serum level of calcium, but it lowers serum level of phosphorus, and increases BMD, in a rat model of osteoporosis. However, the dose-dependent effect of porcine bone protein was not observed within the present experimental dosage. In addition, porcine bone protein can also reduce the marrow cavity of the tibia of rats with osteoporosis.

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INTRODUCTION

Osteoporosis, a major public health problem, is becoming increasingly prevalent with the aging of the world population. This kind of disease is a skeletal disorder characterized by the reduction of bone strength, which increases the risk of fractures of the hip, spine, and other skeletal sites. Many risk factors are associated with osteoporotic fracture, including low peak bone mass, hormonal factors, the use of certain drugs (*e.g.*, glucocorticoids), cigarette smoking, low physical activity, low intake of calcium and vitamin D, race, small body size, and a personal or a family history of fracture[1]. Osteoporosis results in more than 1.5 million fractures in the United States each year, leading to substantial health care costs and loss of quality of life[2]. There are more than 200-million osteoporotic subjects in the world. Osteoporosis easily develops in the people over 50 years of age. Only one quarter of cases of osteoporosis are cured[3]. The main features of this disease is low bone mineral density (BMD), which results in increased susceptibility to bone fracture[4]. Calcitonin secretion in young women is gradually increased with age. After the menopause, calcitonin deficiency is an important factor in the pathogenesis of postmenopausal bone loss, and induces excessive bone resorption[5]. Lower levels of endogenous sex steroids or declines may contribute to the increased rate of bone loss, and have been observed in older adults experiencing weight loss[6]. Insufficiency of 25-OH vitamin D is very common in older population. Despite calcium and vitamin D supplement, 25-OH vitamin D concentrations fail to reach the normal level in a significant proportion. Maintaining vitamin D and calcium intake at the current recommended doses may not be sufficient to ensure adequate 25-OH vitamin D stores[7].

Phosphorus is an important component in bone tissue. However, osteoporosis may be the consequence of progressive dissolution of bone apatite crystals. So, it is necessary to maintain normal or sub-normal blood phosphorus level[8]. Evidence exists that many American women who are at high risk of developing osteoporosis is typically high in serum level of phosphorus and low in calcium[9]. It indicates that osteoporosis can be prevented and cured by decreasing the level of phosphorus and increasing the level of calcium in serum.

The osteoclast is a kind of cell that is unique in its ability to resorb bone, and can expose to unusually high millimolar Ca^{2+} concentration, and can “sense” changes in their ambient Ca^{2+} concentration. This triggers a sharp cytosolic Ca^{2+} increase through both Ca^{2+} release and Ca^{2+} influx. The change in cytosolic Ca^{2+} is transduced finally into inhibition of bone resorption[10].

Osteoblasts have bone-forming function, and they are either entrapped in bone matrix and become osteocytes or remain on the surface as lining cells. Osteoblasts can undergo apoptosis. However, the process can be modulated by growth factors and cytokines (some protein molecules), which are produced in the bone microenvironment[11]. In bone tissue, some proteins and peptides also influence the function of osteocytes. In porcine bone tissue, there also exists a protein[12]. To confirm several

uncertainnesses that whether protein can change the serum levels of calcium, phosphorus, and BMD in a rat model of osteoporosis, the preliminary research results are reported here.

MATERIALS AND METHODS

Design: A controlled animal experiment.

Time and setting: The experiments were performed at the Department of Biochemistry and Molecular Biology, Basic Medical College of Zhengzhou University from September 2007 to June 2009.

Materials

Animals

A total of 60 healthy Wistar rats, equal number of males and females, aged 3 months old, weighing (200±20) g, were provided by Henan Laboratory Animal Center, China (license No. SYXK (Henan) 2005-0012).

Preparation of porcine bone protein

Following three distilled water washes, the fresh porcine bones were broken into small pieces and extracted by saline. The extract solution was dehydrated by freeze-drying method, and the extracted protein was purified on Sephadex G-25 and G-50 columns, respectively. The collected solution containing porcine bone protein was dehydrated by freeze-drying. Thus, the purified powder of porcine bone protein was obtained[12].

Methods

Establishment of rat osteoporosis models

According to previously method[13], 60 rats were randomly divided into 6 groups (n = 10): normal control group: rats did not receive any treatments; physiological saline group, rats were daily administered 2 mL physiological saline; *Jiegu Qili* tablet group, rats were daily given 300 mg/kg Jigu Qili tablet, a composition recipe of Chinese herbs(Hunan Jinsha Drug Co.Ltd., Lot No. 041208); 50, 100, 200 mg/kg porcine bone protein groups: rats daily received porcine bone protein at corresponding dosages. All rats were injected with 2.5 mg/kg dexamethasone (Xizheng Branch of Tianjin Drug Co.Ltd., China; Lot No. 050621) twice a week, with the exception of the normal control group. Six weeks later, osteoporosis was successfully induced. Rats in each group were interfered according to above-mentioned medications. After 12-week treatment, all rats were sacrificed, and the blood samples were collected. The serum levels of calcium and phosphorus were detected by biochemistry method. At the same time, the tibias were also harvested to determine BMD by dual energy X-ray absorptiometry and hematoxylin-eosin staining.

Determination of serum level of calcium

In accordance with the method from Wang *et al*[14], serum level of calcium was detected through the use of calcium detection kit (Beijing Zhongshen Beikong Biology Science and Technology, China). The underlying mechanism of calcium detection is that calcium ion can react with o-cresolphthalein complexone and form a red complex compound, which has a specific absorption at 600 nm. The procedures are as follows: Monoethanolamine buffer was mixed up with 0.175 mmol/L o-cresolphthalein complexone and 7.80 mmol/L 8-hydroxyquinoline at the same volume. Standard reagent should be 2.5 mmol/L calcium solution. The mixture of reagents was incubated in a detecting cup with an aperture of 1 cm at 37 ℃ for 5 minutes. Then the absorbance of the sample was measured at 600 nm, and the serum level of calcium was calculated by the formula: Serum level of calcium = $\text{Absorbance}_{\text{sample}}/\text{Absorbance}_{\text{standard calcium solution}} \times 2.5$ mmol/L..

Determination of serum level of phosphorus

According to a previously published method[15], serum level of phosphorus was detected by ammonium molybdate-phosphorus method. The underlying mechanism is that ammonium molybdate reacts with phosphorus in serum, and forms a kind of blue compound. The compound had a special light absorption at 340 nm. Higher serum level of phosphorus leads to higher absorption to light. The procedures were: ① 13 mL of solution 1 (including 23.8 mmol/L sulphuric acid and 1.06 mmol/L ammonium molybdate), 0.5 mL of solution 2 (Toween 80). ②According to above procedure, the standard phosphorus solution was replaced by serum sample, and the absorbance of serum sample was also detected at 340 nm. Phosphorus level in serum sample was calculated by the following formula. Serum level of phosphorus = $\text{Absorbance}_{\text{sample}}/\text{Absorbance}_{\text{standard phosphorus solution}} \times 1.29$ mmol/L.

Determination of BMD

According to the method from Lei *et al*[16], tibia BMD (g/cm^2) was detected by dual energy X-ray absorptiometry.

Stainings of the tibias

Preparation of tibia paraffin sections: after sacrifice, the left tibias of rats were taken, fixed with 10% formalin for 48 hours, and decalcified with 8% nitric acid overnight. Thereafter, the tibias were washed with distilled water 5 times, dehydrated with a series of ethanol (70%, 80%, 90%, and 100%) for 12 hours, respectively. Then, the tibias were immersed in dimethylbenzene, embedded with paraffin, and sliced into 5 μm sections. Subsequently, sections were placed on the glass slide and treated with egg albumen and glycerol. The slide with the sections was stored at 60 ℃ overnight for staining[17].

Hematoxylin-eosin staining

The glass slides with the sections were immersed in dimethylbenzene for 3 minutes, in 95% ethanol for 3 minutes, and in 80% ethanol for 3 minutes. Subsequently, sections were stained with hematoxylin for 8 minutes, immersed in 1% chlorhydric acid-ethanol for 30 sections, and placed in Li_3CO_3 for 1 second. There were tap water washes between each step. Thereafter, sections were stained with eosin for 25 minutes, immersed in a series of ethanol (80% for 3 minutes, 95% for 3 minutes, and 100% for 3 minutes), and treated with dimethylbenzene for 3 minutes. Finally, the marrow cavity of the tibias was observed.

Statistical analysis

All data were processed by hand using SPSS 13.0 software and were expressed as Mean ± SD.

RESULTS

General conditions of experimental rats

Prior to experimentation, rat conditions were normal. After test, all rats survived and exhibited normal activities, water and food intake.

Measurements of serum levels of calcium and phosphorus

The measurement results of serum calcium and phosphorus are shown in Table 1.

Table 1 Serum levels of calcium and phosphorus ($\bar{x}\pm s$, mmol/L)

Group	Calcium	Phosphorus
Normal control	2.54±0.18	2.37±0.22
Physiological saline	2.47±0.19	2.61±0.23[a]
Jiegu Qili tablet	2.60±0.18	2.20±0.31[c]
50 mg/kg porcine bone protein	2.58±0.21	2.28±0.35[b]
100 mg/kg porcine bone protein	2.48±0.20	2.34±0.32[b]
200 mg/kg porcine bone protein	2.51±0.24	2.29±0.30[b]

[a]$P < 0.05$, *vs*. normal control group; [b]$P < 0.05$, *vs*. physiological saline group; [c]$P < 0.01$, *vs*. physiological saline group

Measurements of BMD

The BMD of rat tibias is listed in Table 2.

Table 2 Bone mineral density of the rat tibias ($\bar{x}\pm s$, g/cm^2)

Group	Bone mineral density
Normal control	0.161±0.014
Physiological saline	0.136±0.012[a]
Jiegu Qili tablet	0.153±0.016[b]
50 mg/kg porcine bone protein	0.150±0.015[b]
100 mg/kg porcine bone protein	0.152±0.015[b]
200 mg/kg porcine bone protein	0.148±0.013[b]

[a]$P < 0.01$, *vs*. normal control group; [b]$P < 0.05$, *vs*. physiological saline group

Hematoxylin-eosin staining results

The tibia marrow cavity was smallest in the normal control group (Figure 1) and largest in the physiological saline group (Figure 2). The tibia marrow cavity was larger in the 50, 100, 200 mg/kg porcine bone protein, *Jiegu Qili* tablet groups than in the physiological saline group (Figures 3–6).

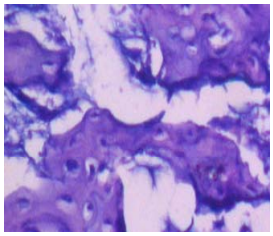

Figure 1 Normal control group, presenting small marrow cavities of the tibia (Hematoxylin-eosin staining, × 200)

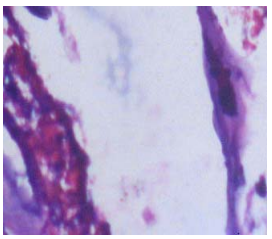

Figure 2 Physiological saline group, showing very big marrow cavities of the tibia (Hematoxylin-eosin staining, × 200)

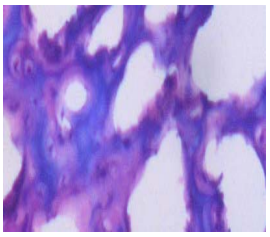

Figure 3 Jiegu Qili tablet group, presenting smaller marrow cavities of the tibia (Hematoxylin-eosin staining, x 200) compared with the physiological saline group

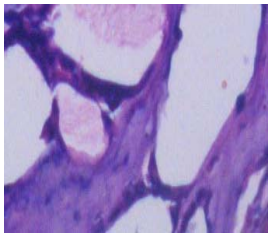

Figure 4 50 mg/kg porcine bone protein group, presenting smaller marrow cavities of the tibia (Hematoxylin-eosin staining, × 200) compared with the physiological saline group

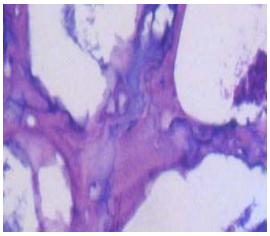

Figure 5 100 mg/kg porcine bone protein group, presenting smaller marrow cavities of the tibia (Hematoxylin-eosin staining, × 200) compared with the physiological saline group

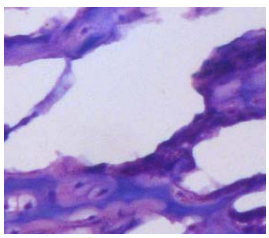

Figure 6 200 mg/kg porcine bone protein group, presenting smaller marrow cavities of the tibia (Hematoxylin-eosin staining, × 200) compared with the physiological saline group

DISCUSSION

A rat model of osteoporosis induced by glucocortical hormone has been accepted a better one[18-19]. The present study investigated porcine bone protein effects on serum levels of calcium and phosphorus, as well as BMD, through the use of rat osteoporosis models. Results showed that there was no significant difference in serum level of calcium among all groups.

Compared with the physiological saline group, serum level of phosphorus was obviously decreased in the *Jiegu Qili* tablet, 50, 100, 200 mg/kg porcine bone protein groups.

Osteoporosis can be induced by progressive decrease of BMD. It is necessary to maintain a normal and low level of serum phosphorous[8]. In human body, serum phosphorus often exists in the manner of phosphate, and the phosphate can bind to serum protein. The present results reveal that porcine bone protein could decrease the serum level of phosphorus. These results indicate that porcine bone protein can bind to serum phosphorus and transfer it to bone tissue so that more phosphate can bind more calcium to form calcium phosphate sediment in bone tissue. By this mechanism, BMD of rats with osteoporosis can be increased, and tibia structure of rats with osteoporosis can get a recovery.

Bone tissue consists of mineral and organic compounds[20], which play a role in supporting the body[21-23]. The mineral compounds can make bone to keep strength and stiffness. But organic compounds can supply toughness for bone. So, detection of BMD can help identify the effects of some drugs on osteoporosis treatment. Osteoporosis most commonly affects the hip and the lumbar vertebrae, but other bones, such as the radius, tibia, and ribs, may also fracture[24]. The risk factors for low bone mass may be important in the etiology of fracture of the shaft of the tibia/fibula in older individuals[25]. In addition, fractures of the tibia are common in children[26]. In this study, the tibia was selected in detecting BMD values. Compared with the physiological saline group, the BMD values in the *Jiegu Qili* tablet, 50, 100, 200 mg/kg porcine bone protein groups were significantly increased ($P < 0.05$). These results indicate that more phosphate had bound more calcium so that more calcium phosphate deposit in bone tissue.

Hematoxylin-eosin staining results from this study show that the tibia marrow cavity was the smallest in the normal control group and the largest in the physiological saline group. These results indicate that the bone structures of osteoporosis rats are very loose, and after porcine bone protein treatment, the marrow cavities of the tibias of rats with osteoporosis have been obviously reduced.

Taken together, porcine bone protein can lower the serum level of phosphorous and increase BMD, in a rat model of osteoporosis, so it can improve the tibia structure and thereby cure osteoporosis. Porcine bone protein appears promising in preventing and treating osteoporosis and needs further investigation.

REFERENCES

[1] Lane NE. Epidemiology, etiology, and diagnosis of osteoporosis. Am J Obstet Gynecol. 2006;194(2 Suppl):S3-11.
[2] White SC, Atchison KA, Gornbein JA, et al. Risk factors for fractures in older men and women: The Leisure World Cohort Study. Gend Med. 2006;3(2):110-123.
[3] Tarantino U, Cannata G, Lecce D, et al. Incidence of fragility fractures. Aging Clin Exp Res. 2007;19(4 Suppl):7-11.
[4] Cauey JA. Osteoporosis in men: prevalence and investigation. Cli Cornerstone. 2006;8 Suppl3:S20-25.
[5] Stevenson JC, Abeyasekera G, Hillyard CJ, et al. Calcitonin and the calcium-regulating hormones in postmenopausal women: effect of oestrogens. Lancet. 1981;1(8222):693-695.
[6] Ensrud KE, Lewis CE, Lambert LC, et al. Endogenous sex steroids, weight change and rates of hip bone loss in older men: the MrOS study. Osteoporos Int. 2006;17(9):1329-1336.
[7] Delappe E, McGreevy C, ni Chadhain N, et al. Vitamin D insufficiency in older female community-dwelling acute hospital admissions and the response to supplementation. Eur J Clin Nutr. 2006;60(8):1009-1015.
[8] Larroche M, Arlet J, Ader JL, et al. Skeletal manifestations of moderate phosphate diabetes. Clin Rheumatol. 1993;12(2):192-197.
[9] Calvo MS. The effects of high phosphorus intake on calcium homeostasis. Adv Nutr Res. 1994;9:183-207.
[10] Zaidi M, Moonga BS, Adbanjo OA. Novel mechanisms of calcium handling by the osteoclast: A review-hypothesis. Proc Assoc Am Physicians. 1999;111(4):319-327.
[11] Jilka RL, Weinstein RS, Bellido T, et al. Osteoblast programmed cell death (apoptosis): modulation by growth factors and cytokines. J Bone Miner Res. 1998;13(5):793-802.
[12] An YH, Yang SH, Jin JL, et al. Anylysis of amino acid in bone protein. Henan Yixue Yanjiu. 2004;13(3):208-209.
[13] Liu HD, Li E. The mechanism of osteoporosis induced by glucocorticoids. Zhonghua Guke Zazhi.1994;14(4):233-235 .
[14] Wang Y, Chen SQ, Wu Q, et al. Detection of serum calcium by the method of o-cresolphthalein complexone and photometer. Anhui Yike Daxue Xuebao. 1996;20(1):68-73.
[15] Fang ZX, Li WH, Zhou H, et al. Experimental exploration inorganic phosphate assay. Bangbu Yixueyuan Xuebao.1998; 23(4):64-65.
[16] Lei GH, Zhou JN, Li K, et al. Effects of passive smoking on bon mineral density and bone metabolism of rats. Zhonghua Shiyan Waike Zazhi. 2002;19(2):127 -129.
[17] Wang MX, Song ZY. Technology and operative procedure of HE dying section. Datong Yixue Zhuanke Xuexiao Xuebao. 2004; 24(2):21-25.
[18] Hu B, Li QN, Li ZY, et al. Experimental study of bone metabolism change induced by retinoic acid to femal rats. Zhongguo Guzhi Shusong Zazhi. 1997;3(1):1214-1216.
[19] Wang YS, Hu XY, Li YB. Studies of mechanism and prevention of osteoporesis induced by glucocorticoid. Henan Yixue Yanjiu. 2006;15(3):193-204.
[20] Hodghinson A,Thommon T. HOP and the different stages of growth. J Clin Pathal. 1982;12:80-81.
[21] Riggs BL,Wahner HW, Dunn WL, et al. Differential changes in bone mineral density of the appendicular and axial skeleton and with aging relationship to spinal osteoporosis. J Clin Invest. 1981;67(2):328-335.
[22] Huang GY. Bone structure and mechanical characteristic of osteoporosis. Guzhi Shusong he Gumidu de Jichu yu Linchuang. 2004;3(4):197-200.
[23] Tang H, Chen H, Luo XZ. New progress of the study of biological mechanics on osteoporosis. Guzhi Shusong he Gumidu de Jichu yu Linchuang. 2004;3(4):2410-2460.
[24] Cauley JA. Osteoporosis in men: prevalence and investigation. Clin Cornerstone. 2006;8 Suppl 3:S20-25.
[25] Kelsey JL, Keegan TH, Prill MM, et al. Risk factors for fracture of the shafts of the tibia and fibula in older individuals. Osteoporosis Int. 2006;17(1):143-149.
[26] Domzalski ME, Lipton GE, Lee D, et al. Fractures of the distal tibial metaphysis in children: patterns of injury and results of treatment. J Pediatr Orthop. 2006;26(2):171-176.

猪骨蛋白对骨质疏松模型大鼠血清磷水平与骨密度的影响**★

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摘要

背景：有报道显示美国处于高风险的骨质疏松妇女中磷水平较高，这是否意味着降低血磷水平的物质将会为防治骨质疏松提供新的有效手段？

目的：观察猪骨蛋白对骨质疏松大鼠骨密度和血清中钙及磷水平的影响。

方法：以肌肉注射地塞米松建立 Wistar 大鼠骨质疏松模型。造模后以数字表法随机分为生理盐水组、接骨七哩片组、50，100，200 mg/kg 猪骨蛋白组，不作任何处置大鼠作为正常对照。治疗 12 周后，分离血清并用生物化学方法测定血清磷和血钙水平，同时收集大鼠胫骨制作成骨切片，以 QDR-4000 双能 X 射线吸收仪测定各组大鼠胫骨吸光度值；苏木精-伊红染色观察胫骨骨髓腔变化。

结果与结论：各组之间血清钙浓度比较差异无显著性意义($P > 0.05$)。与生理盐水组比较，50，100，200 mg/kg 猪骨蛋白组大鼠血清磷浓度下降($P < 0.05$)。50，100，200 mg/kg 猪骨蛋白组、接骨七哩片组骨密度值高于生理盐水组($P < 0.05$)。正常对照组大鼠胫骨的骨髓腔是小的，生理盐水组大鼠胫骨的骨髓腔特别大，50，100，200 mg/kg 猪骨蛋白组、接骨七哩片组大鼠胫骨骨髓腔比生理盐水组大鼠胫骨骨髓腔小。结果提示猪骨蛋白不改变骨质疏松大鼠血清钙的水平，但它能降低骨质疏松大鼠血清磷的浓度，增加骨密度。不过，在该实验浓度范围内，没有显示剂量效应关系；猪骨蛋白也能缩小骨质疏松大鼠胫骨骨髓腔。

关键词：猪骨蛋白；骨质疏松；血清磷；骨密度；大鼠

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