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The calcium supplementation effect of calcium-binding oligopeptides from bonito (*Auxis thazard*) hydrolysate in rats

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Abstract

Sixty Ca-deficient male Wistar rats were divided into six groups to investigate the effect of a calcium-binding bonito hydrolysate (PBH-Ca) on calcium bioavailability in rats, including the normal group, the control groups (PBH + $CaCO_3$ group and the $CaCO_3$ group), and three PBH-Ca groups with doses of 2,000 (PBH-Ca-H group), 1,000 (PBH-Ca-M group), and 500 (PBH-Ca-L group) mg/kg body wt/day. Each group was given the corresponding drug by intragastric administration for 4 weeks. The indices measured included the rate of increase in body weight and body length, apparent calcium absorption rate, serum calcium, serum phosphorus, alkaline phosphatase (ALP) activity, bone volume fraction (BV/TV), bone mineral density (BMD), and trabecular microstructure of the rats scanned by micro-computed tomography. The results showed that PBH-Ca promoted the growth of rats, significantly increased the apparent calcium absorption rate and the number of bone trabeculae, stabilized serum calcium and serum phosphorus content in serum, significantly inhibited ALP activity, decreased the separation of bone trabeculae, accelerated bone growth of rats, and raised the BV/TV and femoral BMD in the rats. These results illustrate that PBH-Ca has promising potential in a high-quality calcium diet.

Keywords: calcium-binding oligopeptides from bonito hydrolysate (PBH-Ca); calcium bioavailability; Ca-deficient rat model.

Practical Application: The study confirmed the method of calcium-binding oligopeptides from bonito (*Auxis thazard*) hydrolysate (PBH-Ca) had a good application in rats, which could be significantly improved calcium bioavailability. It provided evidence in support of PBH-Ca as a potential high-quality calcium food supplement. In addition, it may help to develop high value utilization of bonito (*Auxis thazard*).

1 Introduction

Calcium is an essential nutrient for the human body. Insufficient calcium intake leads to metabolic bone diseases, such as rickets in children and osteoporosis in older adults (Nguyen & Murimi, 2021). Dietary intake is the body's main source of calcium. However, the absorption of calcium is affected by the nature of the chemical matrix of the food source and various factors, such as individual nutrition, metabolism, and physiological status (Wu et al., 2021). Calcium chelates have a good effect on the absorption and bioavailability of calcium and they have potential application value in the treatment of calcium deficiency in humans (Sun et al., 2016). Peptides derived from food proteins can be used as ingredients to develop functional foods and nutraceuticals (Kaur et al., 2021). More data on the calcium-binding peptide mode show that such peptides have higher absorption and bioavailability of calcium than the dietary intake mode (Zhang et al., 2021).

There are two main methods to test calcium bioavailability, such as *in vitro* digestion simulation and an animal test model. Among them, the animal model is *in vivo* verification and has higher reliability. Xu & Dong (2017) studied the effect of the calcium-binding polypeptide from pigskin collagen on BMD in mice by establishing a low calcium model, and the results showed that the calcium-binding polypeptide from pigskin collagen increased the femoral BMD of mice and prevented osteoporosis. Chunhui Liang et al. (2010) established the mice model of osteoporosis and intragastrically administered different doses of calcium-binding collagen. The results showed that BMD was higher in the high dose group of mice than that of calcium carbonate in the control group and the other groups, and that blood calcium content, blood phosphorus content, and alkaline phosphatase (ALP) activity were within the normal range, which confirmed that high doses of calcium-binding collagen protein increased bone density and promoted bone growth in the mice.

Bonito (*Auxis thazard*) is mainly distributed in the East China Sea and South China Sea, and has high market potential due to its high nutritional value, low price, and large fishing volume (Chen & Chen, 2017). However, *A. thazard* products are relatively single, mainly processed as canned food, which has low added value. Tuna protein hydrolysate has attracted much attention as a bioactive substance in functional foods with the increased number of calcium-binding peptides being discovered and characterized. PBH-Ca have been prepared in our previous study (Chen et al., 2019). This study aimed to investigate calcium bioavailability of the PBH-Ca in rats. Thus, a calcium-deficient

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rat model was established. Then, different doses of PBH-Ca were fed to the calcium-deficient rats. Body weight, serum calcium, and phosphorus contents, apparent calcium absorption rate, BV/TV, BMD, and bone trabecular microstructure of the rats were investigated under different dietary conditions, and the effect of PBH-Ca on calcium bioavailability was evaluated.

2 Materials and methods

2.1 Materials and reagents

A. thazard was purchased from Guangdong Xingyi Marine Biological Technology Co. Ltd. (Guangzhou, China). Calcium chloride (GR) was purchased from Dongguan Sparta Chemical Co. Ltd. (Dongguan, Guangdong, China). Calcium diagnostic kits, phosphorus reagent kits, and ALP activity reagent kits were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). The 4% paraformaldehyde, physiological saline, ethanol, and chloral hydrate were purchased from Zhanjiang Kecheng Chemical Co. Ltd. (Zhanjiang, Guangdong, China).

2.2 Instruments and facilities

The FA2104A Analytical Balance was obtained from Shanghai Tianping Instrument Factory (Shanghai, China). The Eyela WFT-700 air supply drying oven was purchased from Tokyo Physicochemical Equipment Co., Ltd. (Tokyo, Japan). The Varioskan Enzyme plate instrument was obtained from Thermo Fisher Co., Waltham, MA, USA). The Quantum GX Micro CT was purchased from Quantum Scientific Instruments, Inc. (Norwich, UK). The Thermo M6 Flame Atomic Absorption Spectrometer was obtained from Thermo Fisher Co. The MDS-6 Microwave Digestion Instrument was from Shanghai Xinyi Microwave Chemical Technology Co., Ltd (Shanghai, China).

2.3 Preparation of PBH-Ca

A. thazard muscle was homogenized and enzymatically hydrolyzed for 3 h under particular conditions (temperature 60°C, pH 8.0, solid-liquid ratio of 1:3, 500 U of papain per gram protein, and 200 U of flavor enzyme per gram protein were added). Then, the hydrolysis reaction was terminated by boiling for 15 min. The enzymatic hydrolysate was centrifuged at 7,654 × g at 4 °C for 20 min, and the supernatant was recovered after de-oiling with absorbent cotton gauze. The samples were filtered through a ceramic membrane (pore size 0.2 μm) and a 1 kDa MW cut-off ultrafiltration membrane. Finally, the ATO power was obtained by concentration and spray drying (Chen et al., 2019; Lukin., 2020).

Two g of bonito oligopeptide powder and 1.0 g of calcium chloride were fully dissolved in 50 mL deionized water. The pH was adjusted to 9.0 with 1.0 mol/L NaOH. The binding reaction was carried out in a water bath shaker at 50 °C. After the reaction, the calcium hydroxide precipitate was removed by centrifugation for 10 min at 4,000 r/min. The supernatant was concentrated by rotating evaporation. Anhydrous ethanol was added to a volume of more than 90% to precipitate the mixture, which was centrifuged for 25 min at 8,000 r/min. Finally, the precipitate was freeze-dried to give the PBH-Ca (Khan et al., 2021).

2.4 Experimental animals

Animals and diets

Wistar rats (SPF males, weight 100 ± 110 g, 4 weeks old) were purchased from Laboratory Animal Center of Southern Medical University (Animal quality certificate number: 0020196, Guangdong, China). The environmental conditions for feeding were temperature (20 ± 1)°C, relative humidity (60 ± 5)%, and a 12 h/12 h light and dark alternating cycle. All diets were prepared based on the AIN-93 diet (Reeves et al., 1993) with either normal calcium (5,000 mg Ca/kg diet, normal diet) or low calcium (1,000 mg Ca/kg diet, low calcium diet) (Chen et al., 2014). All rat experiments were carried out in strict accordance with the Guiding Principles in the Care and Use of the Center for Laboratory Animals, Guangdong Ocean University (Permission No. SYXK (Yue) 2019-0204).

Feeding procedures

Sixty Wistar rats were used in this experiment. The rats were fed a low-Ca diet for 4 weeks to establish the Ca-deficient rat model (Chen et al., 2011). The 60 Ca-deficient rats were randomly divided into six groups of 10 rats per group (Hua et al., 2021). All rats were continuously fed with the low-Ca diet for 4 weeks. The rats in the normal group were fed by gavage with deionized water. Three experimental groups were fed daily by gavage at doses of 500 (PBH-Ca-L group), 1,000 (PBH-Ca-M group), and 2,000 (PBH-Ca-H group) mg/kg body wt/day PBH-Ca for 4 weeks. Two control groups were set in the experiment. The $CaCO_3$ group was fed by gavage daily with doses of 2,000 mg/kg body wt/day $CaCO_3$, and the PBH+$CaCO_3$ group was fed by gavage daily with doses of 1,700 (PBH) + 300 ($CaCO_3$) mg/kg body wt/day. The intake dose calculation was based on 5, 10, and 20 times the recommended daily intake of calcium (800-1,200 mg/60 kg) for adults. The conversion was carried out according to the calcium content (15%) of the calcium-binding peptide. Experimental grouping and feeding of animals were shown in Figure 1.

2.5 Weight and length of the rats

All rats were fasted for 12 h before measurements. The weight and length of the rats were measured every 7 days. The body length of the rats was measured from the middle of the ears to the root of the tail.

2.6 Determining the apparent calcium absorption rate

The calcium metabolism experiment was carried out after continuous gavage of the rats for 2 weeks. The daily food intake and feces were recorded for the next 3 consecutive days, and fecal calcium content was measured. The apparent calcium absorption rate was calculated as follows (Equation 1).

$$\text{Apparent calcium absorption rate } (\%) = \frac{m_1 - m_2}{m_1} \times 100 \quad (1)$$

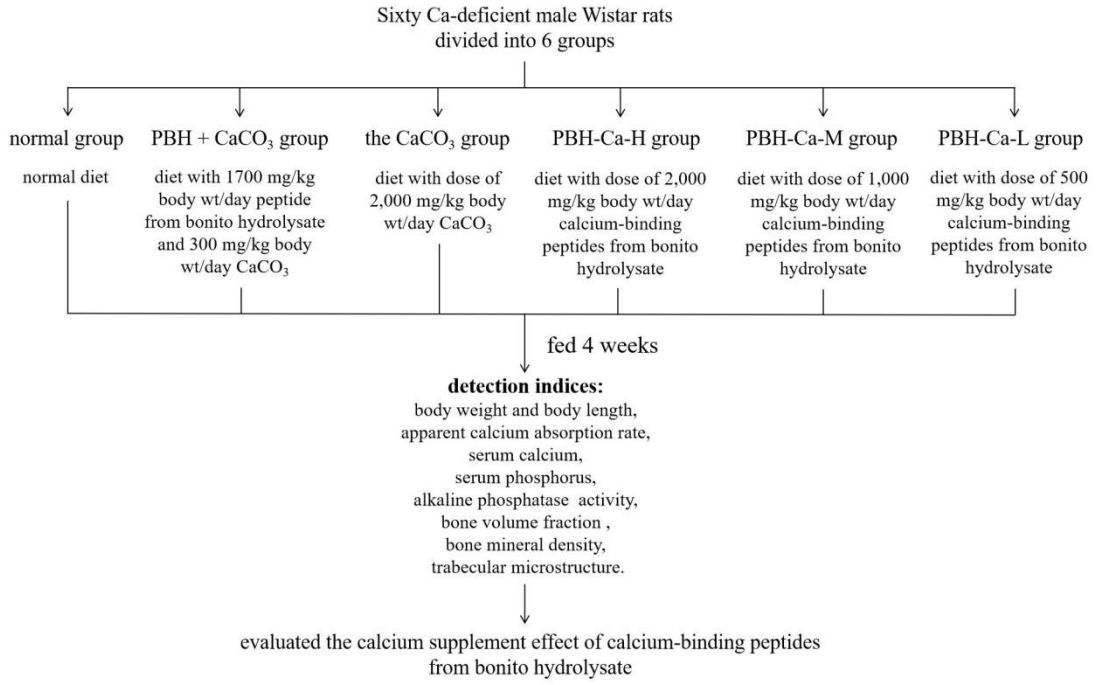

Figure 1. The flow chart of the experiment.

where: m_1 is calcium intake (calcium content in feed × feed quality), g; m_2 is calcium in the feces (calcium content in feces × fecal output), g.

2.7 Determining serum calcium and serum phosphorus contents and ALP enzyme activity

The rats were fasted for 24 h after feeding. Blood was taken from the orbital vein, and the serum was prepared by centrifugation at 4,000 r/min for 20 min. The serum calcium and phosphorus contents and serum ALP activity were determined according to the assay kit instructions.

2.8 Micro-computed tomography (CT)

The X-ray source was fully preheated. Voltage was 90 kV, and the electric current was 88 μA. The imaging field was 9 × 9 × 9 mm, and the resolution was 15 μm. The distal femur of the rats was detected at 100 layers below the growth plate. The three-dimensional regional data of the cortical and trabecular bone tissues were separated and selected with Analyze 12.0 software. The image analysis and storage of the selected region were carried out as well as the calculation of bone-related parameters. BMD, the bone volume fraction (BV/TV), and bone mass (quantity, thickness, separation) of the trabeculae were evaluated based on the scanning results.

2.9 Statistical analysis

Data are expressed as mean ± standard deviation from triplicate measurements of each sample (n = 3). The statistical analysis was performed using SPSS 10.0 software (SPSS Inc., Chicago, IL, USA) with Tukey's multiple comparison test. A P-value < 0.05 was considered significant.

3 Results and discussion

3.1 Effects of PBH-Ca on body weight and body length of the rats

Body weight and body length directly reflect the growth status of rats and were used as the external performance indices for bone growth and development. Some studies (Chen et al., 2011; Peng et al., 2017) have confirmed that animal growth rate is positively correlated with BMD. The effects of PBH-Ca on body weight and length of the rats are shown in Table 1. Body weight and length of the rats increased in each group. The increase of the body weight rate in each PBH-Ca group was higher than that of the normal group, but no significant difference was observed in the rate of increase of body length among the PBH-Ca groups. These results are consistent with those reported previously (Peng et al., 2017), indicating that PBH-Ca promoted the growth of rats.

3.2 Effect of PBH-Ca on the apparent calcium absorption rate

The apparent calcium absorption rate is an important index reflecting calcium bioavailability in rats. As shown in Figure 2, the apparent calcium absorption rate in the normal control was the highest, which may have been due to stimulation of intestinal absorption of calcium ions in rats with a severe calcium deficiency (Zhu et al., 2006). The apparent calcium absorption rates of the three PBH-Ca groups were all significantly higher than those

of the two control groups (PBH+$CaCO_3$ and $CaCO_3$), but there were no significant differences in the PBH-Ca groups.

The PBH-Ca groups were directly absorbed from the intestinal tract through the small peptide active transport pathway, so they had the higher absorption rate (Yang et al., 2021). The apparent calcium absorption rate in the PBH+$CaCO_3$ group was higher than that in the $CaCO_3$ group, indicating that PBH promoted the absorption of calcium in the rats, which was consistent with the results of Xu & Dong (2017).

3.3 Effects of PBH-Ca on serum calcium and phosphorus levels and ALP activity in rats

Stable serum calcium and serum phosphorus levels are necessary to maintain normal activities (Herselman et al., 2010). As shown in Table 2, serum calcium and serum phosphorus levels of rats in the PBH-Ca groups were within the normal range, and there were no significant differences, indicating that PBH-Ca did not lead to abnormal serum calcium or phosphorus concentrations in the rats (Mansilla et al., 2020). ALP is a representative

Table 1. The effect of PBH-Ca on body weight and length of the rats.

Groups	Final body weight of rats/g	Growth rate of body weight/%	Final length of the rats/cm	Growth rate of body length/%
PBH-Ca-L	201.17 ± 5.58	137.65 ± 11.10^{bc}	17.62 ± 0.34	75.25 ± 2.05^{a}
PBH-Ca-M	222.75 ± 8.23	178.18 ± 11.83^{a}	17.67 ± 1.36	79.50 ± 7.27^{a}
PBH-Ca-H	253.81 ± 9.38	184.77 ± 14.42^{a}	18.13 ± 0.75	80.27 ± 6.15^{a}
Normal	187.17 ± 6.54	115.24 ± 13.91^{c}	17.87 ± 0.52	74.25 ± 3.14^{a}
PBH+$CaCO_3$	228.34 ± 9.84	156.51 ± 10.19^{b}	16.92 ± 0.83	74.66 ± 5.04^{a}
$CaCO_3$	212.13 ± 2.55	146.49 ± 12.93^{b}	17.75 ± 0.69	80.50 ± 6.18^{a}

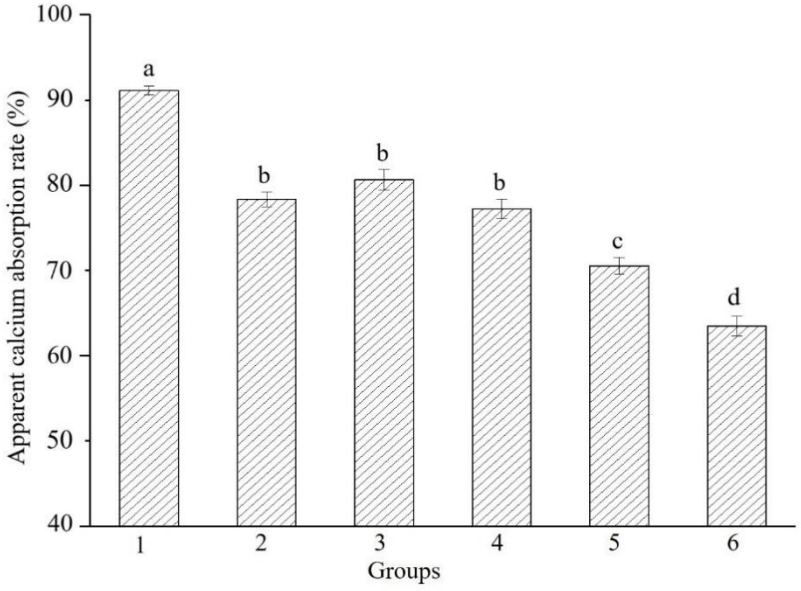

Figure 2. The effect of PBH-Ca on the apparent calcium absorption rate in the rats (1: normal; 2: PBH-Ca-L; 3: PBH-Ca-M; 4: PBH-Ca-H; 5: PBH+$CaCO_3$; 6: $CaCO_3$; the different letters above the bars indicate a significant difference between the groups).

Table 2. The effect of PBH-Ca on serum calcium and phosphorus levels as well as ALP activity in the rats.

Groups	Serum calcium contents(mmol/L)	Serum phosphorus contents(mmol/L)	ALP activity(U/L)
PBH-Ca-L	3.14 ± 0.03	2.68 ± 0.14	95.43 ± 5.12*
PBH-Ca-M	3.13 ± 0.06	2.65 ± 0.11	87.33 ± 6.37*
PBH-Ca-H	3.15 ± 0.04	2.73 ± 0.23	79.56 ± 4.58*
Normal	2.76 ± 0.12	2.45 ± 0.09	143.58 ± 9.31
PBH+$CaCO_3$	3.21 ± 0.13	2.63 ± 0.25	90.12 ± 7.75*
$CaCO_3$	3.14 ± 0.06	2.63 ± 0.25	96.43 ± 5.17*

Note: *indicates a significant difference between the PBH-Ca groups and the normal control ($P < 0.05$).

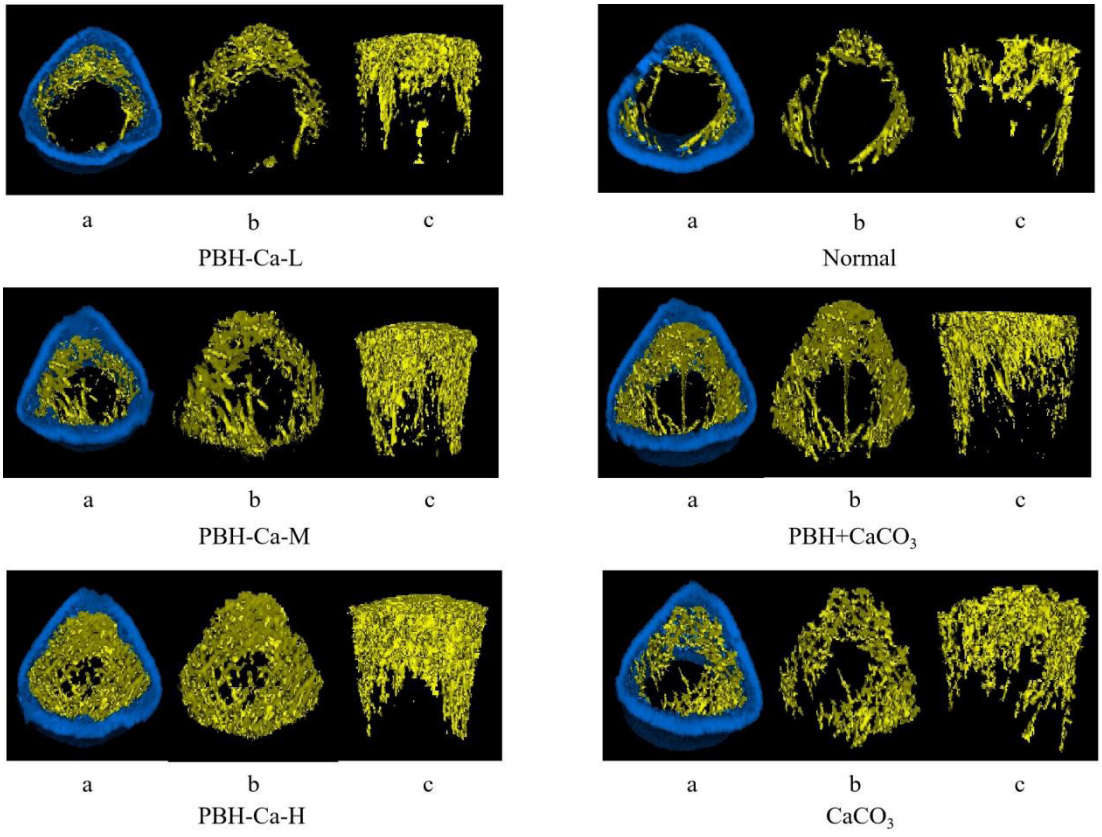

Figure 3. Three-dimensional images of the distal femur of rats in each group. (a) cortical and cancellous bone mass; (b) trabecular bone mass in vertical; (c) trabecular bone mass in horizontal.

osteoblast differentiation enzyme, which accurately reflects the differentiation and maturation of osteoblasts (Wu et al., 2018). ALP activity in the three PBH-Ca groups decreased significantly in a dose-effect relationship compared with that in the normal group ($P < 0.05$), indicating that PBH-Ca effectively inhibited bone metabolism and increased bone stability.

3.4 Effect of PBH-Ca on bone growth in rats

The rat femur is composed of cancellous bone, cortical bone, and the marrow cavity. Cortical bone has strong compressive resistance and is distributed on the surface of the bone where it plays the main supporting role. Cancellous bone is an extension of cortical bone and consists of interwoven bony trabeculae. In the Ca-deficient rat model, the level of bone growth and development is often characterized by the morphological characteristics of the cancellous and cortical bone in the rat femur (Williams et al., 2019). Siu et al. (2004) reported that the growth of cancellous bone is positively correlated with the growth of cortical bone on a quantitative CT scan. The microscopic tissues in cancellous bone are important to maintain normal development of bone. Figure 3 shows that femoral bone growth in the PBH-Ca-H group was significantly better than that in the normal group and the two control groups (PBH+$CaCO_3$ and $CaCO_3$). The cancellous bone mass of the femur increased and its cortical layer thickened with the increase in the intragastric dose of PBH-Ca, indicating that PBH-Ca played an important role in promoting bone growth in rats.

Effect of PBH-Ca on BV/TV in rats

BV/TV is a commonly used index to evaluate the bone mass of cancellous and cortical bone, and directly reflects a change in bone mass. As shown in Table 3, the BV/TV values of the PBH-Ca groups increased to varying degrees compared with the normal group, among which the BV/TV values of the PBH-Ca-H and PBH+$CaCO_3$ groups were 36.29% and 31.23%, respectively, which was significantly higher than that of the normal group (21.17%) ($P < 0.05$). This result was similar to the findings of Yadav et al. (2021), indicating that intake of PBH-Ca and PBH+$CaCO_3$ created a bone anabolic effect in the femur of rats, which increased bone mass.

Effect of PBH-Ca on BMD in rats

BMD is closely related to bone strength and stability of the bone internal structure and is an important indicator to evaluate the low calcium and osteoporosis models. Wang et al. (2020) studied the effects of various calcium supplements on bone metabolism in rats by measuring BMD and reported that L-aspartic acid calcium promotes bone growth in rats with low calcium levels. Miao et al. (2009) compared the bioavailability of a calcium-complex amino-acid chelate and the osteoform multi-

Table 3. Effects of PBH-Ca on BV/TV and BMD in the femur of rats.

Groups	BV/TV(%)	BMD of cortical layer (mg/cc)	BMD of cancellous bone (mg/cc)
PBH-Ca-L	25.96 ± 1.32	5370.43 ± 121.45	3438.34 ± 87.46
PBH-Ca-M	28.14 ± 0.89	6142.12 ± 103.17**	3728.73 ± 75.12
PBH-Ca-H	36.29 ± 1.41*	6303.39 ± 110.78**	4126.09 ± 81.31**
Normal	21.17 ± 0.56	4828.55 ± 213.94	2943.22 ± 98.62
PBH+$CaCO_3$	31.23 ± 1.25*	6016.23 ± 133.28**	4071.65 ± 70.52**
$CaCO_3$	24.77 ± 0.67	5573.11 ± 146.59	3490.93 ± 69.58

Note: *indicates a significant difference between the PBH-Ca groups and the PBH+$CaCO_3$ group ($P < 0.05$); ** indicates a significant difference between the PBH-Ca groups and the normal group ($P < 0.05$).

Table 4. Effect of PBH-Ca on the bone trabecular microstructure in rats.

Groups	Number of bone trabecula (mm^{-1})	Thickness of bone trabecular (mm)	Separation degree of bone trabecular (mm)
PBH-Ca-L	1.94 ± 0.12	0.12 ± 0.01	0.74 ± 0.06
PBH-Ca-M	2.37 ± 0.09*	0.17 ± 0.01	0.69 ± 0.08
PBH-Ca-H	4.87 ± 0.37*	0.18 ± 0.02	0.55 ± 0.10*
Normal	1.06 ± 0.07	0.13 ± 0.04	0.90 ± 0.12
PBH+$CaCO_3$	1.85 ± 0.03	0.18 ± 0.03	0.61 ± 0.09*
$CaCO_3$	3.22 ± 0.21*	0.11 ± 0.03	0.75 ± 0.03

Note: *indicates a significant difference between the PBH-Ca groups and the normal group ($P < 0.05$).

mineral compound V_D capsule and confirmed that these two calcium supplements improve BMD of rats, with BMD values of 0.26 and 0.22 g/cm^2, respectively. As shown in Table 3, the BMD of the cortical layer increased to varying degrees in the PBH-Ca groups compared with that in the normal group. The BMD values of the PBH-Ca-M, PBH-Ca-H, and PBH+$CaCO_3$ groups increased significantly compared with that in the normal group ($P < 0.05$). The BMD of cancellous bone in the PBH-Ca-H and PBH+$CaCO_3$ groups increased significantly compared with that in the normal group ($P < 0.05$). These results show that PBH and PBH-Ca improved BMD in rats.

Effect of PBH-Ca on bone trabecular microstructure in the rats

Bone strength is not only related to bone calcium content and bone density, but also to bone trabecular microstructure. Akhter & Recker (2021) reported that BMD only reflects 60-80% of the change in bone strength and that growth of bone can only be reflected by combining the geometric morphology of bone and the microstructure of the bone trabecular structure. Trabecular number (Tb.N), trabecular thickness (Tb.Th), and trabecular separation (Tb.Sp) are the main indices used to evaluate bone trabecular microstructure. Li et al. (2017) studied the relationship between bone trabecular microstructure and bone fractures in osteoporotic mice by measuring femoral Tb.N, Tb.Th, and Tb.Sp and confirmed that Tb.N, Tb.Th, and Tb.Sp directly and stably reflected the osteoporotic fracture model in mice. The effect of PBH-Ca on bone trabecular microstructure in rats is shown in Table 4. The number of bone trabeculae in the PBH-Ca-M, PBH-Ca-H, and $CaCO_3$ groups increased significantly ($P < 0.05$) compared with that in the normal group. Trabecular separation decreased significantly in the PBH-Ca-H and PBH+$CaCO_3$ groups ($P < 0.05$). No significant difference in the thickness of femoral trabecular bone was observed among the groups ($P > 0.05$). These results are similar with the femoral BV/TV and BMD values obtained in this study.

The results of BV/TV, BMD, and bone trabecular microstructure show that PBH-Ca improved the bioavailability of calcium in rats, increased calcium precipitation in bone, and promoted the growth and development of bone.

4 Conclusions

PBH-Ca improved the apparent calcium absorption rate, bone volume fraction, bone density, and bone growth and inhibited ALP activity, and decreased the trabecular separation in a Ca-deficient rat model, suggesting that PBH-Ca significantly improved calcium bioavailability in rats. These findings provide evidence in support of PBH-Ca as a potential high-quality calcium food supplement.

Ethical approval

On behalf of all co-authors, I would like to declare that all the experiments were implemented in strict accordance with the Guiding Principles in the Care and Use of the Center for Laboratory Animals, Guangdong Ocean University (Permission No. SYXK (Yue) 2019-0204).

Conflict of interest

The authors declare that they have no conflict of interest.

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