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Liprotide-encapsulated vitamin D_3 modulates circulated PTH levels and improved bone microstructure

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ABSTRACT

Background: vitamin D (25(OH)D) is a fat-soluble vitamin that is unstable in the gastrointestinal environment and has low bioavailability. A protein-lipid complex (liprotide) can be used as a shell to increase vitamin D stability and bioavailability. Liprotide can also serve as a delivery system for transporting vitamin D to its intended site. Little attention has been paid to utilizing liprotide as a delivery system for vitamin D and evaluating its functional activity.
Objective: to investigate the effect of liprotide-encapsulated vitamin D_3 on PTH levels and bone microstructure in vitamin D and calcium (VD-Ca) deficient rats.
Materials and Methods: an overall of 24 Wistar rats had been divided into four groups, a normal control group (K), a VD-Ca group without treatment (K-), a VD-Ca group with 180 IU/200 gBW/day free vitamin D_3 (FVD3), and a VD-Ca group with 180 IU/200 gBW/day liprotide-encapsulated vitamin D_3 (LVD3). Before and after 28 days of vitamin D intervention, blood samples were taken and analysed for serum PTH levels. The microstructure of the bone was analyzed using the Scanning Electron Microscope (SEM).
Results: the VD-Ca rats supplemented with vitamin D_3 (FVD3 and LVD3) had a significant decrease in serum PTH levels ($p<0.001$) and improved bone microstructure ($p<0.05$) compared to the (K-) group. The reduction of PTH in the LVD3 group was higher compared to the FVD3 group. The bone microstructure between the FVD3 and LVD3 groups is significantly different, as seen in the Ct.Wi parameter, with the LVD3 group having a higher Ct.Wi than the FVD3 group.
Conclusion: liprotide-encapsulated vitamin D_3 improves the serum PTH level and bone microstructure in a rat model of vitamin D and calcium deficiency.

***Keywords** : bone microstructure; encapsulation; liprotide; PTH; vitamin D_3*

BACKGROUND

Vitamin D is a crucial fat-soluble vitamin for maintaining calcium homeostasis and bone health.[1,2] Several diseases, such as diabetes, hypertension, autoimmune diseases, metabolic syndrome, some cancers, and infectious diseases are all linked to vitamin D.[1] Vitamin D could be obtained externally from the plant in the form of vitamin D_2 (*ergocalciferol*) and from the animal in the form of vitamin D_3 (*cholecalciferol*).[3] The latter is relatively easier to metabolize and therefore is more effective in maintaining vitamin D levels.[4] Despite the sources, it is important to keep vitamin D levels in the body adequate.

Many people are vitamin D deficient (25(OH)D serum levels <20 ng/mL).[5] As many as 60% of adults and 30% of children worldwide have inadequate vitamin D levels.[6] Among the reasons for Vitamin D deficiencies are excessive use of sunblock, covered clothing, minimum outside activities, season, latitude, age, skin pigmentation, and poor intake of foods containing vitamin D and calcium. Vitamin D deficiency raises the possibility of secondary hyperparathyroidism, progressive bone loss, and increased bone turnover.[7,8] Bone is one of the main organ targets for vitamin D. Active vitamin D (1,25(OH)2D) is involved in maintaining bone formation, calcium homeostasis, and neuromuscular function.[9] Calcium homeostasis is maintained by regulating calcium mobilization from bone, renal calcium reabsorption, and intestinal calcium absorption. When serum calcium is low, it triggers the production and secretion of PTH, which induces 1,25(OH)2D synthesis in the kidneys and bone calcium resorption.[10]

Food fortification and vitamin D supplementation have been used to tackle vitamin D deficiency. Unfortunately, oral supplementation is challenged by vitamin D instability in the gastrointestinal tract, as seen by its low solubility and bioavailability and slow absorption into the tissue.[4,11] Several biosystems have been developed to address limitations in vitamin D supplementation, including the lipid-based biosystem.[12] A lipid-based system, such as liposomes, has amazing biocompatibility; however, this system suffers from instability,

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partially controlled particle size, and low encapsulation efficiency.[4] Recently bioconjugation of protein to the lipid-based system has been developed to improve stability, bioavailability and even delivery. Liprotide is a complex consisting of fatty acids surrounded by partially denatured protein.[13,14] This system is promising as an oral delivery system due to its stability and ability to deliver fat-soluble active ingredients to the target.[15] Also, the fatty acid in the system ensures optimal absorption of fat-soluble nutrients in the intestine.[16]

Several studies have documented the optimization strategy of liprotide as a delivery system of various substrates. However, little attention has been paid to utilizing liprotide as a delivery system for vitamin D and evaluating its functional activity, and it has not been widely used in adults who are susceptible to vitamin D deficiency. Therefore, this study's objective was to investigate the effects of liprotide-encapsulated vitamin D_3 on bone microstructure and PTH levels of the model of the vitamin D-deficient rats.

MATERIALS AND METHODS

Research Design

This study used a true experimental pre-post control group design. The experiments in this study were compiled with the bioethical research established by The Medical/Health Research Bioethics Commission, Sultan Agung Islamic University (Approval Number: 29/I/2022/Bioethics Commission).

Sample Vitamin D preparation

The materials used were obtained from Sigma-Aldrich: vitamin D_3/*cholecalciferol* (5.00936.0010), β-lactoglobulin (BLG, L0130), and oleic acid (OA, Y0001479). Other materials used to support this research include potassium hydroxide (KOH, 1310-58-3), PBS OmniPur® liquid concentrate (6506-1LCN), absolute ethanol (1.00983.2500, 99%), and MilliQ water.

Preparation of liprotide begins with 38 mg/mL oleic acid dissolved in ethanol. 6 mg/mL β-lactoglobulin (β-Lg) was dissolved in 1.5 mg/mL oleic acid with 10 mM KOH solution (pH 10.5). The solution was incubated at 45°C for 30 minutes and then terminated. After that, the pH value of β-lactoglobulin and oleic acid (β-Lg-AO) solution was adjusted to pH 7.4 using PBS solution. The next step is vitamin D_3 encapsulated with liprotide, in which 30 mg/mL of vitamin D_3 (*cholecalciferol*) is dissolved in ethanol and diluted with MilliQ water. Afterwards, vitamin D_3 was mixed with the liprotide. The sample was homogenized using a vortex and kept at room temperature.[13] Encapsulation efficiency and vitamin D concentration were seen using HPLC (Shimadzu corp LC20AD® L20105130725 series, Japan) and SEM (JEOL® JSM-6510LA series, Japan). This sample preparation was carried out in the Diponegoro University Integrated Laboratory, Semarang.

Animal Study

This study used a true experimental with pre-post control group design. A total of 24 male Wistar rats, aged eight weeks, weighed 150-200 gr, were housed in individual cages under a typical temperature of 22±2°C, light-controlled (12h/12h alternating light and dark), and humidity 60-70%. They were fed a standard diet AIN-93M[17] and had ad libitum access to water. Experimental animal treatments were carried out at the Experimental Animal Laboratory, Center for Food and Nutrition Studies, Gadjah Mada University, Yogyakarta.

After a one-week acclimatization period, the rats were randomly divided into four groups (six animals per group): a normal control group (K), a VD-Ca group without treatment (K-), a VD-Ca group with 180 IU/200 gBW/day free vitamin D_3 (FVD3), and a VD-Ca group with 180 IU/200 gr BW/day liprotide-encapsulated vitamin D_3 (LVD3). The dosage is based on the Endocrine Society Clinical Practice Guidelines, and it has been proven in previous studies that a daily dose of 10,000 IU of vitamin D can effectively treat vitamin D deficiency in humans without posing any risk of harm.[5,18] This dose was converted into a dose for rats by a conversion factor (0.018) to obtain a dose of 180 IU/200 g BW/day. The normal control group rats were fed a standard diet. The rats in groups K-, FVD3, and LVD3 were fed a VD-Ca-deficient diet. The VD-Ca deficient diets were prepared by modification of AIN-93M; vitamin D_3 and Ca were excluded from the formulation. After two weeks on a diet, VD-Ca deficiency rats were confirmed by measuring serum 25(OH)D levels and serum calcium levels (respectively, control = 82.67±4.15 ng/mL; deficient = 16.02±0.61 ng/mL and control = 12.06±0.18 mg/dL; deficient = 5.80±0.39 mg/dL). Blood samples were taken via retro-orbital plexus. FVD3 was diluted in virgin coconut oil, and LVD3 was diluted in water. Vitamin D supplementation was carried out daily for 28 days. One mL/kg body weight of vitamin D (final dose of 180 IU/200 gr BW) was used in FVD3 and LVD3 groups. Weekly body weight and daily food intake measurements were made. The rats in all groups had free access to water. Rats were fasted for 12 hours, given ketamine anesthesia (100 mg/kg

body weight), and then cervical dislocation was used to finish the experiment. This animal study can be seen in Figure 1.

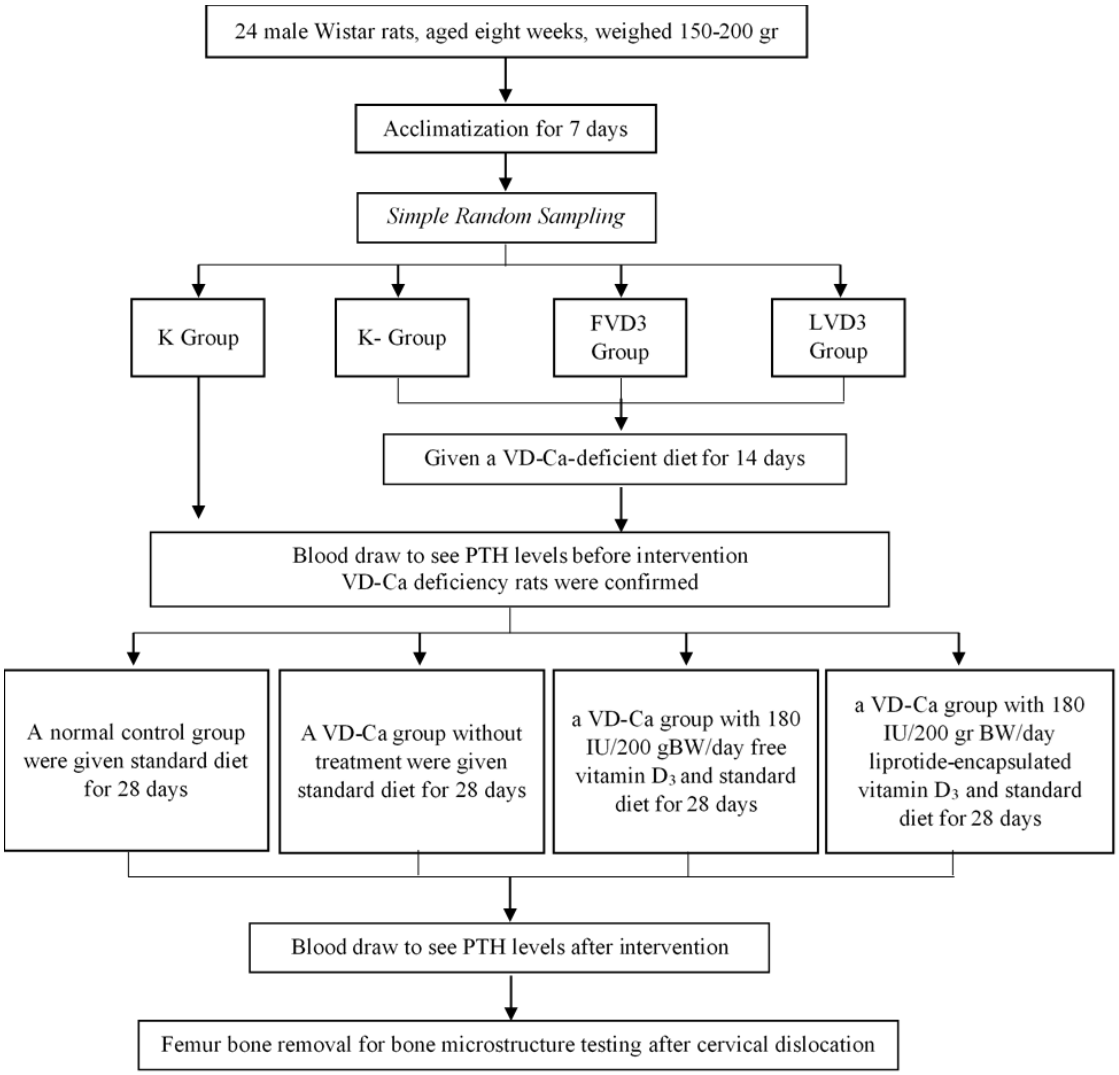

Figure 1. Research Workflow

Analysis of Serum PTH

Blood samples were taken before and after vitamin D_3 treatment. Enzyme-Linked Immunosorbent Assay (ELISA) was used to measure the levels of serum PTH.[19] The assay was performed according to the protocols provided by the ELISA kit (Fine Test).

Analysis of Bone Microstructure Parameters

Left femurs were each sliced longitudinally using a diamond disc saw from the intercondylar line to the diaphysis after being dried to a constant weight at 60°C.[20] Then, the bone samples were coated with a conductive material (gold) using a sputtering machine. Bone measurements were performed on the metaphysis area containing trabecular and cortical bones. The microstructure of the rat's femur (trabecular thickness [Tb.Th], trabecular separation [Tb.Sp], and cortical width [Ct.Wi]) was observed using Scanning Electron Microscope (SEM) with two magnifications (15x and 85x). Tb.Th parameter measurement was carried out by measuring the trabecular diameter in mm; Tb.Sp measures the distance between the segmented trabecular margins in mm; and Ct.Wi measures cortical thickness in mm.[21]

Statistical Analysis

All quantitative data are expressed as mean ± SD (standard deviation). Data analysis was carried out using statistical data processing program (IBM SPSS® Statistics version 25). The Shapiro-Wilk test was used to examine the data's normality. One-Way ANOVA was used to analyze the variations in PTH levels and bone

microstructure parameters between groups, and then a Post-Hoc statistical test was performed. Data were considered statistically significant if the $p<0.05$.

RESULTS

PTH Levels

Table 1. shows the PTH levels of VD-Ca deficient rats before and after two weeks of vitamin D supplementation treatment. VD-Ca deficient rats without treatment (K-) and healthy rats on a standard diet (K) were used as the control experiment. Two weeks after the initiation of the induction of VD-Ca deficiency, the average PTH levels in K-, FVD3, and LVD3 groups (pre-treatment) were above the normal limit (10-65 pg/mL). Post-treatment, PTH levels increased significantly in the normal control group and the VD-Ca deficient without treatment group. The One-Way ANOVA test showed a significant difference in PTH levels after intervention in all groups ($p<0.001$). VD-Ca deficient rats that received FVD3 or LVD3 showed a significant reduction in PTH levels, while the K- group remained high. Notably, the reduction in PTH levels in the LVD3 group was higher than in the FVD3 group. According to similar test results that have been confirmed in earlier studies, VD-Ca deficient rats had vitamin D levels of 15.76 ± 0.36 ng/ml; however, after receiving LVD3, their levels rose to 69.45 ± 3.40 ng/ml.

Table 1. PTH Levels (pg/mL)

Group	Average PTH Levels ± SD			p^r
	Pre-treatment	Post-treatment	Delta pre-post	
K (Normal control)	34.37 ± 3.06	37.17 ± 3.27	2.80 ± 0.63	0.000*
K- (VD-Ca without treatment)	187.38 ± 4.18	189.87 ± 3.27	2.50 ± 0.94	0.001*
FVD3 (VD-Ca with free vitamin D_3)	190.28 ± 3.11	60.83 ± 3.00	-129.45 ± 5.04[ab]	0.000*
LVD3 (VD-Ca with liprotide-encapsulated vitamin D_3)	186.21 ± 1.78	42.78 ± 2.66	-143.43 ± 2.80[abc]	0.000*
p^q			0.000*	

[q]One-Way ANOVA (n=6), followed by Bonferroni Post-Hoc test ([a]Indicates $p<0.05$ versus normal control group; [b]Indicates $p<0.05$ versus VD-Ca group without treatment; [c]Indicates $p<0.05$ versus VD-Ca group with free vitamin D_3); [r]Paired t-test;*significant ($p<0.05$).

Bone Microstructure Parameters

The statistical analysis microstructure parameter of rat's femurs are shown in Figure 2. The lowest Tb.Th and Ct.Wi values were found in the K- group. The Tb.Sp in the K- group is bigger than the K, FVD3, and LVD3 groups. While the Tb.Th and Tb.Sp parameters did not significantly differ between the FVD3 and LVD3 groups, the Ct.Wi parameter did, with the LVD3 group having a higher Ct.Wi than the FVD3 group. The One-Way ANOVA test showed a significant difference in bone microstructure parameters after intervention in all groups ($p<0.05$).

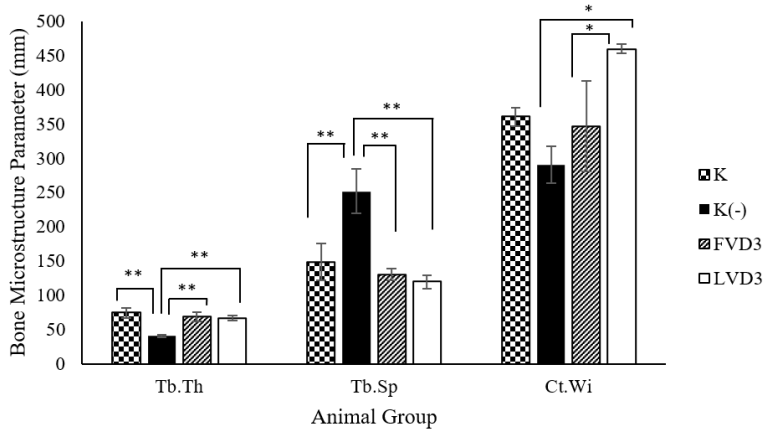

Figure 2. Bone Microstructure Parameters

The bone microstructure parameters of all groups, including Trabecular thickness (Tb.Th), Trabecular separation (Tb.Sp), and Cortical width (Ct.Wi)). Data shown are means; error bars show SD. Data were analyzed by one-way ANOVA ($p<0.05$, n=6), followed by Tamhane's Post-Hoc test. *Indicates $p<0.05$; **Indicates $p<0.001$). K, normal control group; K-, VD-Ca group without treatment; FVD3, VD-Ca group with 180 IU/Kg/day free vitamin D_3; LVD3, VD-Ca group with 180 IU/Kg/day liprotide-encapsulated vitamin D_3.

For comparison purposes, Figure 3. represents the micrograph SEM analysis of the inner part of the left femur. The femoral metaphysis was used to analyze the microstructure of bone. The area that was used to measure Ct.Wi is denoted by white arrows. The squares correspond to Figures 3E–H and show the ranges used to calculate Tb.Th and Tb.Sp. Double-edged arrows and double-edged diamond arrows are used to denote Tb.Th and Tb.Sp. According to these micrographs, the femoral cortical bone of the K- group was thinner than the rest of the groups (Figure 3A-D, see arrows). Figure 3E-H shows femoral metaphysis in detail. The K- group trabecular bone had the highest porosity, indicated by the thinness of Tb.Th and the dimensions of Tb.Sp, compared to the rest of the groups. Although it is not very noticeable, there is a porosity difference between the FVD3 and LVD3 groups. The LVD3 group has lower porosity than the FVD3 group.

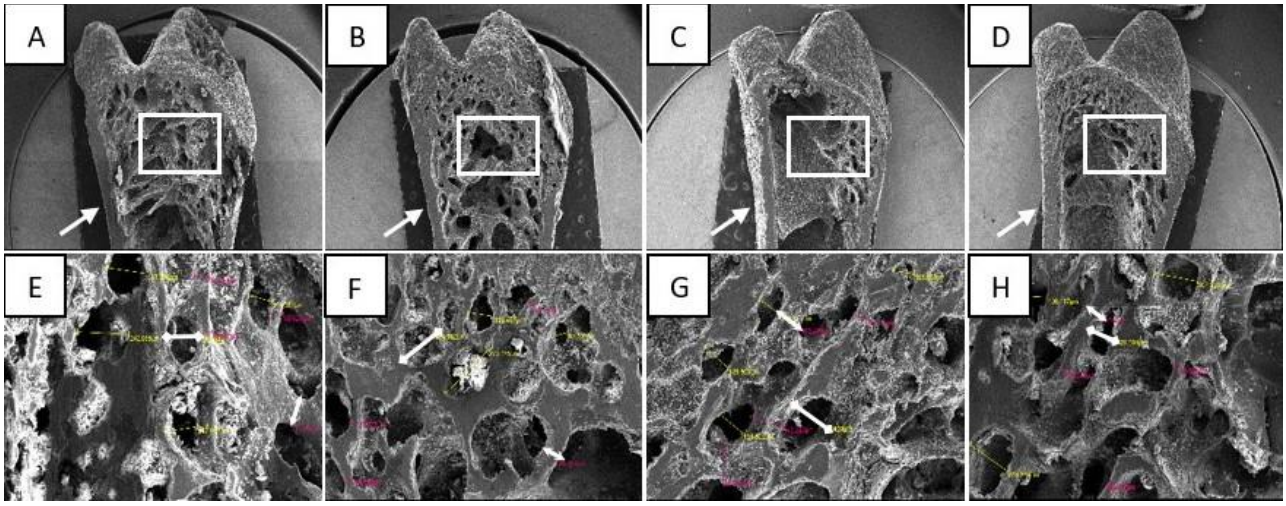

Figure 3. SEM Images of The Inside Left Femur

Left to right: K, normal control group; K-, VD-Ca group without treatment; FVD3, VD-Ca group with 180 IU/Kg/day free vitamin D_3; LVD3, VD-Ca group with 180 IU/Kg/day liprotide-encapsulated vitamin D_3 (**A-D**) shows the interior of the rat's femur that was cut longitudinally from the intercondylar line to the diaphysis (15x), (**E-H**) shows the femoral metaphysis (trabecular area) (85x).

DISCUSSION

This study's main goal was to determine whether LVD3 might lower serum PTH levels and enhance bone microstructure in a rat model of vitamin D and calcium deficiency. We demonstrated that administration of LVD3 for four weeks recovered PTH levels and improved bone microstructure.

Vitamin D and PTH are inversely related.[22] The K- group has high PTH levels, which indicates that vitamin D deficiency increases PTH levels. Similar to a previous study, vitamin D deficiency is frequently associated with high-normal or elevated PTH levels.[23] This was also shown in our previous study, which found that rat with vitamin D deficiency had low vitamin D_3 levels of 15.58 ng/mL and high PTH levels of 187,38 pg/mL. PTH levels increased in the K and K-groups following treatment. It could be because each group has gained weight. Changes in PTH levels can be influenced by environmental factors, such as lifestyle (smoking, alcohol consumption, BMI, diet), pollutants, and genetic factors.[24] Vitamin D_3 supplementation (in the form of FVD3 or LVD3) in VD-Ca deficient rats can significantly reduce PTH levels. Our findings are consistent with a prior investigation that found vitamin D treatment might considerably lower PTH levels and raise serum calcium.[25] The average decrease in PTH levels in the LVD3 group was higher than in the FVD3 group. This trend is most likely due to improved vitamin D_3 stability as LVD3. The previous study showed that encapsulation shields bioactive substances from pH and enzyme breakdown and allows controlled release.[26] Therefore, it is assumed that liprotide serve as a method for delivering vitamin D_3 to its intended recipient in a controlled manner. It also can protect vitamin D_3 from damage, increase absorption efficiency, and increase bioavailability.[27,28] Improvement in stability and bioavailability may strengthen the effect of vitamin D supplementation on PTH levels. As explained in a previous study, the transport and delivery of nutraceuticals in encapsulation is a complex process that involves biological processes, from digestion to their implementation in targeted cells or tissues.[29]

Vitamin D deficiency can affect bone structure. In this study, we observed changes in the microstructure of the femur bone. The femur is the longest and sturdiest bone in the body and has the highest

calcium content.[20,30] The four components of bone structure are the cortical bone, trabecular bone, periosteum, and endosteum.[31] The cortical bone forms a dense outer shell that surrounds the bone, found mainly in the diaphysis and less in the metaphyses and epiphyses.[32] Trabecular bone is found inside the bone, forming a porous network at the epiphyses and being found at the metaphyses separated by growth plates.[33,34] Trabecular bone is a metabolically active tissue associated with calcium homeostasis, unlike cortical bone, which is thick, dense, and metabolically inactive.[20] Bone remodelling can increase porosity and decrease bone mass. Therefore, alterations in the fibers of the cortical and trabecular tissues are correlated with the strength and mechanical characteristics of bone. The K- group had lower Tb.Th and Ct.Wi than the FVD3 and LVD3 group. In contrast, the K- group had a higher Tb.Sp than the FVD3 and LVD3 group. Vitamin D deficiency causes a lower Tb.Th, lower Ct.Wi, and higher Tb.Sp. Similar to the previous study, low vitamin D levels indicate increased bone turnover and weakened bone structure in trabecular bone, where the amount of trabecular bone is low and Tb.Sp is high.[35] From this, it is also known that vitamin D_3 is effective in improving bone structure. The LVD3 group had a higher Ct.Wi than the FVD3 group. The LVD3 group also had the lowest average Tb.Sp value among all groups. Liprotide as a vitamin D_3 delivery system can protect, increase the stability and solubility of vitamin D_3 in water, increasing bioavailability, and deliver vitamins to their targets in a controlled manner.[4,36] LVD3 has the potential to protects and delivers vitamin D_3, and the effect is visible in improving bone structure.

In this study, FVD3 and LVD3 were administered orally. Based on the results of a previous study, oral administration of vitamin D_3 can rapidly improve vitamin D status in patients with vitamin D deficiency conditions.[37] After ingestion, FVD3 and LVD3 pass through the stomach at a pH of 4.5 to 6.0. According to previous research, the β-Lg complex and vitamin D_3 are resistant to digestion by the stomach enzyme pepsin.[38] The hydrophobic nature of the denatured amino acid in β-Lg may inhibit enzymatic hydrolysis of the pepsin β-Lg complex, and vitamin D_3 is susceptible to the entire GI tract, including the presence of proteolytic enzymes.[28] It is reasonable to assume that the liprotide structure that encapsulates vitamin D_3 remains intact and stable during acid digestion in the stomach before degrading in the GI tract implying that liprotide could be an effective vehicle for increasing vitamin D_3 bioavailability. Meanwhile, FVD3 is thought to be almost completely degraded at the end of digestion. An in vitro study demonstrated that 90% of the unprotected vitamin D_3 was lost during six hours in simulated intestinal fluid (SIF).[11]

Vitamin D_3 is absorbed into the small intestine by enterocytes with the help of bile salts, monoglycerides, and free fatty acids.[39,40] Most of the ingested vitamin D is taken up by the chylomicrons into the lymphatic system and stored in adipose tissue and muscle before entering the bloodstream.[41] The remaining portion is moved to the liver by the vitamin D binding protein (VDBP), where it is hydroxylated by the enzyme 25-hydroxylase (CYP27A1) to biologically inactive 25(OH.)D or calcidiol.[42,43] With the aid of the enzyme 1-hydroxylase, 25(OH.)D is rehydroxylated in the kidney to the biologically active 1,25(OH)2D or calcitriol.[42,43] 1,25(OH)2D enters the bloodstream with VDBP and is transported to target tissues like the kidney, bone, and gut.[44] Calcium and phosphorus mobilization, absorption, and reabsorption are all regulated by active vitamin D. In carrying out this biological action, 1,25(OH)2D bind to the VDR found in osteoblasts to release biochemical signals that lead to the formation of mature osteoclasts.[9,45]

PTH is the main stimulator for producing active vitamin D by increasing the activity of CYP27B1.[41,46] 1,25(OH)2D and PTH influence bone homeostasis.[47] When vitamin D and calcium levels are low, the parathyroid glands will release PTH to induce CYP27B1 synthesis, increasing from 1,25(OH)2D.[43] In addition, PTH can also increase the absorption of calcium in the kidneys and intestines, increasing the body's calcium levels. Calcium levels are permanently monitored by calcium-sensing receptors (CaSR) in the parathyroid glands.[40,48] PTH can also increase the release of fibroblast growth factor-23 (FGF-23), produced by mature osteoblasts and bone cells.[47] Prolonged high PTH levels can cause a decrease in trabecular bone and cortical bone microstructure.[49–51] Thus, vitamin D and calcium deficiencies are frequently linked to increased bone turnover, fracture risk, and decreased bone mass.[52]

The age of the Wistar rats used in this study might be comparable to that of an adult human.[53] When comparing the ages of humans and animals, there is no easy way to determine the age difference because the results depend on the factors that are examined.[54] It can be cautiously applied to humans in future research.

CONCLUSIONS

In summary, this study shows that liprotide derived from β-lactoglobulin-oleic acid can protect and deliver vitamin D_3 to the target, as evidenced by a decrease in serum PTH levels and an improvement in bone

microstructure. More research is required to determine how it works in humans and how liprotide-encapsulated vitamin D_3 is absorbed in vitro.

ACKNOWLEDGMENT

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