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ORIGINAL ARTICLE

Impact of vitamin C on teriparatide treatment in the improvement of bone mineral density, strength, and quality in vitamin C-deficient rats

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Abstract
Age-related decreases in serum levels of vitamin C (VC) may negatively affect the efficacy of anti-osteoporotic pharmacotherapy. The purpose of this study was to evaluate the effects of VC and teriparatide (TPTD) on bone mineral density (BMD), strength, and quality in VC-deficient osteogenic disorder Shionogi (ODS) rats. Six-month-old female ODS rats were divided into an untreated ODS control group, a VC group, a TPTD group, and a VC + TPTD group, based on the administration of VC and TPTD ($n = 10$ each). VC was given as 2.0 mg/ml supplemented water. TPTD was administered subcutaneously once a week at 30 μg/kg body weight. After 12 weeks of treatment, BMDs of the femur and lumbar spine, bone strengths of the femoral diaphysis and metaphysis, and cancellous bone quality of proximal tibiae as estimated by Fourier transform infrared spectroscopy (FTIR) were compared between groups. Compared to the ODS control group, the VC group showed significantly higher total femoral BMD, but the TPTD group showed significantly higher femoral and lumbar spinal BMD, maximum load of femoral metaphysis, and hydroxyapatite (HA) crystallinity by FTIR ($p < 0.05$). In addition to the increases shown in the TPTD group, the VC + TPTD group also showed significantly higher stiffness of the femoral diaphysis and breaking energy of the femoral metaphysis compared to the ODS control group ($p < 0.05$). These results indicated that TPTD alone increased cancellous/cortical BMD and cancellous bone strength with improvement of HA crystallinity in ODS rats, but addition of VC supplementation further improved cortical bone strength.

Keywords Ascorbic acid · Bone quality · Bone strength · Teriparatide · Vitamin C

Introduction

Osteoporosis is a skeletal disorder characterized by compromised bone strength predisposing the individual to increased risk of fracture [1]. Osteoporosis is an important worldwide health problem, increasing mortality along with the risk of fragility fractures [2, 3]. Although bone mineral density (BMD) has been used for over 20 years as the primary method of identifying individuals with high fracture risk, its ability to predict fracture has well-recognized limitations [4]. Changes in fracture risk that are greater than expected from BMD are attributed to impaired "bone quality" [4]. Bone strength is thus attributable to not only BMD, but also bone quality [5, 6]. Bone quality is a broad term encompassing factors affecting the structural and material properties of bone. Material properties include mineral, collagen and microdamage, and are regulated by bone tissue turnover rate, cellular activity, and levels of glycation and oxidative stress [7, 8].

Oxidative stress is thought to be a critical factor in the bone loss seen during aging in both women and men [7]. Vitamin C (VC), also known as ascorbic acid, is an important antioxidant and cofactor that is involved in the regulation of development, function, and maintenance of several cell types in the body [9]. VC is known to act as a scavenger of superoxide anion and hydrogen peroxide [10]. VC also exerts a positive effect on trabecular bone formation by influencing expression of bone matrix genes in osteoblasts [9]. As such, although VC is essential for the differentiation of osteoblasts [11, 12], serum levels of VC reportedly decrease with age [13, 14]. Such age-related decreases in

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serum levels of VC may negatively affect anti-osteoporotic pharmacotherapy. However, few studies have examined the efficacy of anti-osteoporotic pharmacotherapy under conditions of VC deficiency.

We have recently investigated the effects of minodronate, a nitrogen-containing bisphosphonate, alone or in combination with VC on BMD, bone strength, and bone quality as determined using Fourier transform infrared spectroscopy (FTIR) in VC-deficient osteogenic disorder Shionogi (ODS) rats [15]. Combined treatment with minodronate and VC, but not minodronate alone, was found to improve BMD and bone strength, but no significant differences in parameters evaluated by FTIR were observed between groups [15]. Those results indicated that VC supplementation was essential in allowing bisphosphonate treatment to increase bone strength in ODS rats, and BMD might be more important than bone quality for bone strength in this rat model.

More recently, we have reported preliminary data for combination therapy with teriparatide (TPTD), as a potent promoter of bone formation, and VC on BMD and bone strength in ODS rats for up to 8 weeks [16]. The results showed that a single administration of VC or TPTD improved BMD, but not bone strength, whereas combined treatment with VC and TPTD improved both BMD and bone strength in ODS rats [16]. However, that preliminary study of VC and TPTD did not evaluate bone quality. The impact and role of VC and TPTD on bone quality and strength under conditions of VC deficiency might differ from those of anti-resorptive agents such as bisphosphonates. The purpose of the present study was thus to evaluate the effects of VC and TPTD on bone quality, as well as BMD and bone strength in VC-deficient ODS rats, using a study protocol lengthened to 12 weeks of treatment to limit the time-frame of the study and ensure physiological conditions are within normal parameters.

Materials and methods

Animals

Four-month-old female ODS rats (Clea Japan, Tokyo, Japan) were used in this study. Unlike normal rats, ODS rats cannot synthesize VC because of a lack L-gulono-γ-lactone oxidase, and develop body weight loss, scurvy, osteoporosis [17, 18]. When ODS rats are fed a VC-free diet, serum VC level decrease to about one-fortieth from 2 to 5 weeks of treatment without VC-supplemented water [19], and polypeptide hydroxyproline levels, which are known to be related to collagen synthesis [20], decrease to below those in normal rats after 1 week and are about one-third of the normal level after 2 weeks [17, 18]. When VC is added to drinking water, scorbutic symptoms resolve within a few days. VC-deficient ODS rats demonstrated impaired bone formation such as decreased serum level of osteocalcin [21], osteoid surface [15] and mineral apposition rate, bone formation rate and mineralizing surface by bone histomorphometry [21]. Furthermore, VC deficiency caused misassembly of the triple helices of collagenous alpha chains in VC-deficient ODS rats [22].

ODS rats were housed in a controlled environment at 22 °C with a 12-h light/dark cycle and were pair-fed and allowed ad libitum access to water and standard VC-free diet (CE-2; Clea Japan) containing 1.14% calcium, 1.06% phosphorus, and 250 IU of vitamin D_3 per 100 g of food, as described previously [23, 24]. Rats received 2.0 mg/ml of VC (Iwaki Pharma, Tokyo, Japan) in drinking water for 16 weeks from birth. At 4 months old, the dose of VC in drinking water was reduced to 0.5 mg/ml [25] for 8 weeks to create a VC-deficient condition.

Experimental design

After the 8-week period of providing VC-deficient (0.5 mg/ml) drinking water, the 6-month-old rats were divided into the following four groups ($n = 10$ per group): (1) VC-deficient ODS control group, given VC-deficient water (0.5 mg/ml); (2) VC group, given VC-supplemented water (2.0 mg/ml); (3) TPTD group, given VC-deficient water and administered TPTD; and (4) VC + TPTD group, given VC-supplemented water and administered TPTD.

TPTD (Asahi Kasei Pharma, Tokyo, Japan) was dissolved in saline containing 0.1% rat serum albumin, and a dosage of 30 μg/kg body weight was administered subcutaneously once a week for 12 weeks based on a previously reported protocol [26]. Animals were euthanized under anesthesia with an intra-abdominal injection of ketamine (Sankyo, Tokyo, Japan) and xylazine (Zenoaq, Fukushima, Japan), and bilateral femora and tibiae and the lumbar vertebrae were harvested. Animal experimental protocols were approved by the Animal Committee of our institute (approval number: a-1-2516), and all animal experiments conformed to the "Guidelines for Animal Experimentation" of our institute.

Sample preparation

The right femur and lumbar vertebrae were fixed in 10% neutral-buffered formalin until preparation for BMD measurement. The right tibia was fixed in 70% ethanol until preparation for FTIR to evaluate bone quality. The left femur was dissected free of soft tissue, wrapped in gauze moistened with saline, and frozen at − 80 °C until biomechanical testing.

BMD measurement

BMDs of the right femur and L4–L5 lumbar vertebrae were measured by dual-energy X-ray absorptiometry (QDR-4500; Hologic, Waltham, MA). We measured BMD of the total femur, distal one-third of the total femur length as the metaphysis (a cancellous bone-rich area) and middle one-third of the total femur length as the diaphysis (a cortical bone-rich area). Vertebral BMDs were determined using manually set regions of interest at L4–L5 and measured based on the previous study [27].

Biomechanical testing

Mechanical testing of the left femur was performed at room temperature using a material testing machine (MZ500S; Maruto, Tokyo, Japan). For stabilization, the mid-diaphysis of the femur was placed on two supports of the test apparatus that were 20 mm apart. The load of a three-point bending test was applied in the anteroposterior direction midway between the two supports. Load–displacement curves were recorded at a crosshead speed of 5 mm/min. Breaking force (N), breaking energy (N mm), maximum load (N), and stiffness (N/mm) were calculated using software for the measurement of bone strength (CTR win. version 1.05; System Supply, Nagano, Japan), as previously described [16]. After the three-point bending test, the distal part of the femur was evaluated by a compression test, as previously described [28]. Load–displacement curves were recorded and the breaking force (N), breaking energy (N mm), maximum load (N), and stiffness (N/mm) were calculated using the same software.

FTIR

FTIR was performed as previously described [15]. The right tibia was embedded in polymethylmethacrylate (PMMA), and cut into 3-μm-thick sections. These undercalcified sections were mounted on barium fluoride infrared windows (SpectraTech, Hopewell Junction, NY), and examined using a Spotlight 400 Infrared Imaging System (PerkinElmer Instruments, Waltham, MA) at a spectral resolution of 4 cm^{-1}. Mineral and matrix properties were then assessed in five different regions of cancellous bone. Mean and standard deviation (SD) for five images in these areas were calculated and then averaged for each area for comparisons among groups. Cross sections were processed for FTIR in a blinded manner and codes identifying groups were not broken until statistical analysis was performed. Spectra were baseline-corrected and the PMMA spectral contribution was subtracted using OMNIC8, TQ Analyst (Thermo Fisher Scientific, Kanagawa, Japan), as previously described in detail [29]. The mineral/matrix ratio, hydroxyapatite (HA) crystallinity, and collagen cross-linking network maturity were evaluated in this study. Mineral/matrix ratio was calculated from the integrated areas of phosphate (907–1183 cm^{-1}) to amide I (1586–1710 cm^{-1}), reflecting bone mineral content. HA crystallinity was measured as the phosphate sub-band 1030/1020 cm^{-1} peak intensity ratio, indicating mineral crystal size and perfection as determined by X-ray diffraction. Collagen cross-linking network maturity was determined as the peak intensity ratio of amide I sub-bands at 1660 cm^{-1}.

Statistical analyses

All values are expressed as mean ± SD. According to the Kolmogorov–Smirnov test, all data were parametric. Differences among groups were evaluated using Scheffe's post hoc test for multiple comparisons with one-way analysis of variance (ANOVA). All statistical analyses were performed using Statistical Package for the Biosciences software (SPBS version 9.4; Akita University Graduate School of Medicine, Akita, Japan) [30]. Values of $p < 0.05$ were considered statistically significant.

Results

BMD

Comparing to ODS control group, TPTD and VC + TPTD groups showed significantly higher BMDs for all the measurement sites in the femur (total, mid 1/3, and distal 1/3) and lumbar spine (Table 1). In addition, TPTD and VC + TPTD groups showed significantly higher BMD than the VC group for the distal femur and lumbar spine, as cancellous bone-rich sites. Significant differences between ODS control and VC groups were only seen for total femoral BMD. No significant differences were seen between TPTD and VC + TPTD groups at any BMD measurement site.

Bone strength by biomechanical testing

In the femoral diaphysis, a cortical bone-rich site, the VC + TPTD group showed significantly higher stiffness than the ODS control group (Table 2). However, no significant differences were seen between these groups in terms of breaking force, breaking energy, or maximum load in the femoral diaphysis.

In the femoral metaphysis, a cancellous bone-rich site, the VC + TPTD group showed significantly higher breaking energy and maximum load than the ODS control and VC groups. The TPTD group also showed significantly higher maximum load than the ODS control and VC groups. Breaking force and stiffness of the

Table 1 Comparison of bone mineral density (g/cm^2) between groups

	ODS	VC	TPTD	VC + TPTD	ANOVA (*p*)
Femur					
Total	0.227 ± 0.009	0.247 ± 0.013[a]	0.263 ± 0.012[a']	0.278 ± 0.020[a']	< 0.001
Mid 1/3 (diaphysis)	0.250 ± 0.009	0.266 ± 0.017	0.285 ± 0.027[a']	0.281 ± 0.014[a]	< 0.001
Distal 1/3 (metaphysis)	0.262 ± 0.008	0.269 ± 0.015	0.312 ± 0.020[a',b]	0.312 ± 0.020[a',b]	< 0.001
Lumbar spine	0.218 ± 0.004	0.223 ± 0.009	0.255 ± 0.011[a',b]	0.256 ± 0.006[a',b]	< 0.001

Values are given as mean ± standard deviation (SD)

ODS vitamin C-deficient osteogenic disorder Shionogi rats as controls; *VC* ODS rats supplemented with vitamin C; *TPTD* ODS rats administered teriparatide; *VC + TPTD* ODS rats administered both vitamin C and TPTD; *ANOVA* one-way analysis of variance

[a] $p < 0.05$, [a'] $p < 0.01$ vs ODS control group by Scheffe's multiple comparison method

[b] $p < 0.01$ vs VC group by Scheffe's multiple comparison method

Table 2 Comparison of femoral bone strength between groups

	ODS	VC	TPTD	VC + TPTD	ANOVA (*p*)
Three-point bending test (diaphysis)					
Breaking force (N)	109.26 ± 20.44	118.65 ± 19.77	127. 05 ± 18.18	132.17 ± 19.96	0.073
Breaking energy (N mm)	70.90 ± 9.67	69.86 ± 13.06	86.37 ± 17.58	86.75 ± 12.24	0.074
Maximum load (N)	124.44 ± 12.40	130.62 ± 21.72	142.19 ± 10.09	146.66 ± 31.53	0.081
Stiffness (N/mm)	273.22 ± 30.85	282.40 ± 30.87	293.93 ± 23.74	320.84 ± 40.65[a]	0.021
Compression test (metaphysis)					
Breaking force (N)	164.86 ± 28.38	136.42 ± 48.84	196.00 ± 41.36	181.24 ± 73.75	0.069
Breaking energy (N mm)	274.69 ± 31.99	275.62 ± 43.72	311.73 ± 71.63	356.90 ± 69.29[a,b]	0.009
Maximum load (N)	201.62 ± 14.13	200.83 ± 31.58	250.32 ± 31.44[a,b]	261.84 ± 41.47[a',b']	< 0.001
Stiffness (N/mm)	209.53 ± 31.97	228.03 ± 59.49	200.37 ± 84.87	253.92 ± 107.36	0.437

Values are given as mean ± standard deviation (SD)

ODS vitamin C-deficient osteogenic disorder Shionogi rats as a control; *VC* ODS rats supplemented with vitamin C; *TPTD* ODS rats administered teriparatide; *VC + TPTD* ODS rats administered both vitamin C and TPTD; *ANOVA* one-way analysis of variance

[a] $p < 0.05$, [a'] $p < 0.01$ vs ODS control group by Scheffe's multiple comparison method

[b] $p < 0.05$, [b'] $p < 0.01$ vs VC group by Scheffe's multiple comparison method

femoral metaphysis showed no significant difference between groups. No significant differences were seen in any parameters from biomechanical testing between ODS control and VC groups, nor between TPTD and VC + TPTD groups.

Bone quality by FTIR

Among the parameters for bone quality of cancellous bone obtained by FTIR, HA crystallinity was significantly higher in the TPTD and VC + TPTD groups than in the ODS control and VC groups (Table 3). Mineral/matrix ratio and collagen maturity showed no significant difference between groups. No significant differences were seen in any parameter from FTIR between ODS control and VC groups, nor between TPTD and VC + TPTD groups.

Discussion

VC and bone

For the management of osteoporosis, nutritional factors are important along with anti-osteoporotic pharmacotherapy. Although calcium and vitamin D have been the primary focus of nutritional factors for osteoporosis, supplementation with vitamins B, C, K, and silicon have also been recommended for proper maintenance of bone health based on a review of the literature [31]. VC is a micronutrient predominantly found in vegetables and fruits. In bone tissue, VC has been known to exert positive effects on trabecular bone formation by influencing expression of bone matrix genes in osteoblasts [9].

Epidemiological studies have reported positive associations between VC intake from foods and supplements and

Table 3 Comparison of parameters for bone quality in cancellous bone of the proximal tibial by Fourier transform infrared spectroscopy between groups

	ODS	VC	TPTD	VC + TPTD	ANOVA (*p*)
Mineral/matrix ratio	7.99 ± 0.77	8.09 ± 0.68	7.82 ± 0.26	8.22 ± 0.67	0.399
HA crystallinity	1.12 ± 0.04	1.11 ± 0.02	1.15 ± 0.02[a,b]	1.16 ± 0.02[a,b]	< 0.001
Collagen maturity	3.18 ± 0.16	3.18 ± 0.17	3.17 ± 0.13	3.14 ± 0.04	0.824

Values are given as mean ± standard deviation (SD)

ODS vitamin C-deficient osteogenic disorder Shionogi rats as a control; *VC* ODS rats supplemented with vitamin C; *TPTD* ODS rats administered teriparatide; *VC + TPTD* ODS rats administered both vitamin C and *TPTD*; *ANOVA* one-way analysis of variance; *HA* hydroxyapatite

[a] $p < 0.05$ vs ODS control group by Scheffe's multiple comparison method

[b] $p < 0.01$ vs VC group by Scheffe's multiple comparison method

higher bone density [32, 33], and inverse associations with bone loss and risk of fractures [34, 35]. A more recent epidemiological study showed that in addition to positive associations between VC intake and heel ultrasound measures, higher plasma VC concentrations were associated with significantly reduced fracture risk in men [36]. The results of these studies indicated that VC can be considered an essential factor for the management of osteoporosis. However, serum levels of VC reportedly decrease with age [13, 14]. Age-related decreases in serum levels of VC thus may also negatively impact the effectiveness of anti-osteoporotic pharmacotherapy.

VC deficiency and bone quality

Several previous studies have demonstrated changes in bone quality by VC-deficient conditions in ODS rats. Togari et al. [19] described that VC deficiency caused marked bone loss and reduction in the bone formation, which was accompanied by the reduction in biomechanics of the femur without macroarchitectural changes in ODS rats. The total level of amino acids in bone collagen and rates of proline and lysine hydroxylation, which are related to the mechanical properties of cortical bone, were significantly lower in the VC-deficient ODS rats [21]. Hasegawa et al. [22] reported that VC deficiency caused misassembly of the triple helices of collagenous alpha chains in VC-deficient ODS rats. We consider FTIR as a newly developed, more generalized method to evaluate bone quality. However, the bone quality changes of VC deficiency or the supplemental effects of VC on bone quality in ODS rats were not significant according to FTIR in the present study. Additional specific methods described in the previous studies might detect an obvious change to bone quality in VC-deficient or supplemented ODS rats.

Effects of TPTD on bone

Among numerous categories of drugs used to treat osteoporosis, TPTD is a unique anti-osteoporotic agent because of its potent bone-forming effects [37, 38]. Randomized clinical trials have shown that TPTD significantly increased BMD in the lumbar spine by 6.7–13.4% from baseline [39–41] and prevented new vertebral fractures with a relative risk reduction of 65–84% compared to placebo controls [40–42].

Previous studies in animals and humans have also shown that TPTD has positive effects on bone quality, which is the counterpart to BMD in contributing to bone strength. TPTD has been reported to affect several parameters of bone quality, including initial mineralization, collagen maturity, and material properties of the bone tissue determined by mineral characteristics such as crystallinity and carbonate substitution [43–45]. Detailed studies of the effects of TPTD on bone quality using FTIR have also been reported [46, 47]. Paschalis et al. [46] demonstrated that TPTD administration in ovariectomized monkeys resulted in a lower degree of mineralization, reduced mineral crystallinity, and decreased collagen cross-link ratio in bone by FTIR analysis [46]. Similar findings have been shown in human biopsy specimens obtained from the iliac crest after TPTD treatment in patients with postmenopausal osteoporosis [47]. Such findings indicate that the bone-forming effects of TPTD result in bone with a molecular profile reminiscent of younger bone [47]. Thus, in addition to the strong effects on BMD, such bone-rejuvenating effects of TPTD are thought to contribute to bone strength in osteoporosis under estrogen-deficient conditions.

TPTD effects on BMD in VC-deficient conditions

The present study assessed the effects of TPTD on femoral and lumbar spinal BMD under VC-deficient conditions in rats. Because most animals, including rats and mice, are capable of de novo synthesis of VC [9], we used ODS rats with a hereditary defect in the ability to synthesize VC [17, 18].

This study showed that TPTD administration in ODS rats increased BMD at all measurement sites on the femur (total, mid 1/3, and distal 1/3) and lumbar spine under conditions both with and without VC administration (i.e., TPTD and VC + TPTD groups) compared to ODS rats without

VC administration (i.e., ODS control group). Furthermore, BMDs in the distal 1/3 of the femur and lumbar spine in ODS rats receiving TPTD with and without VC (TPTD and VC + TPTD groups) were significantly higher than those of ODS rats receiving VC alone (VC group). These results indicated that TPTD is more effective on BMD in cancellous bone-rich sites, in both the presence and absence of VC. Clinical trials have demonstrated that the effects of TPTD on bone were more obvious at cancellous bone-rich regions than at cortical bone-rich regions [39, 41]. Consistent with such clinical evidence, the effects of TPTD on BMD might predominantly act through cancellous bone-rich sites even under VC-deficient conditions.

TPTD effects on bone strength and quality under VC-deficient conditions

The major findings of biomechanical testing in this study were the increased stiffness of cortical bone with VC and TPTD administration and the increased breaking energy and maximum load in cancellous bone with TPTD administration. Although improvement of other parameters did not reach the level of statistical significance, we observed a clear trend in TPTD administration groups compared to non-TPTD administration groups. As with the results of BMD, the effects of TPTD were more obvious in cancellous-rich regions than in cortical-rich regions.

Furthermore, TPTD administration also increased the HA crystallinity of tibial cancellous bone in VC-deficient ODS rats. HA crystallinity indicates the size of mineral crystals. Iwasaki et al. [48] have reported that crystallinity in the femur of rats with chronic kidney disease (CKD) was decreased, and that crystallinity showed a significant positive correlation with parameters of mechanical properties in CKD rats [49]. Considering these results, we speculated that TPTD improved the bone strength in ODS rats by increasing HA crystallinity. However, several previous studies have demonstrated that daily TPTD treatment decreased mineral crystallinity at the iliac crest of periosteal, endosteal, and trabecular sites in patients with postmenopausal osteoporosis [47, 50].

The frequency or dosage of TPTD administration exerts different effects on bone turnover or cortical porosity [26]. The effects of TPTD on the material properties of cancellous or cortical bone might thus differ under the different osteoporotic conditions such as VC-deficient or CKD rats and postmenopausal women. However, one possible reason for the discrepancy between the present study and previous reports about the effects of TPTD on HA crystallinity might be the difference in underlying controls. HA crystallinity in VC-deficient ODS rats might be low compared to normal, non-ODS rats. If so, the present results might be explained by the effects of TPTD on normalization for crystallinity. Further investigations including comparison of ODS rats and normal rats in regard to bone material properties are required.

Limitations

This study is the first to report the effects of TPTD on bone under VC-deficient conditions. However, limitations to this study should also be noted. First, the evaluation sites of cancellous and cortical bone for BMD measurement, for mechanical testing, and for FTIR were different. This was due to the limited samples available for evaluation. Second, the dose or duration of TPTD treatment was single in the present study, even though we performed prior treatment to evaluate the effects of TPTD on BMD or bone strength with 8 weeks of treatment [16]. Longer investigations than the present study might be required, but our preliminary observations suggested that ODS rats without VC administration for more than 12 weeks provide a model of a very pathological condition.

In conclusion, administration of TPTD without VC supplementation increased both cancellous and cortical BMD, cancellous bone strength, and cancellous bone quality in VC-deficient ODS rats. However, TPTD treatment required VC supplementation to improve the stiffness of cortical bone strength and the breaking energy of cancellous bone strength in the ODS rats. VC sufficiency might be an important factor to obtain the effectiveness of TPTD therapy against osteoporosis.

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

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