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25-Hydroxyvitamin D_3 promotes slow-twitch fiber type transition in skeletal muscle

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Abstract

Vitamin D supports musculoskeletal health, including bone mineral density and skeletal muscle function. Vitamin D_3 ($VitD_3$, cholecalciferol) is an inactive form which requires two enzymatic hydroxylation steps, from $VitD_3$ to 25-hydroxyvitamin D_3 ($25OHD_3$, calcifediol) in liver and then to active form 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$, calcitriol) in kidney. Vitamin D status is determined by circulating 25OHD levels. Compared with $VitD_3$, calcifediol bypasses the hepatic 25-hydroxylation step and is more hydrophilic, resulting in higher bioavailability and less sequestration in adipose tissue. Previous studies have linked both $VitD_3$ and $25OHD_3$ to muscle mass, strength or atrophy markers, but their effects on skeletal muscle fiber-type composition remains unclear. We hypothesize that dietary $25OHD_3$ versus $VitD_3$ differentially modulates skeletal muscle fiber composition during postnatal growth. Three-week-old male rats were fed a $VitD_3$–deficient diet for 4 weeks and then assigned for 4 weeks to diets that were deficient or replete with $VitD_3$ or $25OHD_3$. Relative to $VitD_3$ repletion, $25OHD_3$ repletion increased the proportion of slow-twitch type I fibers and decreased fast type IIb fibers in the *bicep femoris*, without altering muscle mass. This shift was accompanied by higher mitochondrial DNA copy number. Transcriptomic profiling indicated enrichment of extracellular signal-regulated kinase (ERK) and calcium signaling pathways, including genes in vascular endothelial growth factor receptor-2 (VEGFR2) and nitric oxide synthase (NOS) pathways and inositol 1,4,5-trisphosphate kinase (IP3K). These data suggest that, following early-life

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vitamin D deficiency, dietary 25OHD$_3$ preferentially promotes slow-oxidative fiber transition compared with VitD$_3$, and is associated with transcriptional enrichment of ERK- and calcium-dependent signaling.

Keywords: Vitamin D$_3$; 25-Hydroxyvitamin D$_3$; Rat; Skeletal muscle fiber type

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Introduction

The benefits of vitamin D for bone health[1,2], cancer[3], and type 2 diabetes[4] are increasingly being reported. Physiological vitamin D levels are associated with skeletal muscle health. Vitamin D signaling induces skeletal muscle cell hypertrophy[5], strengthens muscle[6,7], and plays a key role in modulating the skeletal muscle fiber type[8]. Although the function of vitamin D in modulating the skeletal muscle fiber type remains controversial, elderly individuals deficient in vitamin D have been reported to be susceptible to sarcopenia, possibly because of type IIb fiber-specific atrophy[9]. Clinical trials have shown that dietary $VitD_3$ supplementation alleviates symptoms of myopathy by increasing the fiber cross-sectional area (FCSA) and proportion of type IIb fibers[10]. However, intramuscular administration of calcitriol did not affect the slow and fast fiber-type composition during skeletal muscle regeneration in mouse[11], indicating that the impact of vitamin D on skeletal muscle fiber type composition remains unclear.

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Cholecalciferol is an inactive form, and it requires two enzymatic hydroxylation steps. First, hepatic 25-hydroxylase converts $VitD_3$ to $25OHD_3$. Calcifediol is present in circulation and determines vitamin D status in the body. Subsequently, $25OHD_3$ is further converted to active form $1,25(OH)_2D_3$ by 1α-hydroxylase in kidney[12]. Oral $VitD_3$ supplementation is commonly used in clinical applications. However, the optimal doses and duration of vitamin D supplementation have been reported to vary in clinical trials, with responses differing depending on

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the health status, physical activity, and lifestyle of individuals[13]. Besides $VitD_3$, 25OHD₃, a precursor of $1,25(OH)_2D_3$, which is also found in food, can be orally administered[14]. Intake of $25OHD_3$ increases the circulating levels of 25OHD 3–5 times of the levels achieved with $VitD_3$ intake[15,16]. Moreover, increased muscle fiber size in elderly women with vitamin D insufficiency[10] and improved breast meat yield in broiler chickens[17] upon $25OHD_3$ intake suggest its greater impact on skeletal muscle compared with that of VitD₃. However, comparative effect of $25OHD_3$ and VitD₃ on skeletal muscle fiber type composition are not fully elucidated. Therefore, this study aimed at determining the effect of dietary $25OHD_3$ supplementation on skeletal muscle fiber type distribution of *bicep femoris* in growing rats and comparing the biological responses along with transcriptome profiles.

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Results

Dietary supplementation of VitD₃ and 25OHD₃ did not affect the growth performance and lean content in rats

Experimental scheme for dietary $VitD_3$ and $25OHD_3$ was depicted in **Fig. 1**. Briefly, we aimed to clear the baseline of vitamin D in rats during Period I by providing $VitD_3$-deficient diet for the first 4 weeks. Subsequently, diets were supplemented with $VitD_3$ or $25OHD_3$ for the next 4 weeks. Positive control group (CON) was provided with recommended Vit D_3 (1000 IU/kg diet) throughout 8 weeks of study. No differences in rat body weight, feed intake, and feed efficiency were noted among the

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treatment groups during 8 weeks of the experimental period (**Table 1**). Dietary supplementation of $VitD_3$ up to 2,000 IU/kg diet or of $25OHD_3$ at 400 IU/kg diet after vitamin D depletion did not affect the growth performance of rats. Vitamin D has been reported to affect energy metabolism and body composition[18]. To examine whether dietary $VitD_3$ or $25OHD_3$ supplementation could modulate energy metabolism, lean and fat content of rats was determined at the end of the experiment. No significant differences in the content of lean or fat were observed among the treatments (**Supplementary Fig. S1**). The dietary supplementations did not affect the plasma calcium and phosphate levels and BMD (**Supplementary Table S1**).

Dietary $25OHD_3$ and $VitD_3$ supplementation affected the plasma 25-hydroxyvitamin D levels in a dose-dependent manner

Plasma levels of 25OHD were measured at the beginning of experiment (day 0) and at the end of period I (day 28) (**Fig. 2A**) and II (day 56) (**Fig. 2B**). After the first 4 weeks of vitamin D depletion (period I), plasma 25OHD levels were lower in rats fed the $VitD_3$-deficient diet (D_3 0) than those in CON rats. Plasma 25OHD levels increased linearly with the increase in dietary $VitD_3$ dose over the next 4 weeks (period II) up to 2,000 IU $VitD_3$/kg diet. Moreover, rats supplemented with 400 IU $25OHD_3$/kg had the highest plasma 25OHD levels, which were even higher than in CON rats that were fed 1,000 IU $VitD_3$ for the entire 8-week period. Compared with the same dose (400 IU/kg diet), dietary $25OHD_3$ showed a 2.3-fold

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increase in plasma 25-hydroxyvitamin D levels (23.5 ± 5.1 ng/mL vs. 53.3 ± 1.5 ng/mL) during the 4 weeks of supplementation.

25-Hydroxyvitamin D_3 supplementation increased the percentage of type I fiber at the expense of type IIb fiber in the bicep femoris of rats

To examine the responses of the skeletal muscle to different doses of $VitD_3$ and $25OHD_3$, the mass and fiber type composition of *bicep femoris* were quantified. No differences in skeletal muscle mass or fiber cross sectional area (FCSA) was observed (**Table 2**). However, $VitD_3$-deficient mice showed the lowest type I fiber area (%). Fiber type I area (%) was increased in the rats supplemented with $VitD_3$, including CON, $VitD_3$ 400-2000 IU/kg diet, and was significantly higher in the rats fed $25OHD_3$. Similarly, the fiber type composition of the muscle changed with dietary $VitD_3$ supplementation (**Fig. 3A, Fig. 3B**). Vitamin D-deficient rats had the lowest percentage of type I fiber ($P < 0.05$). Percentage of type I fiber increased with $VitD_3$ repletion compared to Vit D_3-deficient rats, and 4 weeks of supplementation between 400 IU and 2000 IU $VitD_3$/kg diet after deficiency was sufficient to recover type I fiber as the levels were comparable to CON, which had 1,000 IU $VitD_3$/kg diet throughout 8 weeks. However, in the skeletal muscle of rats fed 400 IU $25OHD_3$/kg diet, the percentage of type I fiber was further elevated compared with that in rats fed $VitD_3$ and was even higher than that in CON rats. In contrast, the percentage of type IIb fiber numerically decreased in rats fed 2,000 IU

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$VitD_3$/kg diet compared to CON, and its proportion significantly reduced in the rats fed 400 IU $25OHD_3$/kg diet compared with that in the other groups < 2000 IU $VitD_3$/kg diet, including CON ($P < 0.05$). No difference was observed in the percentage of type IIa fiber among the treatment groups. Moreover, FCSA was not significantly different among the treatment groups (Table 3). Therefore, the fiber type distribution was consistent with the total area of each fiber type.

Relative mtDNA copy number was increased upon dietary 25OHD$_3$ supplementation

The fiber type composition of the skeletal muscle is associated with mtDNA copy number. The oxidative/fiber type I (slow-twitch) requires relatively higher mtDNA copy number, whereas the glycolytic/type IIb (fast-twitch) fiber has lesser numbers[19]. Therefore, we hypothesized that dietary vitamin D supplementation induces changes in fiber type composition during postnatal skeletal muscle development. Relative copy number of *mt-Nd1*, a representative gene for mitochondrial gene involved in oxidative phosphorylation, was elevated in the rats fed $25OHD_3$-replete diets compared with that in the other rats subjected to $VitD_3$-deficient diet during Period I (**Fig. 3C**). This result corroborated with the increased percentage of type I fiber in rats supplemented with $25OHD_3$ whereas CON and vitamin D-deficient rats contained lower percentage of type I fiber and higher of IIb fiber ($P < 0.05$). Notably, plasma 25OHD increased in a saturable manner with dietary vitamin D_3 intake from 0 to 2,000 IU/kg

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with the nonlinear regression (R^2 = 0.73, P < 0.0001) (**Fig. 4A**), and the percentage of type I fibers tended to rise with higher plasma 25OHD, and this relationship was described by a nonlinear regression curve with a modest but significant fit (R^2 = 0.33, P = 0.020) (**Fig. 4B**). In contrast, type IIb fiber percentage showed a slight decline across the vitamin D_3 dose range (R^2 = 0.32, P = 0.024; **Fig. 4C**), together indicating that dietary VitD_3 supplementation during postnatal growth impacts the fiber type composition. When plotted against plasma 25OHD, type IIb fiber percentage showed no statistically significant association (R^2 = 0.14, P = 0.16) (**Fig. 4D**).

Both vitamin D_3 and 25-hydroxyvitamin D_3 supplementation upregulated the expression of slow-twitch fiber type-specific genes in rat bicep femoris compared with that under vitamin D deficiency

In accordance with the increase in mtDNA copy number caused by dietary 25OHD_3 supplementation, transcriptome analysis of the skeletal muscle was conducted to identify corresponding changes in mRNA levels in *bicep femoris*. For the transcriptome analysis, DEGs were compared among the four treatment groups, VitD_3-deficient group (D_3 0) as a negative control (NC, T1), control group (CON) as a positive control (PC, T2), VitD_3-replete group with 1000 IU/kg diet (D_3 1000, T3), and the group replete with 25OHD_3 (25OHD_3 400, T4). We chose the D_3 1000 group as one providing vitamin D levels adequate for growing rats, referring to the NRC prescribed levels. To understand the differences in the skeletal

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muscle transcriptome in the four treatment groups, we compared each group with NC and PC. As illustrated in volcano plots, each comparison exhibited different numbers of DEGs (**Fig. 5, Supplementary Table S3**). Irrespective of the comparison with NC and PC, both $VitD_3$ and $25OHD_3$ treatment was associated with a greater number of upregulated genes than downregulated genes. Notably, the scarce number of DEGs between T2 and T3 suggested that after depletion, $VitD_3$ supplementation at 1000 IU for 4 weeks was sufficient to reach a comparable level in the transcriptome of rat skeletal muscle (data not shown).

Functional enrichment analysis of DEGs revealed upregulation of genes for slow- and fast-fiber transition, muscle contraction, and structural proteins in skeletal muscle, including myosin heavy chain (*Myh*), ATPase sarcoplasmic/endoplasmic reticulum Ca^{2+} transporting 2 (*Atp2a2*), and troponin C (*Tnnc1*), which contribute to slow-twitch fiber, in all the three groups of vitamin D-replete groups (PC, $VitD_3$, and 25OHD) (**Fig. 5A-C**). In addition, the expression levels of genes involved in innate immune responses were elevated, supporting the notion that vitamin D modulates the immune responses[20]. Compared with NC or PC, $25OHD_3$ was associated with more robust defense mechanism toward viral responses, including upregulation of interferon regulatory factor 7 (*Irf7*) and DEXH-box helicase 58 (*Dhx58*), also known as RIG-I-like receptor 2 (LGP2) (**Fig. 5C, 5D**). **Fig. 5E** depicts the distinct biological processes enriched upon $25OHD_3$ supplementation that potentially enhanced the contribution toward slow-twitch fiber type transition compared with that for $VitD_3$

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supplementation. Notably, $25OHD_3$ increased the expression of genes involved in angiogenesis (ERK signaling pathway), including vascular endothelial growth factor receptor 2 (*Vegfr2*) and nitric oxide synthase 3 (*Nos3*), which are also responsible for slow fiber type transition[21]. Detailed results of the functional enrichment analysis are provided in **Supplementary Table S4**.

25-Hydroxyvitamin D_3 supplementation modulated the expression of genes in the motor protein and calcium signaling pathways during slow fiber type transition in skeletal muscle

Skeletal muscles are composed of various types of structural components. Upon mapping of the DEGs (shown in **Fig. 5**) onto the KEGG pathway module for skeletal muscle motor proteins, a distinct pattern was observed for all the VitD-replete groups, with elevated levels of dynein (*Dnah*), myosin light chain (*Myl2* and *Myl3*), and troponin subtypes (*Tnnc1*, *Tnni1*, and *Tnnt1*), which are associated with slow skeletal muscle fiber type (**Fig. 6A**). Skeletal muscle contraction is driven by the intracellular calcium signaling pathway, and calcium flux is regulated at both membrane and endoplasmic reticulum (ER) levels[22,23]. As DEGs were mapped onto the calcium signaling pathway, SERCA, troponin C (*TnC*), and myosin light chain kinase (*Mlck*) were found to be significantly increased in all the Vit D-replete groups (**Fig. 6B**), consistent with significance of vitamin D in modulating skeletal muscle via intracellular calcium flux[7,24]. Furthermore, $25OHD_3$ supplementation was associated

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with the upregulation of genes encoding receptor tyrosine kinase (RTK), NOS3, and inositol-1,4,5-trisphosphate 3-kinase (IP3K), which correspond with upregulated ERK signaling pathway (VEGFR2 and NOS), a part of the RTK superfamily, when compared with the expression under $VitD_3$ supplementation (**Fig. 5E**). Overall, these results indicated that dietary supplementation of $VitD_3$ and $25OHD_3$ modulated skeletal muscle fiber composition by regulating motor proteins and calcium flux regulation at the transcriptome level. Additionally, dietary $25OHD_3$ supplementation preferentially promotes a shift toward a slow-oxidative fiber-type profile compared with $VitD_3$ and is associated with increased mitochondrial biogenesis and transcriptional enrichment of calcium-related pathways and ERK signaling pathways during postnatal development.

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Discussion

In this study, we examined the effects of dietary $VitD_3$ and $25OHD_3$ supplementation on skeletal muscle characteristics in growing rats. Prior to evaluating the $VitD_3$ effect in skeletal muscle, rats were fed $VitD_3$-deficient diet for 4 weeks to induce a basal, equivalent vitamin D status in all animals. The plasma levels of 25OHD, an index for vitamin D status, confirmed the effectiveness of feeding $VitD_3$-deficient diet for 4 weeks in inducing vitamin D deficiency. However, even 8 weeks of feeding $VitD_3$-deficient diet did not cause growth retardation in 3- to 11-week-old male Wistar rats. Additionally, body composition (lean and fat content), calcium, phosphate, and BMD remained unchanged across all treatment groups,

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including CON, in which the rats were fed the recommended doses (1000 IU/kg diet) of vitamin D[25] . These findings align partially with those of Stavenuiter et al. (2015)[26], who did not notice any growth difference between rats fed vitamin D-deficient diet or a diet containing the recommended levels of $VitD_3$. The period for inducing vitamin D deficiency in the study by Stavenuiter et al. (2015) was shorter (3 weeks) compared with that in the present study (4 weeks)[26]; however, the decreased plasma levels of 25OHD, with no growth difference, could be comparable in inducing vitamin D deficiency.

Plasma 25OHD levels responded to dietary supplementation of $VitD_3$ and $25OHD_3$. The levels increased linearly with the increase in $VitD_3$ dose from 0 ($VitD_3$-deficient diet) to 1000 IU/kg diet. As shown in Fig. 2B, supplementation of 2000 IU $VitD_3$ or 400 IU $25OHD_3$ per kg diet for 4 weeks restored the plasma 25OHD to levels similar to those in the CON group. We found a similar trend for the increase in plasma 25OHD levels as pharmacodynamics of vitamin D and 25OHD supplementation in human also requires 3 to 4 weeks to reach the maximal level[16].

Effects of dietary $VitD_3$ supplementation on muscle mass remain controversial as evident from several clinical trials in human subjects reviewed by Beaudart et al. (2014)[13]. However, in our study, the fiber type composition was changed in *bicep femoris* (Fig. 3A-B), which is primarily composed of fibers containing MHC IIb. Dietary $25OHD_3$ supplementation increased the percentage of type I fiber 2.8-times of the percentage achieved with the same dose of $VitD_3$ at 400 IU, and the increase in the

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proportion of type I fiber corresponded with the decrease in that of type IIb fiber. Thus, dietary $25OHD_3$ supplementation was effective inducing fast-to-slow fiber type transition in the skeletal muscle of young rats. Previously, the percentage of type IIb fiber was reported to increase with higher expression of the vitamin D receptor[27]. However, those findings were for *vastus lateralis*, a muscle primarily composed of type I fiber (>50 %). Nonetheless, because *bicep femoris* and *vastus lateralis* make up a large part of the limb muscle, the findings of Ceglia et al. (2013) provide additional evidence in support of the effects of vitamin D upon the skeletal muscle[27]. Although the fiber type transition in skeletal muscle is generally expected[28], it often requires a specific type of exercise (endurance or resistant)[29]. Therefore, dietary $VitD_3$ could impact fiber-type determination during development and prevention of fiber-type specific atrophy or weakness by increasing the proportion of slow-twitch fibers, which are more resistant to atrophy[30]. Notably, the percentage of type I fiber positively correlated with plasma 25OHD levels (Fig. 4B). Therefore, supplementation of both $VitD_3$ and $25OHD_3$ can be used to increase the plasma 25OHD levels, and is associated with fiber type proportion of *bicep femoris* in growing rats.

We also analyzed the *gastrocnemius* muscle in this cohort (data not shown) but did not find clear differences in fiber-type composition among the $VitD_3$ and $25OHD_3$ treatments, unlike in *bicep femoris*. This result may be due to the intrinsic difference between these two muscles rather than a real absence of effect. *Bicep femoris* is more type IIb- dominant and

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relatively homogeneous, whereas the *gastrocnemius* contains a mixed distribution of type I, IIa, IIx, and II fibers and shows greater heterogeneity. Small changes in fiber type may be more difficult to detect, which could blunt the response to $VitD_3$ or $25OHD_3$ in this muscle.

Slow-twitch fiber, with its higher oxidative capacity, is rich in mtDNA[19] (Fig. 3C). Corresponding to an increased percentage of type I fiber in this study, the mtDNA copy number was significantly higher in *bicep femoris* of rats fed 400 IU $25OHD_3$/kg diet compared with that in the other groups which underwent vitamin D_3-deficiency during period I, corroborating the increase in the percentage of oxidative fiber type I in *bicep femoris* (Fig. 3B). These recent findings report that VDR regulates mtDNA transcription by interacting with mtDNA transcription factor A (TFAM)[31], and that vitamin D receptor (VDR) ablation disrupts mitochondrial biogenesis (mtDNA copy number)[32], supporting the impact of vitamin D on mitochondrial properties. Notably, the D_3 2000 group achieved 25OHD and type I fiber proportions similar to CON, yet displayed lower mtDNA copy number, consistent with evidence that muscle mitochondrial biogenesis and function are also shaped by VDR signaling, mitochondrial dynamics, and local metabolic cues, and are not determined solely by circulating 25OHD.

Given the differential effects observed between $VitD_3$ and $25OHD_3$ supplementation in this study, our findings suggest that both mitochondrial and fiber-type adaptations in skeletal muscle are more pronounced at higher circulating 25OHD levels, which support the

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possibility that structural remodeling may require vitamin D status to exceed a certain range in rodents. In the present study, rats supplemented with 400 IU 25OHD$_3$/kg diet achieved higher plasma 25OHD levels (53.3 ± 1.5 ng/mL) than that of 2000 IU VitD$_3$/kg diet (39.1 ± 2.3 ng/mL), and only the rats fed 25OHD$_3$ showed clear increases in type I fiber proportion and mtDNA copy number. This pattern aligns with prior human studies suggesting that optimal 25OHD concentrations of 75-100 nmol/L (30-40 ng/mL) for musculoskeletal outcomes[33,34]. Ceglia et al. observed increased muscle fiber size and VDR expression when levels reached 32 ng/mL in older women[27], while lack of fiber type changes in some studies may reflect insufficient circulating 25OHD levels (< 30 ng/mL) [35]. However, the optimal vitamin D concentration and whether it varies by species, muscle type, age, developmental stage or other physiological contexts remains to be clarified.

Our observations regarding fiber type and mitochondrial responses to vitamin D supplementation differ in some respects from previous reports that emphasized higher VDR expression in fast-twitch fibers and preferential fast-fiber atrophy during vitamin D deficiency[27]. In the present study, we did not directly measure fiber type–specific VDR protein or localization, and our VDR assessment was limited to whole-muscle lysates (data not shown), which may mask differences between individual fiber populations. In addition, several factors may contribute to the discrepancy with earlier work, including the specific muscle examined (*biceps femoris* rather than predominantly type I or mixed muscles), the age of the animals

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and the dosing regimen on depletion–repletion. These considerations suggest that our results should not be interpreted as contradicting the role of VDR in fast-twitch fibers, but rather highlight that vitamin D_3 and $25OHD_3$ may act in a muscle- and context-dependent manner, and that detailed fiber type–specific VDR localization and signaling remain important topics for future studies.

In a previous study, RNA-seq analysis of the effects of $VitD_3$ on skeletal muscle revealed that lack of vitamin D signaling drives skeletal muscle toward oxidative metabolism[36]. In another study, supplementary 25OHD has no effect on the transcriptome of skeletal muscle in vitamin D-deficient frail and old adults[37]. However, the timing and design of our study differed from those in previous studies. Das et al. (2023) ablated VDR in a mouse model, whereas in this study, we examined the effects focusing on the effects of exogenous vitamin D supplementation during postnatal development in rats[36]. Moreover, because we focused on growing rats, from 4 to 12 weeks of age, unlike Hangelbroek et al. (2019) who used older adults, dietary supplementation of $25OHD_3$ apparently has a larger impact on the skeletal muscle fiber type transition during development[37]. Given that VDR expression varies with age, being higher in neonatal muscle compared with that in adult muscle[38], the developmental timing of our intervention may have contributed to the observed effects on the fiber type and mitochondrial biogenesis. Additionally, our transcriptome data indicated that increase in the percentage of type I fiber type and mitochondrial copy number induced by dietary $25OHD_3$ was associated

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with the upregulation *Kdr*, *Nos3* and IP3K-related genes, in addition to slow-twitch fiber specific motor proteins. The active form of vitamin D, $1,25(OH)_2D_3$ binds to VDR and regulates VEGF production by interacting with vitamin D response element in the VEGF promoter[39,40]. ERK1/2, a downstream signaling molecule of VEGF2R, is reported to be associated with a shift toward type I fiber in skeletal muscle fiber type determination, as mice lacking myofiber ERK1/2 exhibit atrophy of type I fiber in the soleus muscle, denervation, and impaired mitochondrial function[41]. Additionally, constitutive ERK activation induces slow-twitch fiber formation in both adult and growing mice[21]. Also, IP3K plays a key role in calcium homeostasis by regulating IP3 and inositol 1,3,4,5-tetrakisphopsphate (IP4) concentrations[42]. Elevated cytosolic calcium levels in complex with calmodulin can promote Ca^{2+}/calmodulin-dependent kinases (CaMKs), including ERK pathway and calcineurin-NFAT. These pathways activate transcription factors that regulate the expression of mitochondria and nucleus-encoded genes with resultant adaptations for both muscle fiber type and oxidative capacity[43]. Further studies will delineate whether the effects of $25OHD_3$ are mediated primarily through direct VDR activation or involve alternative signaling pathways that converge on muscle fiber transition and mitochondrial biogenesis.

This study demonstrates that dietary supplementation of $VitD_3$ or $25OHD_3$ modulates fiber type composition in *bicep femoris* of growing rats. Over the 8-week period, no significant differences in body weight, skeletal

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muscle mass, and calcium and phosphorus levels were noted among the groups. Notably, $25OHD_3$ supplementation increased the proportion of slow/oxidative type I fiber, mtDNA copy number, and the expression of genes associated with angiogenesis (VEGFR2 and NOS3) and calcium homeostasis (IP3K). Overall, these findings suggest that $25OHD_3$ supplementation promotes slow-twitch fiber transition in the *bicep femoris* more effectively than does $VitD_3$, possibly through mechanisms involving enhanced plasma 25OHD levels, mitochondrial biogenesis, and promoting enrichment of genes in ERK and calcium signaling pathways. Therefore, dietary $25OHD_3$ supplementation could be a potential alternative to $VitD_3$ in supporting musculoskeletal health. Future studies will be needed to determine whether ERK- and calcium-signaling pathways causally mediate the effects of $25OHD_3$ on muscle fiber type and mitochondrial biogenesis.

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Materials and methods

Animals and dietary treatments

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Korea University (KUIACUC-2012-178). All experiments adhered to the ARRIVE guidelines, and all methods were conducted in accordance with the relevant guidelines and regulations. Seventy male Wistar rats (3-week-old) were purchased from Samtako (Daejeon, South Korea). Diets were formulated for vitamin D deficiency, based on AIN-76A from National Research Council (NRC)[25,44]. All food were given in mashed form. The composition of the basal experimental diet

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is shown in **Table 3**. For supplementation, $VitD_3$ and $25OHD_3$ were substituted for dextrose. Each treatment was performed in 8 or 10 replicates. Rats were fed and housed in individual cages for 8 weeks. Except for the control group (CON) rats, which were fed a diet containing 1,000 IU $VitD_3$/kg diet for 8 weeks of the experiment, rats were fed $VitD_3$-deficient diet (0 IU $VitD_3$/kg diet) for the first 4 weeks (period I, week 1 to 4), followed by vitamin D-supplemented diet for the next 4 weeks (period II, week 5 to 8). Vitamin D doses were 0, 400, 700, 1,000, and 2,000 IU/kg diet for $VitD_3$ and 400 IU/kg diet for $25OHD_3$, as shown in the experimental scheme in **Fig. 1**. The rats were housed under the controlled conditions (22 °C, 60% relative humidity, 12 h light and 12 h dark cycle), allowed free access to food and water, and were not exposed to sunlight during the experiment period. Body weight and feed intake were measured every week. At the end of the experiment, lean and fat content of each animal was determined using Minispec LF-90, an NMR-based body composition analyzer (Bruker, USA). At the culmination of treatments, the rats were anesthetized with Zoletil® (tiletamine-zolazepam, 20-40 mg/kg, intramuscular injection) and confirmed the absence of pain reflexes. Then rats were euthanized by CO_2 inhalation at a concentration exceeding 7.5% CO_2, in accordance with the approved institutional animal care and use protocol. After confirmation of death, blood was immediately collected via cardiac puncture in heparinized VACUTAINER tubes (BD Bioscience, CA, USA) and the collected blood was centrifuged at 1,800 × *g* for 15 min at 4 °C to obtain plasma. *Bicep femoris* was isolated from both hindlimbs,

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weighed, snap frozen in liquid nitrogen and stored at −80 °C until analysis. Plasma and other tissue samples were also stored at −80 °C until analysis.

Chemicals and materials

$VitD_3$ and $25OHD_3$ were purchased from Sigma-Aldrich (St. Louis, MO, USA). Vitamin-free test casein (VFC), Ca- and P-deficient mineral mixture (Cat No. 79055), and vitamin mixture without vitamin A, D, E, and choline (Cat. No. 83171) were obtained from Teklad diets (WI, USA).

Bone mineral density (BMD)

The right femur of rats was collected and stored at −80 °C until analysis. The BMD of the femur was measured using InAlyzer, a Dual energy X-ray Absorptiometry (Medicors, Korea).

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Determination of plasma calcium, phosphorus, and 25-hydroxyvitamin D levels

Plasma calcium and phosphorus levels were measured by using spectrophotometric kit (Embiel, Gunpo, Korea). Plasma 25OHD levels were measured via chemiluminescent immunoassay using the Liaison 25-OH vitamin D total kit (Diasorin, MN, USA) from Green Cross lab (Yongin, Korea).

Skeletal muscle fiber typing and FCSA measurement

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Sections of muscle tissue were prepared using Cryostat CM 1850 (Leica, Wetzlar, Germany). Skeletal muscle fiber typing was performed using myofibrillar adenosine 5′-triphosphatase (ATPase) histochemistry by acid preincubation (pH 4.7) followed by incubation with ATP (pH 9.4)[45]. The fiber types are referred to as MHC I, IIa, and IIb according to the differential activities of ATPase in each fiber. Each fiber population was counted manually, and FCSA was measured using the ImageJ software (NIH).

Determination of mitochondrial DNA (mtDNA) copy number

Whole genomic DNA was extracted from *bicep femoris* using the DNeasy blood & tissue kit (Qiagen, Germany). The relative mtDNA copy number was determined as previously described[46] with SYBR using Stepone Plus real-time PCR by comparing the ratio of mitochondrial to nuclear DNA using Ct levels (ΔCt, nuclear DNA Ct – mitochondrial DNA Ct) of mitochondrial gene (NADH dehydrogenase, *mt-Nd1*) and nuclear gene (β-actin)[47]. The mtDNA copy number was calculated as $N = 2^{\Delta Ct}$. The following primers were used: Mitochondrial NADH dehydrogenase (F: 5′-TGGCCTTCCTCACCCTAGTA-3′; R: 5′-TAGGGGGCGTATGGGTTCTT-3′) and β-actin (F: 5′-GTGTGGTCAGCCCTGTAGTT-3′; R: 5′-CCTAGAAGCATTTGCGGTGC-3′).

RNA isolation and next-generation sequencing

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Sequencing libraries were prepared from 0.5 µg of total RNA for each sample using the Illumina TruSeq Stranded Total RNA Library Prep Globin Kit (Illumina, Inc., San Diego, CA, USA; #20020613). The first step in the workflow involved removing the rRNA from the total RNA using the Ribo-Zero rRNA Removal Kit (Human/Mouse/Rat Gold) (Illumina, Inc.). The mRNA was then fragmented into small pieces using divalent cations under elevated temperature. The cleaved RNA fragments were reverse transcribed into first strand cDNA using SuperScript II reverse transcriptase (Invitrogen; #18064014) and random primers. Second strand cDNA synthesis was performed using DNA polymerase I, RNase H, and dUTP. The cDNA fragments were subjected to end repair, addition of a single “A” base, and ligation of adapters. The products were purified and enriched using PCR to create the final cDNA library. The libraries were quantified using KAPA Library Quantification kit for Illumina Sequencing platform according to the qPCR Quantification Protocol Guide (KAPA BIOSYSTEMS; #KK4854) and qualified using the TapeStation D1000 ScreenTape (Agilent Technologies; #5067-5582). Indexed libraries were then sequenced on an Illumina HiSeq 2000 (Illumina, Inc.) for paired-end (2 × 151 bp) sequencing by Macrogen Incorporated (Seoul, South Korea).

Quality control, read mapping, and quantification of gene expression

A comprehensive analysis of transcriptome data quality was conducted to ensure the reliability of findings. We utilized the FastQC

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software (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) to assess the quality of raw data. Subsequently, we used Trimmomatic version 3.8 to eliminate low-quality reads and adapter sequences from the dataset[48]. Following data preprocessing, we aligned the high-quality sequence reads to the rat reference genome (mRatBN7.2) using the Ensembl Genome Browser (http://www.ensembl.org/Rattus_norvegicus). This alignment was performed using Hisat2 and SAMtools[49,50]. To quantify the expression of genes, we used FeatureCounts, referencing a GTF file from Ensembl (version 110) to count the number of reads mapped to each genomic feature[51].

To improve the overall quality and reliability of the dataset, we excluded genes that were not expressed in all samples. We utilized the trimmed mean of M (TMM) values obtained using the edgeR package for data normalization[52]. Furthermore, we conducted a differential expression analysis between the control and treatment groups by employing the DESeq2 library[53]. Differentially expressed genes (DEGs) were identified based on stringent criteria—a false-discovery rate less than 0.05 and an absolute fold change of at least 2—ensuring the robustness and significance of our results.

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Functional enrichment analysis

To interpret the biological implications of $VitD_3$ and $25OHD_3$ on the dataset, we conducted a functional analysis focusing on the DEGs. To accomplish this, we utilized the Database for Annotation, Visualization,

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and Integrated Discovery (DAVID)[54], applying stringent criteria—a significance threshold of $P < 0.05$ and minimum gene count of two—as filtering parameters for the enrichment analysis. For the gene ontology (GO) analysis [55], we used the REVIGO visualization tool, which allowed us to generate a treemap representing the enriched GO terms in an effectively condensed format[56]. Similarly, for the Kyoto encyclopedia of genes and genomes (KEGG) pathway analysis [57], we visualized the pathways based on the significance of their enrichment. We employed Fisher's exact test to calculate the significance levels and obtained a statistical measure of the findings. For effectively visualizing and presenting the results, we utilized the R software to create volcano plots, tree maps, and depict the KEGG pathway.

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Statistical analysis

All values are presented as means ± SD. All analyses were replicated a minimum of three times. Statistical differences among treatments were evaluated with one-way analysis of variance (ANOVA) using the Tukey's Honestly Significant Difference (HSD) test. All statistical analyses except non-linear regression mitochondrial copy number were performed using the SAS software (version 9.2, SAS institute, NC, USA). For correlation and regression analysis, and mitochondrial copy number, statistical analysis was performed using Graphpad Prism (version 10.6.1, San Diego, CA, USA). For dose–response and association analyses, we modeled the relationships between dietary vitamin D_3 intake, plasma 25OHD, and fiber

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type composition using nonlinear regression. Goodness-of-fit was evaluated by R^2 and the associated P-value for the regression model, and 95% confidence bands for the fitted curves are shown in the figures. For mitochondrial copy number, raw values were log-transformed to stabilize variance and approximate normality. Normality was confirmed using Shapiro–Wilk and Kolmogorov–Smirnov tests. Group comparisons were performed using one-way ANOVA on the log-transformed data, followed by Tukey's Honestly Significant Difference (HSD) post-hoc test for multiple pairwise comparisons.

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Data availability

Raw RNA-seq FASTQ files have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject accession number PRJNA1253676. All other data supporting the findings of this study are available from the corresponding author upon reasonable request.

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Min Young Park: Investigation, Methodology, Formal analysis, Writing - original draft, Visualization. Do-Young Kim: Data curation, Visualization, Software, Writing - review & editing. Min-Ki Seok: Methodology. Kwang-Youn Whang: Conceptualization, Methodology, Resources, Writing - review & editing, Supervision, Project administration. Jun-Mo Kim: Writing - review & editing, Resources, Supervision, Funding acquisition.

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Ethics declarations

Competing interests

The authors declare that there are no conflicts of interest.

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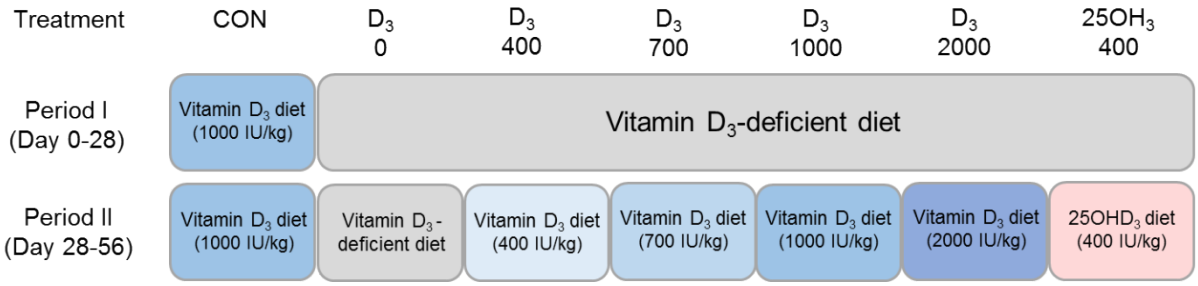


Fig. 1. Schematic of experimental design and dietary treatment. The experiment consisted of two 4-week periods. During period I, all rats except the control group (CON) received a vitamin D₃-deficient diet to establish a common baseline. In period II, rats were allocated to the following treatments: CON (1000 IU vitamin D₃/kg diet throughout the experiment); D₃ 0 (vitamin D₃-deficient diet in both periods); D₃ 400, D₃ 700, D₃ 1000, and D₃ 2000 (vitamin D₃-deficient diet in period I and 400, 700, 1000, or 2000 IU vitamin D₃/kg diet, respectively, in period II); and 25OHD₃ 400 (vitamin D₃-deficient diet in period I and 400 IU 25OHD₃/kg diet in period II).

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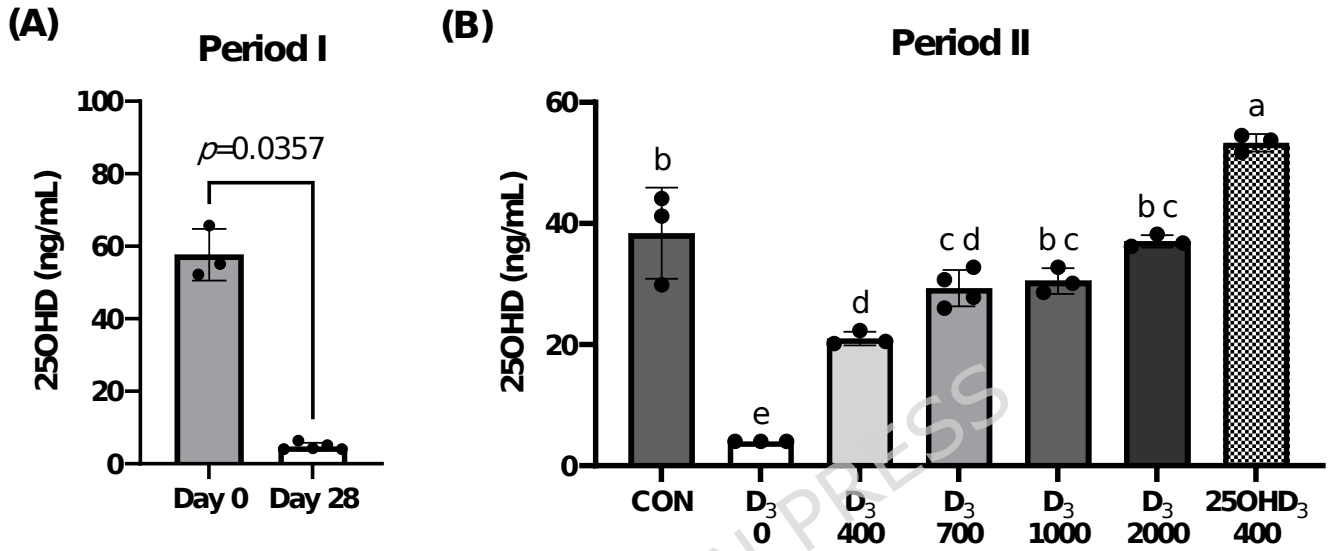
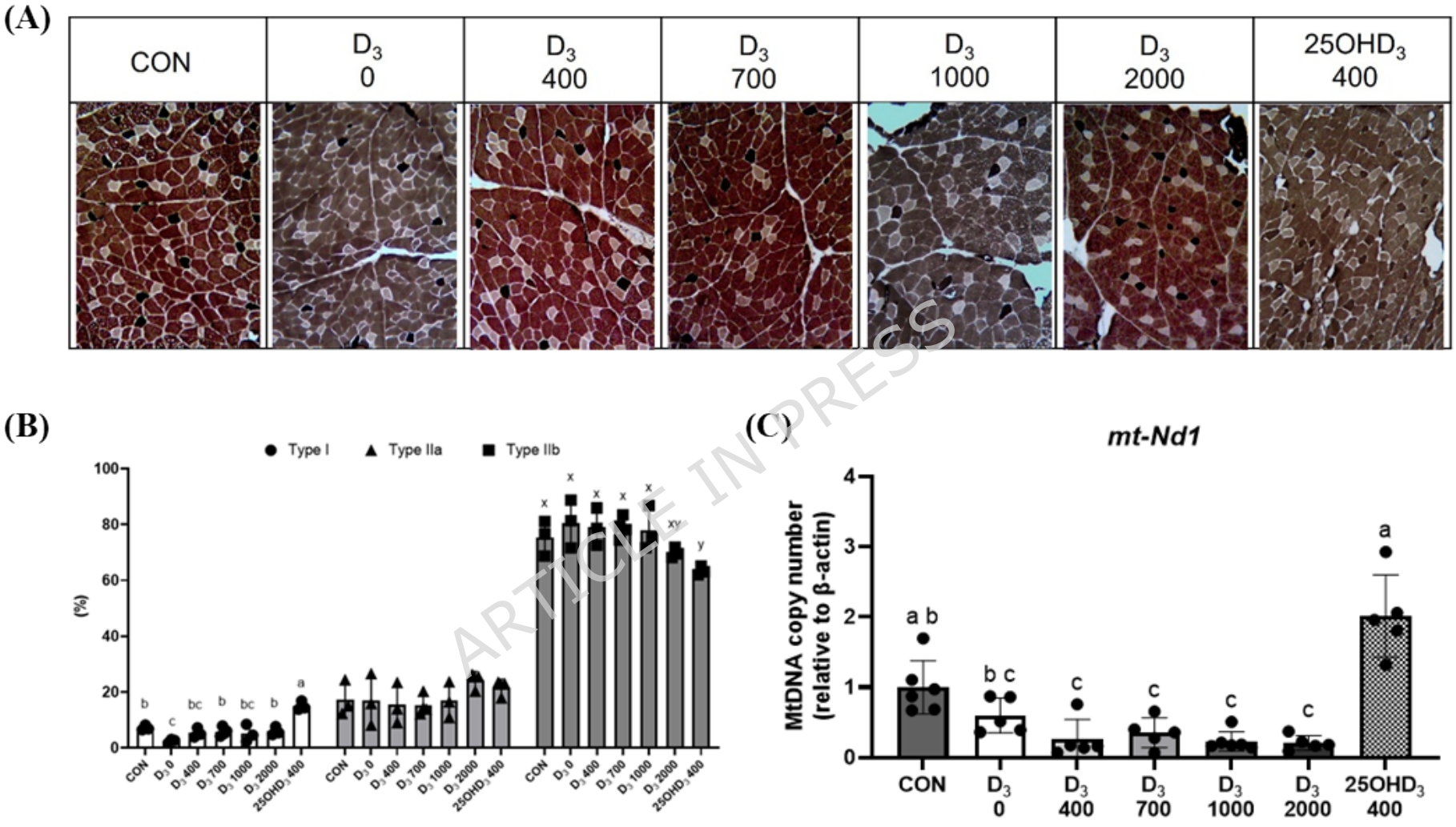


Fig. 2. Plasma 25OHD concentrations in the rats. (A) Initial plasma 25OHD level (Day 0) and level after feeding vitamin D_3-deficient diet during period I (Day 28). (B) Plasma 25OHD levels in all treatments post dietary replenishment of vitamin D_3 after period II (day 28–56). The experiment consisted of two 4-week periods. During period I, all rats except the control group (CON) received a vitamin D_3-deficient diet to establish a common baseline. In period II, rats were allocated to the following treatments: CON (1000 IU vitamin D_3/kg diet throughout the experiment); D_3 0 (vitamin D_3-deficient diet in both periods); D_3 400, D_3 700, D_3 1000, and D_3 2000 (vitamin D_3-deficient diet in period I and 400, 700, 1000, or 2000 IU vitamin D_3/kg diet, respectively, in period II); and 25OHD_3 400 (vitamin D_3-deficient diet in period I and 400 IU 25OHD_3/kg diet in period II).
Data are presented as Mean ± SD. Values with different superscript differ significantly ($p < 0.05$).

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Fig. 3. Fiber type distribution in rat *bicep femoris*. (A) Representative images of myofibrillar ATPase staining by acid preincubation (pH 4.7) followed by incubation with ATP (pH 9.4). The fiber types are referred to as MHC I (darkest), IIa (brightest), and IIb (B) The number of individual fiber type per field were represented as percentage to the total number of fibers analyzed (a total of 130-300 fibers per field) (C) Mitochondrial DNA copy number in the rat *bicep femoris*. Relative mtDNA copy number was determined by comparing the ratio of mitochondrial to nuclear DNA using Ct levels (ΔCt, nuclear DNA Ct – mitochondrial DNA Ct) of mitochondrial gene (NADH dehydrogenase, *mt-Nd1*) and nuclear gene (β-actin)[43]. The mtDNA copy number was calculated as $N = 2^{\Delta Ct}$.

The experiment consisted of two 4-week periods. During period I, all rats except the control group (CON) received a vitamin D_3-deficient diet to establish a common baseline. In period II, rats were allocated to the following treatments: CON (1000 IU vitamin D_3/kg diet throughout the experiment); D_3 0 (vitamin D_3-deficient diet in both periods); D_3 400, D_3 700, D_3 1000, and D_3 2000 (vitamin D_3-deficient diet in period I and 400, 700, 1000, or 2000 IU vitamin D_3/kg diet, respectively, in period II); and 25OHD_3 400 (vitamin D_3-deficient diet in period I and 400 IU 25OHD_3/kg diet in period II).

Data are presented as Mean ± SD.

^{a,b,c}Values with different superscript differ significantly for type I fiber percentage or mtDNA copy number ($p < 0.05$).

^{x,y,z}Values with different superscript differ significantly for type IIb fiber percentage ($p < 0.05$).

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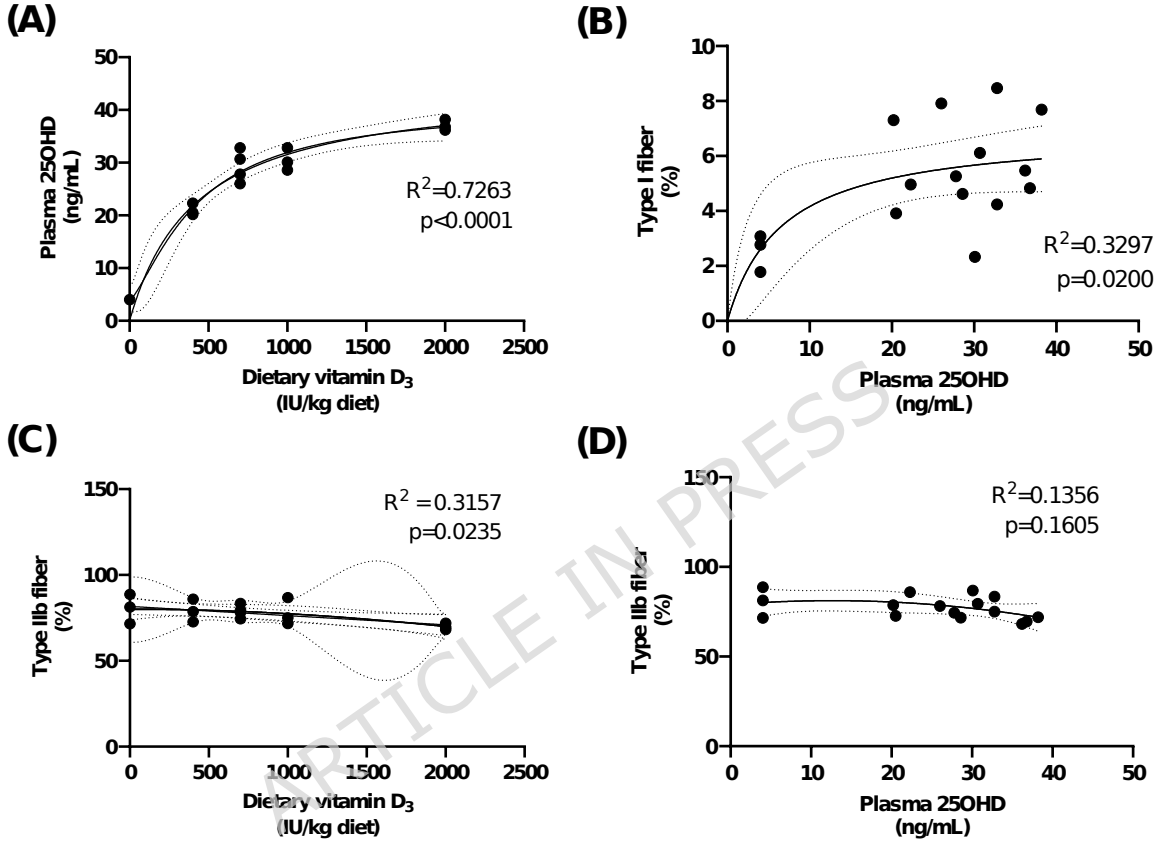


Fig. 4. Correlation between (A) dietary vitamin D_3 level vs. plasma 25OHD level. (B) dietary vitamin D_3 level vs. fiber type IIb (%), (C) plasma 25OHD vs. fiber type I (%), (D) plasma 25OHD vs. fiber type IIb (%) in *bicep femoris*. Each parameter was plotted to the best fitted curve and Spearman correlation was conducted.

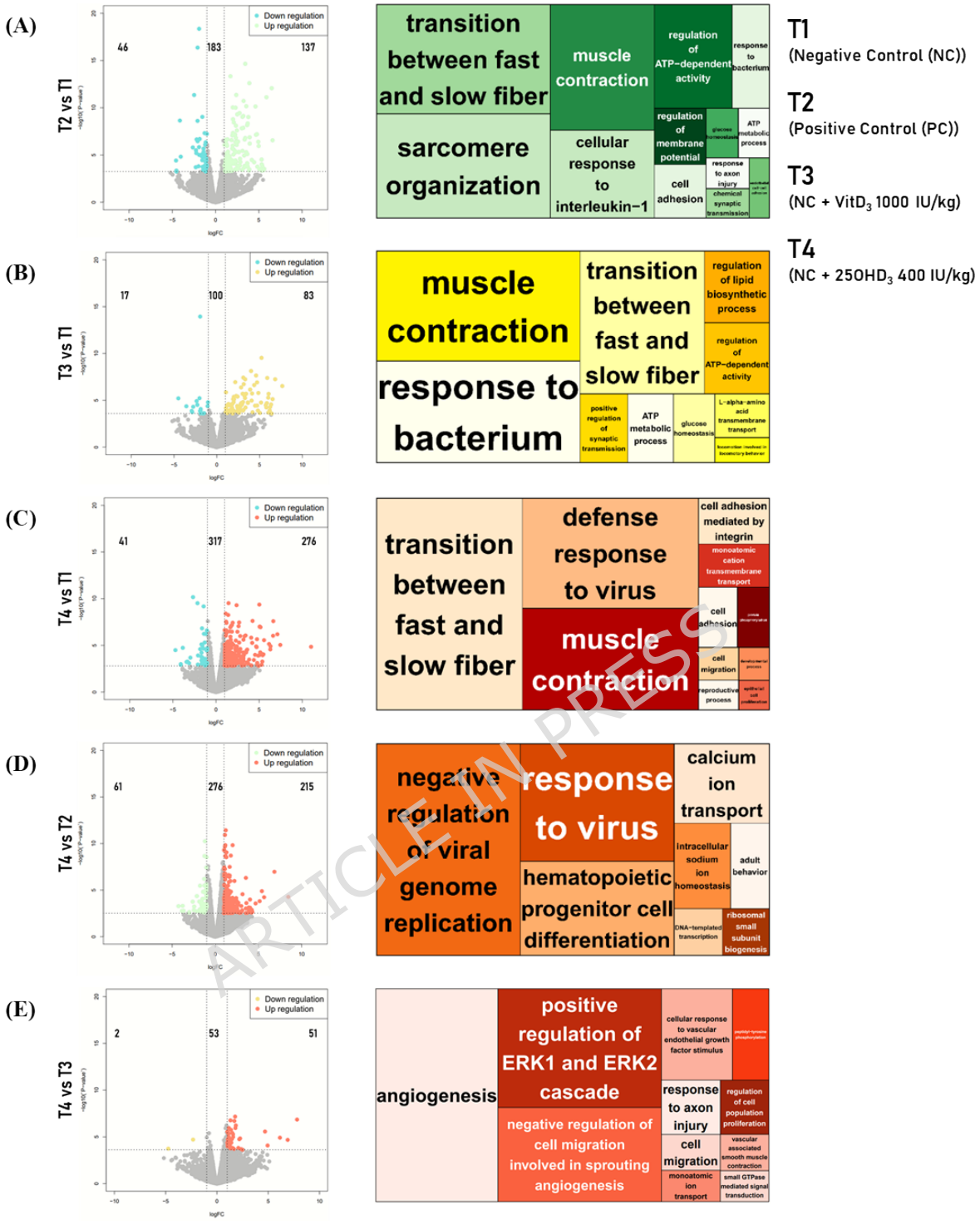


Fig. 5. Differentially expressed genes (DEGs) identified by comparison groups. (Left panels) The X- and Y-axes of the volcano plot represent fold change and significance level, respectively. The colors of dots indicate significant expression changes, whereas gray shows nonsignificant genes. (Right panels) Biological process (BP) of gene ontology enrichment treemap. The size of the rectangle is proportional to the significant value

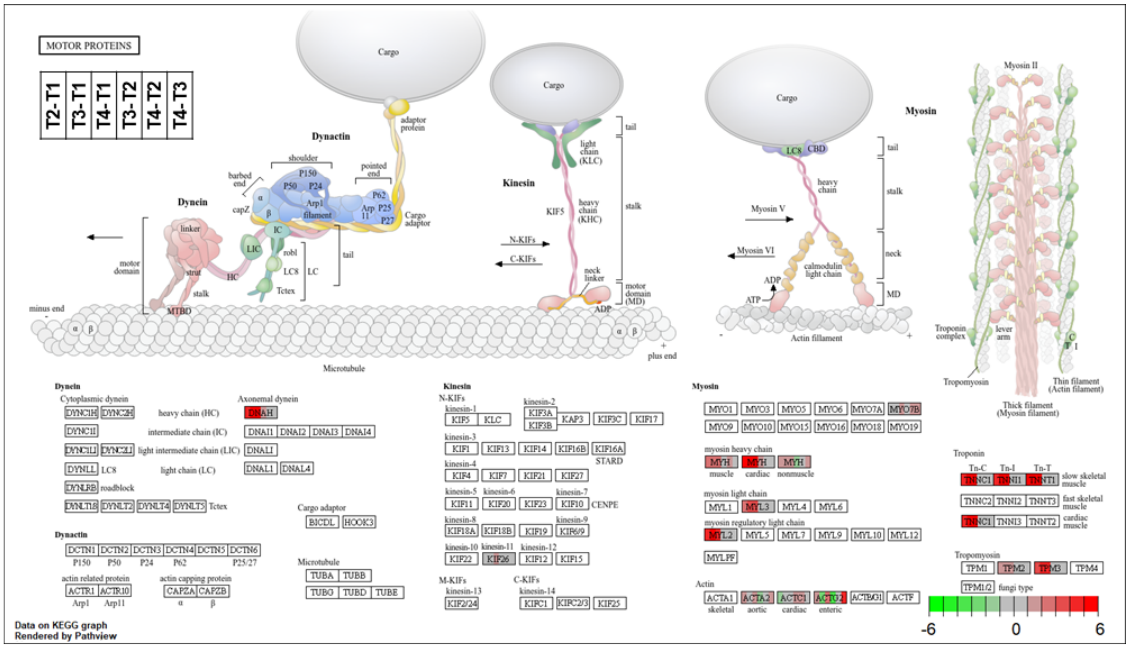
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for that category. Each colored rectangle clusters one or more semantically related representative terms.

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(A)



(B)

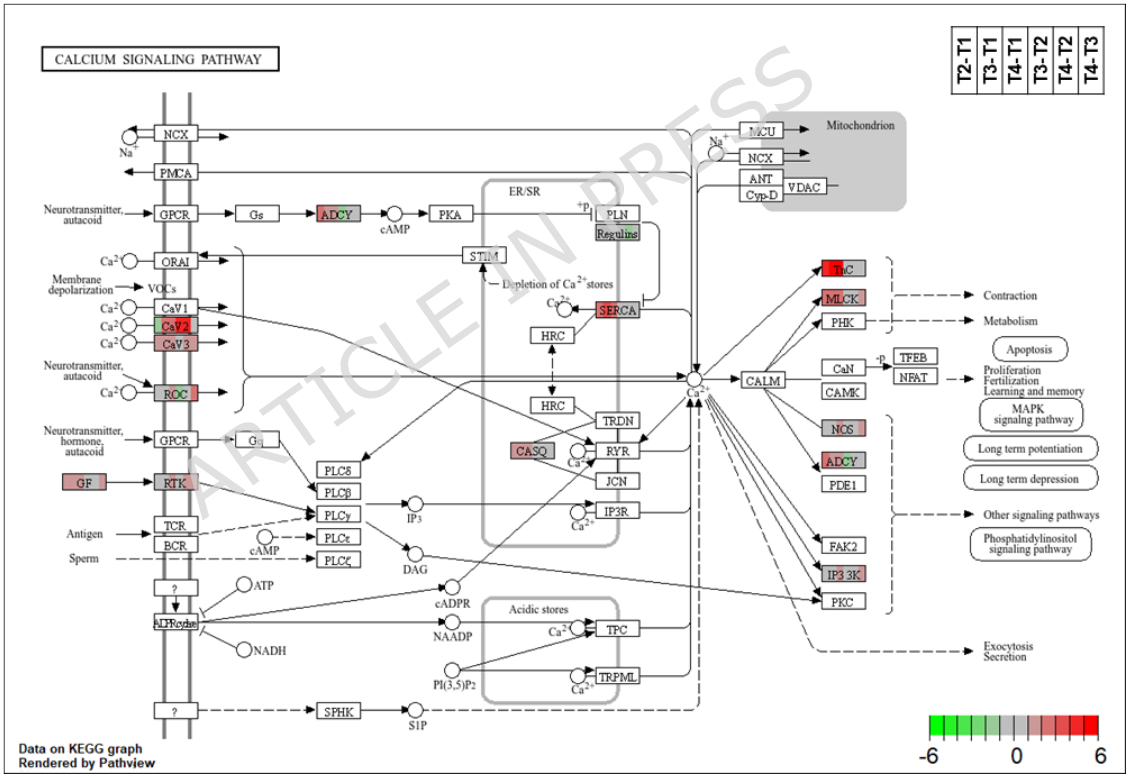


Fig.6. Levels of expressed genes mapped onto the KEGG pathway module. (A) Motor proteins. (B) Calcium signaling pathway. The expression levels of genes for the six groups are shown in each gene box. The gene box colors indicate low (green) and high (red) expression. Undetected genes are shown in white.

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Table 1. Growth performance of rats fed experimental diets

Treatment	CON (*n* = 10)	D_3 0 (*n* = 10)	D_3 400 (*n* = 8)	D_3 700 (*n* = 8)	D_3 1000 (*n* = 8)	D_3 2000 (*n* = 8)	25OHD_3 400 (*n* = 10)
BW, g							
Day 0	54.00±4.37	55.70±4.64	56.38±3.34	53.75±4.13	54.38±5.21	54.38±4.66	53.50±5.21
Day 28	200.00±11.69	208.70±20.97	206.25±19.83	205.75±15.38	205.63±16.84	207.88±15.79	203.30±14.12
Day 56	294.90±18.96	298.00±43.86	306.13±25.47	308.75±24.74	306.25±27.41	308.00±20.70	306.70±16.93
ADG, g/day							
Day 0–28	5.21±0.34	5.46±0.65	5.35±0.68	5.43±0.53	5.40±0.57	5.48±0.46	5.35±0.45
Day 28–56	3.39±0.53	3.19±1.46	3.57±0.36	3.68±0.50	3.59±0.46	3.58±0.44	3.69±0.45
Day 0–56	4.30±0.35	4.33±0.77	4.46±0.45	4.55±0.45	4.50±0.50	4.53±0.33	4.52±0.33
ADFI, g/day							
Day 0–28	13.36±1.06	14.00±1.37	13.95±1.04	13.59±0.62	13.80±0.92	13.89±1.51	13.57±0.97
Day 28–56	17.88±1.48	17.33±2.52	18.75±0.91	18.42±1.46	18.36±1.51	18.36±1.40	18.83±1.72
Day 0–56	15.62±1.15	15.66±1.54	16.35±0.90	16.01±0.94	16.08±1.15	16.12±1.30	16.20±1.16
FE							
Day 0–	0.39±0.0	0.39±0.02	0.38±0.02	0.40±0.02	0.39±0.02	0.40±0.02	0.39±0.

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28	2						02	
Day 28–56	3	0.19±0.0	0.17±0.09	0.19±0.01	0.20±0.02	0.20±0.02	0.19±0.02	0.20±0. 02
Day 0–56	2	0.28±0.0	0.27±0.03	0.27±0.01	0.28±0.02	0.28±0.02	0.28±0.01	0.28±0. 01

Data represent mean±SD of values for 8 or 10 rats. BW, Body weight; ADG, Average daily gain; ADFI, Average daily feed intake; FE, Feed efficiency. The experiment consisted of two 4-week periods. During period I, all rats except the control group (CON) received a vitamin D_3-deficient diet to establish a common baseline. In period II, rats were allocated to the following treatments: CON (1000 IU vitamin D_3/kg diet throughout the experiment); D_3 0 (vitamin D_3-deficient diet in both periods); D_3 400, D_3 700, D_3 1000, and D_3 2000 (vitamin D_3-deficient diet in period I and 400, 700, 1000, or 2000 IU vitamin D_3/kg diet, respectively, in period II); and 25OHD_3 400 (vitamin D_3-deficient diet in period I and 400 IU 25OHD_3/kg diet in period II).

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Table 2. Characteristics of *bicep femoris* in rats at the end of the experiment

Treatment	CON	D_3 0	D_3 400	D_3 700	D_3 1000	D_3 2000	25OHD_3 400
Muscle mass (*g* per 100 *g* body weight)							
	(*n* = 10)	(*n* = 10)	(*n* = 8)	(*n* = 8)	(*n* = 8)	(*n* = 8)	(*n* = 10)
	1.45±0.11	1.45±0.06	1.44±0.06	1.42±0.09	1.30±0.22	1.41±0.20	1.37±0.05
Fiber cross-sectional area (FCSA, μm²)							
	(*n* = 3)	(*n* = 3)	(*n* = 3)	(*n* = 3)	(*n* = 3)	(*n* = 3)	(*n* = 3)
Type I	1114.54±293.82	1330.49±172.98	1539.94±497.40	1365.30±405.78	1336.69±493.11	1223.04±297.18	999.83±316.91
Type IIa	866.98±281.03	1198.39±521.61	1166.33±321.68	1263.16±397.53	1272.87±407.73	1212.56±314.04	1413.47±426.55
Type IIb	2427.70±800.55	2525.32±1013.10	3032.39±1146.79	2914.41±1098.90	2304.85±863.64	2748.39±937.93	3122.52±1014.81
Total area (%)							
	(*n* = 3)	(*n* = 3)	(*n* = 3)	(*n* = 3)	(*n* = 3)	(*n* = 3)	(*n* = 3)
Type I	3.88±0.67[b]	1.42±0.36[c]	3.12±0.78[b]	3.22±1.06[b]	3.03±1.14[b]	3.17±0.50[b]	6.20±0.70[a]
Type IIa	7.53±3.84	9.40±7.47	6.98±3.87	7.50±0.93	10.50±4.32	13.25±3.93	12.48±1.42
Type IIb	88.60±3.67	89.18±7.32	89.90±3.30	89.29±1.07	86.47±4.76	83.59±4.36	81.33±1.25

[a,b,c]Values with different superscript in the same row differ significantly ($P < 0.05$).

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Data represent mean±SD. The experiment consisted of two 4-week periods. During period I, all rats except the control group (CON) received a vitamin D_3-deficient diet to establish a common baseline. In period II, rats were allocated to the following treatments: CON (1000 IU vitamin D_3/kg diet throughout the experiment); D_3 0 (vitamin D_3-deficient diet in both periods); D_3 400, D_3 700, D_3 1000, and D_3 2000 (vitamin D_3-deficient diet in period I and 400, 700, 1000, or 2000 IU vitamin D_3/kg diet, respectively, in period II); and 25OHD_3 400 (vitamin D_3-deficient diet in period I and 400 IU 25OHD_3/kg diet in period II). *Bicep femoris* of rat were collected and measured for its weight. Muscle samples were snap-frozen until it was cryosectioned and each fiber type was characterized by ATPase staining. The data represent mean fiber cross-sectional area (FCSA) of fiber type in each treatment group, and relative area (%) of each fiber type normalized by total area in each treatment group.

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Table 3. Composition of the basal experimental diet (g/kg) (Vitamin D_3-deficient diet)

Ingredient	**g/kg of diet**
Vitamin-free test (VFT) casein	180.00
Cornstarch	1.22
Cellulose	30.00
Corn oil	100.00
Dextrose, monohydrate	643.59
L-Cysteine•HCl	2.62
Ca, P-deficient mineral mix[1]	13.37
Calcium carbonate	11.63
Potassium phosphate, dibasic	4.90
Potassium phosphate, monobasic	3.90
Vitamin mix without choline, A, D, E[2]	5.00
Vit A palmitate (500,000 IU/g)	0.04
Vit E, DL-α tocopheryl acetate (500 IU/g)	0.24
Choline bitartrate	3.42
Ethoxyquin (25 %)	0.08
Total	**1000.00**

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The experiment consisted of two 4-week periods. During period I, all rats except the control group (CON) received a vitamin D_3-deficient diet to establish a common baseline. In period II, rats were allocated to the following treatments: CON (1000 IU vitamin D_3/kg diet throughout the experiment); D_3 0 (vitamin D_3-deficient diet in both periods); D_3 400, D_3 700, D_3 1000, and D_3 2000 (vitamin D_3-deficient diet in period I and 400, 700, 1000, or 2000 IU vitamin D_3/kg diet, respectively, in period II); and 25OHD_3 400 (vitamin D_3-deficient diet in period I and 400 IU 25OHD_3/kg diet in period II).

[1]Mineral mix contained the following components per kilogram mixture: sodium chloride (193.73 g); potassium citrate, monohydrate (575.49 g); potassium sulfate (136.14 g); magnesium oxide (62.83 g); manganous carbonate (95% Mn) (9.65 g); ferric citrate (16–17% Fe; 15.71 g); zinc carbonate (70% ZnO; 4.19 g); cupric carbonate (53–55% Cu; 0.79 g); potassium iodate (0.03 g); sodium selenite (0.02 g); chronium potassium sulfate (1.44 g).

[2]Vitamin mix contained the following components per kilogram mixture: vitamin K (50% menadione; 19.84 g); thiamine•HCl (B1, 98%; 4.49 g); riboflavin (B2, 80%; 5.50 g); nicotinic acid (niacin, B3, 100%; 19.82 g); d-calcium pantothenate (B5, 98%; 13.49 g); pyridoxine•HCl (B6, 99%; 4.44

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g); biotin (d-biotin, B7, 2%; 4.00 g); cyanocobalamin (B12, 1%; 0.60 g); folic acid (91%; 0.44 g); inositol (100%; 22.02 g); *p*-aminobenzoic acid (99%; 22.24 g); vitamin C, ascorbic acid, coated (95%; 208.67 g); corn starch (674.45 g).

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