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Original

Effect of deficient vitamin D levels on muscular activity and vascular health in an experimental model

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Abstract

Introduction: previous studies show that adequate levels of calcidiol are associated with greater muscle strength, maintenance of daily activities and less progression of aortic calcification. The objective of this study was to assess the effect of vitamin D deficiency on muscular activity and vascular health in an experimental model.

Material and methods: 18-month-old FVB/N mice were used. One group received a diet without vitamin D (deficient group, $n = 20$) and another group received a normal diet (control group, $n = 17$) for 8 weeks. To measure muscular activity, a wooden rod fixed to a table with a mark 10 cm from the supported end was used to indicate the finish line. Mice were placed on the "open" end of the rod with their backs to the target. After a previous training process, the animals were evaluated three times measuring two parameters: orientation time (time necessary to turn 180° from the initial position and look towards the supported end) and transition time (time necessary to reach the goal). At sacrifice, blood and tissues were removed.

Results: calcidiol levels were higher in the control group (23.3 ± 3.9 *vs* 12.7 ± 3.1 ng/mL, $p < 0.001$). Orientation times (43 ± 46 *vs* 15 ± 21 seconds, $p = 0.020$) and transition times (62 ± 51 *vs* 31 ± 37 seconds, $p = 0.041$) were much higher in the deficient group compared to the control group. At the aortic level, the gene expression of α-actin and miR-145 were highly compromised in the deficient group. Runx2 expression was not altered.

Conclusions: these experimental results confirm previous clinical results where maintaining adequate levels of vitamin D prevents the loss of muscular functionality and vascular damage.

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Vitamin D.
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INTRODUCTION

The endocrine system of vitamin D modulates the expression of more than 3 % of all genes in the body, thus regulating various physiological processes in other organs and systems, such as the muscle (1). In recent years, there has been an emphasis on maintaining an adequate vitamin D status to optimize muscle strength and reduce falls and fractures in the elderly population (2-4).

Vitamin D stimulates the absorption of calcium in the intestine and maintains serum calcium levels required for muscle function (5). Several in vivo studies suggest the role of vitamin D in regulating muscle mass and function. Observational studies demonstrate that vitamin D deficiency in elderly individuals is associated with reduced muscle mass and strength (6-8), lower physical performance (6,9), and an increased risk of falls (10). Furthermore, a meta-analysis of 17 clinical trials showed that vitamin D supplementation in subjects with baseline calcidiol levels < 10 ng/mL had a positive effect on hip muscle strength (11). These studies suggest that vitamin D may affect muscle mass and function; however, it remains unclear whether vitamin D plays a direct or indirect role.

In recent years, increasing attention has been given to the local conversion of calcidiol to calcitriol, the most active metabolite of vitamin D, which is synthesized mainly in the kidney through its precursor, calcidiol (5). This local synthesis has been demonstrated in several cell types, such as osteoblasts (12-15) and monocytes (16), reinforcing the importance of maintaining adequate calcidiol levels in the body.

The vitamin D endocrine system also regulates the cardiovascular system (17). In fact, data from our group, in a cohort of an unselected general population, have shown that adequate calcidiol levels are associated not only with greater muscle strength and maintenance of daily activities but also with less progression of aortic calcification (18,19).

Therefore, the objective of this study was to evaluate the effect of vitamin D deficiency on motor activity and vascular health in an experimental model. This type of study allows for greater precision in limiting and controlling potential biases inherent in epidemiological studies.

MATERIALS AND METHODS

Male and female FVB/N mice, 18 months of age, were used. One group received a vitamin D-deficient diet (Teklad 2014, Vitamin D omitted, Envigo, Spain) (deficient group, *n* = 20), while the other remained on a normal diet (Teklad 2014, standard version, Envigo) (control group, *n* = 17) for 8 weeks.

To induce the expression of CYP24A1 or renal 1-α hydroxylase and accelerate the catabolism of endogenous calcidiol and calcitriol reserves, the animals received intraperitoneal injections of 3 ng of 19-nor-1,25-dihydroxyvitamin D2 (paricalcitol; Zemplar, kindly provided by Abbott, now AbbVie, Lake Bluff, IL, USA) on days 1, 3, 5, 8, 10, and 12, following a protocol used by other authors to accelerate vitamin D catabolism (20).

At the end of the last week of treatment with the deficient or normal diet, motor activity was measured using a wooden rod (60 cm long and 28 mm in diameter) fixed to a table 60 cm above a cushioned surface, with a mark 10 cm from the supported end to indicate the finish line (21). Mice were placed at the "open" end of the rod, facing away from the finish line (Fig. 1). Two weeks before conducting the motor activity study, a training process was conducted to acclimate the animals to the rod, with a treat placed at the finish line to reward their effort. During the study, animals were evaluated three times, measuring two parameters: orientation time (time needed to turn 180° from the initial position to face the supported end) and transition time (time required to reach the finish line). If a mouse fell twice during the orientation or transition times, 25 seconds were added to the time recorded. Each mouse had a total of 2 minutes to complete the test. At sacrifice, blood and aortic tissue were collected and stored at -80 °C until processing.

Serum biochemical determinations were performed using specific Quanti-Chrom™ kits (Bioassay Systems, Hayward, CA, USA) for BUN, Ca, and P. For other biochemical markers, specific ELISA kits were used: PTH (Immutopics, Inc., San Clemente, CA, USA), intact FGF23 (Immutopics), and calcidiol (Immunodiagnostic Systems Ltd, Scottsdale, AZ, USA).

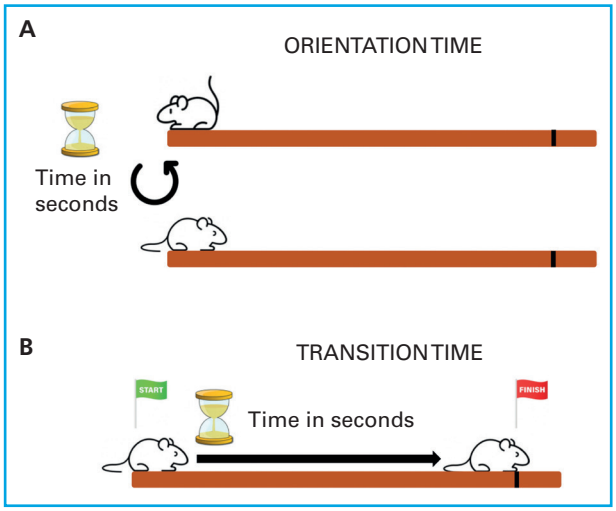

Figure 1. Diagram representing motor activity. A. Orientation time is defined as the time (in seconds) it takes for the animal to turn around on the rod to change the direction of movement. B. Transition time represents the time (in seconds) the animal takes to traverse the rod to the finish line.

The aortas of the animals were not included in paraffin to assess calcifications, as mice are not a good model for observing calcifications (22) unless they are ApoE mutants (23). Additionally, in our case, the mice had normal renal function, so finding calcifications at this level, even in aged mice, was unlikely.

Total RNA from kidney and aorta was extracted using TRI reagent (Sigma-Aldrich, St. Louis, MO, USA). Two µg of total RNA was reverse-transcribed into cDNA using the High-Capacity Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA). Gene expression in the aorta (α-actin, Runx2, and microRNA (miR)-145) and kidney (1-α hydroxylase and 25-α hydroxylase) was assessed using qPCR with pre-designed assays (Applied Biosystems) on a Stratagene Mx3005P QPCR System (Agilent Technologies, Santa Clara, CA, USA). All reactions were performed in triplicate. Relative gene expression was quantified using the ΔΔCT method, with GAPDH and U6 as housekeeping genes for microRNA, and expressed in relative units (RU) (24).

STATISTICAL ANALYSIS

Data analysis was performed using SPSS version 25.0 for Windows. Quantitative variables were analyzed using Student's t-test with a significance threshold of $p < 0.05$ to highlight statistically significant differences.

RESULTS

At biochemical level, there were no differences between mice fed a vitamin D-deficient diet and those fed a normal vitamin D diet in renal function measured by BUN, or in serum levels of calcium, phosphorus, PTH, or FGF23 (Table I). As expected, calcidiol levels were significantly higher in the group receiving the normal vitamin D diet compared to those on the deficient diet (23.3 ± 3.9 vs 12.7 ± 3.1 ng/mL, p < 0.001) (Table I).

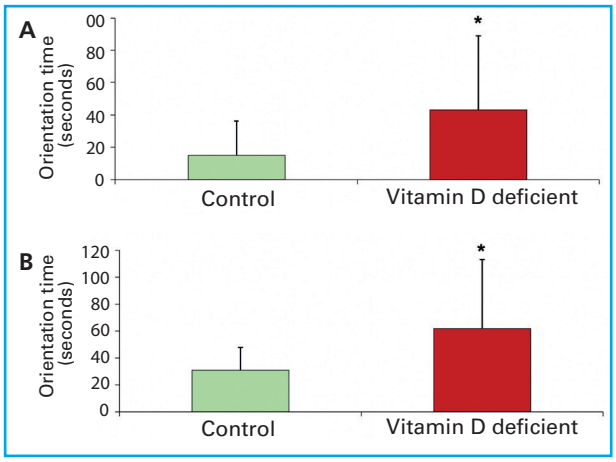

Figure 2. Motor activity measurements of orientation (A) and transition times (B). Data show measurements taken on a wooden rod in groups of mice fed a vitamin D-deficient or normal diet. *$p < 0.05$ *vs* the control group (normal vitamin D diet group).

When studying muscle activity parameters, it was observed that orientation (43 ± 46 vs 15 ± 21 seconds, $p = 0.020$) and transition times (62 ± 51 vs 31 ± 37 seconds, $p = 0.041$) were significantly higher in the group on the vitamin D-deficient diet vs the normal diet group (Fig. 2). The minimum time required for orientation was 2 seconds, and for transition, it was 6 seconds. In both cases, the maximum time was 120 seconds. In the vitamin D-deficient group, 35 % of mice were unable to complete the task vs 17.6 % in the normal diet group.

At aortic level, gene expression of α-actin, a vascular phenotype marker, was markedly impaired in the vitamin D-deficient group (2.05 ± 1.48 vs 0.98 ± 0.51 RU, $p = 0.041$). Expression of miR-145, the key microRNA regulating contractile phenotype, also showed a similar pattern to α-actin, with a significant reduction in its expression in vitamin D-deficient mice (1.70 ± 1.72 vs 0.76 ± 0.34 RU, $p = 0.048$). Runx2 expression remained unaltered (Fig. 3).

Table I. Biochemical markers in the experimental study of mice fed a normal or vitamin D-deficient diet

Biochemical markers	Normal diet group ($n = 17$)	Deficient diet group ($n = 20$)	*p*-value
BUN (mg/dL)	23.2 ± 4.5	22.7 ± 3.8	0.846
Calcium (mg/dL)	9.1 ± 0.6	8.9 ± 0.8	0.563
Phosphorus (mg/dL)	5.3 ± 1.2	5.4 ± 1.3	0.929
PTH (pg/mL)	173 ± 58	202 ± 104	0.513
FGF23 (pg/mL)	239 ± 124	288 ± 58	0.398
Calcidiol (ng/mL)	23.3 ± 3.9	12.7 ± 3.1	*< 0.001*

At renal level, gene expression of 1-α hydroxylase and 25-α hydroxylase showed no statistically significant differences between vitamin D-deficient and non-deficient animals. However, there was a 39 % reduction in 1-α hydroxylase gene expression in the vitamin D-deficient group (1.83 ± 2.19 vs 3.00 ± 4.77 RU, $p = 0.413$). This reduction was 23 % for 25-α hydroxylase (1.60 ± 2.39 vs 2.08 ± 2.00 RU, $p = 0.586$).

DISCUSSION

These experimental results confirm previous clinical findings that maintaining adequate vitamin D levels prevents motor function loss and vascular damage.

First, in aged mice, it was shown that a lack of vitamin D affects mobility, including orientation and completing specific tasks. Several years ago, our group published an article in the general population showing that vitamin D deficiency was associated with reduced handgrip strength and greater difficulty in performing daily activities. This appears to be corroborated by this experimental study (18).

Vitamin D deficiency is common in older adults and may contribute to muscle deterioration through sarcopenia (25,26). This results in devastating effects, increasing care needs and morbidity/mortality among older individuals (25). Additionally, older adults face a higher risk of vitamin D deficiency due to age-related factors such as decreased synthesis or reduced sunlight exposure (27). Although a recent meta-analysis questions the utility of vitamin D supplements for reducing fall risk, bone mineral density loss, and fractures, there is ample evidence of the importance of vitamin D for muscle and bone health (28).

While there is debate about whether the vitamin D receptor (VDR) is expressed in muscle, some authors have found significantly higher VDR levels in young mice muscle, supporting a pleiotropic effect in this tissue (29-31). Moreover, VDR expression in muscle is known to decline with age (32,33), making the musculoskeletal system more vulnerable to low vitamin D levels in the elderly. Skeletal muscle appears to act as a significant storage site for vitamin D, which may diffuse back into circulation or to adjacent areas under specific signals (34,35).

We were unable to detect a reduction in renal 1-α hydroxylase expression in vitamin D-deficient mice, though there was a trend toward reduction, likely due to the lack of calcidiol substrate for conversion to its active form, calcitriol. It should be noted that muscle expresses the 1-α hydroxylase enzyme, facilitating local calcitriol synthesis (30,36,37), particularly in mice with adequate calcidiol levels.

Although mice are not ideal models for studying vascular calcification due to their resistance to calcification, molecular alterations indicating vascular changes were observed. The significant reductions (over 50 %) in α-actin expression, the most specific marker of contractile phenotype, in vitamin D-deficient mice suggest potential signs of a shift from vascular to osteogenic phenotype. However, Runx2 levels were not elevated. Interestingly, miR-145 expression, the key regulator of vascular phenotype, was markedly reduced. Recent findings from our group suggest that the loss of miR-145 precedes α-actin reductions or intracellular calcium deposits in the vascular-to-osteogenic phenotype transition (38).

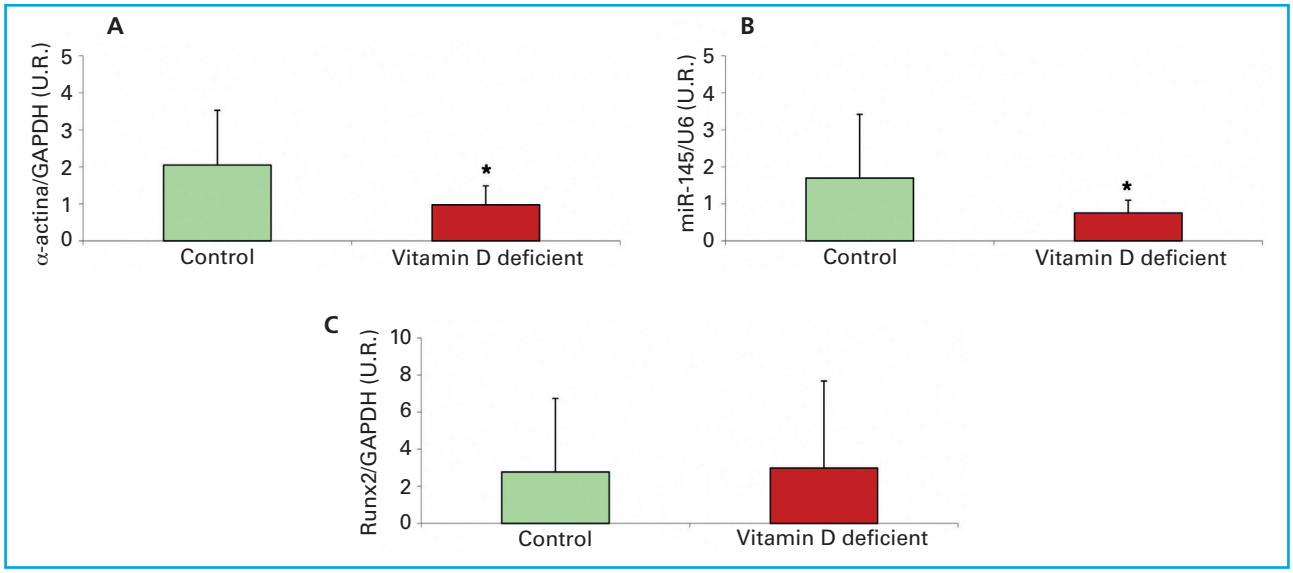

Figure 3. Relative gene expression in the aortas of mice fed normal and vitamin D-deficient diets. A. α-actin expression. B. miR-145 expression. C. Runx2 expression. *$p < 0.05$ vs the control group (normal vitamin D diet group).

Thus, maintaining adequate miR-145 levels appears essential for vascular health. Among the inducers of miR-145 expression is vitamin D, which mediates its antiproliferative and gene-regulating effects, potentially benefiting the prognosis and development of therapeutic strategies for gastric cancer (39). In a recent study, our group demonstrated that vitamin D administration prevented reductions in miR-145 and α-actin in vascular smooth muscle cells induced by uremia, thereby reducing vascular contractility and osteogenic differentiation alterations (40).

As other authors have pointed out (41), several mechanisms explain the link between vitamin D deficiency and cardiovascular diseases. Vitamin D deficiency stimulates PTH gene transcription, promoting myocyte hypertrophy and inflammatory mechanisms involving vascular smooth muscle cells. The vitamin D endocrine system also suppresses inflammatory processes, a key pathogenic mechanism in atherosclerosis, and exerts antiproliferative effects, reducing myocardial hypertrophy and damage (41).

It is essential to consider the challenges of extrapolating rodent model results to humans. However, these findings align with clinical studies while controlling confounding factors inherent to patient studies. Additionally, mice are not optimal models for studying vascular calcification due to their resistance to it. Nonetheless, the observed vascular alterations may indicate an initial vascular phenotype change leading to calcification.

This study shows that vitamin D deficiency in an aged mouse model impairs motor functionality and induces vascular phenotype changes. These vascular alterations include a marked loss of α-actin, the primary contractile protein in vascular smooth muscle cells, and miR-145, the main regulator of vascular phenotype.

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