Basic Medical Research

Formulation of mice diet with low cholecalciferol content

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pISSN: 0853-1773 • eISSN: 2252-8083
https://doi.org/10.13181/mji.oa.257859
Med J Indones. 2025;34:221–6

Received: November 02, 2024
Accepted: April 29, 2025
Published online: August 04, 2025

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ABSTRACT

BACKGROUND Vitamin D deficiency has been linked to autoimmune diseases, cancer, and cardiovascular diseases. Although 1 study attempted to elucidate the ingredients required to make this diet, the process remained unclear. Hence, this study aimed to customize a low cholecalciferol diet with good tolerability in mice.

METHODS We customized a diet containing a normal cholecalciferol content (1 IU/g diet) and another with a low cholecalciferol content (0.05 IU/g diet). Samples from both diets were sent to an independent laboratory to ensure that the levels of cholecalciferol, phosphorus, and calcium present in the custom diets matched our calculations. 5 mice were fed the customized normal cholecalciferol diet for 1 week to assess tolerability. Tolerability was assessed by measuring the amount of food consumed, weight gained, and the presence of any adverse events.

RESULTS Cholecalciferol, phosphorous, and calcium levels in both diets satisfactorily matched our calculations. The diet was well tolerated without any adverse events or mortalities. The mice consumed an adequate amount of food (mean: 5.34 [0.08] g diet/day, 95% confidence interval [CI]: 5.12–5.56; 19.38 kcal, fat: 0.43 g, protein: 0.14 g, carbohydrates: 3.16 g, and cholecalciferol: 0.007 mg) and gained a slight amount of weight by the end of the experiment (mean: 1.86 [0.46] g, 95% CI: 0.58–3.14).

CONCLUSIONS This study successfully created 2 custom diets with quantified cholecalciferol contents. This animal model may prove valuable for studies involving vitamin D.

KEYWORDS diet, food safety, methods, mice, vitamin D

Vitamin D, a steroid hormone, plays a vital role in regulating the innate and adaptive immune system.[1] The Institute of Medicine defines vitamin D deficiency as <12 ng/ml, while the Endocrine Society sets the threshold at <29 ng/ml.[2] Vitamin D deficiency has been linked to autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis, systemic sclerosis, psoriatic arthritis, ankylosing spondylitis, inflammatory bowel disease, and autoimmune thyroid diseases.[1,3] One way in which vitamin D deficiency contributes to the pathogenesis of autoimmune diseases is by regulating dendritic cell (DC) maturation. Immature DCs have a higher capacity for antigen uptake and processing, whereas mature DCs have a lower capacity and function as antigen-presenting cells, triggering an overreactive inflammatory response. Vitamin D inhibits DC maturation and suppresses the inflammatory response.[1,4]

Several studies have linked vitamin D to cancer, potentially reducing its risk through anti-

inflammatory effects, as chronic inflammation can trigger carcinogenesis. Several single nucleotide polymorphisms affecting the vitamin D pathway, such as rs731236 (*TaqI*), rs1544410 (*BsmI*), and rs2228570 (*FokI*), have also been found to be associated with increased cancer risk.[5] Additionally, vitamin D plays a role in cardiovascular health by inhibiting angiotensin I and II in the myocardium, kidneys, and renal arteries, while promoting the production of angiotensin-converting enzyme 2, which helps break down angiotensin II. These actions endow vitamin D with antihypertensive, antifibrotic, and antihypertrophic properties.[6]

While human studies have linked vitamin D deficiency to various diseases, most are observational studies that cannot confirm causation.[3,7–15] Animal models, such as mice fed a low cholecalciferol diet, may be pivotal in proving this hypothesis. Vitamin D levels are influenced by many factors, including diet, sunlight, gastrointestinal absorption, and the hydroxylation capacity of the liver and kidneys.[16] Although previous studies have assessed the role of vitamin D deficiency in animal models, they lack a detailed, step-by-step method for preparing a low cholecalciferol mouse diet.[17–19] Many steps are needed to create a validated low cholecalciferol mouse diet with the desired cholecalciferol content; this involves calculating the correct amount of cholecalciferol needed, as well as evenly distributing cholecalciferol to the chow. Thus, this study aimed to formulate a well-tolerated low cholecalciferol diet for mice.

METHODS

Diet

We designed two diets with different amounts of cholecalciferol: one with normal recommended dietary intake (RDI) cholecalciferol content (1 IU/g of diet) and another with a low cholecalciferol content (0.05 IU/g).[2] A study by Mallya et al[2] confirmed that 0.05 IU/g cholecalciferol is sufficient to induce vitamin D deficiency. All ingredients except cholecalciferol were identical in both diets. Since Mallya et al[2] had proven its effectiveness in lowering vitamin D levels,

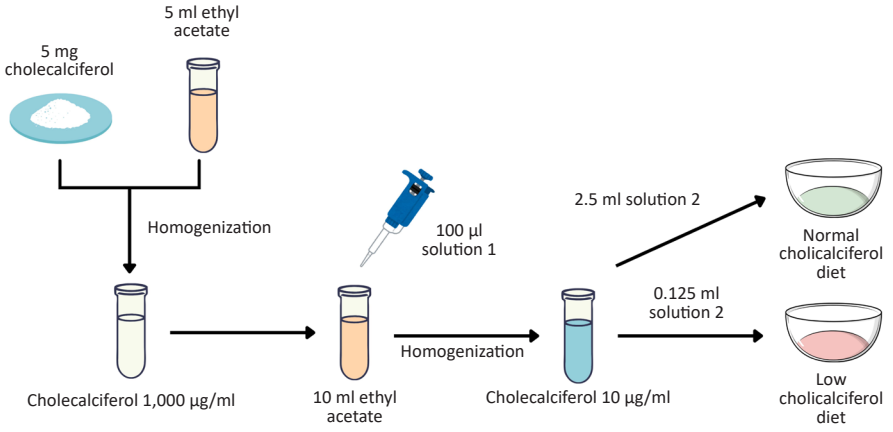

Figure 1. Procedure for making cholecalciferol solution

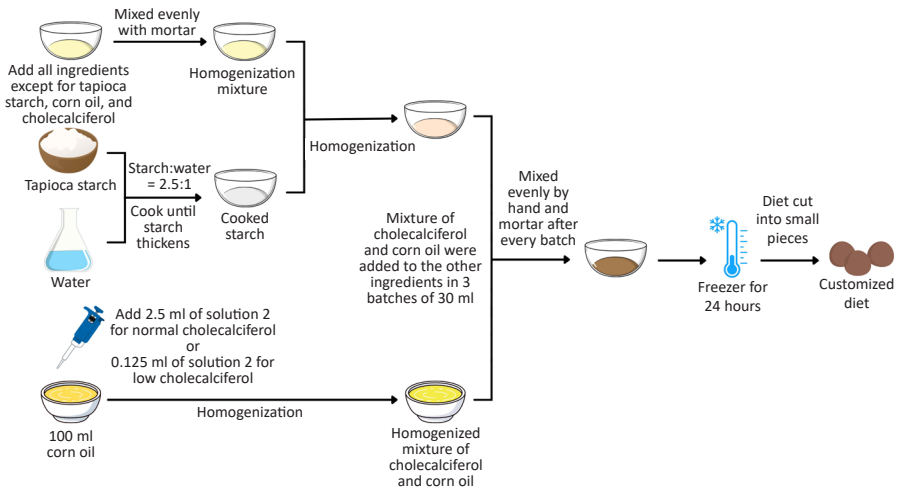

Figure 2. Procedure for creating a customized diet with measured cholecalciferol content

our study focused on the process of creating a low cholecalciferol diet instead of manipulating vitamin D levels in mice.

Preparation of cholecalciferol solution

We used a cholecalciferol concentration of 1 IU/g diet for the normal diet and 0.05 IU/g diet for the low cholecalciferol diet. Because 1 IU of vitamin D equals 0.025 µg, normal and low diets required 25 µg/kg and 1.25 µg/kg, respectively. To facilitate extraction, we first prepared a 1,000 µg/ml of cholecalciferol solution (solution 1) by dissolving 5 mg of cholecalciferol in 5 ml of ethyl acetate. Next, we diluted 100 µl of solution 1 in 10 ml of ethyl acetate to create 10 µg/ml (solution 2). We then mixed 2.5 ml of solution 2 into 1,000 g of normal diet and 0.125 ml of solution 2 into 1,000 g of low cholecalciferol diet (Figure 1).

Procedure for making the diet

All ingredients, as shown in Table 1, except tapioca starch, corn oil, and cholecalciferol solution, were evenly mixed using a mortar. Water was added to tapioca starch (2.5:1 ratio) and slowly cooked until the starch thickened into a transparent, mochi-like texture. Cooked starch was then added to the previously mixed ingredients and mixed thoroughly. The cholecalciferol solution was first homogenized with corn oil, before being gradually and evenly added to the mixture. The final mixture was weighed, portioned, and placed in a freezer for 24 hours, before being cut into small pieces for feeding (Figure 2). To verify the accuracy of the concentrations, diet samples were sent to an independent commercial laboratory (Regional Health Laboratory) to ensure that the concentrations of cholecalciferol, phosphorus, and calcium remained within <15% of the target values (RDI: calcium, 10 g/kg; phosphorus, 3 g/kg).[2]

Animals and housing

Five female BALB/c mice (4–6 weeks old) obtained from Inolabs, Indonesia, were used in this study. After a 1-week acclimatization period to a standard diet, the mice were switched to a customized normal cholecalciferol diet to assess tolerability. This was defined as a sufficient diet intake (3.5–3.75 g diet/day), with adequate weight gain (≥85.7 g/day) and no major adverse events (e.g., death).[20,21] Daily measurements recorded food consumption and body weight before and after the study. The mice were also monitored for acute toxicity to ethyl acetate, such as vomiting, salivation, tremor, lethargy, irritation to the eyes, loss of coordination, and death.[22] According to the Food and Drug Administration guidelines, a 1–2-week observation period was deemed sufficient to detect any treatment-related effects.[23] Another study using ethyl acetate in mice also used a 1-week observation period.[24] Thus, we deemed a 1-week observation period to be sufficient in this study. All mice were housed in standard cages with *ad libitum* access to water and food. Statistical analyses were performed using SPSS software version 22 (IBM Corp., USA). The protocol for this study was approved by the Ethics Committee of the Faculty of Medicine, Universitas Indonesia – Cipto Mangunkusumo Hospital (No: KET-589/UN2.F1/ETIK/PPM.00.02/2022).

RESULTS

Levels of cholecalciferol, calcium, and phosphorus in the custom diet

After preparing the custom diets (Table 1, Supplementary Table 1), samples were sent to the

Table 1. Composition of custom diets

Ingredients	Amounts (g/kg)
Casein, vitamin-free	180.00
L-cystine	2.00
Dextrose	580.78
Corn oil	100.00
Cellulose fiber, microcrystalline	30.00
Modified AIN-93M mineral mix without calcium and phosphorus	13.37
Calcium carbonate anhydrous	29.93
Phosphorus	1.03
Potassium phosphate, dibasic anhydrous	4.90
Potassium phosphate, monobasic	3.90
Corn starch	13.22
Choline dihydrogen citrate	3.50
Vitamin E, DL-alpha tocopheryl acetate (500 IU/g)	0.24
Vitamin A palmitate (500,000 IU/g)	0.04
Vitamin mix AIN-93-VX, without vitamin A, D, and E	5.00
6-ethoxy-2,2,4-trimethyl-1,2-dihidroquinoline	0.02
Tapioca starch (5%) (water:tapioca starch = 2.5:1)	50.00
Cholecalciferol*	
Normal vitamin D diet (1 IU/g = 1,000 IU/kg)	25.00
Low cholecalciferol diet (0.05 IU/g = 50 IU/kg)	1.25

*Cholecalciferol is in µg/kg

Table 2. Cholecalciferol, calcium, and phosphorus levels in custom diets

	Mineral	RDI (mg/kg)	Measured content (mg/kg)
Normal vitamin D diet	Calcium 1%	10,000	11,430
	Phosphorus 0.3%	3,000	5,641
	Cholecalciferol*	25.00	26.39
Low cholecalciferol diet	Calcium 1%	10,000	11,485
	Phosphorus 0.3%	3,000	6,219
	Cholecalciferol*	1.25	1.24

*Cholecalciferol is in µg/kg
RDI=recommended dietary intake

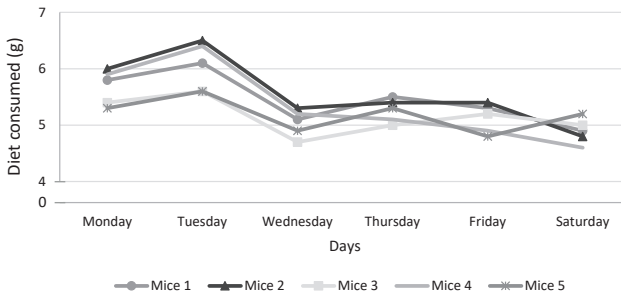

Figure 3. Amount of diet consumed each day

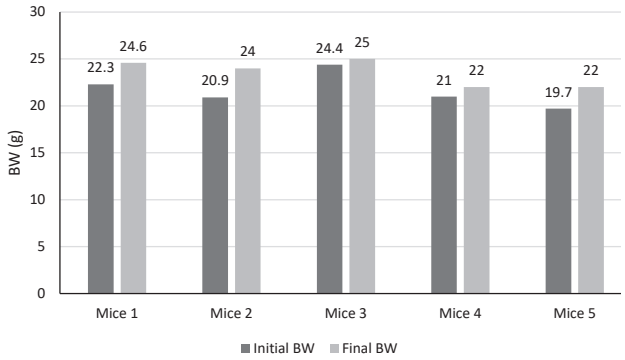

Figure 4. Changes in body weight (BW) during the experimental period

Regional Health Laboratory to measure vitamin D, calcium, and phosphorus levels. The results confirmed that the calculated levels of all three minerals were similar (Table 2).

Tolerance of mice to the custom diet

The low cholecalciferol diet was well tolerated, with no deaths or adverse events. On average, the mice consumed a satisfactory amount of food (mean: 5.34 [0.08] g diet/day, 95% confidence interval [CI]: 5.12–5.56; 19.38 kcal, fat: 0.43 g, protein: 0.14 g, carbohydrates: 3.16 g, and cholecalciferol: 0.007 µg) (Figure 3, Supplementary Figure 1) and gained weight (mean: 1.86 [0.46] g; 95% CI: 0.58–3.14) (Figure 4).

DISCUSSION

In this study, we successfully customized a low cholecalciferol diet with good tolerability in mice, with no adverse events or deaths. The mice consumed adequate food and gained sufficient weight. While Mallya et al[2] provided a formula, they did not report the steps required to create the diet, giving our study an advantage in methodology documentation.[2]

Vitamin D is associated with autoimmune diseases, cancer, and cardiovascular diseases, highlighting its role in immune regulation. Vitamin D is mainly obtained via diet or cutaneous synthesis under ultraviolet light exposure, in which 7-dehydrocholesterol is converted to pre-cholecalciferol, which is later isomerized into cholecalciferol. Once absorbed or synthesized, cholecalciferol undergoes hydroxylation in the liver to form 25-hydroxycholecalciferol, which is then converted in the kidney to calcitriol, the active metabolite of vitamin D, with the help of 1α-hydroxylase.[25]

Vitamin D levels are influenced by the calcium and phosphorus balance. In vitamin D deficiency, intestinal calcium absorption declines, leading to hypocalcemia, which triggers parathyroid hormone (PTH) production to increase serum calcium levels through the increased renal production of vitamin D. Elevated phosphorus levels can further stimulate PTH production, underscoring the need for balanced calcium and phosphorus levels in vitamin D metabolism. It is important to ensure sufficient calcium and phosphorus levels to accurately induce vitamin D deficiency using a low cholecalciferol diet.[26] In this study, we accounted for this by ensuring adequate amount of calcium and phosphorus in the customized diets, which were confirmed to be within the required levels in the finalized product.

The diet formulation involved evenly mixing ingredients, grinding the mixture to the correct

density, moistening, drying, and cooling before packaging and feeding. The diet may also undergo irradiation or autoclaving for sterilization, but these steps require specialized factory-level equipment that may not be available in a standard laboratory.[20] However, our method is simpler, cost-effective, and feasible in a laboratory with minimal equipment.

Throughout this study, we made three attempts before obtaining the correct levels of cholecalciferol, phosphorus, and calcium in the custom diets, according to our calculations (Supplementary Table 2). In the first attempt, the levels in the sampled custom diets did not match our calculations. To exclude measurement errors due to calibration issues, we sent samples from both diets to an independent laboratory (Regional Health Laboratory) for confirmation; however, the results did not match. Next, we assumed that the ingredients were not evenly mixed and changed from manual to mortar mixing. Although this change reduced the discrepancy between our calculations and the levels detected in the custom diets, it was still unsatisfactory. To address this, we changed the concentration of the cholecalciferol solution from 10,000 µg/ml to 10 µg/ml. We made this change as we hypothesized that using a higher concentration of cholecalciferol (10,000 µg/ml) required adding only a very small volume (20 µl for normal cholecalciferol diet and 0.65 µl for low cholecalciferol diet) during production. It may be difficult to distribute such a small volume evenly throughout the mixture. In addition, the mixing process was slightly altered. Previously, cholecalciferol solution was added to corn oil and then directly incorporated into the mixture without homogenization. This could have led to uneven mixing owing to the high viscosity of the corn oil. Thus, we altered this step by ensuring the homogenization of the cholecalciferol solution with corn oil before adding it to the mixture. Ultimately, the desired levels of cholecalciferol, phosphorus, and calcium were successfully achieved.

AIN-93M, a standard mouse chow, typically contains soybean oil as the required source of fat in the mouse diet. However, soybean oil contains vitamin D, which may affect the final vitamin D levels when formulating a low cholecalciferol diet. To minimize this effect, it is essential to use oil with a minimal vitamin D content to ensure that the final vitamin D levels of the customized diet are aligned with the desired amount and remain unaffected by additional ingredients. This study addressed this issue by substituting soybean oil with corn oil, which has a minimal vitamin D content. Furthermore, vitamin D, a fat-soluble vitamin, requires an oil-based carrier. Corn oil, an excellent carrier of vitamin D, achieved >80% bioaccessibility of vitamin D, making it an ideal choice for this study.[27]

After successful diet preparation, we tested its tolerability in mice. In addition to monitoring adverse events, we assessed the food consumption to determine whether the custom diets were adequately consumed. Additionally, the weight gain was evaluated to confirm that the diet provided sufficient nutrition.

In this study, mice tolerated the custom diet well, with a slight weight gain after consumption. Although we successfully customized a low cholecalciferol diet, this study still has several limitations. One limitation is that we did not measure the serum levels of vitamin D, calcium, or phosphorus in the experimental mice after consuming the diet. In addition, we used only a small sample of animals and a short observation period. Studies with a larger number of samples and longer observation periods may be required to further validate our findings. In conclusion, we successfully developed two custom diets with quantified cholecalciferol content. The custom diet was well tolerated in the mice without any reported adverse events.

Conflict of Interest

The authors affirm no conflict of interest in this study.

Acknowledgment

We would like to acknowledge the Department of Nutrition, Faculty of Health Sciences, Universitas Brawijaya, Malang; *Laboratorium Penelitian Hewan Coba* (LPHC), Faculty of Medicine, Universitas Brawijaya, Malang; Regional Health Laboratory, Jakarta; and Faculty of Medicine, Universitas Indonesia, Cipto Mangunkusumo Hospital for the support throughout this research.

Funding Sources

None.

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