

Original Article

Dose-Dependent Effects of Cholecalciferol Supplementation on Hepatocyte Ballooning in Sprague–Dawley Rats

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ABSTRACT

Background: Obesity is characterized by excessive fat accumulation resulting from an imbalance between caloric intake and energy expenditure. Low serum vitamin D concentrations correlate with reduced high-density lipoprotein concentrations, elevated triglyceride levels, and increased lipid deposition within hepatocytes through mechanisms involving impaired lipid metabolism and hepatic inflammation. Vitamin D supplementation improves lipid profiles, body mass index, and waist circumference. **Objective:** This study aimed to analyze histopathological changes in the livers of male Sprague–Dawley rats induced with a high-fat high-fructose diet supplemented with cholecalciferol. **Methods:** Thirty male Sprague–Dawley rats aged 6–8 weeks were randomly divided into five groups. The normal control group (KN) received BR-2 pellets and a PAM solution. The negative control group (K-) received a high-fat, high-fructose (HFHF) diet for 28 days. The treatment groups (P1, P2, and P3) received an HFHF diet for 28 days, followed by supplementation with 2500 IU, 5000 IU, or 10000 IU cholecalciferol for 56 days. Histopathological examination was used to assess hepatic steatosis, lobular inflammation, and hepatocyte ballooning according to the NAFLD activity score (NAS) criteria after cholecalciferol administration. **Results:** Unexpectedly, no hepatic steatosis developed in any group, including the HFHF-fed controls, indicating inadequate model establishment. Cholecalciferol did not significantly affect lobular inflammation ($p>0.05$). However, a significant dose-dependent reduction in hepatocyte ballooning occurred ($p<0.05$), with 10,000 IU resulting in near-complete elimination. **Conclusion:** Cholecalciferol (2,500–10,000 IU) did not significantly reduce liver steatosis or lobular inflammation but significantly reduced hepatocyte ballooning in a dose-dependent manner. The absence of steatosis limits the interpretation of the effects of cholecalciferol on established NAFLD pathology.

KEYWORDS

Cholecalciferol;
nonalcoholic fatty
liver disease; obesity;
diet, high-fat;
hepatocytes; rats,
Sprague–Dawley

ARTICLE INFO

Received Jul 13, 2025
Revised Des 03, 2025
Accepted Jan 21, 2026
Online Jan 23, 2026

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CITE THIS ARTICLE

Veonika, DA. (2025). Dose-dependent effects of cholecalciferol supplementation on hepatocyte ballooning in Sprague–Dawley rats. *Svāsthya: Trends in General Medicine and Public Health*, 2(6): e134. <https://doi.org/10.70347/svsthya.v2i6.134>.



Supplemental data for this article can be accessed online at <https://doi.org/10.70347/svsthya.v2i6.134>
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INTRODUCTION

Obesity is a condition characterized by abnormal or excessive fat accumulation that leads to health risks. Obesity is defined as a body mass index of more than 30. The World Health Organization reported that 39% of adults are overweight and that 13% are obese. In Indonesia, the prevalence of obesity increased from 18.8% in 2007 to 26.6% in 2013. In 2018, the prevalence of obesity in Indonesia slightly decreased to 21.8% [1,2]. Obesity increases the risk of various health problems, such as cardiovascular disease, type 2 diabetes mellitus, hypertension, nonalcoholic fatty liver disease (NAFLD), cancer, hyperlipidemia, chronic kidney disease, obstructive sleep apnea, depression, and osteoarthritis. Obese individuals may have an elevated risk of all-cause mortality of 21–108% [3,4].

Several risk factors that increase the risk of obesity can be nonmodifiable, such as genetics, and modifiable factors, such as physical inactivity, excessive caloric intake, obesity from the mother, insufficient sleep, certain drugs, certain ethnicities, and psychosocial stress [5]. Vitamin D deficiency has also been linked to obesity. A meta-analysis reported an inverse association between vitamin D levels and body weight [6]. Low serum 25-hydroxyvitamin D concentrations predict subsequent weight gain and visceral adiposity accumulation, establishing a temporal relationship beyond mere associations. Vitamin D deficiency has also been correlated with childhood obesity, although the mechanism remains unclear [7]. Vitamin D regulates adipogenesis through vitamin D receptor (VDR)-mediated suppression of PPAR γ expression and modulation of Wnt/ β -catenin signaling while promoting adipocyte apoptosis via upregulation of proapoptotic genes, including BAX and caspases. At the hepatic level, VDR activation upregulates peroxisome proliferator-activated receptor alpha (PPAR α), enhancing fatty acid β -oxidation and reducing lipid accumulation in hepatocytes [8].

Vitamin D levels have also been reported to affect lipid levels. Higher serum vitamin D levels are associated with decreased low-density lipoprotein (LDL) levels. Individuals with high triglyceride levels, low high-density lipoprotein (HDL) levels, and high total cholesterol levels also have low vitamin D levels [9,10]. Vitamin D deficiency has also been linked to the development of NAFLD, independent of metabolic syndrome [11]. Mechanistically, vitamin D deficiency promotes hepatic inflammation through increased NF- κ B activation and elevated proinflammatory cytokines, including TNF- α , IL-6, and IL-1 β , accelerating the progression from simple steatosis to steatohepatitis. Vitamin D supplementation has been shown to decrease the risk of obesity and correct triglyceride levels in patients with obesity. It has also been shown that vitamin D supplementation improves indices of body composition, such as fat mass, fat mass index, and body fat percentage [12–14].

Despite the established association between vitamin D deficiency and NAFLD, critical knowledge gaps remain regarding optimal dosing strategies for hepatic protection in obese individuals. Research has reported conflicting effects on hepatic steatosis reversal, with studies employing widely varying doses (400–4000 IU/day) and durations (6 weeks to 12 months), limiting clinical translation. No dose-response evaluation of cholecalciferol (2,500–10,000 IU) has been conducted via standardized histopathological assessments in diet-induced obesity models. This study addresses these gaps by systematically evaluating the effects of three different cholecalciferol doses on hepatic histopathology via the validated NAFLD activity score system, providing evidence-based guidance for therapeutic vitamin D supplementation in obesity-related liver disease.

MATERIALS AND METHODS

Study design and setting

This study used a true experiment with a preposttest control group design, which compared every dependent variable in each obesity model rat via simple randomization. This study employed a randomized controlled experimental design to compare histopathological outcomes across five groups [15,16]. The study was conducted at the Central Laboratory of Food and Nutrition Studies at Gadjah Mada University, Yogyakarta. Histopathological analysis was

performed at the Anatomical Pathology Laboratory of the Faculty of Medicine, Public Health, and Nursing of Universitas Gadjah Mada. The study was conducted from January to April 2023.

Population and sample

The study population comprised white Sprague–Dawley rats aged 6–8 weeks. The rats were male to ensure homogeneity and avoid hormonal factors such as estrogen. The samples in this study consisted of 30 rats divided into five intervention groups, with six animals in each group. The sample size was calculated via Federer's formula for animal experiments, with $n=6$ per group, providing adequate statistical power while adhering to the ethical principles of minimizing animal use. The groups included a normal control (KN), a negative control (K-), and three treatment groups labeled P1, P2, and P3.

Inclusion and exclusion criteria

The inclusion criteria for the animal samples were as follows: (1) Sprague–Dawley rats, (2) male sex, (3) aged 6–8 weeks, (4) weighing 150–200 grams, and (5) healthy with normal behavior. The exclusion criteria for the animal samples were as follows: (1) rats with diarrhea, as shown by the informed fecal matter of the rat, and (2) rats that did not meet the obesity criteria according to the Lee index after induction. The Lee index was calculated on day 29 following the HFHF dietary intervention. Animals with Lee indices <300 were excluded and replaced to maintain a group size of $n=6$.

Study intervention and data collection procedure

Prior to the intervention, the rats underwent a seven-day acclimation period and were provided unrestricted access to drinking water. To induce obesity, the rats were fed a high-fat, high-fructose (HFHF) diet composed of 32 g of B2-2 feed, 28 g of duck egg yolk, 40 g of beef, 12 g of chicken liver, 4 g of butter, and 10% fructose solution. The dietary composition was calculated to provide a high-fat ($>40\%$ energy from fat) and high-fructose content to induce metabolic dysfunction. Cholecalciferol was administered daily in a soft gel form at 08:00 AM.

Table 1. Summarizes the intervention timeline for all the experimental groups

Group	Day 1-7	Day 8-35 (28 days)	Day 36-91 (56 days)	Duration
KN	Acclimation	BR-2 pellet + PAM	BR-2 pellet + PAM	91 days
K(-)	Acclimation	HFHF diet	HFHF diet	91 days
P1	Acclimation	HFHF diet	HFHF diet + Cholecalciferol 2500 IU	91 days
P2	Acclimation	HFHF diet	HFHF diet + Cholecalciferol 5000 IU	91 days
P3	Acclimation	HFHF diet	HFHF diet + Cholecalciferol 10000 IU	91 days

Remarks: HFHF, high-fat high-fructose diet; PAM, drinking water; cholecalciferol was administered daily at 08:00 AM in soft gel form via oral gavage.

Obesity in the rats was determined via the Lee index formula. The Lee index was measured at baseline (day 0), post-HFHF induction (day 29), and at termination (day 92). Body weight was recorded via a digital scale (precision ± 0.1 g), and nasoanal length was measured via a flexible measuring tape while the animals were under light isoflurane anesthesia to ensure accurate measurement. The rats were classified as obese when the Lee index exceeded 300. The Lee index was measured by recording the body weight and nasoanal length (the distance from the nose to the anus) and then calculating the value via the following formula: histopathological evaluation was performed in a blinded manner by two independent pathologists. The pathologist who assessed the liver sections was not informed of the group assignments or treatment conditions to minimize observer bias. The histopathological assessment in this study involved microscopic evaluation of liver tissue from obese model rats following 56 days of cholecalciferol administration.

Liver sections were processed via standard paraffin embedding techniques, sectioned to a thickness of 4–5 μm , and stained with hematoxylin and eosin (H&E) for evaluation. All

histological evaluations were performed in a blinded fashion. The pathologist responsible for scoring the slides was not informed of the group assignments or treatment conditions to minimize observer bias. Each slide was coded prior to the assessment. Liver injury was evaluated via the NAFLD Activity Scoring (NAS) system established by the NASH Clinical Research Network. The NAS system includes three microscopic components: steatosis (score 0–3), lobular inflammation (score 0–3), and hepatocyte ballooning (score 0–2). The total NAS was calculated as the sum of the three parameters to provide an overall measure of disease activity.

Statistical analysis

The data are presented as the means $\pm$ standard deviations (SDs). Data normality was assessed via the Shapiro–Wilk test, and homogeneity of variance was evaluated via Levene's test. Cholecalciferol administration and liver histopathology data were analyzed via the nonparametric kruskal–wallis test. Nonparametric tests were employed due to the violation of normality assumptions ($p < 0.05$ for the Shapiro–Wilk test) and the ordinal nature of the histopathological scores. If the results were significant, a Mann–Whitney test was performed. For multiple pairwise comparisons, the Bonferroni correction was applied with an adjusted significance threshold of $\alpha = 0.005$ ($0.05/10$ comparisons). Statistical significance was set at $p < 0.05$ for all analyses, except for post hoc comparisons. All the statistical analyses were performed via SPSS version 16.0 (SPSS Inc., Chicago, IL, USA).

Ethical considerations

All experimental procedures were approved by the Research Ethics Committee of the Faculty of Medicine, Universitas Sebelas Maret (approval number: 17/UN27.06.11/KEP/EC/2023, protocol ID 01/02/12/2022/191 date: January 24, 2023) and conducted in accordance with the ARRIVE guidelines for animal research.

RESULTS

Degree of steatosis

This study evaluated the histopathological changes in rat livers following 56 days of cholecalciferol supplementation at three doses. Histopathological assessment was performed via the NAFLD activity score (NAS) to evaluate steatosis, lobular inflammation, and hepatocyte ballooning. The results are presented sequentially for each parameter. Unexpectedly, hepatic steatosis was absent in all the experimental groups (Table 2). The mean steatosis score for all groups was 0.00 ± 0.00 , indicating $<5\%$ lipid accumulation in hepatocytes according to the NAS criteria. This finding was observed despite the 28-day HFHF dietary intervention in the K(-), P1, P2, and P3 groups. The absence of steatosis in the HFHF-fed negative control group (K-) indicates that the dietary protocol failed to induce the expected hepatic lipid accumulation characteristic of diet-induced obesity models. The kruskal–wallis test confirmed that there were no significant differences between the groups ($p = 1.000$). Visual inspection of H&E-stained liver sections from all groups revealed preserved hepatic architecture without vacuolated hepatocytes (Figure 1).

Table 2. Degree of steatosis in histopathological images

Group	Mean $\pm$ SD	P value
KN	0,00 $\pm$ 0,00	1,000
K(-)	0,00 $\pm$ 0,00	
P1	0,00 $\pm$ 0,00	
P2	0,00 $\pm$ 0,00	
P3	0,00 $\pm$ 0,00	

Remarks: There was no significant difference ($P > 0.05$), 95% confidence level.

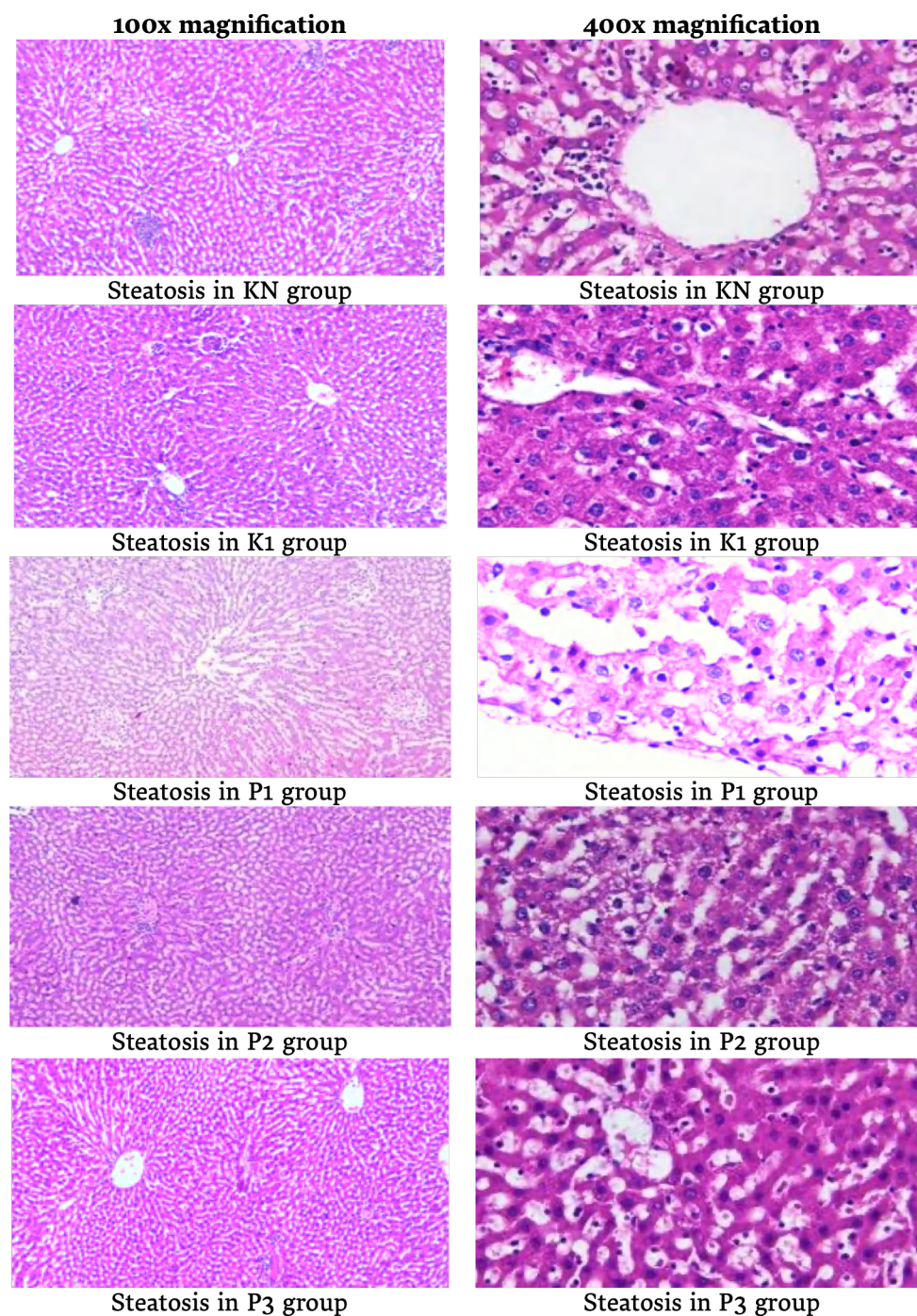


Figure 1. The histopathology of the livers of the rats at 100x and 400x magnification was assessed for steatosis. Remarks: All groups showed no significant degree of steatosis.

Lobular inflammation

Lobular inflammation scores varied across groups but did not reach statistical significance (Table 3). The kruskal-wallis test revealed a trend toward significance ($p=0.058$), approaching the conventional threshold of $p<0.05$. The highest mean lobular inflammation score occurred in the K(-) group, indicating that the animals received the HFHF diet without cholecalciferol supplementation. Among the cholecalciferol-treated groups, P2 (5,000 IU) presented the highest inflammation score, whereas P1 (2,500 IU) and P3 (10,000 IU) presented identical mean scores. The normal control group (KN) presented the lowest level of inflammation, which was consistent with the absence of dietary challenge. Histological examination revealed inflammatory foci primarily comprising lymphocytes and occasional neutrophils in the periportal and intralobular regions (Figure 2).

Table 3. Lobular inflammation on liver histopathology

Group	Mean±SD	P value
KN	0,33±0,52	0,058
K(-)	1,67±0,82	
P1	1,00±0,63	
P2	1,50±0,84	
P3	1,00±0,89	

Remarks: There was no significant difference ($P>0.05$), 95% confidence level.

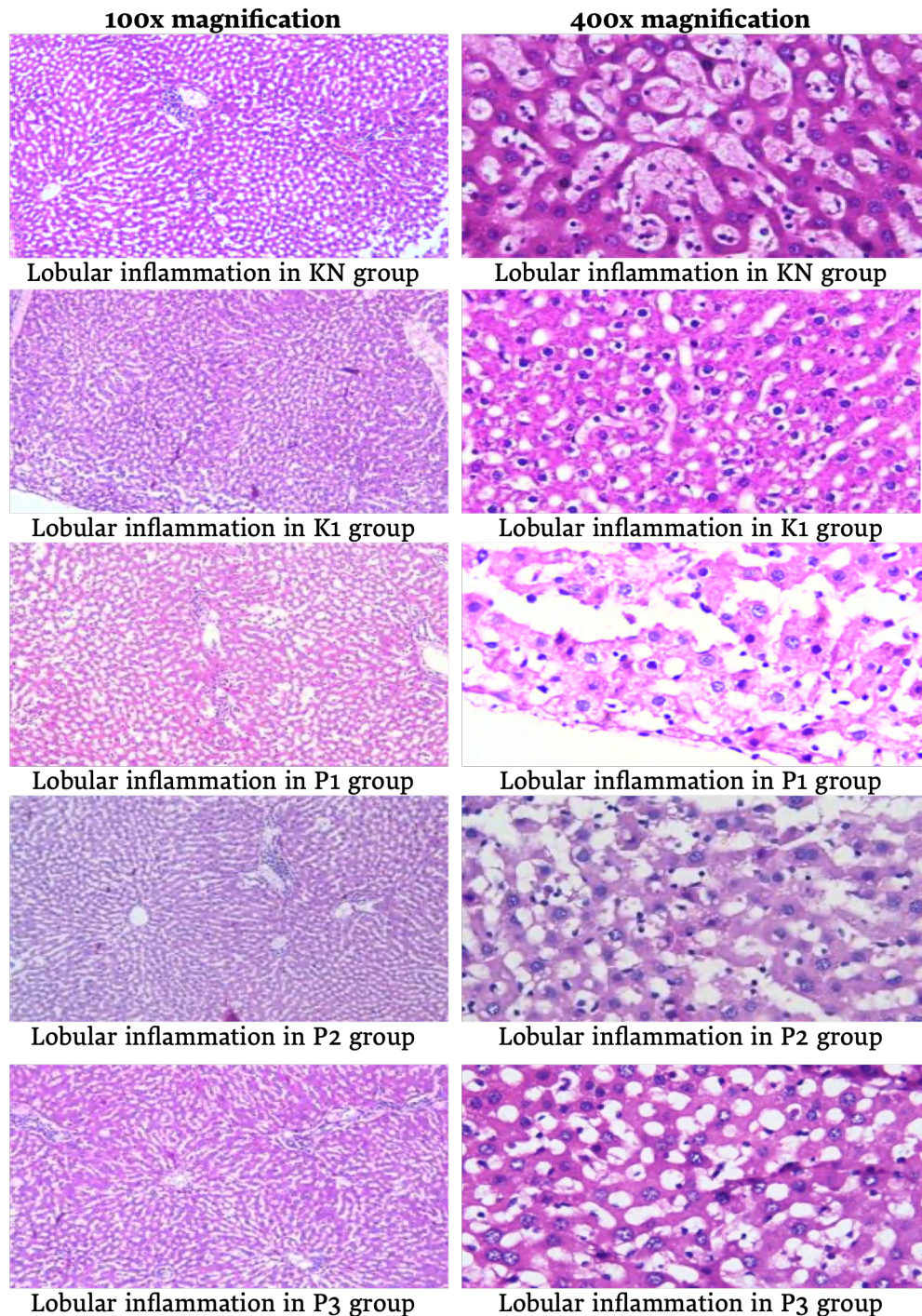


Figure 2. Histopathology of the rat liver at 100x and 400x magnification was performed to assess lobular inflammation. Remarks: There was no significant difference in lobular inflammation between the groups.

Ballooning in liver histopathology

Hepatocyte ballooning varied significantly across the experimental groups (Table 4). Kruskal-wallis analysis revealed significant differences between the groups ($p=0.003$). Unexpectedly, the highest ballooning score occurred in treatment group P1, which received the lowest cholecalciferol dose, followed by the normal control group KN. This paradoxical finding in the KN group, where healthy animals on a standard diet exhibited substantial hepatocyte ballooning comparable to that of the obesity-induced groups, warrants careful interpretation and suggests potential scoring inconsistencies or technical artifacts. The negative control group K(-), which received a HFHF diet without supplementation, presented moderate ballooning. Treatment group P2 (5,000 IU) demonstrated intermediate ballooning, whereas P3 (10,000 IU) showed a complete absence of ballooning. Histopathological examination confirmed that ballooned hepatocytes presented characteristic features, including cellular enlargement, pale cytoplasm with rarefaction, and the loss of distinct cytoplasmic borders (Figure 3).

Table 4. Ballooning in liver histopathology

Group	Mean $\pm$ SD	P value
KN	0,83 $\pm$ 0,75	0,003*
K-	1,67 $\pm$ 0,52	
P1	1,83 $\pm$ 0,41	
P2	1,17 $\pm$ 0,98	
P3	0,00 $\pm$ 0,00	

Remarks: * There was significant difference ($P<0.05$), 95% confidence level.

Ballooning comparison of liver histopathology

Post hoc pairwise comparisons via the Mann-Whitney test revealed specific group differences in hepatocyte ballooning (Table 5). Before applying the multiple comparison correction, significant differences ($p<0.05$) were observed for KN vs. P1, KN vs. P3, K(-) vs. P3, P1 vs. P3, and P2 vs. P3. After Bonferroni correction for 10 pairwise comparisons (adjusted $\alpha=0.005$), only two comparisons maintained statistical significance: K(-) vs. P3 and P1 vs. P3. These results confirm that the highest cholecalciferol dose (10,000 IU) significantly reduced hepatocyte ballooning compared with both the HFHF-fed negative control and the lowest dose of cholecalciferol supplementation. The comparison between the KN group and the other groups did not survive multiple comparison correction, reducing confidence in the elevated ballooning scores observed in the normal control group.

Table 5. Ballooning comparison of liver histopathology

Group comparison	U	Z	P value
KN $\times$ K-	7,00	-1,903	0,057
KN $\times$ P1	5,00	-2,272	0,023*
KN $\times$ P2	14,00	-0,677	0,489
KN $\times$ P3	6,00	-2,309	0,021*
K- $\times$ P1	15,00	-0,638	0,523
K- $\times$ P2	13,00	-0,903	0,367
K- $\times$ P3	0,00	-3,14	0,002*
P1 $\times$ P2	11,00	-1,340	0,180
P1 $\times$ P3	<0,001	-3,207	0,001*
P2 $\times$ P3	6,00	-2,309	0,021*

Remarks: * There was significant difference ($P<0.05$), 95% confidence level.

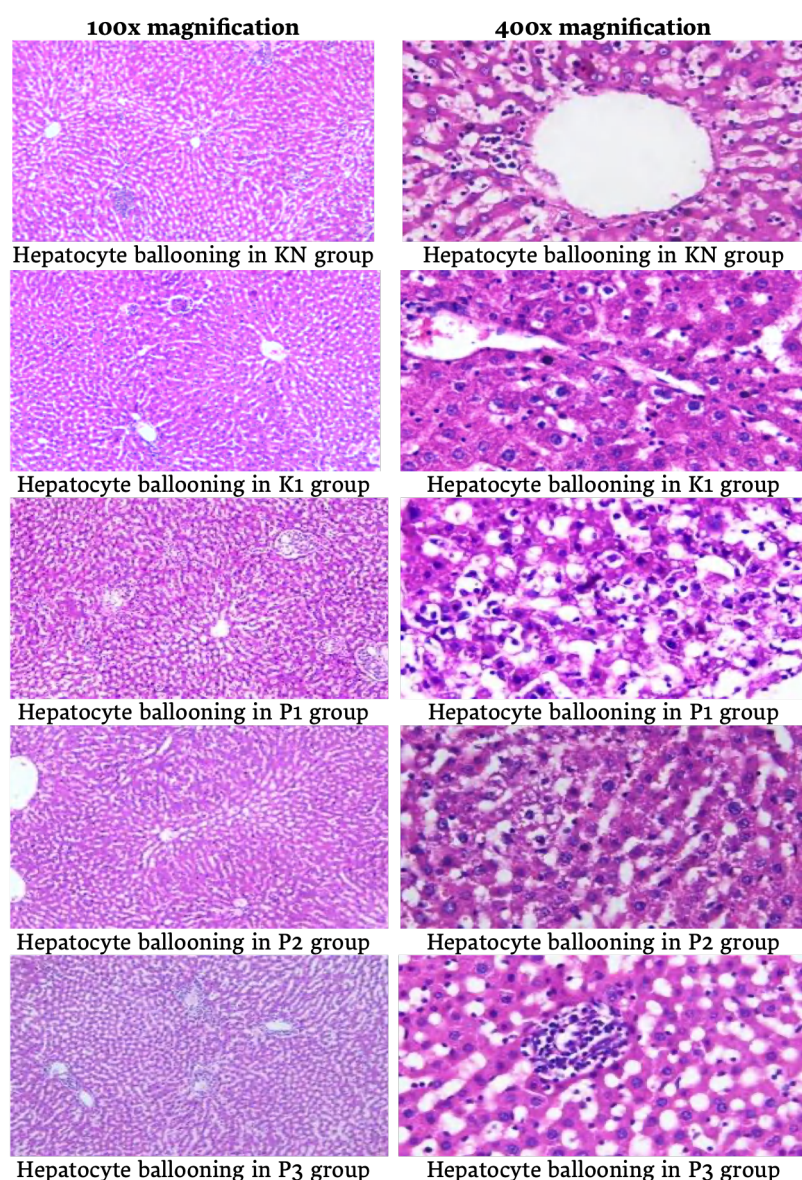


Figure 3. Histopathology of the rat liver at 100x and 400x magnification was assessed for ballooning. There was a significant difference in lobular inflammation between the groups.

NAS score

Total NAFLD Activity Score (NAS) was calculated as the sum of steatosis, lobular inflammation, and hepatocyte ballooning scores for each animal (Table 6). Group P3 receiving the highest cholecalciferol dose demonstrated the lowest total NAS among HFHF-fed groups, approaching the level of the normal control. However, none of the groups met the threshold for definitive NAFLD diagnosis (NAS ≥ 5) or NASH diagnosis, primarily due to absence of steatosis, which is a prerequisite criterion.

Table 6. Total NAS scores

Group	Steatosis	Lobular Inflammation	Hepatocyte Ballooning	Total NAS (Mean $\pm$ SD)	Interpretation
KN	0.00 $\pm$ 0.00	0.33 $\pm$ 0.52	0.83 $\pm$ 0.75	1.17 $\pm$ 0.98	No NAFLD
K(-)	0.00 $\pm$ 0.00	1.67 $\pm$ 0.82	1.67 $\pm$ 0.52	3.33 $\pm$ 1.03	Borderline*
P1	0.00 $\pm$ 0.00	1.00 $\pm$ 0.63	1.83 $\pm$ 0.41	2.83 $\pm$ 0.75	Borderline*
P2	0.00 $\pm$ 0.00	1.50 $\pm$ 0.84	1.17 $\pm$ 0.98	2.67 $\pm$ 1.37	Borderline*
P3	0.00 $\pm$ 0.00	1.00 $\pm$ 0.89	0.00 $\pm$ 0.00	1.00 $\pm$ 0.89	No NAFLD

Remarks: *Borderline: NAS 3-4 without steatosis does not meet NAFLD diagnostic criteria; NAFLD requires steatosis $\geq 5\%$ (score ≥ 1) as prerequisite.

DISCUSSIONS

Steatosis is a histological characteristic that is closely associated with NAFLD. Pathologists divide NAFLD into NAFL or “simple steatosis” (predominantly macrovesicular steatosis with or without specific inflammation) and nonalcoholic steatohepatitis (NASH). According to the NAFLD activity score (NAS) scoring system, NAFLD is confirmed if steatosis is found in $\geq 5\%$ of hepatocytes with causes other than alcohol, drugs, and viral induction. The steatosis score ranged from 0 to 3. A score of 0 indicated steatosis $< 5\%$, a score of 1 or mild indicated steatosis 5–33%, a score of 2 or moderate indicated steatosis 33–67%, and a score of 3 or severe indicated steatosis $> 67\%$. [15,16] A score of at least 1 is required to diagnose NAFL or NASH. The differentiation between NAFLD and NASH is based on lobular inflammation and ballooning scores [17].

The complete absence of hepatic steatosis in all experimental groups represents a fundamental limitation of this study. No animals, including those receiving a 28-day HFHF diet without cholecalciferol supplementation, developed measurable lipid accumulation in hepatocytes. This outcome indicates that the dietary intervention failed to establish the intended obesity-related liver pathology. Several methodological factors may explain this finding. First, the 28-day HFHF diet duration may have been insufficient for Sprague-Dawley rats. Successful NAFLD induction protocols typically employ 12–16 weeks of high-fat feeding, as demonstrated by Rahimi et al. [14], who required 12 weeks to achieve consistent steatosis in similar rat models. Additionally, Aboujassoum et al. [18] reported that Sprague-Dawley rats require 16–18 weeks of a cafeteria diet to develop significant hepatic steatosis and metabolic dysfunction.

Maciejewska et al. [19] demonstrated that a high-fat high-cholesterol diet induced gradual NAFLD development, with steatosis progressing from stage 0/1 after 4 weeks to stage 2/3 after 20 weeks in Sprague-Dawley rats. Second, the dietary fat composition may have been suboptimal. While we provided high fat content through duck egg yolk, beef, and butter, the total energy percentage from fat may not have reached the 45–60% threshold typically required for metabolic dysfunction [20]. Third, Sprague-Dawley rats exhibit considerable metabolic heterogeneity, with some individuals showing resistance to diet-induced hepatic lipid accumulation despite obesity. Without baseline steatosis, evaluating the protective effects of cholecalciferol on this parameter becomes impossible. Studies reporting vitamin D-mediated steatosis reduction, such as Du et al. [21] and Emam et al. [22], employed longer intervention periods (12–16 weeks) and confirmed the presence of steatosis before supplementation, which is fundamentally different from our experimental conditions.

Cholecalciferol supplementation at all the tested doses did not significantly reduce lobular inflammation, although a trend toward significance was observed ($p=0.058$). The HFHF-fed negative control group presented the highest inflammation score (1.67 ± 0.82), whereas the cholecalciferol-treated groups presented variable responses without clear dose-dependent patterns. This finding contrasts with the recent findings of Liu et al. [23], who reported a significant reduction in inflammation following vitamin D₃ supplementation in Wistar rats with established NAFLD. Similarly, El Amrousy et al. [24] demonstrated improved lobular inflammation in children with biopsy-confirmed NAFLD after 6 months of vitamin D supplementation. The discrepancy between our findings and those of these studies likely stems from differences in baseline pathology.

Both Liu et al. [23] and El Amrousy et al. [24] intervened in subjects with confirmed hepatic inflammation, whereas our model lacked significant baseline steatosis—the primary trigger for inflammatory cascade activation. Hepatic inflammation in NAFLD patients can develop secondary to lipotoxicity, oxidative stress, and mitochondrial dysfunction caused by lipid accumulation [25,26]. Without substantial steatosis, the inflammatory stimulus remains minimal, potentially explaining why the anti-inflammatory effects of vitamin D were not observable in our model. Additionally, our 8-week supplementation period may have been insufficient to modulate inflammatory pathways, as studies showing positive results employed longer durations (12–24 weeks).

Compared with the HFHF-fed control diet, cholecalciferol supplementation significantly reduced hepatocyte ballooning in a dose-dependent manner, with the 10,000 IU dose achieving complete elimination. The lowest dose (2,500 IU) paradoxically resulted in the highest ballooning

score, whereas intermediate dosing (5,000 IU) resulted in moderate ballooning. These findings suggest a threshold effect, where higher vitamin D doses provide superior hepatoprotection against cellular injury [17,27]. However, the unexpected observation of substantial ballooning in the normal control group raises concerns regarding potential technical variability in histopathological scoring or biological factors unrelated to dietary intervention.

Hepatocyte ballooning is a morphological marker of cellular stress characterized by cytoplasmic swelling, organelle dysfunction, and loss of cytoskeletal integrity [27]. This finding contrasts with that of El Amrousy et al. [24], who reported no significant effect of vitamin D (2000 IU/day for 6 months) on hepatocyte ballooning in pediatric NAFLD patients. The dose used in that study was substantially lower than our highest dose (2000 IU vs. 10,000 IU), which may explain these differential outcomes. Our results suggest that hepatoprotective effects against ballooning require higher vitamin D doses than those required for metabolic improvements. The mechanism underlying ballooning reduction likely involves the effects of vitamin D on hepatocyte membrane stability, mitochondrial function, and endoplasmic reticulum stress reduction, independent of its effects on lipid metabolism [28,29].

Vitamin D exerts hepatoprotective effects through multiple molecular pathways, which may explain the observed reduction in hepatocyte ballooning despite the absence of steatosis reversal. At the cellular level, vitamin D receptor (VDR) activation modulates hepatocyte survival and function via several mechanisms. VDR signaling upregulates peroxisome proliferator-activated receptor alpha (PPAR α), a master regulator of fatty acid β -oxidation and lipid metabolism. Du et al. [21] demonstrated that vitamin D supplementation increased hepatic PPAR α protein expression in high-fat diet-fed mice, enhancing mitochondrial fatty acid oxidation and reducing lipotoxicity. While this mechanism was unable to reverse established steatosis in our model, it may protect hepatocytes from lipid-induced cellular stress. Vitamin D has direct antiapoptotic effects on hepatocytes by inhibiting the p53-p21 signaling pathway, which regulates cellular senescence and programmed cell death. Yilmaz [30] reviewed evidence showing that vitamin D suppresses hepatocyte apoptosis and reduces oxidative stress by modulating the mitochondrial membrane potential and reducing reactive oxygen species production.

This antiapoptotic effect may directly prevent hepatocyte ballooning, which represents a preapoptotic state. Li et al. [27] comprehensively reviewed the mechanisms underlying hepatocyte ballooning, highlighting that increased endoplasmic reticulum stress and the unfolded protein response, along with cytoskeletal rearrangement and mitochondrial dysfunction, collectively contribute to the ballooned hepatocyte phenotype. Ajoolabady et al. [28] further reported that ER stress activates multiple pathways, including the PERK, IRE1 α , and ATF6 pathways, which regulate hepatocyte survival and contribute to the cellular swelling characteristic of ballooning degeneration [32]. VD modulates the ER stress response in hepatocytes. Hepatocyte ballooning is partly mediated by ER stress-induced cytoskeletal disruption and organelle dysfunction [31,32].

VDR activation reduces the expression of ER stress markers and preserves the cellular architecture, potentially explaining the reduction in ballooning, which is independent of lipid content. Fourth, vitamin D influences hepatic inflammation by suppressing nuclear factor-kappa B (NF- κ B) signaling, reducing the production of proinflammatory cytokines, including tumor necrosis factor-alpha (TNF- α), interleukin-6 (IL-6), and interleukin-1 beta (IL-1 β) [33]. Zhang et al. [32] demonstrated that vitamin D supplementation modulates the gut microbiota composition and reduces systemic inflammation, indirectly protecting hepatocytes from inflammatory injury [26]. The dose-dependent nature of ballooning reduction in our study suggests that these protective mechanisms operate on a threshold basis, requiring higher vitamin D concentrations to achieve measurable cellular protection.

Clinical implications

The dose-dependent reduction in hepatocyte ballooning observed with high-dose cholecalciferol (10,000 IU) suggests potential hepatoprotective effects that merit careful consideration. While this finding indicates that vitamin D may protect against hepatocellular injury, the absence of established steatosis in our model prevents conclusions about its therapeutic efficacy in clinical NAFLD. The reduction in ballooning may represent a preventive

rather than a therapeutic effect, protecting hepatocytes from initial injury under metabolically stressful conditions. This distinction is important for clinical applications. Recent meta-analyses have provided conflicting evidence regarding vitamin D supplementation in patients with established NAFLD [26,31,34]. These findings suggest that the hepatoprotective effects of vitamin D may be independent of systemic metabolic improvements, which operate through direct cellular mechanisms rather than through the correction of metabolic dysfunction [8,35]. For clinical application, our results suggest that high-dose vitamin D (10,000 IU) may prevent hepatocellular injury in individuals at risk for NAFLD; however, it cannot be recommended for reversing established disease on the basis of current evidence. The optimal timing, dose, and patient selection for vitamin D supplementation require further investigation via properly validated disease models and clinical trials.

Limitations

This study had several important limitations. The 28-day HFHF feeding period was insufficient to induce hepatic steatosis, preventing the evaluation of the effects of cholecalciferol on established NAFLD pathology. Successful protocols typically require 12–20 weeks in Sprague–Dawley rats. The small sample size ($n=6$ per group) limited the statistical power to detect modest treatment effects. The exclusion of female rats prevented the assessment of sex-specific differences in vitamin D metabolism, as estrogen modulates VDR expression and vitamin D-metabolizing enzymes. Serum 25-hydroxyvitamin D concentrations were not measured, preventing the confirmation of vitamin D deficiency or adequate repletion. The absence of molecular analyses (VDR expression, PPAR α levels, and inflammatory cytokines) limits the mechanistic understanding of the observed ballooning reduction. These limitations substantially constrain interpretation, and findings cannot be extrapolated to clinical NAFLD treatment without validation in properly established disease models with confirmed steatosis, larger sample sizes, longer interventions, inclusion of both sexes, and comprehensive mechanistic analyses.

CONCLUSIONS

High-dose cholecalciferol supplementation (10,000 IU) significantly reduced hepatocyte ballooning in Sprague–Dawley rats in a dose-dependent manner, suggesting potential hepatoprotective effects against cellular injury. However, dietary intervention failed to induce hepatic steatosis, indicating inadequate establishment of the obesity-NAFLD model and fundamentally limiting the interpretation of these findings. No significant effects on steatosis or lobular inflammation were detected. The reduction in ballooning may represent preventive rather than therapeutic effects, protecting hepatocytes from initial injury under metabolic stress rather than reversing established disease. While these preliminary results suggest that cholecalciferol may protect against hepatocellular injury through mechanisms involving endoplasmic reticulum stress reduction and antiapoptotic pathways, definitive conclusions regarding its therapeutic efficacy in the treatment of NAFLD in the clinic cannot be drawn. Future research should employ validated long-duration dietary protocols with confirmed steatosis development, larger sample sizes, mechanistic analyses, and the inclusion of both sexes to comprehensively evaluate the therapeutic potential of cholecalciferol in obesity-related liver disease.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Resources, Data Curation, Writing - Original Draft, Writing - Review & Editing, Visualization, and Supervision: DAV.

FUNDING

This study did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

DECLARATION OF ARTIFICIAL INTELLIGENCE USE

We hereby confirm that no artificial intelligence (AI) tools or methodologies were utilized at any stage of this study, including during data collection, analysis, visualization, or manuscript preparation. All work presented in this study was conducted manually by the authors without the assistance of AI-based tools or systems.

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