

Calcium and vitamin D₃ combinations improve fatty liver disease through AMPK-independent mechanisms

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Received: 15 August 2016 / Accepted: 6 December 2016
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Abstract

Purpose Some research findings suggest that calcium plus vitamin D (VitD) might have a preventive effect on non-alcoholic fatty liver disease development. Moreover, contradictory evidence also exists regarding calcium and VitD deficient diets. This study aimed to evaluate the effect of four different dietary calcium and VitD₃ (cholecalciferol) levels on the development of high-fat, high-fructose (HFHFr) diet-induced nonalcoholic fatty liver disease and AMP-activated protein kinase (AMPK) phosphorylation.

Methods Thirty male Wistar rats were fed with normal or HFHFr diet containing low calcium (0.2%) and VitD₃ (250 IU/kg) (LCD), normal calcium (0.5%) and VitD₃ (1000 IU/kg) (CN), high calcium (1.2%) and VitD₃ (4000 IU/kg) (HCD) or very high amount of calcium (2.4%) and VitD₃ (10,000 IU/kg) (VHCD). After 60 days, anthropometric, metabolic and hepatic parameters were evaluated. The effect of the experimental diets on liver AMPK phosphorylation was also investigated.

Results Rats fed on high calcium plus VitD₃ diets, especially VHCD, demonstrated lower adiposity, serum liver enzymes, hepatic lipid accumulation and steatosis. The LCD diet also decreased hepatic lipid content and fatty

changes. No evidence indicating the involvement of AMPK in the observed associations was found (P value = 0.51).

Conclusions The results showed high calcium plus VitD₃ intakes considerably prevent biochemical and hepatic changes induced by HFHFr diet, probably via an insulin and AMPK-independent pathway. A low intake of these two nutrients was also linked with a significant decrease in HFHFr diet-induced hepatic steatosis.

Keywords Nonalcoholic fatty liver disease · Calcium · Vitamin D₃ · AMP-activated protein kinase

Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most common form of chronic liver disease in the world and is correlated with obesity, insulin resistance and metabolic syndrome [1]; however, its pathogenesis is yet to be fully understood. Excessive hepatic triglyceride accumulation >5% of liver volume or liver weight is the major hallmark of NAFLD [2]. The growing number of NAFLD incidence worldwide and the absence of an effective treatment for this disease have led to more interest in the protective role of dietary components, such as calcium and vitamin D (VitD) in the development of NAFLD and other relevant metabolic disorders.

There are some epidemiological studies indicative of an inverse relationship between dietary calcium and VitD intake or VitD status and adiposity [3–5], insulin resistance [6, 7], metabolic syndrome [8, 9] and NAFLD [10, 11]. Some studies in rodent models have also demonstrated that calcium, VitD₃ (cholecalciferol) or calcitriol administration alone could prevent fat accumulation, insulin resistance [12, 13] and hepatic fatty changes [14, 15]. A systematic

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review of studies suggested that calcium and VitD, especially in combination, may promote pancreatic β cell function or ameliorate insulin resistance and systemic inflammation, and that the deficiency of these two nutrients could have adverse effects [16]; however, the existing data are controversial [17–20].

A key regulator of hepatic carbohydrate and lipid metabolism is AMP-activated protein kinase (AMPK). The AMPK is a multisubstrate serine/threonine protein kinase which functions as an intracellular energy sensor activated by an increase in the AMP/ATP ratio or calcium-mediated signaling. Activation of AMPK could both induce catabolic pathways, such as fatty acid oxidation, and suppress anabolic pathways like lipogenesis [21, 22]. Calcium and VitD are considered to be related to AMPK activation in different tissues. It has been reported that postweaning low calcium diet decreases AMPK activation in skeletal muscle [23]. Gao et al. [24] also demonstrated that calcitriol increases AMPK phosphorylation in the heart. Nonetheless, the critical question on the effect of a combination of these two nutrients on hepatic AMPK phosphorylation levels remains unanswered.

Due to inconsistency of data and the possibility of a stronger effect in optimizing glucose metabolism, body weight and adiposity when calcium and VitD are combined, compared to the highest intake of each nutrient separately [12, 16], we aimed to determine whether increased or decreased dietary calcium and VitD₃ intakes could differently affect the anthropometric, metabolic and hepatic parameters in rats fed on a high-fat, high-fructose (HFHFr) diet. We also aimed to determine the effect of NAFLD and different amounts of dietary calcium and VitD₃ intakes on AMPK phosphorylation and serum complement C1q/tumor necrosis factor- α -related protein-3 (CTRP3) levels, a new adipokine and an important regulator of lipid and glucose metabolism.

Materials and methods

Animals and diets

Male Wistar rats (216 ± 19 g) were used in this study. Rats were housed at a constant temperature of 21 °C with a 12-h light–dark cycle. After acclimation, weight-matched rats were randomly assigned to feed on either a standard chow (control, $n = 6$) or HFHFr diet ($n = 24$) for a period of 60 days. This diet has previously been shown to induce NAFLD [25]. The rats in the HFHFr-fed group were also randomly divided into four separate groups to receive sub-optimal calcium (0.2%) and VitD₃ (250 IU/kg) (LCD), normal calcium (0.5%) and VitD₃ (1000 IU/kg) (CN), high calcium (1.2%) and VitD₃ (4000 IU/kg) (HCD) or a very

Table 1 Macronutrient composition and energy contents of the control and high-fat, high-fructose (HFHFr) diets

Constituent	Control	HFHFr
Macronutrients (% by weight)		
Carbohydrate	52.8	47.9
Starch	52.8	30.4
Fructose	0	17.5
Fat	5	27.8
Soybean oil	5	2.8
Hydrogenated oil	0	5
Sheep tallow	0	20
Protein	20	11.5
Macronutrients (% Kcal)		
Carbohydrate	59.7	37.3
Fat	14.5	53
Protein	25.8	9.7
Energy (Kcal/g)	3.1	4.72

high amount of calcium (2.4%) and VitD₃ (10,000 IU/kg) (VHCD) diets. These HFHFr groups had continuous access to a bottle with 20% fructose. The standard chow also contained sufficient amounts of calcium (0.5%) and VitD₃ (1000 IU/kg), in accordance with the recommended levels [26]. The highest amount of vitamin D₃ (10,000 IU/kg diet) used in the present study was selected as the optimal level of vitamin D₃ intake with no adverse effects [27]. Details of control and HFHFr diets used in this study are provided in Table 1. Throughout the experiment, the animals had free access to assigned water and food; their intakes were recorded every day. This study was performed in accordance with the Guide for the Care and Use of Laboratory Animals of Urmia University of Medical Sciences. In addition, all animal protocols were approved by the Ethics Committee of Urmia Medical Sciences University (ir.umsu.rec.1393.281).

Serum analysis

At the end of the 60th day of the diet, the rats were anesthetized after an overnight food deprivation, and their blood samples were taken via portal vein. After serum separation, the samples were used for determining fasting glucose, alanine aminotransferase (ALT), aspartate aminotransferase (AST), triglycerides, total and high-density lipoprotein (HDL) cholesterol, using individual commercial kits (Pars Azmun, Tehran, Iran) and an automatic biochemical analyzer (BT 4500, Biotechnica, Italy). Serum insulin (Bioassay Technology Laboratory, China) and CTRP3 (zellBio, Germany) levels were quantified by ELISA kits. Non-HDL-C was calculated as total cholesterol minus HDL-C.

Evaluation of animal and organ weights

All animals were weighed on a weekly basis until the end of the experimental protocol. At the end of the experiment, body length (nose–anus length) was measured in all rats and after blood sampling, the livers, epididymal and perirenal fat depots were rapidly removed and weighted. Lee index was also calculated according to the following formula: cube root of body weight (g)/length (cm). The livers were stored at -80°C for further analysis.

Histological examination

After blood sampling, the rats' livers were removed immediately and washed with physiological saline. Furthermore, fragments of liver tissues were cut and kept in a solution of 10% buffered formaldehyde. Formalin-fixed and paraffin-embedded tissues were processed for hematoxylin and eosin staining; this was done to semi-quantitatively assess the fatty degeneration using the NAFLD activity score (NAS). The histological features were numerically scored according to the percentage of distributions, while pathologists were blinded to the experimental groups. Scores for steatosis (score 0–3), lobular inflammation (score 0–3) and ballooning (score 0–2) were also summed to produce the NAS; thus they ranged from 0 to 8.

Hepatic lipid quantitation and lipid peroxidation

The levels of hepatic lipids including triglycerides and cholesterol were measured according to the method described by Folch et al. [28] using commercial kits (Pars Azmun, Iran). The oxidant status in tissues was determined, as described by Yousefi et al. [29], by quantifying concentrations of malondialdehyde (MDA), a lipid peroxidation marker. The principle of this method is based on the formation of a pink-colored product resulting from the reaction of thiobarbituric acid with MDA.

Western blot analysis

Proteins from livers were extracted by a homogenization buffer containing 50 mM Tris-HCl, 150 mM NaCl, 5 mM sodium pyrophosphate (NaPPi), 50 mM NaF, 1 mM EDTA, 1 mM dithiothreitol (DTT), 0.1% SDS (w/v), 1% TXT-100 (v/v) and protease inhibitor cocktail (Roche, Mannheim, Germany). After completion of homogenization, samples were centrifuged at 10,000 rpm at 4°C for 10 min. The protein level in each supernatant was determined using a Bradford Protein Assay kit. Moreover, 50 μg of the homogenate protein was subjected to SDS-PAGE and then transferred to an Immobilon-P membrane (Millipore, Billerica, MA). After the membranes were blocked by Tris-buffered saline

(TBS) containing 5% nonfat milk, they were probed with anti-phospho-AMPK primary antibodies and anti-AMPK primary antibodies (diluted 1:1000) at 4°C overnight. The membranes were extensively washed and incubated with peroxidase-conjugated secondary antibodies. After three 5-min washes, antibodies were visualized using the BM Chemiluminescence kit (Roche, Mannheim, Germany). The densitometric analysis of the immunoblots was performed using an image j software (Wayne Rasband, National Institute of Health, USA) [30].

Statistical analysis

Data were analyzed performing a one-way analysis of variance (ANOVA) or analysis of covariance (ANCOVA) with energy intake as a covariate. The Tukey multiple comparisons test was performed when significant differences were observed (SPSS19). Data are expressed as mean \pm standard error (SE), and differences are considered to be statistically significant at $P < 0.05$.

Results

Food and energy intake

At the end of the experimental period, there was a marked reduction in food and water intakes, but an elevation of energy intake in the CN compared to the control group. The VHCD rats consumed significantly more water, whereas the LCD and HCD rats had lower food consumption (data not shown) and subsequently lower energy intakes compared to the rats in the CN group (Fig. 1).

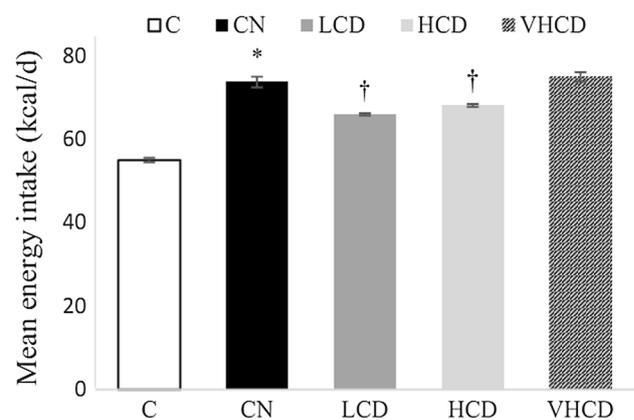


Fig. 1 Mean energy intake for all groups throughout the experiment. C control, CN positive control NAFLD group, LCD low calcium and vitamin D₃ group, HCD high calcium and vitamin D₃ group, VHCD very high calcium and vitamin D₃ group. * $P < 0.05$ versus the control group. † $P < 0.05$ versus the CN group. Data are mean \pm SE

Table 2 Body and tissue weight-related measurements

	C	CN	LCD	HCD	VHCD	P value
Length (cm)	20.4 ± 0.52	20.6 ± 0.45	19.91 ± 0.22	19.86 ± 0.21	19.34 ± 0.11	0.11
Initial body weight (g)	213.33 ± 6.52	220.33 ± 8.28	214 ± 8.77	209.33 ± 8.57	221.16 ± 9.03	0.83
Final body weight (g)	278.6 ± 11.71	278.83 ± 11.15	258.16 ± 7.94	246.4 ± 7.52	262.33 ± 6.53	0.1
BMI (g/cm ²)	0.71 ± 0.017	0.67 ± 0.015	0.66 ± 0.015	0.62 ± 0.016*	0.69 ± 0.027	0.049
Lee index	0.326 ± 0.004	0.319 ± 0.004	0.321 ± 0.002	0.316 ± 0.003	0.329 ± 0.004	0.19
Liver/body weight (%)	2.93 ± 0.098	2.92 ± 0.056	2.86 ± 0.078	3.06 ± 0.1	2.89 ± 0.081	0.5
Visceral fat weight (g)	3.41 ± 0.45	7.5 ± 1.21*	6.23 ± 0.97	3.76 ± 0.68 [†]	5.11 ± 0.57 [§]	0.009
Visceral fat/body weight (%)	1.16 ± 0.13	2.63 ± 0.33**	2.37 ± 0.3	1.65 ± 0.26	1.94 ± 0.19 [§]	0.003

Data are mean ± SE

C control, CN positive control NAFLD group, LCD low calcium and vitamin D₃ group, HCD high calcium and vitamin D₃ group, VHCD very high calcium and vitamin D₃ group, BMI body mass index

* $P < 0.05$ versus the control group

** $P < 0.005$ versus the control group

[†] $P < 0.05$ versus the CN group using one way ANOVA

[§] $P < 0.05$ versus the CN group after controlling for energy intake using ANCOVA

Body and tissue weights

The initial body weight was similar among the five groups (P value = 0.83). After 60 days, the HFHFr diet augmented the visceral fat weight in the CN group compared to that in the control group (P value = 0.013). Visceral fat/body weight percentage was also significantly higher in the CN group (P value = 0.003), whereas the HCD group showed a significant decrease in the visceral fat weight compared to the CN group (P value = 0.034). After energy intake was controlled, significant differences in visceral fat weight and visceral fat/body weight percentages were found only between the VHCD and the CN groups. At the end of the study, no significant differences in the body weight, body mass index (BMI), Lee index and liver/body weight percentages were observed between the groups (Table 2).

Serum biochemistry

There were no significant differences in the levels of serum triglycerides, cholesterol and non-HDL-C among the five groups (P value >0.05). The HCD and VHCD rats, however, exhibited a significant elevation in serum HDL-C compared to the CN group.

The CN group showed significantly higher levels of serum ALT compared to the control group ($P < 0.005$). Calcium and VitD₃ supplementation attenuated the HFHFr diet-induced elevation in serum concentrations of ALT and AST significantly. Moreover, compared to the CN group, the LCD group had slightly and nonsignificantly lower

levels of serum ALT and AST. After 60 days, the HFHFr diet yielded higher levels of serum glucose, and the rats fed on VHCD showed significantly lower levels of insulin; although, after controlling for energy intake, these observed changes disappeared (Table 3). The HFHFr diet and different doses of calcium and VitD₃ did not induce any significant difference in the serum levels of CTRP3.

Hepatic lipid content and lipid peroxidation

As expected, hepatic triglyceride and cholesterol were significantly elevated in the HFHFr-fed rats, while they were significantly reduced in rats fed on HCD and VHCD diets. However, suboptimal levels of calcium and VitD₃ not only did not exacerbate HFHFr diet-induced elevation of lipid content, but also nonsignificantly reduced it. The concentration of hepatic MDA was also significantly decreased in the HCD and VHCD groups compared to that in the CN group (Fig. 2).

Liver histology

As it is shown in Fig. 3, there was no evidence for fat deposition in the sections of livers obtained from the control group, but the histology of the livers from the HFHFr-fed groups showed evidence of fatty changes. Consistent with the results of hepatic lipid content, steatosis and NAS scores were attenuated in all the other doses of dietary calcium and VitD₃, especially in the VHCD compared to that in the CN group (nonsignificantly for steatosis in LCD). In addition, control for energy intake revealed that a low

Table 3 Serum biochemical parameters

	C	CN	LCD	HCD	VHCD	P value
Triglycerides (mg/dl)	46.4 ± 3.5	43.5 ± 4.4	41.16 ± 3.8	35 ± 4.1	38 ± 6.8	0.62
Cholesterol (mg/dl)	63 ± 4.7	57.33 ± 2.4	68.83 ± 2.7	67.83 ± 5.6	65.83 ± 3.8	0.26
HDL-C (mg/dl)	20 ± 0.9	20.3 ± 0.4	23.16 ± 1.2	24.16 ± 0.9 [†]	26.66 ± 0.8 ^{††§}	0.002
Non-HDL-C (mg/dl)	43.7 ± 4.2	37 ± 2.2	45.6 ± 1.9	43.6 ± 4.9	39.1 ± 3.3	0.36
ALT(IU/l)	65.22 ± 6.2	131.95 ± 17.6 ^{**}	104.5 ± 12.5	79.12 ± 6.4 ^{†§}	73.06 ± 5.3 ^{††§}	0.002
AST (IU/l)	171.5 ± 9.3	239.8 ± 27.9	163.7 ± 24.6	113.76 ± 15.1 ^{††§}	109.74 ± 5.4 ^{††§}	0.001
Glucose (mg/dl)	114 ± 8.3	170.5 ± 9.05	196.5 ± 22.1 [*]	198 ± 29.5 [*]	213.2 ± 6.04 [*]	0.01
Insulin (mIU/l)	12.62 ± 1.01	12.69 ± 0.1	12.93 ± 0.4	11.23 ± 0.4	10.38 ± 0.5 [†]	0.01
CTRP3 (ng/ml)	129.3 ± 47.5	128.72 ± 40.07	130.94 ± 38.9	94.12 ± 42.5	100.54 ± 37.8	0.47

CTRP3: complement C1q/tumor necrosis factor- α related protein-3. Data are mean \pm SE

C control, CN positive control NAFLD group, LCD low calcium and vitamin D₃ group, HCD high calcium and vitamin D₃ group, VHCD very high calcium and vitamin D₃ group, HDL-C high-density lipoprotein cholesterol, ALT alanine aminotransferase, AST aspartate aminotransferase

* $P < 0.05$ versus the control group

** $P < 0.005$ versus the control group

[†] $P < 0.05$ versus the CN group

^{††} $P < 0.005$ versus the CN group using one-way ANOVA

[§] $P < 0.05$ versus the CN group after controlling for energy intake using ANCOVA

calcium and VitD₃ diet significantly reduced hepatic fatty deposition.

Western blot analysis

The HFHFr diet did not significantly alter the phosphorylation state of AMPK. It was detected that the levels of p-AMPK in the liver tissues were slightly increased (non-significant) following LCD and VHCD diets (Fig. 4).

Discussion

This study revealed that calcium and VitD₃ have the potential to ameliorate the adverse effects of HFHFr diet on some components of NAFLD, through insulin and AMPK-independent mechanisms. Indeed, our results showed that suboptimal levels of calcium and VitD₃ could significantly attenuate hepatic cholesterol content and fatty deposition. These findings could have implications for human NAFLD and provide some direction for future research.

Hepatic excessive triglyceride accumulation is considered to be a pathogenic trigger to NAFLD development [31]. In this study, improvements in the hepatic lipid content, steatosis, adiposity, lipid profile and liver enzymes were seen due to combined administration of dietary calcium and VitD₃, particularly in high-doses. According to the best of our knowledge, no report exists on the effect of combined administration of these two nutrients on hepatic steatosis; although, in some studies the ameliorative effects

of calcitriol [14, 32] and dietary calcium [15] have been reported. Calcitriol has been linked to the inhibition of lipogenesis and promotion of lipid oxidation in rats' livers [14]. Some studies [33, 34], but not all of them [35], have also found that dietary calcium exerts anti-obesity effects on mouse preadipocytes through similar mechanisms [33]. In addition, calcium and VitD₃ may activate the Ca²⁺-mediated apoptotic pathway in adipose tissues or improve insulin sensitivity and therefore increase the anti-lipolytic effect of insulin [12].

Insulin resistance seems to have a fundamental role in the etiology of NAFLD [36]. Furthermore, insulin resistance is not only caused by obesity, but it also contributes to the development of obesity [37]. Our hypothesis was based on the literature, suggesting that among high-risk populations, combined calcium and VitD supplementations may have more favorable effects on insulin resistance than the highest intake of each nutrient separately [16]. Nevertheless, we concluded that dietary calcium plus VitD₃ could alleviate the adverse anthropometric, metabolic and hepatic effects of the HFHFr diet, via an insulin-independent pathway. Sergeev et al. [12] reported that both calcium (1.2%) and VitD₃ (10,000 IU/kg), especially in combination, improved markers of adiposity, plasma glucose, insulin and adiponectin in a diet-induced obesity (DIO) mouse model. However, other than our study, there are several studies on humans [38, 39] and some on animals that have not found any effect exerted by dietary calcium [35] or VitD [40, 41] on glucose intolerance. In our study, the calcium and vitamin D₃ co-supplementation resulted in nonsignificantly

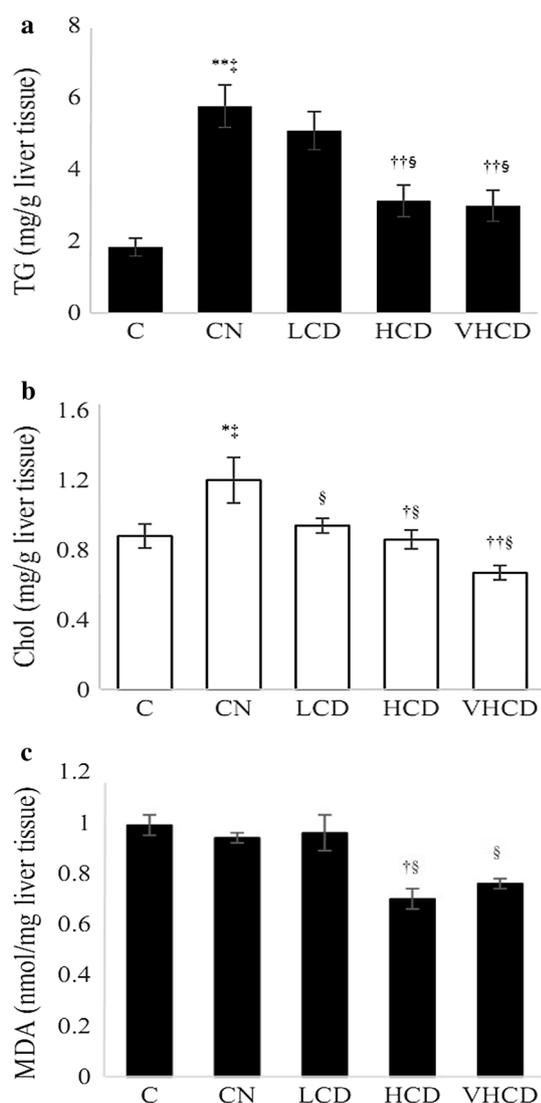


Fig. 2 Effect of experimental diets on hepatic lipid content and malondialdehyde. **a** Hepatic TG, **b** hepatic Chol, **c** hepatic MDA content. C control, CN positive control NAFLD group, LCD low calcium and vitamin D₃ group, HCD high calcium and vitamin D₃ group, VHCD very high calcium and vitamin D₃ group, TG triglyceride, Chol cholesterol, MDA malondialdehyde. Data are mean \pm SE. * $P < 0.05$ versus the control group, ** $P < 0.005$ versus the control group, † $P < 0.05$ versus the CN group, †† $P < 0.005$ versus the CN group using one-way ANOVA. ‡ $P < 0.05$ versus the control group, § $P < 0.05$ versus the CN group after controlling for energy intake using ANCOVA

lower insulin but higher glucose levels, despite unaltered insulin sensitivity (data not shown). Although there are no data available to us to support this, a possible explanation for the aforementioned observation on a high dietary calcium and vitamin D₃ intake might be a decreased absorption of magnesium due to the formation of an insoluble calcium–magnesium–phosphate complex in the intestinal lumen [42]. In a previous study, changes in magnesium

intake were connected to glucose intolerance [43]. Moreover, Jeddi et al. [44] reported that preincubation of rat islets with calcitriol reduced insulin release in cocubation with high levels of glucose. However, further research is needed to gain more insight into this phenomenon.

CTRP3, an adiponectin paralog, is a secreted plasma protein from the C1q family with several effects such as lowering glucose levels and diet-induced hepatic steatosis by regulating triglyceride metabolism [45], inhibiting the gluconeogenesis in the liver [46] and increasing angiogenesis [47]. Except for Choi et al. [48] study, reduced circulating and tissue expression levels of CTRP3 are reported in human and rodent models of obesity and diabetes [46, 49–51]. In this study, however, neither the HFHF diet nor calcium and VitD₃ did not cause any significant differences in the circulating levels of this adipokine.

In the current study, the CN rats consumed significantly lower food, but had a higher energy intake compared to the control rats. This was associated with a larger visceral fat weight in the CN rats, suggesting that the excess energy intake leads to a greater adiposity, but not to a higher body weight (Table 2). Moreover, the LCD and HCD rats exhibited nonsignificantly lower final body weight compared to the CN group, in concert with their lower energy intakes.

Over the years, the concomitance of NAFLD and VitD deficiency has been reported by some [52–54] but not all [55, 56] human studies. However, the directionality of this association is still not clear. According to some animal reports, VitD deficiency could exacerbate hepatic fatty changes by elevation of hepatic lipogenesis and inflammation [57, 58], and that calcium deficiency is related to an increased insulin resistance and a higher liver weight [59]. By contrast, our results interestingly showed that diet with suboptimal levels of calcium and VitD₃ not only did not exacerbate NAFLD, but also led to a meaningful decline in HFHF diet-induced hepatic fatty changes and hepatic cholesterol content. In line with our results, Liu et al. [20] indicated that VitD deficiency significantly attenuated HFD-induced overweight, hyperinsulinemia and hepatic lipid accumulation through promotion of fatty acid β -oxidation and elevation of energy expenditure in adipose tissues. Moreover, Bhat et al. [60] reported that VitD deficiency, in an AIN-93-based diet, could decrease body weight, total fat and visceral adiposity by decreasing the expression of genes involved in adipogenesis (such as peroxisome proliferator-activated receptor and fatty acid synthase) and increasing the expression of uncoupling protein genes in white adipose tissue. It should be noted that contrary to these studies, we used a diet containing suboptimal levels of dietary VitD, not a deplete one, which may explain this minor inconsistency. Recently, Voznesenskaya et al. [19] also revealed that under calcium-deficient conditions, the detrimental effects of fructose overconsumption on glucose

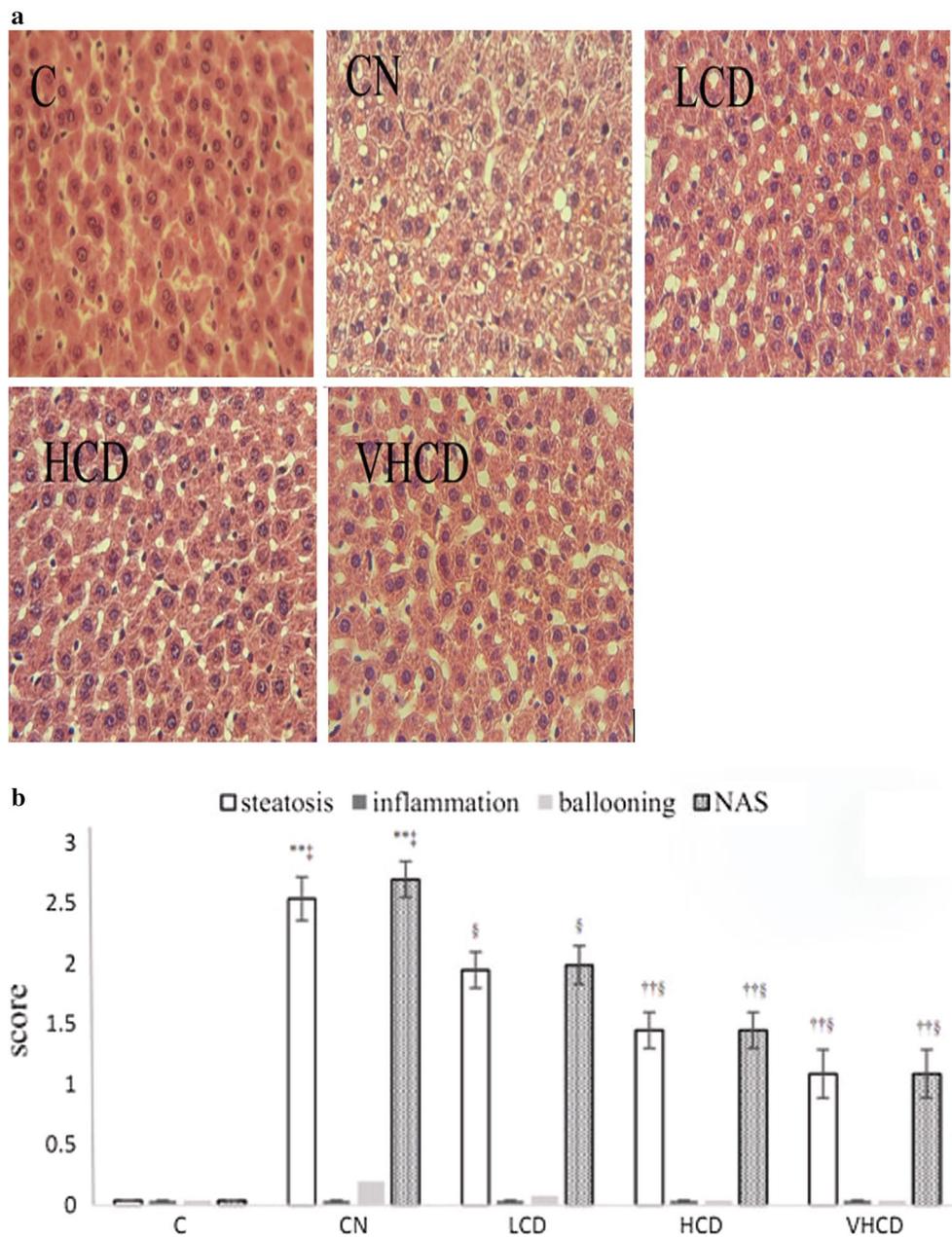


Fig. 3 a Histopathological features after hematoxylin–eosin staining of liver sections. Sections from liver tissues of the control rats showed no significant steatosis, inflammation or ballooning degeneration. The HFHF_r diet-fed rats showed hepatic steatosis and slightly ballooning degeneration (CN). Other three groups, especially the VHCD, showed lower fat accumulation and ballooning (magnification $\times 400$). **b** The NAFLD activity score (NAS) was determined based on histopathological analysis (steatosis, inflammation and ballooning). *C* control,

CN positive control NAFLD group, *LCD* low calcium and vitamin D group, *HCD* high calcium and vitamin D₃ group, *VHCD* very high calcium and vitamin D₃ group. Data are mean \pm SE. * $P < 0.05$ versus the control group, ** $P < 0.005$ versus the control group, † $P < 0.05$ versus the CN group, †† $P < 0.005$ versus the CN group using one-way ANOVA. ‡ $P < 0.05$ versus the control group, § $P < 0.05$ versus the CN group after controlling for energy intake using ANCOVA

handling could be alleviated through the classic effect of low calcium diet, to increase the levels of 1,25-hydroxyvitamin D₃.

Our observations of the reduced fat mass and hepatic lipid content with high calcium and VitD₃ intakes prompted

us to examine the involvement of AMPK in the observed association. AMPK, a metabolic master switch, has been proposed to suppress the hepatic glucose production and lipogenic processes [61, 62], and that it could be a target for the treatment of NAFLD. Nonetheless, in the present study,

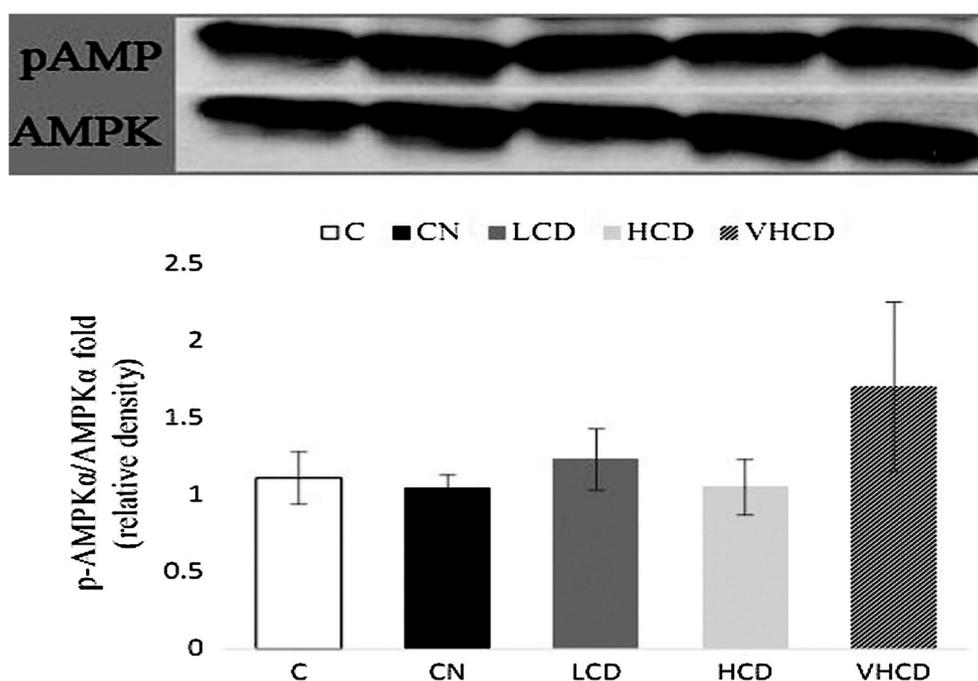


Fig. 4 Western blotting analysis of AMPK and p-AMPK expression in the livers of experimental rats. *Image* shows demonstrative bands of the analyzed proteins in the livers. *Bars* represent the ratio of phosphorylated AMPK α to total AMPK α . *C* control, *CN* positive control

NAFLD group, *LCD* low calcium and vitamin D₃ group, *HCD* high calcium and vitamin D₃ group, *VHCD* very high calcium and vitamin D₃ group data are mean \pm SE. There were no significant differences between groups

measurement of p-AMPK/AMPK levels showed no difference between the control and CN rats, nor there was any effect observed on the phosphorylation of AMPK caused by different doses of calcium and VitD₃. This indicates that beneficial effects of these two nutrients are AMPK-independent. Nevertheless, we found a nonsignificant elevation in the phosphorylation of AMPK in the LCD and VHCD groups. Our results are contrary to the literature increasingly supporting the idea that a high-fat diet could significantly decrease hepatic p-AMPK [63, 64]. In addition, two recent studies have shown that milk has a role in reducing the levels of liver phosphorylated AMPK [65] and increasing the phosphorylated AMPK in the gastrocnemius muscle, compared to the rodent control groups [66]. Low calcium diets [23] and calcitriol [24] administration have also been linked to a decrease and increase in phosphorylation levels of AMPK in skeletal muscles and the heart, respectively. In the present study, we did not evaluate inflammatory pathways and fatty acid metabolism-related gene expressions, which could provide a direction for future research.

In conclusion, our data demonstrated that dietary calcium plus VitD₃, especially in high-doses, could alleviate adverse anthropometric, metabolic and hepatic effects of the HFHFr diet via an insulin and AMPK-independent pathway. The findings in our study revealed that, in rats, suboptimal levels of calcium and VitD, despite their own

well-known harmful effects, can decrease the HFHFr diet-induced hepatic lipid content and fatty degeneration.

Acknowledgements This article is extracted from a master's thesis in Nutrition and supported by Urmia University of Medical Sciences. The authors would like to express their gratitude toward Dr. Amir Abbas Farshid and Dr. Ali Asghar Tehrani for conducting the pathological assessments. The authors would also like to thank the Pharma chemie and Daana Pharmaceutical Companies and Beyza 21 Feed Mill Company.

Compliance with ethical standards

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

Ethical standards All animal protocols were approved by the Ethics Committee of Urmia Medical Sciences University (ir.umsu. rec.1393.281).

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