Prophylactic and therapeutic effects of different doses of vitamin C on high-fat-diet-induced non-alcoholic fatty liver disease in mice

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Research

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Abstract

Background: Epidemiological studies support the association between inadequate vitamin C (Vc) intake and non-alcoholic fatty liver disease (NAFLD). However, the intervention dose of Vc and the mechanisms of its action in NAFLD are unclear. This study aimed to investigate the prophylactic and therapeutic effects of low, medium and high doses of Vc on NAFLD.

Methods: C57BL/6 mice were randomly assigned to prophylactic groups or therapeutic groups. Each group had five subgroups: control subgroup (C), high-fat subgroup (HF), low-dose Vc subgroup (15 mg/kg per day, LVc), medium-dose Vc subgroup (30 mg/kg per day, MVc), and high-dose Vc subgroup (90 mg/kg per day, HVc). In prophylactic groups, mice received high-fat diet (HFD) and simultaneously supplied with different doses Vc for 12 weeks. In therapeutic groups, mice were fed HFD for 6 weeks to form NAFLD model, and then treated with different dose Vc for 12 weeks.

Results: Prophylactic LVc and MVc administration reduced the risk of NAFLD development in HFD-fed mice, as evidenced by significantly lowered body weight, perirenal adipose tissue mass, and steatosis, whereas prophylactic HVc administration did not prevent HFD-induced NAFLD development. Furthermore, therapeutic MVc administration significantly ameliorated HFD-induced increase in body weight, perirenal adipose tissue mass and steatosis, whereas therapeutic LVc and HVc administration did not ameliorate NAFLD symptoms. In fact, therapeutic HVc administration significantly increased body weight, perirenal adipose tissue mass and lobular inflammation. Moreover, prophylactic LVc administration was more effective than therapeutic LVc administration as evidenced by significantly lower body weight, perirenal adipose tissue mass, steatosis, ballooned hepatocytes, and lobular inflammation in the prophylactic LVc subgroup. And same trends were observed between prophylactic HVc administration and therapeutic HVc administration. In addition, all Vc-administered mice exhibited low blood glucose, triglycerides, and homeostasis model assessment of insulin resistance values and high adiponectin levels compared to HF mice.

Conclusion: MVc was beneficial for HFD-induced NAFLD prophylaxis and therapy. LVc prevented HFD-induced NAFLD development, while HVc for NAFLD management was risky. This study offers valuable insight into the effect of various Vc doses on NAFLD management.

Background

Non-alcoholic fatty liver disease (NAFLD) is a hepatic manifestation of metabolic syndrome associated with obesity, insulin resistance (IR), type 2 diabetes mellitus, and dyslipidemia[1, 2]. NAFLD has rapidly become the most prevalent cause of liver dysfunction in many developed and developing countries, in line with the increasing prevalence of obesity and IR as lifestyles become increasingly sedentary and dietary patterns change[3]. Lifestyle modification that includes weight loss and structured exercise remains the cornerstone of NAFLD prevention and treatment. Oxidative stress, defined as an imbalance between the production of reactive oxygen species production and protective antioxidants, is a common

final pathway in free fatty acid lipotoxicity, which is considered as a major mechanism of hepatocellular injury and disease progression in patients with NAFLD[4]. Adiponectin is an adipocyte-derived secretory protein that acts by binding and activating two different receptor isoforms (AdipoRI and AdipoRII) and exerts beneficial systemic metabolic effects by acting on adipogenesis, atherosclerosis, insulin sensitivity, and inflammation[5]. Furthermore, it is strongly associated with the risk of metabolic syndrome as well as the development and progression of NAFLD[6–8].

Vitamin C (Vc) is a remarkably safe and water-soluble vitamin, even at 10–100 times the recommended dietary allowance (RDA) when taken orally[9]. The RDA for Vc set by different health agencies around the world has traditionally been intended to prevent Vc deficiency and ranges widely from 40–110 mg/day for adults; however, 200 mg/day is the optimum dietary intake for the majority of the adult population to maximize the potential health benefits of Vc[10]. Vc exerts pleiotropic pharmacological effects and exhibits antioxidant properties such as scavenging reactive oxygen species and reducing oxidative stress in vitro and in vivo[11]. It has potential effects in alleviating inflammatory conditions by reducing hs-CRP, IL-6, and fasting blood glucose in hypertensive and/or diabetic obese patients[12]. Moreover, Vc may be associated with glucose and lipid homeostasis[13, 14] and can suppress high fat diet (HFD)-induced visceral obesity and NAFLD by activating the peroxisome proliferator-activated receptor α (PPAR α)[15]. Unlike most animals, humans cannot synthesize Vc because they lack the terminal enzyme required in the Vc biosynthetic pathway; therefore, humans must acquire Vc dietarily[16]. Plasma Vc is inversely associated with islet autoimmunity and type 1 diabetes risk[17] and Vc supplementation may be of beneficial in reducing hyperglycemia and blood pressure in individuals with type 2 diabetes mellitus[18, 19]. Recently, a number of epidemiological studies indicated a Vc intake below the RDA in NAFLD patients, suggesting an association between dietary habits, disease, and Vc deficiency[20]. However, whether Vc supplementation is associated with preventing NAFLD development or the improvement of patients that already have NAFLD remains unclear and current literature on optimal Vc doses in NAFLD patients is scant.

This study aimed to explore the prophylactic and therapeutic effects of different doses of Vc on HFD-induced NAFLD in mice. Low, medium and high doses of Vc (15, 30, and 90 mg/kg per day, respectively) were applied in the prophylactic and therapeutic groups. Visceral fat, biochemical variables and liver histology were analyzed to explore the intervention effects based on different doses of Vc.

Materials And Methods

Animal protocols

Male C57BL/6 mice (6–8 weeks, 20 ± 2 g) were purchased from the Laboratory Animal Center of the Academy of Military Medical Sciences (Beijing, China) and housed in an animal room with a controlled temperature of $23^{\circ}\text{C} \pm 2^{\circ}\text{C}$, a relative humidity of $50\% \pm 10\%$, and a 12 h light/dark cycle. The mice were fed either a normal chow (NC) or an HFD containing 18% fat, 12% casein, and 1.0% cholesterol ad libitum and allowed free access to water.

All mice were acclimatized for one week before conducting further experiments, then randomly assigned to one of two groups: the prophylactic group (P) or the therapeutic group (T). Each group had five subgroups: control (C), high-fat group (HF), low-dose Vc group (15 mg/kg per day, LVc), medium-dose Vc group (30 mg/kg per day, MVc), and high-dose Vc group (90 mg/kg per day, HVc). The control subgroup was fed NC, while the HF and all Vc-administered subgroups were fed an HFD. In the P groups, Vc-administered subgroups (P-LVc, P-MVc or P-HVc) were prophylactically supplied with different doses of Vc for 12 weeks, the control subgroup (P-C) and high-fat subgroup (P-HF) were supplied with an equal volume of normal saline (NS) instead of Vc for 12 weeks. In the T groups, mice were fed either NC (T-C) or an HFD for 6 weeks. Two mice from each group were euthanized to assess the presence of steatosis. Upon confirmation of steatosis, the HFD-fed mice were assigned to high-fat (T-HF) and Vc-administration subgroups. Vc-administered subgroups (T-LVc, T-MVc, or T-HVc) were treated with different doses of Vc for another 12 weeks, T-C and T-HF subgroups were treated with equal volume of NS as a vehicle for 12 weeks. All treatments were provided via intragastric administration and the doses were calculated according to mouse weight. Previous studies indicate that Vc inhibits the development of experimental liver steatosis induced by choline-deficient diet in rats when treated orally at doses of 30 mg/kg per day[21]. Therefore, Vc administration dosages of 15, 30, and 90 mg/kg per day were chosen for this study. Experimental groups and technical routes are recorded in Fig. 1.

Food intake and body weight were recorded weekly until the mice were sacrificed. After an 8-h fast on the last day of the study, the mice were sacrificed under diethyl ether anesthesia and blood samples were collected. Liver and visceral adipose tissues were harvested, weighed, snap-frozen in liquid nitrogen, and stored at -80°C. Additional sections of liver were immediately fixed in freshly prepared 4% paraformaldehyde for histological analyses. The liver index was calculated as the ratio of liver weight to body weight. This animal experimental protocol was approved by the Ethics Committee of Nankai University and was carried out in accordance with the Guide for the Care and Use of Laboratory Animals.

Serum analysis

Serum concentrations of alanine transaminase (ALT), aspartate transaminase (AST), triglycerides (TG), and total cholesterol (TC) were estimated using an automatic biochemical analyzer (Hitachi, Tokyo, Japan). The mouse insulin and adiponectin enzyme-linked immunosorbent assay (ELISA) kit (Cusabio Biotech Co., Ltd., Wu Han, China) were used to determine the insulin and adiponectin concentrations. Insulin resistance was determined using the homeostasis model assessment of insulin resistance ($\text{HOMA-IR} = [\text{fasting insulin (mU/L)}] \times [\text{fasting plasma glucose (mmol/L)}] / 22.5$).

Histological analysis

Liver sections fixed in 4% paraformaldehyde and embedded in paraffin wax were sectioned at a 5 μm thickness before staining with hematoxylin and eosin (H&E). Histological variables were scored on a scale from 0 to 8 (steatosis 0-3, lobular inflammation 0-3, and hepatocyte ballooning 0-2) according to the NAFLD activity score[22]. Hepatic neutral lipid deposition was evaluated by performing Oil Red O staining. Gun-sucrose-fixed liver sections (10 μm thickness) were frozen in optimal cutting temperature

compound and stained with Oil Red O for lipid analysis. Images were captured using a upright microscope (Leica DM3000, Wetzlar, Germany). Stained sections were independently assessed by two liver histopathologists experienced in evaluation of histology in clinical trials and translational models of NAFLD who had no knowledge of the origin of the slides.

Immunohistochemistry

Liver expression of adiponectin and adiponectin receptor II (adipoRII) were detected by immunohistochemistry. Paraffin-embedded tissue samples were dewaxed, rehydrated, and incubated with anti-adiponectin antibody and anti-adipoRII (Abcam, Cambridge, UK) overnight at 4°C. The slides were incubated with secondary antibody (Bioss, Beijing, China) according to the manufacturer's instructions. Brown staining was considered antibody-positive. Staining was semi-quantitatively evaluated by measuring the mean optical density of the brown areas with Image-Pro Plus.

Statistical analysis

Data with a normal distribution were expressed as the mean $\pm$ standard deviation (SD) and comparisons among groups were performed by one-way ANOVA followed by Tukey's multiple comparison test. Data with a skewed distribution were expressed as the median $\pm$ quartile interval and comparisons among groups were performed by Kruskal-Wallis H followed by Bonferroni's multiple comparison *post hoc* tests. $P < 0.05$ was considered significant. All calculations were performed with SPSS 23.0 software (Chicago, IL, USA).

Results

Effects of different Vc doses on body weight and perirenal adipose tissue mass

The Body weight and perirenal adipose tissue mass are shown in Fig. 2; both significantly increased in HF mice compared to control mice ($P < 0.05$). P-LVc mice showed significantly decreased body weight and perirenal adipose tissue mass compared to P-HF mice ($P < 0.05$), while T-LVc mice tended to show increased body weight and perirenal adipose tissue mass compared to T-HF mice (although no statistical difference was observed; $P > 0.05$). Meanwhile, T-LVc mice showed significantly increased body weight and perirenal adipose tissue mass compared to T-MVc mice (Fig. 2A, B, D, E). Furthermore, the body weight and perirenal adipose tissue mass of P-LVc mice were significantly decreased compared to T-LVc mice ($P < 0.05$) (Fig. 2C, F), suggesting that LVc exhibited better performance in NAFLD prophylaxis than in NAFLD therapy.

Body weight and perirenal adipose tissue mass were significantly decreased both in P-MVc and T-MVc mice compared to those of HF mice ($P < 0.05$) (Fig. 2A, B, D, E). Hence, MVc administration effectively resulted in the reduction of body weight and perirenal adipose tissue mass for both prophylaxis and therapy groups.

P-HVc mice showed decreased body weight and perirenal adipose tissue mass compared to P-HF mice ($P > 0.05$). In contrast, T-HVc mice exhibited markedly increased body weight and perirenal adipose tissue mass compared to T-HF mice ($P < 0.05$). Meanwhile, T-HVc mice showed significantly increased body weight and perirenal adipose tissue mass compared to T-MVc mice (Fig. 2A, B, D, E). Furthermore, the body weight and perirenal adipose tissue mass of P-HVc mice were significantly decreased compared to T-HVc mice (Fig. 2C, F). HVc could not protect HFD-fed mice from weight and visceral fat gain, and furthermore, may have contributed to further weight gain in obese mice.

Effects of different Vc doses on serological index

ALT, AST and TC levels are shown in Fig. 3. Compared to control mice, all HFD-fed mice exhibited a significantly elevated serum TC level; Vc administration did not appear to affect TC level. An HFD increased ALT and AST serum enzyme levels, which are considered liver damage markers. Mice fed an HFD for 12 weeks merely showed simple steatosis. Although ALT and AST levels were increased in HF mice, no statistical difference was observed compared to control mice. LVc and MVc administration were accompanied by slightly decreased serum enzyme levels. However, HVc did not reverse the elevated ALT and AST levels induced by HFD. Instead, it increased the enzyme levels (Fig. 3A, B, D, E). Moreover, compared to P-HVc mice, T-HVc mice exhibited significantly elevated ALT and AST levels ($P < 0.05$) (Fig. 3C, F). Meanwhile, significantly elevated ALT and AST levels were also observed in T-HVc mice compared to T-MVc mice (Fig. 3B, E). These results suggested that HVc administration in obese mice may be associated with an increased risk of liver injury.

Glu, TG, adiponectin levels and HOMA-IR index are shown in Fig. 4. Significantly increased Glu and TG levels and a decreased adiponectin level were observed in HF mice compared to control mice. The HFD-induced increase in TG and Glu levels was attenuated by Vc administration in a dose-dependent manner. In addition, the HFD-induced decrease in adiponectin level was prevented by Vc administration. LVc-administered mice exhibited significantly decreased TG level and **increased** adiponectin level compared to HF mice ($P < 0.05$). The same trends were observed in MVc-administered mice. MVc-administered mice also showed significantly lower Glu levels ($P < 0.05$). Significantly decreased Glu and TG levels were also observed in HVc mice ($P < 0.05$). However, although HVc administration was accompanied by slightly decreased adiponectin values, they did not significantly differ from those of HF mice ($P > 0.05$) (Fig. 4A, B, D, E, G, H). HF mice exhibited a significantly increased HOMA-IR index compared to control mice, which indicated a higher degree of insulin resistance. VC administration reduced the HFD-induced increase in HOMA-IR index but there was no statistical difference compared to the reductions in HF mice (Fig. 4J, K). These findings collectively suggested that Vc administration improved the metabolic disorders of HFD-induced NAFLD in mice by reducing Glu and TG levels and increasing adiponectin expression, especially under MVc administration.

Immunohistochemistry of adiponectin and adipoRII

The expression and distribution of adiponectin and adipoRII are shown in Fig. 5 and 6. Adiponectin protein expression was localized primarily in the endothelial cells of portal vessels and liver sinusoids.

AdipoRII protein was localized in hepatocytes showing a predominantly cytoplasmic staining pattern. The expression of adiponectin and adipoRII in LVc and MVc mice were higher than that in HF mice ($P < 0.05$). This was in accordance with the increased serum adiponectin levels. HVc administration increased adiponectin and adipoRII expression but there was no significant difference compared to HF mice ($P > 0.05$).

Effects of different Vc doses on hepatic histopathology

H&E and Oil Red O liver staining revealed severe lipid deposition inside liver parenchyma cells and liver steatosis in HF mice but no obvious lobular inflammation or ballooned hepatocytes were observed (Fig.7 and 8, Table 1). Compared to P-HF mice, P-LVc mice exhibited significantly ameliorative liver steatosis ($P < 0.05$) (Fig. 7C). No alleviation of steatosis was observed in T-LVc mice compared to T-HF mice (Fig. 7H). Meanwhile, T-LVc mice exhibited significantly increased steatosis compared to T-MVc mice ($P < 0.05$) (Table 1). Furthermore, compared to P-LVc mice, T-LVc mice exhibited significantly increased steatosis, ballooned hepatocytes, and lobular inflammation ($P < 0.05$) (Table 1), these changes in histopathology further demonstrated that the prophylactic effect of Vc is superior to the therapeutic effect. These results indicated that LVc could prevent HFD-induced intrahepatic lipid deposition but could not improve notable steatosis.

Table 1
Histological findings of the liver specimens in prophylactic and therapeutic groups

	P (n = 6)					T (n = 6)				
	P-C	P-HF	P-LVc	P-MVc	P-HVc	T-C	T-HF	T-LVc	T-MVc	T-HVc
Steatosis	0.0 ± 0.0	1.0 ± 1.0 ^a	0.0 ± 0.0 ^b	0.0 ± 0.0 ^b	1.0 ± 0.0 ^a	0.0 ± 0.0	2.0 ± 1.0 ^a	1.5 ± 1.0 ^{ac}	0.5 ± 1.0 ^b	2.5 ± 1.0 ^{ac}
Inflammation	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 1.0	0.5 ± 1.0	0.0 ± 0.0	1.0 ± 1.0 ^{abc}
Ballooning	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 1.0	0.0 ± 0.0	0.5 ± 1.0	1.0 ± 0.0 ^a	0.0 ± 0.0	1.0 ± 0.0 ^{ac}

P, prophylactic groups; T, therapeutic groups. P-C, control prophylactic subgroup; P-HF, high-fat prophylactic subgroup; P-LVc, low-dose Vc prophylactic subgroup (15 mg/kg Vc per day); P-MVc, medium-dose Vc prophylactic subgroup (30 mg/kg Vc per day); P-HVc, high-dose Vc prophylactic subgroup (90 mg/kg Vc per day); T-C, control therapeutic subgroup; T-HF, high-fat therapeutic subgroup; T-LVc, low-dose Vc therapeutic subgroup; T-MVc, medium-dose Vc therapeutic subgroup; T-HVc, high-dose Vc therapeutic subgroup. All values are expressed as the (M ± Q) (n = 6 per group). ^a $P < 0.05$ compared to the C group, ^b $P < 0.05$ compared to the HF group, ^c $P < 0.05$ compared to the MVc group. Steatosis, inflammation and ballooning were significant between P-LVc and T-LVc groups. Steatosis and inflammation were significant between P-HVc and P-HVc groups.

Significantly improved liver steatosis was observed in both P-MVc and T-MVc mice compared to HF mice ($P < 0.05$) (Fig. 7D, I and Table 1) and was confirmed by Oil Red O staining (Fig. 8D, I). MVc administration tended to counteract HFD-induced steatosis. In accordance with ameliorated steatosis, ballooned hepatocytes and lobular inflammation were rare in P-MVc and T-MVc mice; this indicated that MVc administration significantly improved HFD-induced liver histological changes.

HVc could not alleviate HFD-induced liver steatosis. Compared to P-HF mice, steatosis in P-HVc mice increased and a similar result was observed between T-HVc and T-HF mice (Fig. 7E, J and Fig. 8E, J). Prominent lobular inflammation and ballooned hepatocytes were present in T-HVc mice, and lobular inflammation was more severe in T-HVc mice than in T-HF mice ($P < 0.05$). Meanwhile, T-HVc mice exhibited significantly increased steatosis, lobular inflammation, and ballooned hepatocytes compared to T-MVc mice ($P < 0.05$). Furthermore, compared to P-HVc mice, T-HVc mice exhibited significantly increased liver steatosis and lobular inflammation ($P < 0.05$) (Table 1). These results indicated that HVc could not ameliorate HFD-induced pathologic changes in mice liver.

Discussion

Our results showed that different doses of Vc had different prophylactic and therapeutic effects on HFD-induced NAFLD, and that prophylactic administration appeared to be more effective than therapeutic administration. Both LVc and MVc administration were beneficial in preventing NAFLD development in HFD-fed mice. MVc treatment also ameliorated HFD-induced NAFLD and had a hepatocellular protective effect, while LVc administration did not improve NAFLD. In contrast, HVc was ineffective in both NAFLD prophylaxis and therapy, and furthermore, appeared to increase hepatic injury risk in HFD-fed mice (Table 2).

Table 2
Prophylactic and therapeutic effects of different Vc doses on HFD-fed mice

	P (n = 6)			T (n = 6)		
	P-LVc	P-MVc	P-HVc	T-LVc	T-MVc	T-HVc
Body weights (g)	++	++	±	-	++	-
Perirenal adipose (g)	++	++	±	-	++	-
Liver weight (g)	+	+	±	±	+	-
Liver index	+	+	±	±	+	-
Adiponectin (µg/ml)	++	++	±	++	++	+
Glu (mmol/L)	+	++	++	+	++	++
TG (mmol/L)	++	++	++	++	++	++
ALT (U/L)	+	+	±	+	+	-
AST (U/L)	+	+	±	+	+	-
TC (mmol/L)	+	+	±	±	+	-
Steatosis	++	++	-	-	++	-
Inflammation	+	+	±	-	+	-
Ballooning	+	+	-	-	+	-
P, prophylactic groups; T, therapeutic groups; P-LVc, low-dose Vc prophylactic subgroup (15 mg/kg Vc per day); P-MVc, medium-dose Vc prophylactic subgroup (30 mg/kg Vc per day); P-HVc, high-dose Vc prophylactic subgroup (90 mg/kg Vc per day); T-LVc, low-dose Vc therapeutic subgroup; T-MVc, medium-dose Vc therapeutic subgroup; T-HVc, high-dose Vc therapeutic subgroup; GLU, glucose; TG, triglycerides; ALT, alanine transaminase; AST, aspartate transaminase; TC, total cholesterol; -, negative effect on NAFLD amelioration; -, significantly negative effect on NAFLD amelioration (P < 0.05); +, positive effect on NAFLD amelioration; ++, significantly positive effect on NAFLD amelioration (P < 0.05); ±, no apparent effect on NAFLD.						

Inadequate dietary Vc intake and low circulating Vc concentration are associated with IR and NAFLD; NAFLD patients showed suboptimal dietary Vc intake and an appropriate increasement of Vc intake have been associated with a significantly lower risk of developing NAFLD[23–25]. In the present study, the lower body weight, perirenal adipose tissue mass, and ameliorative liver steatosis observed in P-LVc mice were not observed in T-LVc mice. It is plausible that prophylactic supplementation of LVc to HFD-fed mice slows down NAFLD development, while the inability of LVc to treat NAFLD might be related to the Vc dose or NAFLD severity. Concomitantly, MVc administration was associated with decreased body weight and perirenal adipose tissue mass, and resulted in reduced steatosis, inflammation, and ballooning both in prophylaxis and therapy in accordance with other research[21, 26]. These results indicated the hepatoprotective effects of MVc administration against HFD-induced NAFLD. In addition, T-LVc mice

exhibited more severe metabolic and pathological changes than P-LVc mice. P-MVc mice also exhibited a slightly stronger inhibitory effect on HFD-induced NAFLD than T-MVc mice. Therefore, it is conceivable that the prophylactic efficacy of Vc may be superior to its therapeutic efficacy in HFD-induced NAFLD. Taken together, Vc administration may be feasible as a preventive strategy for NAFLD.

Glu, TG and HOMA-IR levels were lowered in all Vc-administered mice than HF mice, particularly in the case of MVc-administered mice. Vc may be involved in glucose regulation; this is supported by a meta-analysis of randomized controlled trials reporting that long-term supplementation of Vc in diabetic patients reduces fasting blood glucose levels[27]. This could be a result of the capacity of Vc to increase high molecular weight adiponectin levels[28, 29]. Similarly, we observed that Vc could increase the expression of adiponectin and adipoRII in HFD-fed mice. Adiponectin and its receptors (adipoRI/RII) are associated with various metabolic disorders such as obesity and diabetes[30], which are important regulators of adiposity and IR and have been implicated in NAFLD development[31]. Vc might play a key role in mediating adiponectin/adipoRII signaling, which may be a novel functional property of Vc. Ashor et al[32] found that Vc supplementation in patients with diabetes and hyperlipidemia significantly improved TG levels. Two possible mechanisms may be involved. In the first, Vc, a cofactor for the 7- α -hydroxylase enzyme (the rate-limiting enzyme in bile acid synthesis), may facilitate the conversion of cholesterol into bile acids, thereby lowering blood cholesterol levels. Another possible mechanism is that as an antioxidant, Vc can inhibit oxidative stress and improve IR, thereby regulating lipid metabolism and reducing serum TG levels. The suggestion of these mechanisms may be somewhat limited by the cross-sectional nature of these studies and our limited data. Further studies investigating how Vc regulates glycolipid metabolism in HFD-fed mice are warranted.

Surprisingly, HFD-fed mice cannot be prevented from developing NAFLD by prophylactic HVc administration, as evidenced by the slightly increased liver steatosis in P-HVc mice whose body weight and perirenal adipose tissue mass were similar to those of P-HF mice. Furthermore, therapeutic HVc administration increased body weight, perirenal adipose tissue mass and aggravated liver histology. Namely, HFD-induced NAFLD cannot be counteracted by treatment with HVc. In addition, T-HVc mice exhibited markedly increased body weight, liver weight, perirenal adipose tissue mass, ALT, AST, liver steatosis and lobular inflammation compared to P-HVc mice, which further confirmed that HVc as unfeasible for NAFLD therapy. In humans, plasma Vc concentrations have a sigmoid relation to dose[33] and Vc supplementation dose-dependently increases plasma Vc concentrations [34]. In addition to acting as an antioxidant, Vc can also function as a pro-oxidant. Vc spontaneously oxidizes at millimolar plasma concentrations, at high pH, or in the presence of redox-active transition metals such as iron, thereby acting as a source of hydrogen peroxide (H_2O_2)[35]. Hepatic iron overload prior to the onset of liver steatosis and insulin resistance has been observed in high-fat high-fructose diet-fed mice[36], and NAFLD is frequently associated with hepatic iron overload[37]. Therefore, long-term HVc management and HFD-induced iron overload in our study could potentially lead to Vc autooxidation that could in turn generate excessive H_2O_2 . This cytotoxic H_2O_2 could undergo further reactions to exacerbate oxidative stress, thus exacerbating NAFLD progression. Therefore, HVc should be used with caution in NAFLD. However, owing

to the limited bioavailability and poor chemical stability of Vc, a high plasma concentration (> millimolar) of Vc can only be achieved by intravenous injection[38]. Unfortunately, mice were given Vc via daily intragastric administration and plasma Vc concentrations were not measured in our present study. Therefore, the proposed mechanism above requires further exploration.

Three doses of VC were applied in our study. Compared to LVc and HVc, MVc showed better performance in alleviating HFD-induced NAFLD. T-LVc and T-HVc mice had higher levels of body weight, visceral fat, and lipid deposition than T-MVc mice. Moreover, hepatic injury was more pronounced in T-HVc mice as evidenced by the significantly elevated ALT and AST levels and increased lobular inflammation and ballooned hepatocytes compared to those of T-MVc mice. Taken together, our results suggested that MVc might be more suitable for NAFLD management. However, the major limitation of this study is that baseline Vc concentrations, as well as plasma and hepatic Vc status were unclear. Therefore, the putative effects of Vc supplementation could not be assessed in mice already saturated with Vc due to its non-linear absorption kinetics. Consequently, the role of Vc in NAFLD should be investigated in future research, while paying close attention to plasma and hepatic Vc status.

Conclusions

This is the first study to investigate the prophylactic and therapeutic effects of different Vc dosages on NAFLD mice. MVc supplementation could be beneficial for both NAFLD prophylaxis and therapy. However, LVc was only effective in terms of NAFLD prophylaxis, while HVc administration for NAFLD management was risky. This study offers valuable insight into the effect of various Vc doses on NAFLD management. The utilization of MVc administration as a prophylactic strategy for NAFLD was feasible and the possible involvement of adiponectin/adipoRII signaling should be further explored. However, as a therapeutic option for NAFLD, Vc should be used with caution. Future research should focus on the molecular mechanisms mediated by Vc in NAFLD.

Abbreviations

Vc: Vitamin C; NAFLD: Non-alcoholic fatty liver disease; RDA: Recommended daily allowance; IR: Insulin resistance; T2DM: Type 2 diabetes mellitus; ROS: Reactive oxygen species; P: Prophylactic groups; T: Therapeutic groups; C: Control subgroup; HF: High-fat diet subgroup; LVc: Low-dose Vc subgroup; MVc: Medium-dose Vc subgroup; HVc: High-dose Vc subgroup; ALT: Alanine transaminase; AST: Aspartate transaminase; Glu: Blood glucose; TG: Triglycerides; TC: Total cholesterol; HOMA-IR: Homeostasis model assessment of insulin resistance; AdipoRII: Adiponectin receptor II.

Declarations

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Authors' Contributions

QZ and LZ conducted all experimental work and contributed to drafting the manuscript; CM and XZ helped and guided the detection of serological indicators; YL and RS helped and guided histopathological analysis; XH helped prepare the manuscript and interpret the results; TW and JL contributed to the research design, edited the manuscript and take primary responsibility for the final content. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this article.

Ethics approval and consent to participate

This animal experimental protocol was approved by the Ethics Committee of Nankai University and was carried out in accordance with the Guide for the Care and Use of Laboratory Animals.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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References

- 1 Stahl EP, Dhindsa DS, Lee SK, Sandesara PB, Chalasani NP, Sperling LS. Nonalcoholic Fatty Liver Disease and the Heart: JACC State-of-the-Art Review. *J Am Coll Cardiol*. 2019;73(8):948-63.
- 2 Hardy T, Oakley F, Anstee QM, Day CP. Nonalcoholic Fatty Liver Disease: Pathogenesis and Disease Spectrum. *Annu Rev Pathol*. 2016;11:451-96.
- 3 Younossi ZM, Koenig AB, Abdelatif D, Fazel Y, Henry L, Wymer M. Global epidemiology of nonalcoholic fatty liver disease-Meta-analytic assessment of prevalence, incidence, and outcomes. *Hepatology*. 2016;64(1):73-84.
- 4 Chalasani N, Younossi Z, Lavine JE, Charlton M, Cusi K, Rinella M, et al. The diagnosis and management of nonalcoholic fatty liver disease: Practice guidance from the American Association for the Study of Liver Diseases. *Hepatology*. 2018;67(1):328-57.
- 5 Turer AT, Scherer PE. Adiponectin: mechanistic insights and clinical implications. *Diabetologia*. 2012;55(9):2319-26.
- 6 Polyzos SA, Kountouras J, Zavos C, Tsiaousi E. The role of adiponectin in the pathogenesis and treatment of non-alcoholic fatty liver disease. *Diabetes Obes Metab*. 2010;12(5):365-83.
- 7 Moschen AR, Wieser V, Tilg H. Adiponectin: key player in the adipose tissue-liver crosstalk. *Curr Med Chem*. 2012;19(32):5467-73.
- 8 Boutari C, Mantzoros CS. Adiponectin and leptin in the diagnosis and therapy of NAFLD. *Metabolism*. 2019;154028.
- 9 Khoshnam-Rad N, Khalili H. Safety of vitamin C in sepsis: a neglected topic. *Curr Opin Crit Care*. 2019;25(4):329-33.
- 10 Frei B, Birlouez-Aragon I, Lykkesfeldt J. Authors' perspective: What is the optimum intake of vitamin C in humans? *Crit Rev Food Sci Nutr*. 2012;52(9):815-29.
- 11 Padayatty SJ, Katz A, Wang Y, Eck P, Kwon O, Lee JH, et al. Vitamin C as an antioxidant: evaluation of its role in disease prevention. *J Am Coll Nutr*. 2003;22(1):18-35.
- 12 Ellulu MS, Rahmat A, Patimah I, Khaza'Al H, Abed Y. Effect of vitamin C on inflammation and metabolic markers in hypertensive and/or diabetic obese adults: a randomized controlled trial. *Drug Des Devel Ther*. 2015;9:3405-12.
- 13 Tabatabaei-Malazy O, Nikfar S, Larijani B, Abdollahi M. Influence of ascorbic acid supplementation on type 2 diabetes mellitus in observational and randomized controlled trials; a systematic review with meta-analysis. *J Pharm Pharm Sci*. 2014;17(4):554-82.

- 14 McRae MP. Vitamin C supplementation lowers serum low-density lipoprotein cholesterol and triglycerides: a meta-analysis of 13 randomized controlled trials. *J Chiropr Med.* 2008;7(2):48-58.
- 15 Lee H, Ahn J, Shin SS, Yoon M. Ascorbic acid inhibits visceral obesity and nonalcoholic fatty liver disease by activating peroxisome proliferator-activated receptor alpha in high-fat-diet-fed C57BL/6J mice. *Int J Obes (Lond).* 2018.
- 16 Nishikimi M, Fukuyama R, Minoshima S, Shimizu N, Yagi K. Cloning and chromosomal mapping of the human nonfunctional gene for L-gulono-gamma-lactone oxidase, the enzyme for L-ascorbic acid biosynthesis missing in man. *J Biol Chem.* 1994;269(18):13685-8.
- 17 Mattila M, Erlund I, Lee HS, Niinisto S, Uusitalo U, Andren AC, et al. Plasma ascorbic acid and the risk of islet autoimmunity and type 1 diabetes: the TEDDY study. *Diabetologia.* 2019.
- 18 Mason SA, Rasmussen B, van Loon L, Salmon J, Wadley GD. Ascorbic acid supplementation improves postprandial glycaemic control and blood pressure in individuals with type 2 diabetes: Findings of a randomized cross-over trial. *Diabetes Obes Metab.* 2019;21(3):674-82.
- 19 Das UN. Vitamin C for Type 2 Diabetes Mellitus and Hypertension. *Arch Med Res.* 2019;50(2):11-4.
- 20 Ipsen DH, Tveden-Nyborg P, Lykkesfeldt J. Does vitamin C deficiency promote fatty liver disease development? *Nutrients.* 2014;6(12):5473-99.
- 21 Oliveira CP, Gayotto LC, Tatai C, Della NB, Lima ES, Abdalla DS, et al. Vitamin C and vitamin E in prevention of Nonalcoholic Fatty Liver Disease (NAFLD) in choline deficient diet fed rats. *Nutr J.* 2003;2:9.
- 22 Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology.* 2005;41(6):1313-21.
- 23 Donin AS, Dent JE, Nightingale CM, Sattar N, Owen CG, Rudnicka AR, et al. Fruit, vegetable and vitamin C intakes and plasma vitamin C: cross-sectional associations with insulin resistance and glycaemia in 9-10 year-old children. *Diabet Med.* 2016;33(3):307-15.
- 24 Wei J, Lei GH, Fu L, Zeng C, Yang T, Peng SF. Association between Dietary Vitamin C Intake and Non-Alcoholic Fatty Liver Disease: A Cross-Sectional Study among Middle-Aged and Older Adults. *Plos One.* 2016;11(1):e147985.
- 25 Han JM, Jo AN, Lee SM, Bae HS, Jun DW, Cho YK, et al. Associations between intakes of individual nutrients or whole food groups and non-alcoholic fatty liver disease among Korean adults. *J Gastroenterol Hepatol.* 2014;29(6):1265-72.
- 26 Rezazadeh A, Yazdanparast R, Molaei M. Amelioration of diet-induced nonalcoholic steatohepatitis in rats by Mn-salen complexes via reduction of oxidative stress. *J Biomed Sci.* 2012;19:26.

- 27 Ashor AW, Werner AD, Lara J, Willis ND, Mathers JC, Siervo M. Effects of vitamin C supplementation on glycaemic control: a systematic review and meta-analysis of randomised controlled trials. *Eur J Clin Nutr.* 2017;71(12):1371-80.
- 28 Rose FJ, Webster J, Barry JB, Phillips LK, Richards AA, Whitehead JP. Synergistic effects of ascorbic acid and thiazolidinedione on secretion of high molecular weight adiponectin from human adipocytes. *Diabetes Obes Metab.* 2010;12(12):1084-9.
- 29 Gu X, Luo X, Wang Y, He Z, Li X, Wu K, et al. Ascorbic acid attenuates cell stress by activating the fibroblast growth factor 21/fibroblast growth factor receptor 2/adiponectin pathway in HepG2 cells. *Mol Med Rep.* 2019;20(3):2450-8.
- 30 Yamauchi T, Iwabu M, Okada-Iwabu M, Kadowaki T. Adiponectin receptors: a review of their structure, function and how they work. *Best Pract Res Clin Endocrinol Metab.* 2014;28(1):15-23.
- 31 Boutari C, Mantzoros CS. Adiponectin and leptin in the diagnosis and therapy of NAFLD. *Metabolism.* 2019:154028.
- 32 Ashor AW, Siervo M, van der Velde F, Willis ND, Mathers JC. Systematic review and meta-analysis of randomised controlled trials testing the effects of vitamin C supplementation on blood lipids. *Clin Nutr.* 2016;35(3):626-37.
- 33 Levine M, Dhariwal KR, Welch RW, Wang Y, Park JB. Determination of optimal vitamin C requirements in humans. *Am J Clin Nutr.* 1995;62(6 Suppl):1347S-1356S.
- 34 Polidori MC, Mecocci P, Levine M, Frei B. Short-term and long-term vitamin C supplementation in humans dose-dependently increases the resistance of plasma to ex vivo lipid peroxidation. *Arch Biochem Biophys.* 2004;423(1):109-15.
- 35 Du J, Cullen JJ, Buettner GR. Ascorbic acid: chemistry, biology and the treatment of cancer. *Biochim Biophys Acta.* 2012;1826(2):443-57.
- 36 Tsuchiya H, Ebata Y, Sakabe T, Hama S, Kogure K, Shiota G. High-fat, high-fructose diet induces hepatic iron overload via a hepcidin-independent mechanism prior to the onset of liver steatosis and insulin resistance in mice. *Metabolism.* 2013;62(1):62-9.
- 37 Fujita N, Takei Y. Iron overload in nonalcoholic steatohepatitis. *Adv Clin Chem.* 2011;55:105-32.
- 38 Chakraborty A, Jana NR. Vitamin C-Conjugated Nanoparticle Protects Cells from Oxidative Stress at Low Doses but Induces Oxidative Stress and Cell Death at High Doses. *ACS Appl Mater Interfaces.* 2017;9(48):41807-17.

Figures

Figure 1

Experimental groups and technical route. NC, normal chow diet, HFD, high-fat diet; NS, normal saline; LVc, low-dose (15 mg/kg) Vc; MVc, medium-dose (30 mg/kg) Vc; HVc, high-dose (90 mg/kg) Vc. P, prophylactic groups; T, therapeutic groups. P-C, control prophylactic subgroup; P-HF, high-fat prophylactic subgroup; P-LVc, LVc prophylactic subgroup; P-MVc, MVc prophylactic subgroup; P-HVc, HVc prophylactic subgroup; T-C, control therapeutic subgroup; T-HF, high-fat therapeutic subgroup; T-LVc, LVc therapeutic subgroup; T-MVc, MVc therapeutic subgroup; T-HVc, HVc therapeutic subgroup.

Figure 2

Effects of different Vc doses on body weight, perirenal adipose tissue mass, liver weight, and liver index in HFD-fed mice. Effects of different Vc doses in prophylactic subgroups (A, D, G, J). Effects of different dose Vc in therapeutic subgroups (B, E, H, K). Comparison of the effects of Vc between prophylactic and therapeutic groups (C, F, I, L). All values are expressed as the mean $\pm$ SD (n = 6 per group). $aP < 0.05$

compared to C subgroup, bP < 0.05 compared to the HF subgroup, cP < 0.05 compared to the MVc subgroup, *P < 0.05 significant differences between the groups.

Figure 3

Effects of different Vc doses on ALT, AST, and TC in HFD-fed mice. Effects of different Vc doses in prophylactic subgroups (A, D, G). Effects of different Vc doses in therapeutic subgroups (B, E, H). Comparison of the effects of Vc between prophylactic and therapeutic groups (C, F, I). ALT, alanine transaminase; AST, aspartate transaminase; TC, cholesterol; P, prophylactic groups; T, therapeutic groups. All values are expressed as the mean ± SD (n = 6 per group). aP < 0.05 compared to C subgroup, bP < 0.05 compared to the HF subgroup, cP < 0.05 compared to the MVc subgroup, *P < 0.05 significant differences between the groups.

Figure 4

Effects of different Vc doses on adiponectin, Glu, TG, and HOMA-IR in HFD-fed mice. Effects of different Vc doses in prophylactic groups (A, D, G, J). Effects of different Vc doses in therapeutic groups (B, E, H, K). Comparison of the effects of Vc between prophylactic and therapeutic groups (C, F, I, L). P, prophylactic group; T, therapeutic group; Glu, blood glucose; TG, triglycerides; HOMA-IR, $[\text{fasting insulin (mU / L)}] \times [\text{fasting plasma glucose (mmol/L)}] / 22.5$. All values are expressed as the mean $\pm$ SD (n = 6).

per group). aP < 0.05 compared to C group, bP < 0.05 compared to HF group, *P < 0.05 significant differences between the groups.

Figure 5

Immunohistochemistry for adiponectin expression and distribution in C57BL/6 mice liver specimens (×400). Expression and distribution of adiponectin in prophylactic subgroups (A-E) and therapeutic

subgroups (G-K). Mean optical density (MOD) of adiponectin in prophylactic (F) and therapeutic (L) groups.

Figure 6

Immunohistochemistry for adiponectin receptor II (adipoR2) expression and distribution in C57BL/6 mice liver specimen (x400). Expression and distribution of adipoR2 in prophylactic subgroups (A-E) and

therapeutic subgroup (G-K). Mean optical density (MOD) of adipoRII in prophylactic (F) and therapeutic (L) groups.

Figure 7

Representative H&E-stained sections of liver tissues in C57BL/6 mice. (×400). Liver sections from prophylactic sgroups (A-E). (A) P-C, normal hepatic lobule. (B) P-HF, swollen hepatocytes with some microvesicular fat droplets. (C) P-LVc, relatively normal liver morphology. (D) P-MVc, relatively normal liver morphology.(E) P-HVc, slightly swollen hepatocytes with microvesicular fat droplets. Liver sections from therapeutic groups (F-J). (F) T-C, normal hepatic lobule. (G) T-HF, macrovesicular steatosis with some microvesicular fat droplets. (H) T-LVc, slightly macrovesicular steatosis and ballooned hepatocytes. (I) T-MVc, relatively normal liver morphology. (J) T-HVc, pronounced macrovesicular steatosis with some focal necrosis.

Figure 8

Representative Oil Red O staining of liver specimens in C57BL/6 mice. (×200). Liver sections in prophylactic subgroups (A-E) and therapeutic subgroups (F-J).