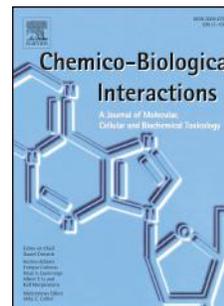# Accepted Manuscript

Vitamin D nanoemulsion enhances hapatoprotective effect of conventional vitamin D in rats fed with a high-fat diet

Mohamed El-Sherbiny, Mohamed Eldosoky, Mohamed El-Shafey, Gamal Othman, Hany A. Elkattawy, Tamer Bedir, Nehal Mohsen Elsherbiny

Please cite this article as: M. El-Sherbiny, M. Eldosoky, M. El-Shafey, G. Othman, H.A. Elkattawy, T. Bedir, N.M. Elsherbiny, Vitamin D nanoemulsion enhances hapatoprotective effect of conventional vitamin D in rats fed with a high-fat diet, *Chemico-Biological Interactions* (2018), doi: 10.1016/j.cbi.2018.04.010.

Vitamin D nanoemulsion enhances hapatoprotective effect of conventional vitamin D in rats fed with a high-fat diet

Mohamed El-Sherbiny[1, 6], Mohamed Eldosoky[2], Mohamed El–Shafey[1,7], Gamal Othman [3, 6], Hany A. Elkattawy[4, 6]Tamer Bedir[5], Nehal Mohsen Elsherbiny[8,9,*]

[1]Anatomy Department, Mansoura Faculty of Medicine – Egypt,[2]Medical physiology Department, Mansoura Faculty of Medicine – Egypt, [3]Medical Biochemistry Department, Mansoura Faculty of Medicine – Egypt, [4]Medical Physiology Department, Zagazig obesity management and research unit, Zagazig Faculty of Medicine – Egypt, [5]Medical microbiology and immunology Department, Mansoura Faculty of Medicine – Egypt, [6]Almaarefa College of Medicine, Riyadh, Saudi Arabia, [7]Fakeeh college for medical sciences, Jeddah, Saudi Arabia;[8]Department of Pharmaceutical sciences, Faculty of Pharmacy, University of Tabuk, Tabuk, Saudi Arabia; [9]Department of Biochemistry, Faculty of Pharmacy, Mansoura University, Egypt

* Corresponding author

Nehal Elsherbiny, PhD

University of Tabuk, Tabuk, Saudi Arabia

Mansoura University-Egypt

Email: nelsherbiny@ut.edu.sa

Abstract

Background. Non-alcoholic fatty liver disease (NAFLD) is associated with hyperlipidemia, obesity and type II diabetes. Due to increasing prevalence of these diseases globally, NAFLD is considered as a common form of chronic liver diseases. Vitamin D is a fat soluble vitamin with reported anti-inflammatory, anti-oxidant and immune modulating activity. Hypovitaminosis D often coexists with NAFLD and various studies reported beneficial role of vitamin D in modulating NAFLD. However, variable oral bioavailability, poor water solubility, and chemical degradation hinder the clinical application of vitamin D. **Purpose.** We evaluated the potential protective effect of Vitamin D nanoemulsion (developed by sonication and pH-Shifting of pea protein isolate and canola oil) compared to conventional vitamin D against liver injury in rats fed with high fat diet (HFD). **Methods.**We analyzed liver function enzymes, lipid profile, lipid metabolism, levels and histopathology of inflammation and fibrosis in rat liver tissues. **Results.** HFD fed rats exhibited deterioration of liver function, poor lipid profile, decreased fatty acid oxidation and up-regulation of inflammatory cytokines and extracellular matrix deposition. Vitamin D administration reduced elevated liver enzymes, improved lipid profile, enhanced fatty acid oxidation and attenuated liver inflammation and fibrosis. Interestingly, vitamin D nanoemulsion was superior to conventional vitamin D with remarkable hepatoprotective effect against HFD-induced liver injury. **Conclusion.**This study demonstrated vitamin D nanoemulsion as a more efficient formulation with more prominent hepatoprotective effect against HFD-induced liver injury compared to conventional oral vitamin D.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is a pathological condition characterized by diffused steatosis and accumulation of lipid droplets in hepatocytes without excessive alcohol consumption. It is a progressive disease with spectrum of disorders ranging from simple steatosisor nonalcoholic fatty liver (NAFL) to nonalcoholic steatohepatitis (NASH) [1]. NASH is a typical alcoholic hepatitis without the history of drinking. It is associated with steatosis, inflammation, and fibrosis [2]. Progression of NASH resulted in development of advanced fibrosis or cirrhosis in 30% to 50% of patients within a decade [3].NAFLD is associated with hyperlipidemia, obesity and type II diabetes. The prevalence of these diseases is increasing worldwide. As a result, NAFLD affects about 25% of people worldwide. Hence, NAFLD is considered one of the most common forms of chronic liver diseases and a major cause of morbidity from liver diseases with insulin resistance as a cardinal player in its pathophysiology [4]. In addition, NAFLD has been recently linked to cardiovascular diseases [5]. Therefore, management of NAFLD is an alarming public health problem [6].

The pathogenesis of NAFLD was firstly explained by "double hit theory", where the first hit is associated with accumulation of lipids in liver which is believed to be triggered by insulin resistance and the second one is accompanied by oxidative stress and lipid peroxidation leading to activation of hepatic stellate cells, hepatocellular ballooning, inflammation, and fibrosis. These events may lead eventually to serious hepatic conditions including more liver failure and hepatocellular carcinoma [7]. However, a multiple hit theory has recently been established to recapitulate the complexity of NAFLD pathogenesis, where multiple synergistically acting factors are involved in disease incidence and progression.These factors include genetic predisposition, dietary factors, altered gut microbiota and Insulin resistance- induced alteration in production and secretion of adipokines, mitochondrial dysfunction, and endoplasmic reticulum stress. These factors not only contribute to the development of steatosis and steatohepatitis but also reflect different disease patterns among NAFLD patients [8]. Indeed, it was first thought that steatosis always precedes inflammation; however it is now believed that inflammation can be the initial liver insult [9].

Currently, there is no approved therapy for NAFLD. The available therapeutic strategies are based on targeting cellular stress, apoptosis, liver metabolism, inflammation or fibrosis. Thiazolidinediones are the currently used pharmacological candidates. However, they are not satisfying due to potential long- term safety concerns. Therefore, there is a great demand to developsafe and effective therapy for management and treatment of NAFLD [10].

Vitamin D (cholecalciferol) is a fat soluble vitaminthat is either produced from 7-dehydrocholesterol in the skin or ingested from dietary sources. Calcitriol (1,25(OH)2VD3), the active form of vitamin Dacts mainly as regulator of skeletal and mineral homeostasis. This role is accomplished via binding to vitamin D receptor [11]. However, recent studies link vitamin D-Vitamin D receptor axis to a wide range of biological activities including anti-inflammatory, anti-oxidant and immune modulating activity [12].

Vitamin D deficiency is a common mineral deficiency and is currently considered as a risk factor for development of various diseases including cardiovascular diseases, cancer, autoimmune diseases and insulin resistance[13]. Specifically, hypovitaminosis D, Vitamin D receptor polymorphisms and altered expression of Vitamin D receptor have been associatedwith various liver diseases such as autoimmune liver diseases and livercancer [14]. Interestingly, hypovitaminosis D often coexists with NAFLD independent of obesity and insulin resistance. Moreover, epidemiologic evidence has shown that both of these conditions share several cardiometabolic risk factors[15].In addition, a recent study reported association of vitamin D with liver steatosis, necro-inflammatory damage and mortality in NAFLD [16]. On the other hand, various studies reported beneficial role of vitamin D supplementation in modulating NAFLD [17]. The anti-oxidant, anti-inflammatory and anti-apoptotic properties of vitamin Dmay explain its ability to halt disease progression [18].

Nanoemulsions have been recently introduced to various applications including food and industry as efficient delivery system for nutrients and drugs. Nanoemulsions are liquid dispersions in which the size of droplets ranges from (50 to 500 nm) [19]. Compared to other conventional delivery systems, nanoemulsions show higher stability against phase separation, better bioavailability and absorptive capacity of hydrophobic compounds [20].

From the pharmaceutical point of view, many challenges hinder the clinical application as well as food and beverage fortification with vitamin D. Among which are variable oral bioavailability, poor water solubility, and chemical degradation upon exposure to light, oxygen, or elevated temperatures [21]. Therefore, nano-systems for vitamin D may provide an efficient way to overcome the aforementioned challenges, due to its ability to enhance vitamin D bioavailability and maximize its therapeutic potential.

However, to the best of our knowledge, the use of nano-system of vitamin D for management of NAFLD has not yet been reported. Therefore, the current study was designed to evaluate the potential protective effect of Vitamin D nanoemulsion compared to conventional oral vitamin D in NAFLD using rats fed with high fat diet.

2. Materials and Methods

2.1. Vitamin D nanoemulsion

We used novel vitamin D (cholecalciferol) containing nanoemulsion (VDN) developed by sonication and pH-Shifting of pea protein isolate and canola oil (Pea Protein Nano-Particles(VD) powder, university of Illinois at Urbana-Champaign). Briefly, Pea protein isolate (PPI, NUTRALYS, S85F, 85% pea protein based on dry basis) was provided by Roquette (Geneva, IL, USA), and was produced using a wet extraction process from dry yellow peas. Soluble pea protein were examined for water solubility, particle size, solution turbidity, surface hydrophobicity, free sulfhydryl group content, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The PPI samples (10 mg/ml) treated with pH-shifting at pH 12 in combination with ultrasound (pH12+U5), which had highest solubility, were used to prepare nanoemulsions (0.25% oil) and nanocomplexes loaded with vitamin D3. The loading capacity of the PPI-based nanoparticles was 1.5 ± 0.2 μg/mg pea proteins [22].

2.2. Animal experiment

All experimental procedures were approved by the Research Ethical Committee, Faculty of Medicine, Mansoura University, Egypt which conforms to the international guidelines set by National Institutes of Health guide for the care and use of Laboratory animals. Twenty four adult male albino rats weighing 180-200gm were used in this study. The rats were kept under standard conditions of humidity and temperature. After acclimatization for one week, the rats were randomly assigned into two groups. The first group (Group I) is normal healthy control group (6 rats) and the second group (group II) is high fat diet fed group (18 rats). All rats in group II were fed with fat with high amounts of corn oil, containing > 98% ω-6 poly unsaturated fat acid (PUFA) (21.4 % fat, 17.5 % protein, 50 % carbohydrate, 3.5 % fiber, and 4.1 % ash) for 12 weeks for induction of NAFLD [23].Rats in group II were further classified into three groups: group IIA (non-treated group), group IIB (commercial vitamin D treated group in a dose 1 μg (40 IU)/kg), orally/daily) [24] and group IIC (vitamin D nanoemulsion-treated group): 9 ug nano-vitamin D powder was dissolved in 1 ml distilled deionized water to produce (3.240 IU/ml). The

volume of vitamin D nanoemulsion administrated was calculated so that each rat received a daily oral dose of 40 IU/Kg. Treatment was started along with initiation of high fat diet (HFD).

At the end of the experimental study, animals were sacrificed by decapitation and blood was collected for biochemical analyses. Liver was dissected and cut into three pieces. The first part was immediately flash frozen in liquid nitrogen and then kept in -80° C for RT-PCR and ELISA experiments. The second part was cut longitudinally and fixed in buffered formalin 10%, for further sectioning and histopathological assessments. The third part was processed for electron microscopy.

2.3.   Assessment of plasma biochemical parameters

The separated blood was allowed to clot and then centrifuged at 3000rpm for 10 min. The separated serum was used to measure sGPT (MAK052), sGOT (MAK055), GGT (MAK089) using commercially available kits purchased from Sigma Aldrich according to manufacturer's instruction. Moreover, serum levels of cholesterol, high density lipoprotein (HDL), TGs were assayed using kits from SPINREACT (Ctra.Santa Coloma, SPAIN) following manufacturer's protocols.

2.4.   Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted from collected liver tissues (30 mg) according to the manufacturer's instructions (RNeasy mini kit , Qiagen , Hilden, Germany), then Nano Photometer P-330 (Munchen ,Germany) was used to measure RNA concentration and quality, after that SensiFAST TM cDNA Synthesis Kit (Bioline, UK) was used to reverse transcript one mg of RNA. Real –time PCR was done with a 20 µL total reaction volume containing cDNA of volume 4 µL and 1 µL for each primer pair and a volume of 10 µL of SYBR green (SensiFAST TM SYBR ® N0-ROX kit; Bioline , UK). RT-PCR primers used in this work are listed in **Table1**. Act-b was used as endogenous controls that target mRNA levels were adjusted as the values compared to it, PikoReal TM Real-Time PCR system (Finland) was used to perform PCR.

2.5.   Enzyme-Linked Immunosorbent Assay (ELISA)

Serum TNF- α and IL-10 protein concentrations were measured by ELISA using commercial kits purchased from ALPCO (45-TNFRT-E01.1) and LsBio (LS-F2482), respectively. Absorbance was measured at 450 nm.

2.6.   Oil Red O Staining:

Frozen liver tissues, were cut at thickness of 5 μm in an ideal cutting temperature, mounted on slides and left to dry for 1-2 hours then fixed in 10% formalin for 10 min. After fixation, slides were rinsed with PBS (PH 7.4), kept till dry, dipped in 100% propylene glycol for about 2 min then stained in 0.5% Oil Red O solution for 30 min, shifted to 85% propylene glycol solution for 1 min. finally, the slides were washed in distilled water for 2 times, and counter stain with hematoxylin was used as a final phase [25].

2.7.   Histopathological and immunohistochemical analysis of liver tissue

After fixation of isolated rat livers in buffered formalin 10%, liver tissues were dehydrated, cleared and embedded in paraffin wax. Five micrometer-thickness sections were cut with a microtome and deparaffinated with xylene. A total of threesets of slides were prepared for the following histopathological and immunohistochemical evaluation.

Hematoxylin and eosin (H&E) staining

The first set was stained with standard H&E staining to evaluate the major liver histopathological changes.

Scoring of NASH

NAFLD activity score (NAS) was made after histological analysis by summation of single scores of steatosis (0–3), lobular inflammation (0–2), and hepatocellular ballooning (0–2). NASH was diagnosed if rats have total score of ≥5 [26].

Histopathological evaluation of liver fibrosis

The secondset was stained with Sirus red stain for evaluation of collagen and liver fibrosis.

Immunohistochemical staining for anti-caspase-3 antibody

The thirdset of slides was stained with rabbit anti-Caspase-3 antibody (cat# ab4051, Abcam, USA) for detecting the areas of apoptosis. The Deparaffinized sections were rinsed in phosphate buffered saline and endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide for 10 minutes at room temperature. The sections were then incubated overnight with a primary antibody: biotinylated goat anti-rabbit antibody diluted 1:200 for 1 hr at room temperature. Liver sections were counterstained with hematoxylin, dehydrated in ethanol and cleared with xylene.

The slides for sirus red and anti-caspase-3 antibody were photographed using Olympus® digital camera installed on Olympus® microscope with 1/2 X photo adaptor, using 400 X objective. The result images were analyzed on Intel® Core I3® based computer using VideoTest Morphology® software (Russia) with a specific built-in routine for area, % area measurement and object counting.

2.8. Transmission Electron Microscopy

The parts of liver tissues were fixed and washed in cacodylate buffer 0.1 M, and 1% osmium tetroxide for one hour. The specimens were dehydrated by Ethanol gradient series and embedded in Epon. Ultra-thin sections were cut at thickness of 80 nm, mounted on copper grids and stained with uranyl acetate 5 % for 15 min followed by lead citrate for 8 min and examined by transmission electron microscope; (JEOL, JEM-2100, Electron Microscopic Unit, Mansoura University) [27].

2.9. Statistical analysis

Data are represented as mean±SE. Statistical analysis was performed using SPSS 13.0. ANOVA test was used for comparison between groups followed by the Tukey's *post hoc* test. The differences will be considered statistically significant at P<0.05.

3. Results

3.1. Effect of conventional oral vitamin D and vitamin D nanoemulsion on liver function tests

As indicated in **Figure 1**, HFD group showed marked increase in serum liver enzymes including GPT, GOT and GGT (by 0.25, 1.07 and 0.72 fold, respectively) compared to normal control group (P<0.001). Oral vitamin D administration significantly attenuated elevated liver enzymes (by 12%, 28.9%, and 34.5%, respectively) compared to HFD group. Moreover, Vitamin D nanoemulsion-treated group demonstrated greater reductions in levels of serum liver enzymes (by 10.6%, 16% and 35.8%, respectively) compared to those demonstrated by conventional vitamin D-treated rats (P<0.05).

3.2. Effect of conventional oral vitamin D and vitamin D nanoemulsion on lipid profile

The serum values of total cholesterol and triglycerides were significantly higher by 0.24 and 0.38, respectively in HFD group compared to normal control group (P<0.05, P<0.01, respectively). However, the lipid profile was markedly improved in Oral vitamin D-treated group compared to HFD group (P<0.01, P<0.001, respectively). Moreover, serum lipid profile of Vitamin D nanoemulsion-treated rats was lower (by 19.9% and 24.6%, respectively) than that observed in conventional oral vitamin D-treated rats(P<0.05), **Figure 2a, 2b**. Regarding HDL, HFD rats showed marked decrease in serum HDL (13%, P<0.05) compared to normal control group, but this decrease was markedly restored by conventional vitamin D treatment (P<0.01). Vitamin D nanoemulsion-treated rats showed more increase by 0.22 fold in serum HDL levels compared with conventional vitamin D-treated group (P<0.001), **Figure 2c**.

3.3.   Effect of conventional oral vitamin D and vitamin D nanoemulsion on hepatic VDR

As shown in **Figure 3a,** our results demonstrated significant decrease in mRNA levels of hepatic VDR in HFD rats compared to normal control group (P<0.05). Oral vitamin D administration markedly increased mRNA levels of hepatic VDR (P<0.001). It is noteworthy that Vitamin D nanoemulsion-treated rats demonstrated a significantly greater enhancement of mRNA levels of hepatic VDR than conventional vitamin D-treated rats (P<0.001).

3.4.   Effect of conventional oral vitamin D and vitamin D nanoemulsion on nuclear factor, erythroid 2-like 2 (Nrf2)

Figure 3b indicated that hepatic mRNA level of Nrf2 was significantly decreased in HFD group compared to normal control group (P<0.001). However, hepatic mRNA level of Nrf2 was significantly increased by oral vitamin D administration (P<0.001). Interestingly, Vitamin D nanoemulsion-treated group showed more up-regulation in hepatic mRNA level of Nrf2 compared to conventional oral vitamin D-treated group (P<0.001).

3.5.   Assessment of inflammatory markers

The serum TNF-α level in HFD group was significantly higher by 0.53 fold than that of normal control group (P<0.001).However, serum TNF-α level in oral vitamin D-treated group was significantly lower by 16.4% than HFD group (P<0.001). Vitamin D nanoemulsion-treated group demonstrated a significantly greater reduction in the level of TNF-α by 20% than oral vitamin D-treated group (P<0.05), **Figure 4a**. On the other hand, serum level of the anti-inflammatory cytokine IL-10 was significantly reduced by 25% in HFD group compared to normal control group (P<0.05). However, treatment with conventional oral vitamin D and Vitamin D nanoemulsion significantly increased serum IL-10 compared to HFD group with more significant elevation in Vitamin D nanoemulsion-treated group (P<0.05), **Figure 4b**. In hepatic tissue, mRNA level of TNF-α was markedly reduced in HFD group compared to normal group (P<0.001). Treatment with conventional oral vitamin D and Vitamin D nanoemulsion markedly reduced hepatic TNF-α mRNA levels with greater inhibitory effect induced by Vitamin D nanoemulsion (P<0.05), **Figure 4c**.

3.6.   Histopathological study

H & E stained liver sections of control group showed normal intact liver architecture with cords of hepatocytes radiating from central vein **Figure 5a, a1**. Whereas liver sections of HFD group showed, swollen hepatocytes filled with multiple fat droplets with central nuclei (micro-vesicular hepatic steatosis), other hepatocytes were ballooned and filled with large fat droplet with peripheral nuclei (Macro-vesicular hepatic steatosis) and inflammatory cell infiltration were seen also in the periportal region and between hepatic lobules, (steatosis score: 1–2 to the maximum 3, NASH score 5-6, according to Kleiner et al. [26], **Figure 5b, b1**,whereas, the Vit-D group and nano Vitamin D were protected against the HFD-induced hepatic steatosis and appeared more or less intact. On the other hand, the liver architecture of Nano group was quiet preserved against such observations in the HFD group with mild microvesicular steatosis, **Figure 5 c, c1 and d, d1**.

3.7. Conventional oral vitamin D and vitamin D nanoemulsion improved lipid metabolism and reduced Liver Content of TG

As shown in **Figure 6a**, HFD markedly reduced hepatic Cpt1a mRNA levels compared to normal control group (P<0.001). Conventional oral vitamin D treatment significantly increased Cpt1a mRNA levels in hepatic tissue compared to HFD group (P<0.001). Moreover, vitamin D nanoemulsion treatment showed more significant increase in hepatic Cpt1a mRNA levels compared to conventional oral vitamin D-treated group (P<0.001). Histopathological studies showed increased oil red staining in liver sections from HFD group. This was markedly ameliorated by conventional oral vitamin D administration (P<0.001). In addition, vitamin D nanoemulsion-treated group showed more significant reduction in oil red staining compared to that of hepatic tissue isolated from conventional oral vitamin D-treated rats (P<0.001), **Figure 6b, 6c**.

3.8. Effect of conventional oral vitamin D and vitamin D nanoemulsionon liver injury and fibrosis

Sirus red staining demonstrated marked fibrosis in liver tissues isolated from HFD rats (P<0.001). Treatment with both conventionaloral vitamin D and vitamin D nanoemulsion showed marked reduction in hepatic sirus red staining compared to normal control group.

However, vitamin D nanoemulsion treatment showed more significant effect compared to conventional oral vitamin D treatment (P<0.05, P<0.001, respectively) **Figure 7**.

3.9. Conventional oral vitamin D and vitamin D nanoemulsion inhibited hepatic apoptosis

Our results showed that immunostaining of hepatic caspase-3 in HFD group was significantly higher than in normal control group (P<0.001). Caspase-3 immunostaining in liver tissue of both conventional oral vitamin D and Vitamin D nanoemulsion-treated rats was markedly decreased compared with HFD group with the difference between conventional oral vitamin D and Vitamin D nanoemulsion-treated rats being statistically significant (P<0.001), **Figure 8**.

3.10. Electron microscopy study

In sections examined with TEM in control group, normal hepatic structures were prominent (normal euchraomatic nucleus, normal hepatocytes cytoplasm with elongated mitochondria andabundant microvilli of hepatocytes are seen in the lumen of the bile canaliculus with junctional complex). In HFD group,hepatocytes cytoplasm with large abnormal lipid droplets, irregular mitochondria and microvilli of hepatocytes are disrupted in the lumen of the bile canaliculus with no abundant junctional complexwere observed. While in the Vitamin D treated group showed improvement when compared to HFD group in the form of decrease lipid droplet but the bile canaliculi still widened with loss of microvilli in it. Nano vitamin D group showed significant improvement apart from scanty lipid droplet were seen, **Figure 9.**

4. Discussion

Drug delivery systems in the nanoscale have been successfully employed to potentiate the therapeutic efficacy, enhance drug bioavailability and reduce toxic effects of many bioactive molecules [28]. Herein, we demonstrated that vitamin D Nano formulation enhanced its hepatoprotective effect in HFD model of NAFLD.

HFD has been widely used to induce NAFLD in rats. As obesity is a major cause of NAFLD, this model is more commonly used than genetic and pharmacologically-induced NAFLD [29]. Indeed, HFD model of NAFLD is characterized by typical hepatic lesions accompanied by elevated serum levels of TG, cholesterol and biochemical l indexes of liver dysfunction [30]. In the present study, HFD worsened lipid profile, increased serum liver enzymes and induced hepatic cellular injury in rats, indicating incidence of hyperlipidemia and impaired liver function. Indeed, pathological results revealed that our model faithfully mimicked the tissue changes associated with NAFLD. Vitamin D treatment attenuated hyperlipidemia and ameliorated hepatic pathological injuries and dysfunction. This effect was in coincidence with many previous studies that established efficacy of vitamin D supplementation in NAFLD [17, 18 and 31]. Nano delivery of vitamin D resulted in more decline in liver enzymes, better lipid profile, greater up-regulation of serum HDL and more preservation of hepatic cellular structure. Moreover, vitamin D nanoemulsion more effectively attenuated hepatic ultrastructural abnormalities. This implies the superiority of vitamin D nanoemulsification of over regular vitamin D treatment in preservation of liver cellular structure and function.

Long lasting high fat diet leads to disturbance of lipid metabolism in liver, with subsequent hepatic lipid accumulation and steatosis. Dyslipidemia in NAFLD patients is characterized by increased Triglycerides, decreased HDL levels, and increased low density lipoprotein levels

[32].In our study, we observed increased fat accumulation in HFD treated rats as evidenced by increased oil red staining. However, vitamin D treatment reduced hepatic fat accumulation and vitamin D nanoemulsion showed greater reduction. To further explain this finding, we analyzed the effect of both conventional vitamin D and vitamin D nanoemulsion on mRNA levels of hepatic cpta1, a key enzyme in hepatic lipid oxidation. Cpt1a initiates β oxidation of fatty acids by activation of malonyl CoA in mitochondrial matrix. Up-regulated mRNA levels of Cpt1a decreases lipid accumulation and TG synthesis in the liver [33]. Reduced hepatic Cpt1a was reported in HFD-induced lived injury [34 and 35] and targeting Cpt1 to enhance mitochondrial fatty acid oxidation has been suggested as a promising treatment strategy for obesity-related disorders including NAFLD [36]. In the present study, although vitamin D treatment markedly restored hepatic Cpt1a mRNA levels, nanoemulsification of vitamin D showed enhanced gene expression of fatty acid oxidation enzyme via more prominent elevation of hepatic cpt1a mRNA levels.

NAFLD is seemingly a multifactorial condition characterized by various pathological end points. Molecular mechanisms underlying NAFLD are not yet well understood. However, oxidative stress coupled with apoptosis of hepatic cells is believed as crucial player in pathogenesis of NAFLD [37]. Nrf2 is an emerging regulator of cellular resistance to oxidative stress. Nrf2 is activated in fatty liver as a defense mechanism against stress-related injury [38]. Once activated, it is translocated to nucleus where it activates transcription of various antioxidant response elements [39]. Vitamin D has been reported as an activator of Nrf2. This effect mediates vitamin D antioxidant activity in various diseases such as diabetic nephropathy [40], asthma [41] and leptin-induced endothelial dysfunction. Recently, vitamin D has been reported to reduce hepatic oxidative stress in NAFLD via induction of Nrf2/ARE pathway [42]. Similarly, our study revealed upregulation of hepatic mRNA levels of Nrf2 in vitamin D-treated rats. Interestingly, this effect was markedly enhanced with nanoemulsification of vitamin D, suggesting that nanoemulsification potentiates the antioxidant effect of vitamin D.

Apoptosis of hepatocytes is a hallmark of NAFLD and has been reported in both patients and experimental animals [43]. It is implicated as one of possible mechanisms of oxidative stress-induced hepatocyte transition from fatty liver to steatohepatitis. Targeting hepatocyte

apoptosis could be effective in modulating NAFLD [44]. Vitamin D has been previously reported to inhibit caspase-3 dependent apoptosis of both neuronal cells and hepatocytes [45 and 46]. Similarly, our data demonstrated inhibition of hepatic cleaved caspase-3 expression with vitamin D treatment. However, the antiapoptotic effect of vitamin D was markedly increased via nanoemulsification.

Further, we aimed to study the effect of vitamin D and its nanoemulsion on inflammatory burden in HFD fed rats. Accumulation of fats is assumed as potential activator of adipocytes to secrete various adipocytokines including TNF-α [47]. This leads to imbalance between serum pro and anti-inflammatory cytokines which establishes chronic inflammatory conditions with subsequent hepatocytes injury and ultimately development of NAFLD [48]. In addition to systemic inflammation, fat deposition in hepatic tissues activates NF-κB that stimulates gene expression of proinflammatory cytokines [49]. Indeed, over-expression of hepatic TNF-α mRNA was found in hepatic tissue of NAFLD patients and was correlated with histological severity [50]. Similarly, we found increased serum TNF-α protein, increased hepatic mRNA levels as well as reduced serum IL-10 in HFD fed rats. On the other hand, our results indicated that vitamin D mitigated inflammatory burden associated with HFD and restored serum IL-10 suggesting an anti-inflammatory potential of vitamin D. Current findings are matched with other studies that reported anti-inflammatory activity of vitamin D [51, 52 and 53]. Nevertheless, nanoemulsification of vitamin D potentiated its anti-inflammatory effect.

Upregulated proinflammatory cytokines produce all of the classical features of NAFLD including recruitment of inflammatory cells and fibrosis. Liver fibrosis is the end stage of NAFLD that involves activation of hepatic stellate cells along with accumulation of extracellular matrix [54]. Blocking fibrosis in NAFLD is essential to prevent the development of irreversible cirrhosis. Various studies reported anti-fibrotic properties of vitamin D in NAFLD. This antifibrotic effect of vitamin D has been suggested to be mediated via inhibition of HSCs activation and proliferation and suppression of ECM production [17]. In consistent, we found that vitamin D treatment reduced ECM deposition in hepatic tissues. Interestingly, Nano-delivery of vitamin D improved its anti-fibrotic properties in HFD fed rats

Accumulating evidence highlighted ameliorative effect of vitamin D on oxidative stress, inflammation and fibrosis likely through the binding to specific VDR [55].Our results revealed that nanoemulsification of vitamin D increased mRNA levels of hepatic VDR than conventional vitamin D. This enhancement of hepatic VDR expression is thought to amplify tissue actions of vitamin D.

In summary, nanoparticles of vitamin D were successfully prepared using sonication and pH-Shifting of pea protein isolate and canola oil. Although administration of vitamin D protected against liver injury in HFD rats, our results clearly revealed that vitamin D nanoemulsion displayed better results and almost restored the normal state. This enhanced therapeutic efficacy of vitamin D against NAFLD could be attributed to better absorption of vitamin D with subsequent enhancement of hepatic VDR expression. Indeed, Almajwal et al., 2016 [56] reported increased bioavailability and enhancement of intestinal absorption of vitamin D nanoemulsion compared to oral conventional vitamin D using the same way of preparation of vitamin D nanoemulsion. Eventually, the present study may open the door to pursuing nanotechnology as an effective strategy for vitamin D delivery in NAFLD and other therapeutic purposes.

5. References

1. Liu, H., Zhong, H., Leng, L., Jiang, Z. (2017). Effects of soy isoflavone on hepatic steatosis in high fat-induced rats. *Journal of clinical biochemistry and nutrition*, *61*(2), 85-90.

2. Nan Y, Wang R, Zhao S, Han F, Wu WJ, Kong L, Fu N, Kong L, Yu J (2010). Heme oxygenase-1 prevents non-alcoholic steatohepatitis through suppressing hepatocyteapoptosis in mice. *Lipids Health Dis*. 9:124.

3. Takai S, Jin D. (2018). Chymase Inhibitor as a Novel Therapeutic Agent for Non-alcoholic Steatohepatitis.*Front Pharmacol.* 9: 144.

4. Zarzour, A., Hamdan, R., Ahmad, M., Asmawi, M. Z., Kaur, G., Saeed, M. A. A., Al-Mansoub, M.A., Saghir, S.A.M., Usman, N.S., Al-Dulaimi, D.W., Yam, M. F. (2017). PhyllanthusNiruri Standardized Extract Alleviates the Progression of Non-Alcoholic Fatty Liver Disease and Decreases Atherosclerotic Risk in Sprague–Dawley Rats. *Nutrients*, 9(7): 766.

5. Anstee, Q. M., Targher, G., Day, C. P. (2013). Progression of NAFLD to diabetes mellitus, cardiovascular disease or cirrhosis.*Nature Reviews Gastroenterology and Hepatology*, 10(6), 330

6. Ding, S., Jiang, J., Zhang, G., Bu, Y., Zhang, G., Zhao, X. (2017). Resveratrol and caloric restriction prevent hepatic steatosis by regulating SIRT1-autophagy pathway and alleviating endoplasmic reticulum stress in high-fat diet-fed rats. *PloS one*. 12(8), e0183541.

7. Day, C. P. (2011). Non-alcoholic fatty liver disease: a massive problem. *Clinical medicine*, 11(2), 176-178

8. Buzzetti E, Pinzani M, Tsochatzis EA (2016). The multiple-hit pathogenesis of non-alcoholic fatty liver disease (NAFLD). *Metabolism*. 65 (8): 1038-48.

9. Yilmaz Y (2012). Review article: is non-alcoholic fatty liver disease a spectrum, or are steatosis and non-alcoholic steatohepatitis distinct conditions? *Aliment PharmacolTher.* 36(9):815-23.

10. Issa D, Patel V, Sanyal AJ.(2018). Future therapy for non-alcoholic fatty liver disease.*Liver Int.* 38 Suppl 1:56-63.

11. Barchetta, I., Cimini, F. A., Cavallo, M. G. (2017). Vitamin D Supplementation and Non-Alcoholic Fatty Liver Disease: Present and Future. *Nutrients*, *9*(9), 1015.

12. Han, H., Cui, M., You, X., Chen, M., Piao, X., Jin, G. (2015). A role of 1, 25 (OH) 2D3 supplementation in rats with nonalcoholic steatohepatitis induced by choline-deficient diet. *Nutrition, Metabolism and Cardiovascular Diseases*, *25*(6), 556-561

13. Nakano, T., Cheng, Y. F., Lai, C. Y., Hsu, L. W., Chang, Y. C., Deng, J. Y., Huang, Y.Z., Honda, H., Chen, K.D., Wang, C.C., Chiu, K.W., Jawan, B., Eng, H.L., GotoSChiu, K. W. (2011). Impact of artificial sunlight therapy on the progress of non-alcoholic fatty liver disease in rats. *Journal of hepatology*, 55(2), 415-425.

14. Zúñiga, S., Firrincieli, D., Housset, C., Chignard, N. (2011). Vitamin D and the vitamin D receptor in liver pathophysiology. *Clinics and research in hepatology and gastroenterology*, *35*(4), 295-302.

15. Barchetta, I., Angelico, F., Del Ben, M., Baroni, M. G., Pozzilli, P., Morini, S., Cavallo, M. G. (2011). Strong association between non alcoholic fatty liver disease (NAFLD) and low 25 (OH) vitamin D levels in an adult population with normal serum liver enzymes. *BMC medicine*, *9*(1), 85.

16. Kim, H. S., Rotundo, L., Kothari, N., Kim, S. H., Pyrsopoulos, N. (2017). Vitamin D Is Associated with Severity and Mortality of Non-alcoholic Fatty Liver Disease: A US Population-based Study. *Journal of clinical and translational hepatology*, *5*(3), 185

17. Eliades, M. and Spyrou, E. (2015). Vitamin D: a new player in non-alcoholic fatty liver disease?.*World journal of gastroenterology: WJG*, *21*(6), 1718.

18. Nobili, V. and Reif, S. (2014). Vitamin D and liver fibrosis: let's start soon before it's too late. *Gut*, gutjnl-2014

19. Aboalnaja, K. O., Yaghmoor, S., Kumosani, T. A., McClements, D. J. (2016). Utilization of nanoemulsions to enhance bioactivity of pharmaceuticals, supplements, and nutraceuticals: nanoemulsion delivery systems and nanoemulsion excipient systems. *Expert opinion on drug delivery*, *13*(9), 1327-1336.

20. Walia, N., Dasgupta, N., Ranjan, S., Chen, L., Ramalingam, C. (2017). Fish oil based Vitamin D nanoencapsulation by ultrasonication and bioaccessibility analysis in simulated gastro-intestinal tract. *Ultrasonicssonochemistry*, *39*, 623-635

21. Guttoff, M., Saberi, A. H., McClements, D. J. (2015). Formation of vitamin D nanoemulsion-based delivery systems by spontaneous emulsification: factors affecting particle size and stability. *Food Chemistry*, *171*, 117-122.

22. Jiang, S. (2015). *Enhanced physicochemical and functional properties of pea (Pisum sativum) protein by pH-shifting and ultrasonication combined process* (Master dissertation). Retrieved from Graduate Dissertations and Theses at Illinois. URI: http://hdl.handle.net/2142/88229.

23. Lozano, I., Van der Werf, R., Bietiger, W., Seyfritz, E., Peronet, C., Pinget, M., Jeandidier, N., Maillard, E., Marchioni, E., Sigrist, S., Dal, S. (2016). High-fructose and high-fat diet-induced disorders in rats: impact on diabetes risk, hepatic and vascular complications. Nutrition & metabolism, 13(1), 15.

24. Yin, Y., Yu, Z., Xia, M., Luo, X., Lu, X., Ling, W. (2012). Vitamin D attenuates high fat diet–induced hepatic steatosis in rats by modulating lipid metabolism. European journal of clinical investigation, 42(11), 1189-1196

25. Pittenger, M. F., Mackay, A. M., Beck, S. C., Jaiswal, R. K., Douglas, R., Mosca, J. D., Moorman, M.A., Simonetti, D.W., Craig, S., Marshak, D. R. (1999). Multilineage potential of adult human mesenchymal stem cells. Science, 284(5411), 143-147.

26. Kleiner, D. E., Brunt, E. M., Van Natta, M., Behling, C., Contos, M. J., Cummings, O. W., Ferrell, L.D., Liu, Y.C., Torbenson, M.S., Unalp-Arida, A., Yeh, M., McCullough, A.J., Sanyal, A.J. (2005). Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology, 41(6), 1313-1321.

27. Woods, A.E., Stirling, J.W., 2008. Electron microscopy. in: Bancroft, J.D., Gamble, M., (eds), Theory and practice of histological techniques, 6th ed. Churchill Livingstone Elsevier, Edinburgh, pp 601-636.

28. Conte, R., Marturano, V., Peluso, G., Calarco, A., Cerruti, P. (2017). Recent advances in nanoparticle-mediated delivery of anti-inflammatory phytocompounds. *International journal of molecular sciences*, *18* (4), 709

29. Ma, Z., Chu, L., Liu, H., Wang, W., Li, J., Yao, W., Yi, J., Gao, Y. (2017). Beneficial effects of paeoniflorin on non-alcoholic fatty liver disease induced by high-fat diet in rats. *Scientific reports*, *7*, 44819.

30. Chien, M. Y., Ku, Y. H., Chang, J. M., Yang, C. M., Chen, C. H. (2016). Effects of herbal mixture extracts on obesity in rats fed a high-fat diet. *Journal of food and drug analysis*, *24*(3), 594-601.

31. Barchetta, I., Cimini, F. A., Cavallo, M. G. (2017). Vitamin D Supplementation and Non-Alcoholic Fatty Liver Disease: Present and Future. *Nutrients*, *9*(9), 1015.

32. Zhang, Q. Q., and Lu, L. G. (2015). Nonalcoholic fatty liver disease: dyslipidemia, risk for cardiovascular complications, and treatment strategy. *Journal of clinical and translational hepatology*, *3*(1), 78

33. Zhang, Y., Meng, T., Zuo, L., Bei, Y., Zhang, Q., Su, Z., Huang, Y., Pang, J., Xiang, Q., Yang, H. (2017). Xyloketal B Attenuates Fatty Acid-Induced Lipid Accumulation via the SREBP-1c Pathway in NAFLD Models. Marine drugs, 15(6), 163.

34. Lee, K. C., Hsieh, Y. C., Yang, Y. Y., Chan, C. C., Huang, Y. H., & Lin, H. C. (2016). Aliskiren reduces hepatic steatosis and epididymal fat mass and increases skeletal muscle insulin sensitivity in high-fat diet-fed mice. *Scientific reports*, *6*, 18899.

35. Heritage, M., Jaskowski, L., Bridle, K., Campbell, C., Briskey, D., Britton, L., Fletcher L, Vitetta, L., Subramaniam, V.N., Crawford, D. (2017). Combination curcumin and vitamin E treatment attenuates diet-induced steatosis in Hfe-/-mice. *World journal of gastrointestinal pathophysiology*, *8*(2), 67.

36. Serra, D., Mera, P., Malandrino, M. I., Mir, J. F., Herrero, L. (2013). Mitochondrial fatty acid oxidation in obesity. *Antioxidants & redox signaling*, *19*(3), 269-284

37. Sinha-Hikim, I., Sinha-Hikim, A. P., Shen, R., Kim, H., French, S. W., Vaziri, N. D., Crum, A.C., Rajavashisth, T.B. , Norris, K. C. (2011). A novel cystine based antioxidant attenuates oxidative stress and hepatic steatosis in diet-induced obese mice. *Experimental and molecular pathology*, *91*(1), 419-428.

38. Hardwick, R. N., Fisher, C. D., Canet, M. J., Lake, A. D., Cherrington, N. J. (2010). Diversity in antioxidant response enzymes in progressive stages of human nonalcoholic fatty liver disease. *Drug metabolism and disposition*, *38*(12), 2293-2301

39. Lee, J. M. and Johnson, J. A. (2004). An important role of Nrf2-ARE pathway in the cellular defense mechanism. *BMB Reports*, *37*(2), 139-143

40. Nakai, K., Fujii, H., Kono, K., Goto, S., Kitazawa, R., Kitazawa, S., Hirata, M., Shinohara, M., Fukagawa, M., Nishi, S. (2013). Vitamin D activates the Nrf2-Keap1 antioxidant pathway and ameliorates nephropathy in diabetic rats. American journal of hypertension, 27(4), 586-595

41. Wang, Z., Zhang, H., Sun, X., Ren, L. (2016). The protective role of vitamin D3 in a murine model of asthma via the suppression of TGF-β/Smad signaling and activation of the Nrf2/HO-1 pathway. *Molecular medicine reports*, *14*(3), 2389-2396.

42. Zhu, C. G., Liu, Y. X., Wang, H., Wang, B. P., Qu, H. Q., Wang, B. L., Zhu, M. (2017). Active form of vitamin D ameliorates non-alcoholic fatty liver disease by alleviating oxidative stress in a high-fat diet rat model. *Endocrine journal*, *64*(7), 663-673

43. Xiao, J., Guo, R., Fung, M. L., Liong, E. C., Chang, R. C. C., Ching, Y. P., &Tipoe, G. L. (2013). Garlic-derived S-allylmercaptocysteine ameliorates nonalcoholic fatty liver disease in a rat model through inhibition of apoptosis and enhancing autophagy. *Evidence-Based Complementary and Alternative Medicine*, *2013*: 642920.

44. Alkhouri, N., Carter-Kent, C., Feldstein, A. E. (2011). Apoptosis in nonalcoholic fatty liver disease: diagnostic and therapeutic implications. *Expert review of gastroenterology &hepatology*, *5*(2), 201-212.

45. Yuan, J., Guo, X., Liu, Z., Zhao, X., Feng, Y., Song, S., Cui, C., Jiang, P. (2018). Vitamin D receptor activation influences the ERK pathway and protects against neurological deficits and neuronal death. *International journal of molecular medicine*, *41*(1), 364-372.

46. Lisakovska, O., Shymanskyy, I., Mazanova, A., Khomenko, A., Veliky, M. (2016). Vitamin D3 protects against prednisolone-induced liver injury associated with the impairment of the hepatic NF-κB/iNOS/NO pathway. *Biochemistry and Cell Biology*, *95*(2), 213-222.

47. Schäffler, A., Schölmerich, J., Büchler, C. (2005). Mechanisms of disease: adipocytokines and visceral adipose tissue—emerging role in nonalcoholic fatty liver disease. *Nature Reviews Gastroenterology and Hepatology*, *2*(6), 273.

48. Song, L., Qu, D., Zhang, Q., Zhou, H., Jiang, R., Li, Y., Zhang, Y., Yan, H. (2017). Phytosterol esters attenuate hepatic steatosis in rats with non-alcoholic fatty liver disease rats fed a high-fat diet. *Scientific reports*, *7*, 41604

49. Xie, Y., Zhang, Y., Guo, Z., Zeng, H., Zheng, B. (2016). Effect of Alkaloids from NelumbinisPlumula against Insulin Resistance of High-Fat Diet-Induced Nonalcoholic Fatty Liver Disease in Mice. *Journal of diabetes research*, *2016:* 3965864

50. Crespo, J., Cayón, A., Fernández-Gil, P., Hernández-Guerra, M., Mayorga, M., Domínguez-Díez, A., Fernández-Escalante, J.C., Pons-Romero, F. (2001). Gene expression of tumor necrosis factor α and TNF-receptors, p55 and p75, in nonalcoholic steatohepatitis patients. *Hepatology*, *34*(6), 1158-1163.

51. Liu, L., Lv, G., Ning, C., Yang, Y. E., Zhu, J. (2016). Therapeutic effects of 1, 25-dihydroxyvitamin D3 on diabetes-induced liver complications in a rat model. *Experimental and therapeutic medicine*, *11*(6), 2284-2292.

52. Farhangi, M. A., Mesgari-Abbasi, M., Nameni, G., Hajiluian, G., Shahabi, P. (2017). The effects of vitamin D administration on brain inflammatory markers in high fat diet induced obese rats. *BMC neuroscience*, *18*(1), 81

53. Yuan, Y., Das, S. K., Li, M. (2018). Vitamin D ameliorates impaired wound healing in streptozotocin induced diabetic mice by suppressing NF-κB mediated inflammatory genes expression. *Bioscience reports*, BSR20171294.

54. Zhang, Q. Z., Liu, Y. L., Wang, Y. R., Fu, L. N., Zhang, J., Wang, X. R., Wang, B. M. (2017). Effects of telmisartan on improving leptin resistance and inhibiting hepatic fibrosis in rats with non-alcoholic fatty liver disease. *Experimental and therapeutic medicine*, *14*(3), 2689-2694

55. Gil, Á., Plaza-Diaz, J., Mesa, M. D. (2018). Vitamin D: Classic and Novel Actions. *Annals of Nutrition and Metabolism*, *72*(2), 87-95.

56. Almajwal, AM, Abulmeaty, MMA, Andrade, JE, Elsadek, MF, Razak S (2016). MON-LB273: Novel Vitamin D-Nanoemulsion Developed by Sonication and PH-Shifting of Pea Protein. Isolate Enhances Intestinal Absorption of Vitamin D in Rats. Clinical Nutrition 35, S253-S254

Figure legends

Figure 1:Effect of treatments on biochemical parameters of liver dysfunction.A) Serum glutamic pyruvic transaminase (SGPT). **B)** Serum glutamic-oxaloacetic transaminase. **C)** Gamma-glutamyl transferase (GGT).***significant compared to normal control group p<0.001. $^{\#\#\#}$significant compared to HFD group p<0.001. $^{\#\#}$significant compared to HFD group p<0.01. $^{@}$ significant compared to conventional vitamin D p<0.05.

Figure 2: Effect of treatments on biochemical parameters of liver dysfunction. A) Serum total cholesterol. **B)** Serum triglycerides. **C)** Serum high density lipoprotein (HDL). **significant compared to normal control group p<0.01. *significant compared to normal control group p<0.05. $^{\#\#\#}$significant compared to HFD group p<0.001. $^{\#\#}$ significant compared to HFD group p<0.01. $^{@@@}$significant compared to conventional vitamin D p<0.001. $^{@}$ significant compared to conventional vitamin D p<0.05.

Figure 3: Effect of treatments on A) Hepatic vitamin D receptor mRNA level. **B)**Hepatic nuclear factor erythroid 2 (NFE2)-related factor 2 (Nrf2) mRNA level. ***significant compared to normal control group p<0.001. *significant compared to normal control group p<0.05. $^{\#\#\#}$significant compared to HFD group p<0.001. $^{\#\#\#}$significant compared to HFD group p<0.001. $^{@@@}$significant compared to conventional vitamin D p<0.001.

Figure 4: Effect of treatments on inflammation. A) Serum TNF-α level. **B)** Serum IL-10 level. **C)** Hepatic mRNA level of TNF-α. ***significant compared to normal control group p<0.001. *significant compared to normal control group p<0.05.$^{\#\#\#}$significant compared to HFD group p<0.001. $^{\#\#}$ significant compared to HFD group p<0.01. $^{@}$ significant compared to conventional vitamin D p<0.05.

Figure 5: Histological image of liver sections stained with H&E. A) Control group: showing intact hepatic lobules. The central veins (CV) are found at the center of the lobules and the portal tracts (PT) are present at some angles. **B)** HFD group: showingextensive steatosis in its two forms; micro-vesicular steatosis (B) macro-vesicular steatosis (LD), and lobular inflammation (arrows). **C)** Vitamin D treated group: showingdecrease in micro-vesicular

steatosis (B), macro-vesicular steatosis (ballooning) (LD), and lobular inflammation (arrow). **D)** Nano vitamin D treated group: The liver architecture is more or less intact with significant improving of inflammation and steatosis which mainly of micro-vesicular type.

Figure 6: Effect of treatment on lipid metabolism. A) Hepatic mRNA carnitin epalmitoyl transferase 1A (cpt1a) levels. **B)** Histological image of liver sections stained with Oil Red O staining.A) Control group: showing little numbers of hepatocyte with lipid droplets (< 5%). B) HFD group: showing full lipid droplets (arrow heads) of different sizes in almost of hepatocytes, while vitamin D treated group (C) and Nano vitamin D treated group (D) treated groups showing diffuse distribution of lipid droplets of hepatocytes but fewer when they are compared with HFD group. **C)** Cpt1a positive areas were measured and expressed as a percentage of totalanalyzed area.

Figure 7: Histological image of liver sections stained with sirus red staining. A) HFD group(B): showing significant increase in the amount of fibrous tissue around the central vein, around hepatocytes, in the periportal region, and between hepatic lobules (arrow heads), when compared to other groups (A, C, D) which reveal minimal to mild amount of fibrous tissue. **B)** Quantitative analysis of the total area and percentage of area of fibrosis.

Figure 8: Immunohistochemical image of liver sections stained with of anti-caspase-3 antibody. A) HFD group (B): showing significant increase of caspase-3 expression when compared to other groups (A, C, D) which reveal weakly positive brownish staining. **B)** Caspase-3 positive areas were measured and expressed as a percentage of total analyzed area.

Figure 9: Electron microscopic image of liver tissues. A) Control group: showing normal euchraomatic nucleus, normal hepatocytes cytoplasm with elongated mitochondria. Abundant microvilli of hepatocytes are seen in the lumen of the bile canaliculus with junctional complex. **B)** HFD group: showing hepatocytes cytoplasm with large abnormal lipid droplets (LD), irregular mitochondria (M) and microvilli of hepatocytes are disrupted in the lumen of the bile canaliculus with no abundant junctional complex (BL). **C)** Vitamin D treated group: showing improvement when compared to HFD group in the form of decrease lipid droplet but the bile

canaliculi still widened with loss of microvilli in it. **D):** Nano vitamin D group: showing significant improvement compared to HFD group apart from scanty lipid droplet are seen.

(N: euchraomatic nucleus; Nu: nucleolus; M: mitochondria; BL: bile canaliculi; LD: lipid droplet; BS: blood sinusoid).

Table 1; Sequences of primers for Quantitative RT-PCR

Gene	Gene Bank Accession No.	Forward sequence	Reverse sequence	Expected product size (bp)
Nrf-2	NM_031789.2	CTGCCATTAGTCAGTCGCTCTC	TCGGCTGGGACTTGTGTTC	2305
Tnf-α	NM_012675.3	TCTTCAAGGGACAAGGCTGC	CTTGATGGCAGAGAGGAGGC	1687
Vdr	NM_017058.1	GACTTTGACCGGAACGTGCC	CATCATGCCGATGTCCACAC	2043
Cpt1a	NM_031559.2	ACAATGGGACATTCCAGGAG	AAAGACTGGCGCTGCTCA	4356
Act-b	NM_031144.3	CACCATGTACCCAGGCATTG	CACACAGAGTACTTGCGCTC	1293

Figure 1
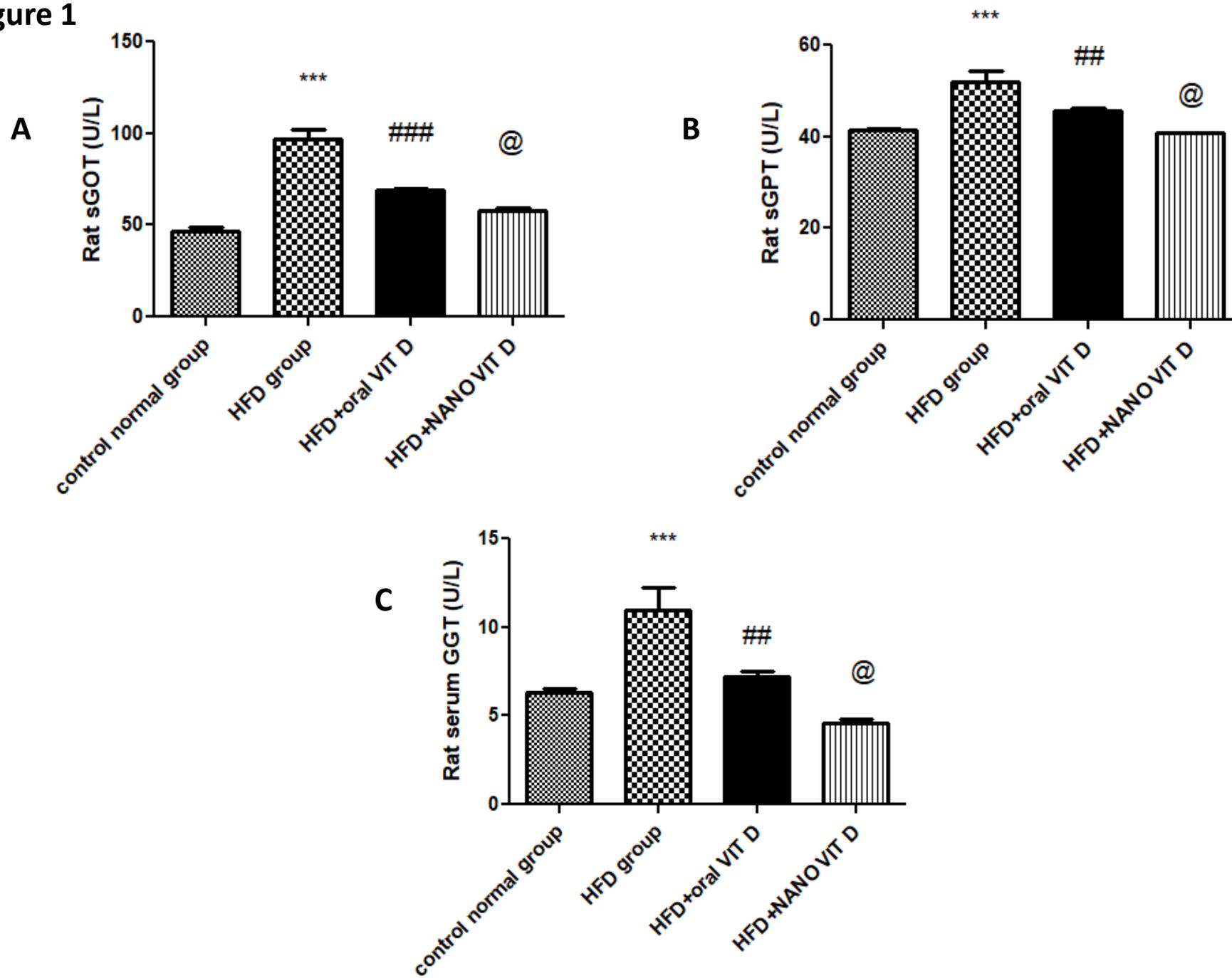
Figure 2
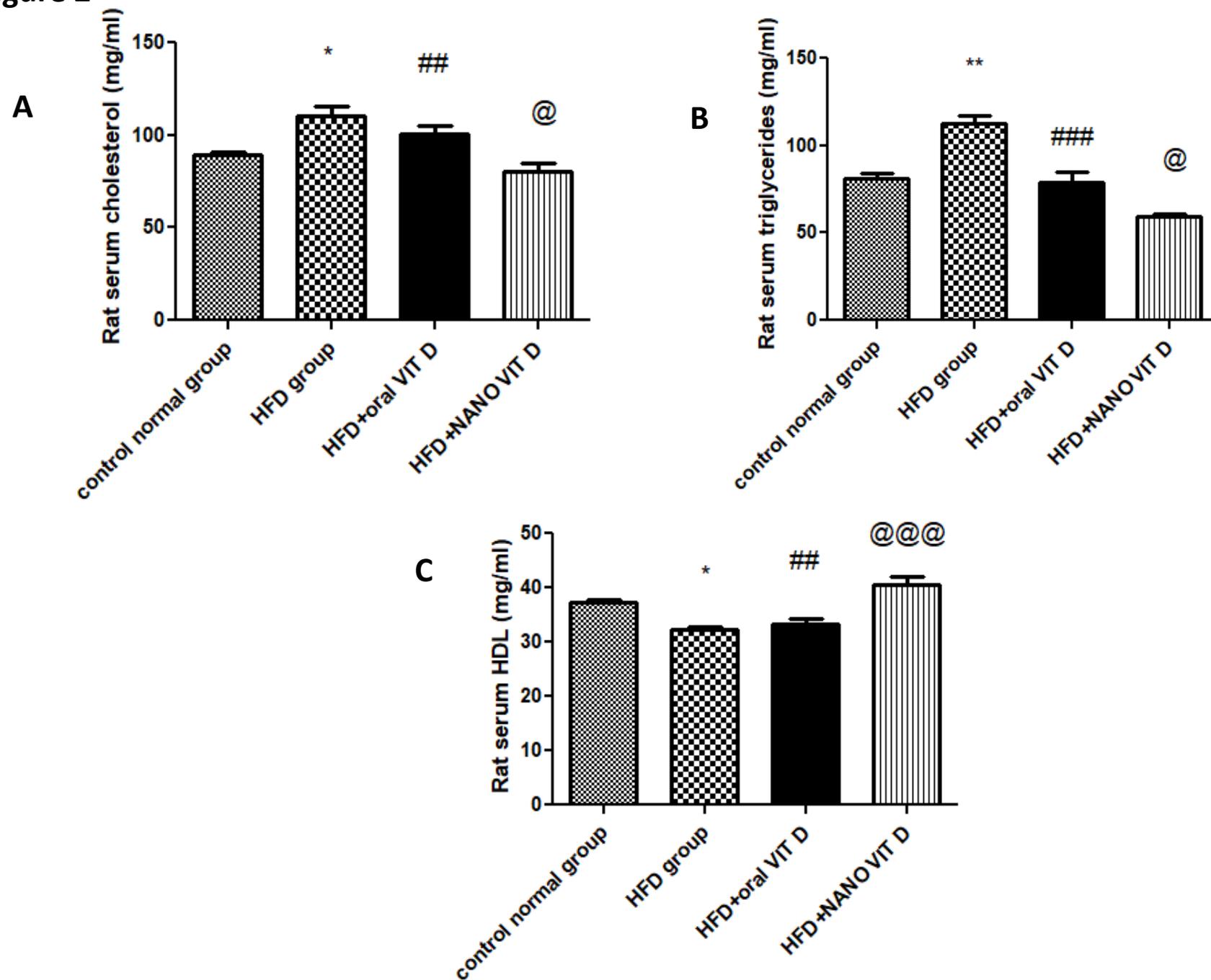
Figure 3
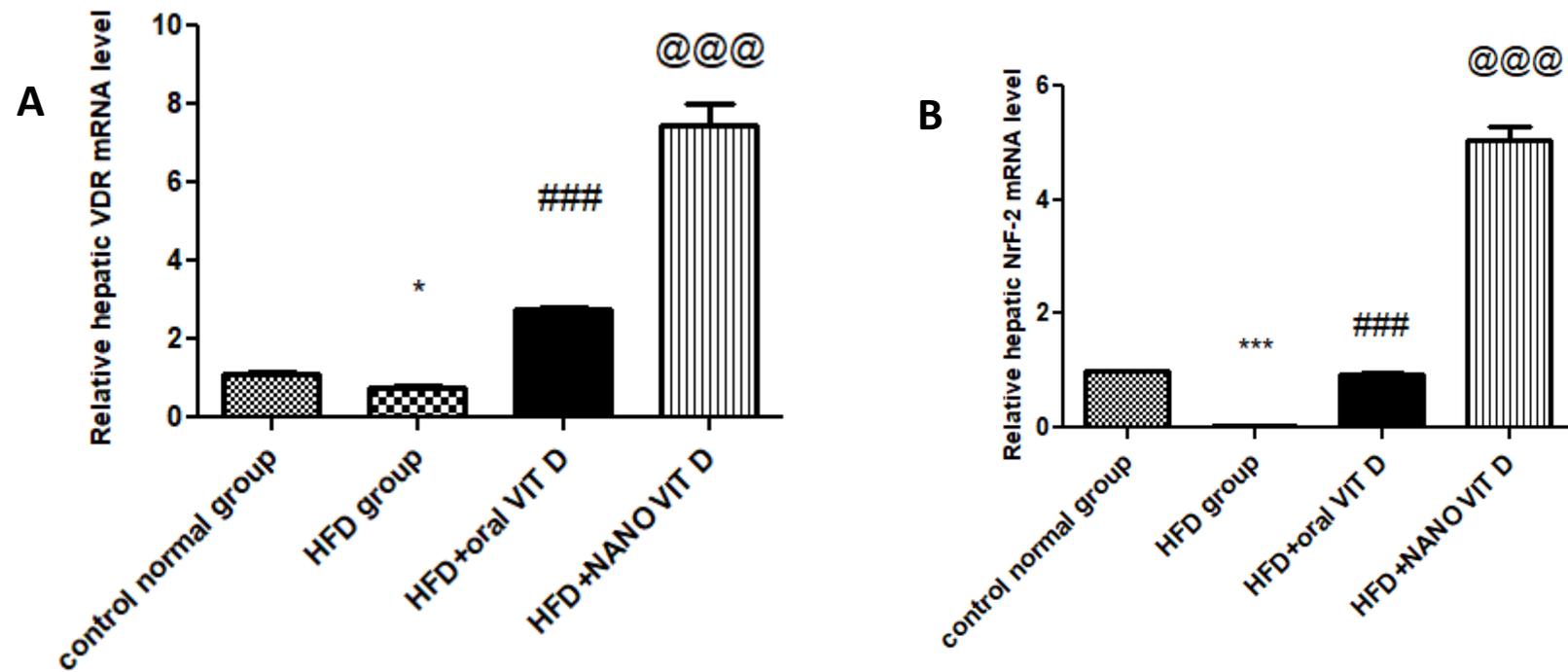
Figure 4
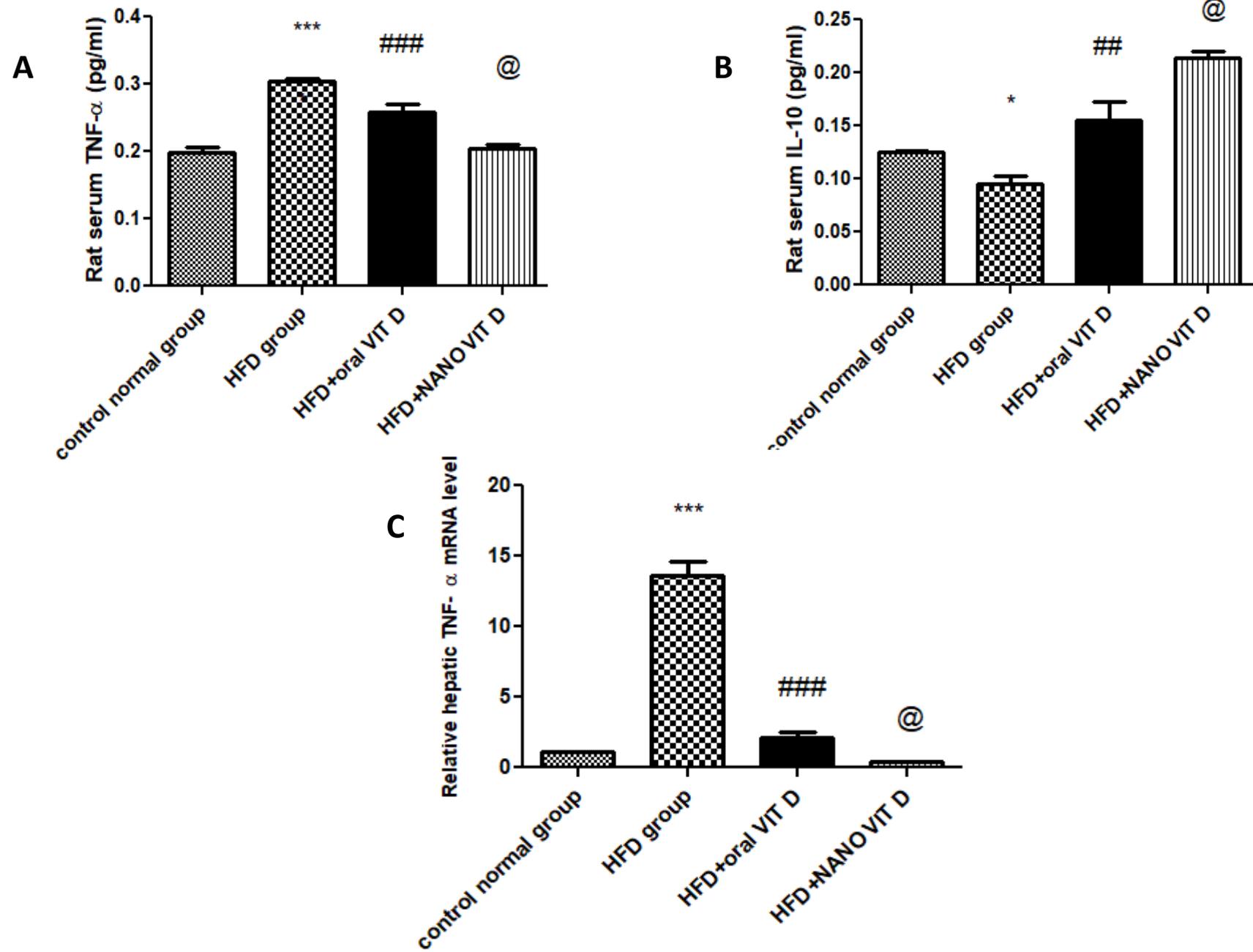
Figure 5
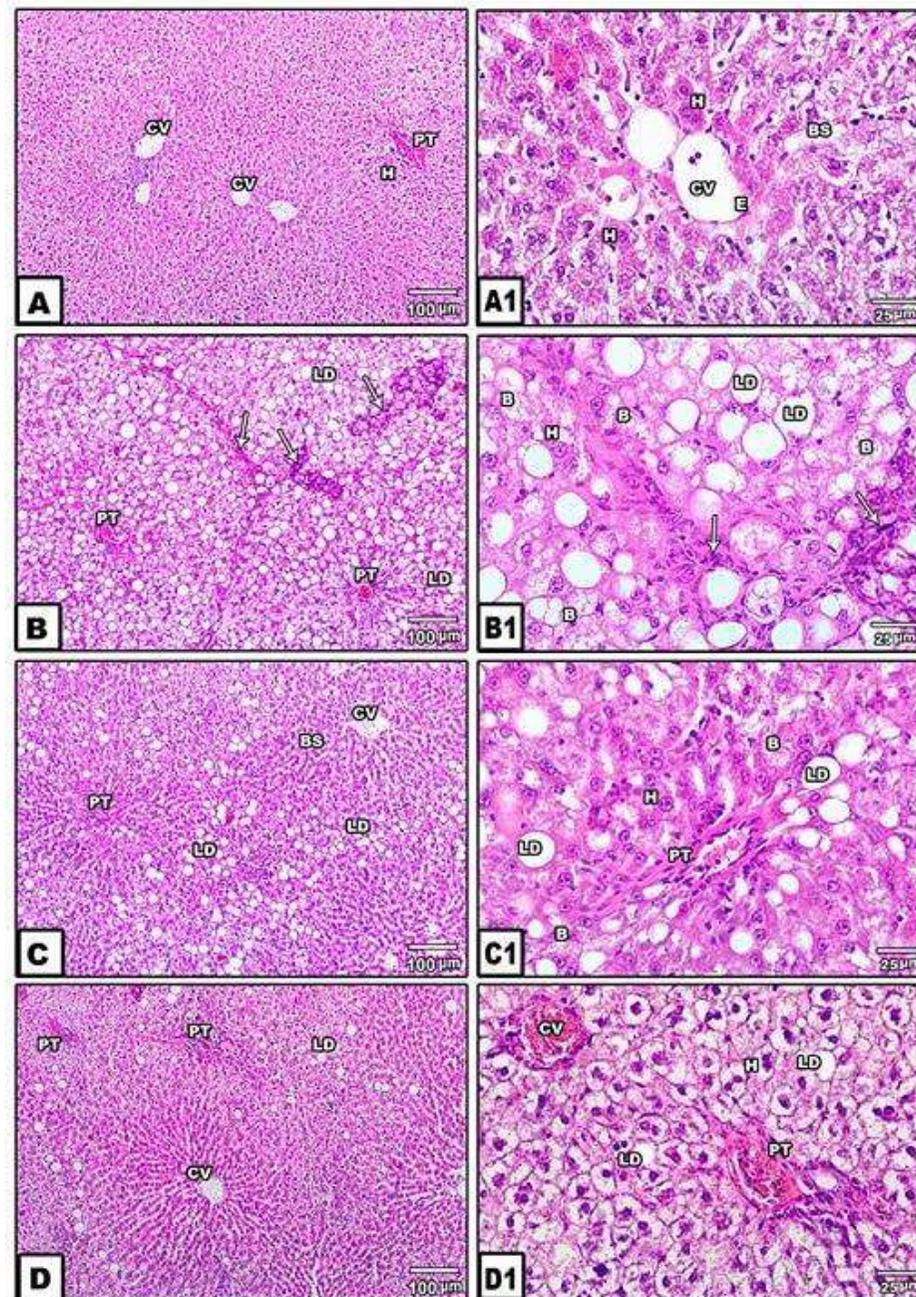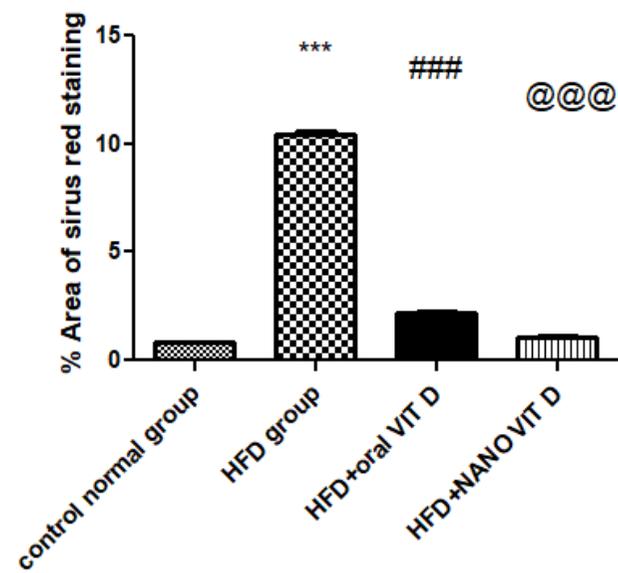
Figure 6
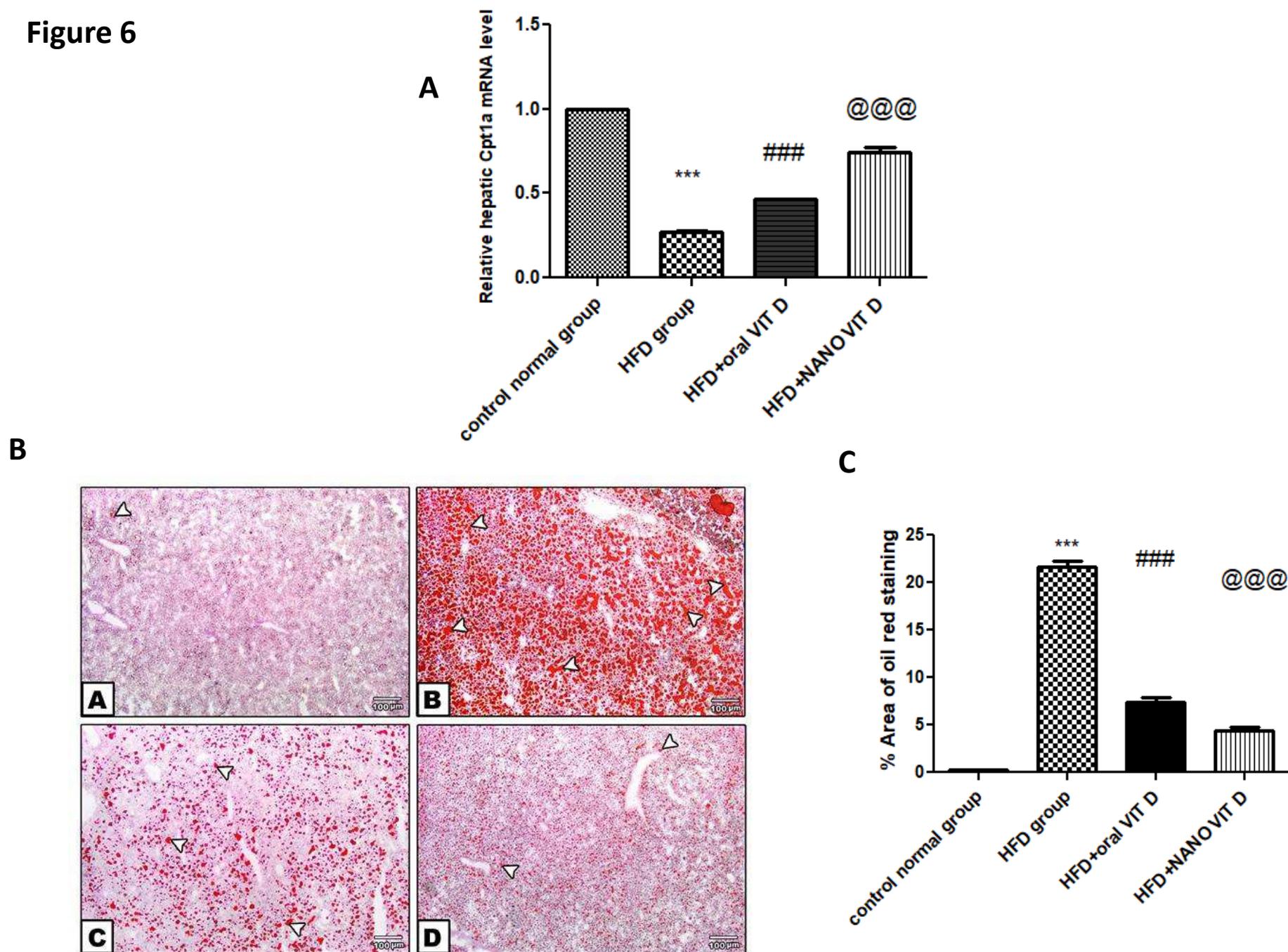
Figure 7
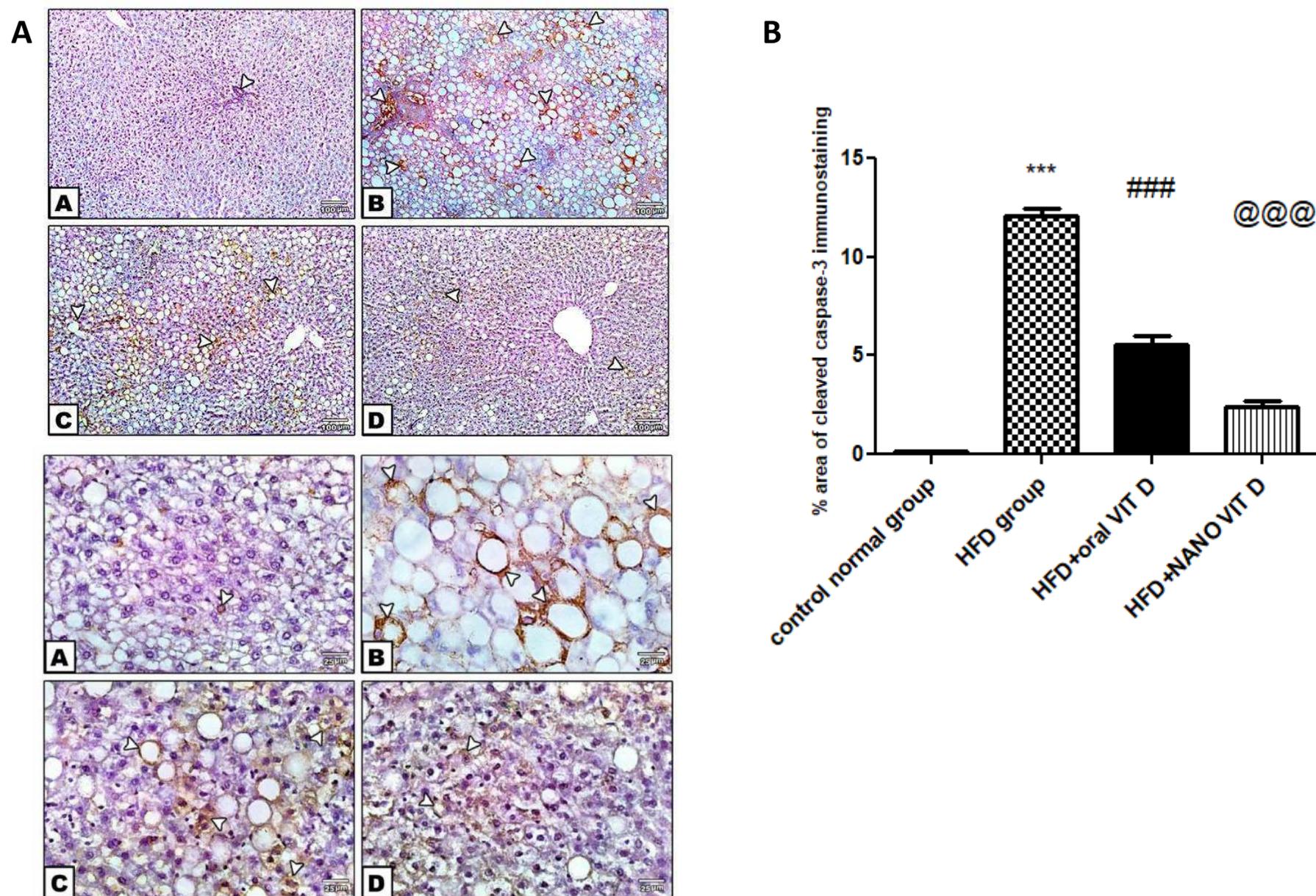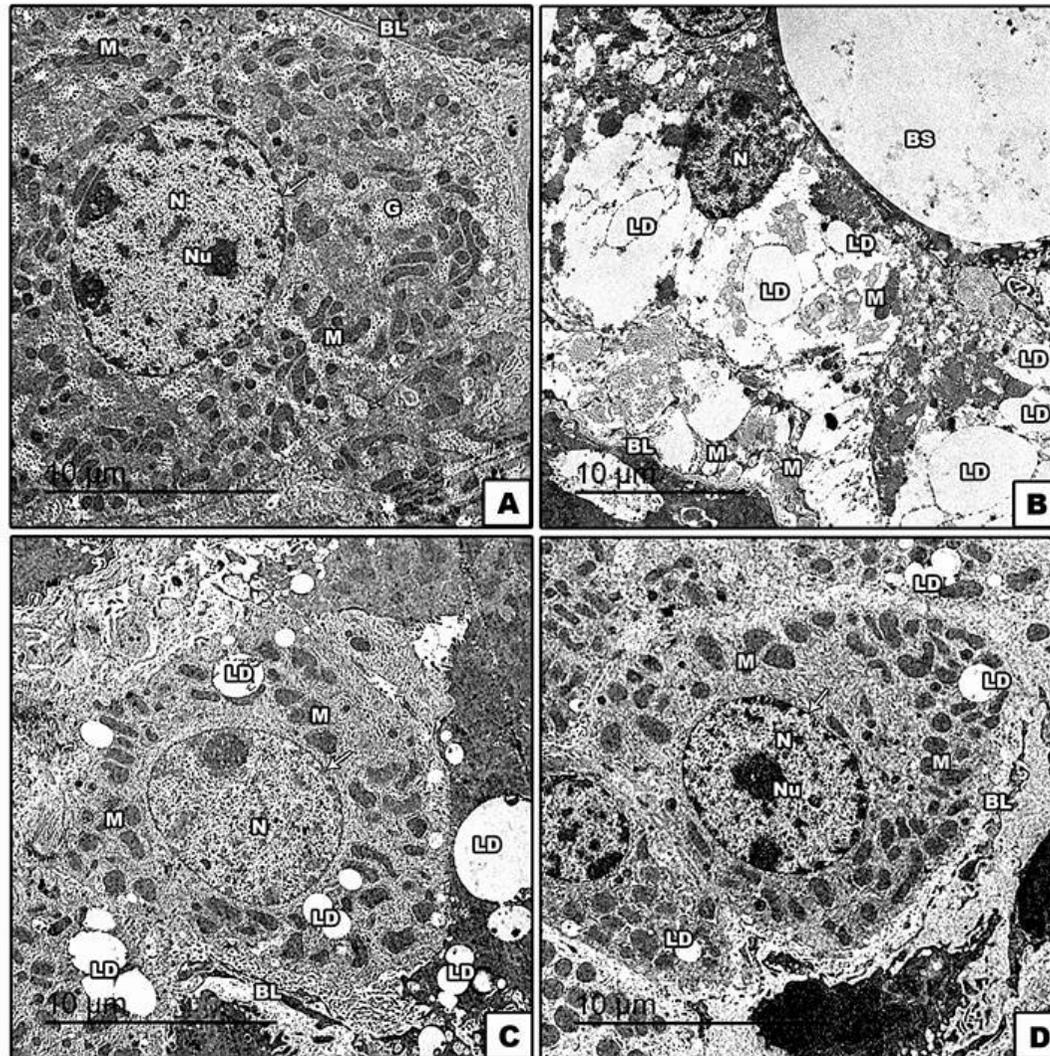
A
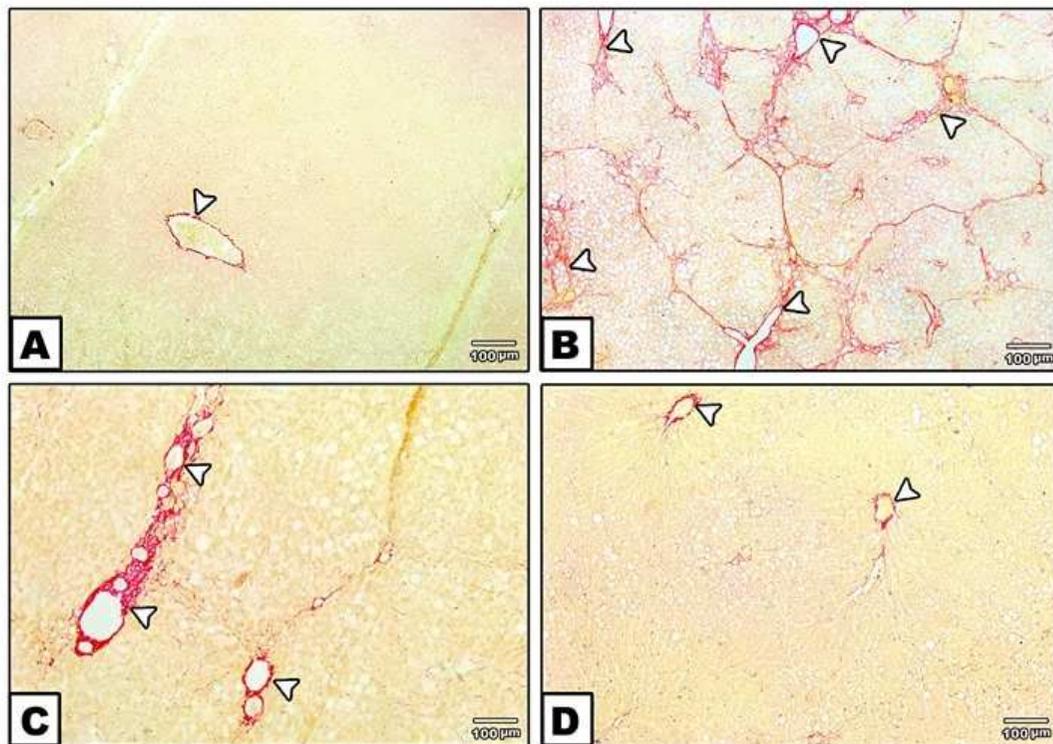
B

Figure 8

Figure 9

- Hypovitaminosis D often coexists with non alcoholic fatty liver disease
- Vit D nanoemulsion attenuated HFD-induced liver injury, inflammation and fibrosis
- Vit D nanoemulsion showed more hepatoprotective effect than conventional vit D