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Lipolysis-stimulating peptide-VHVV ameliorates high fat diet induced hepatocyte apoptosis and fibrosis

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ABSTRACT

Nonalcoholic fatty liver disease (NAFLD) is a common outcome of obesity characterized with the accumulation of triacylglycerols, increase in tissue apoptosis and fibrosis. This study was performed to determine the effect of a lipolysis stimulating peptide-VHVV to reduce the NAFLD related effects in mice liver induced by high fat diet (HFD). Five groups of mice (n = 8), control fed with standard chow; HFD-induced obese control and three treatment groups with high/moderate/low dose VHVV-treatment, were used to evaluate the effect of VHVV. HFD fed mice exhibited heavier body weight than those with control chow diet. However, the levels of triacylglycerol and low density lipoprotein-cholesterol were significantly reduced in mice administered with low, moderate and high doses of VHVV. VHVV-administration also suppressed the HFD-induced hepatic apoptosis and fibrosis related proteins. Our results indicate that VHVV attenuate hepatic lipid accumulation and ameliorate inflammation related events in HFD fed mice to suppress apoptosis and fibrosis.

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1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is one of the most common metabolic syndromes of the developed and the de-

veloping world and is known to cause chronic liver disease in obese adults (Bogdanova et al., 2006; Yilmaz, 2012). NAFLD is characterized with significant amount of triacylglycerols (TAG) accumulation in the liver with potential to progress into non-alcoholic steatohepatitis, fibrosis, cirrhosis and even carcinoma.

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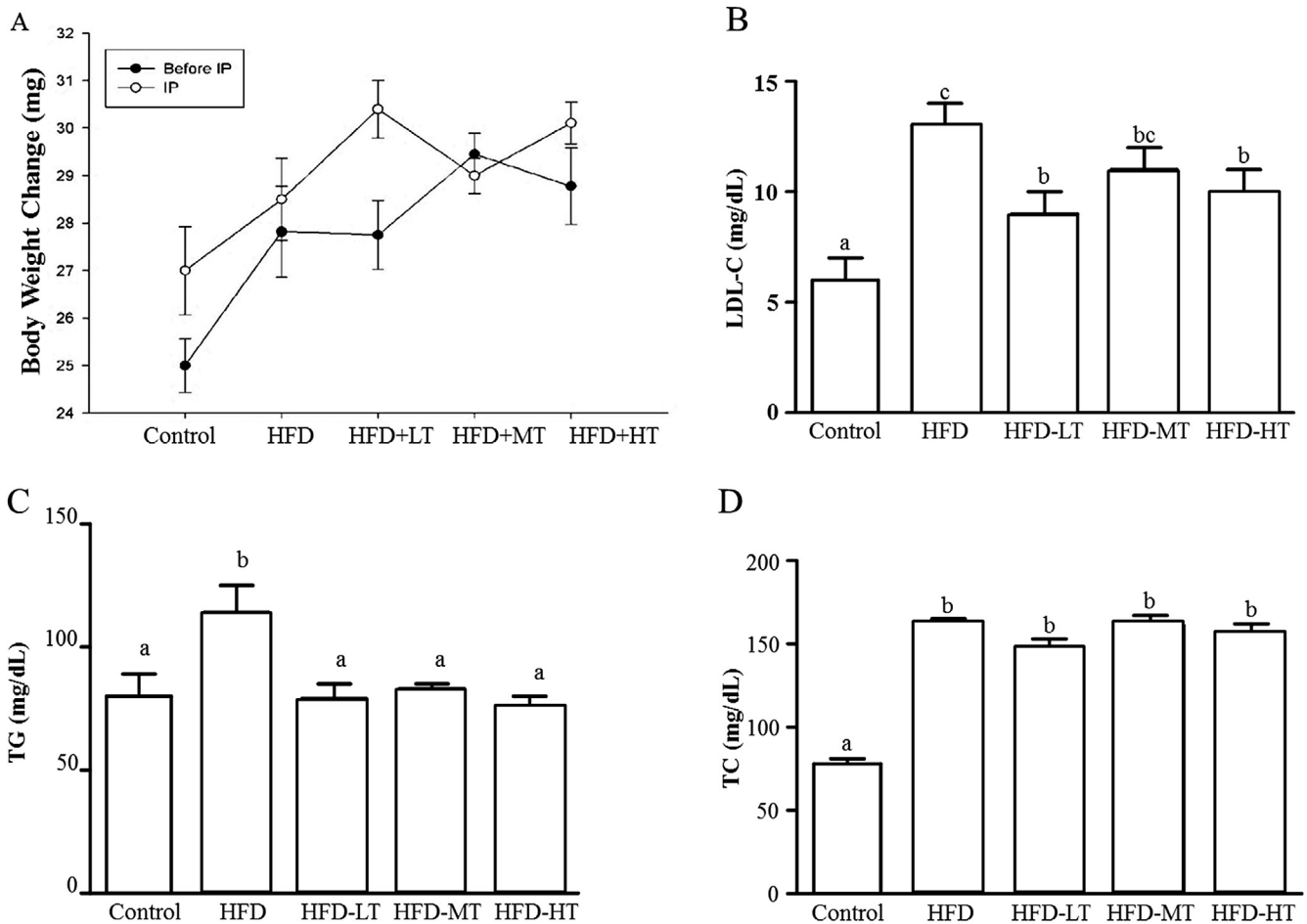


Fig. 1 – Effect of VHVV on total body weight and on plasma triglycerides and cholesterol levels. The changes in body weight and in the levels of low density lipoprotein- cholesterol (A), triglycerides (B) and total cholesterol (C) were measured in mice fed with regular diet (control) mice fed with high fat diet (HFD), mice fed with high fat diet with low dose VHVV (HFD+LT) treatment, mice fed with high fat diet with moderate dose VHVV (HFD+MT) treatment and mice fed with high fat diet with high dose VHVV (HFD+HT) treatment after 8 weeks of respective feeding. The results represent mean \pm SD of three independent experiments. Values bearing the same letter are not significantly different by one-way ANOVA analysis.

Because the liver does not store fat, the concentration of TAG in the liver remains low under normal physiological conditions. However, there is a considerable movement of TAGs and fatty acids into and out of the liver in response to feeding and fasting. Conditions of hypernutrition or insulin resistance causes an imbalance in hepatic lipid uptake and removal, this results in excess accumulation of TAG in the liver (Kawano & Cohen, 2013). Apoptosis is believed to play a crucial role in NAFLD-induced liver injury and progression of non-alcoholic steatohepatitis and cirrhosis (Jiang, Zhao, & An, 2011; Xiao et al., 2013). Moreover, the degree of apoptosis is closely associated with the circumstances for fibrosis. Therefore, inhibition of hepatic apoptosis may be a convenient strategy to treat NAFLD. As there are no drugs to effectively treat NAFLD, diet control and diet related treatment becomes more crucial for prevention of NAFLD (Kawano & Cohen, 2013).

Flavourzyme is a fungal peptide complex containing both endo-peptidase as well as exo-peptidase and it has been used to produce protein hydrolysates with low bitterness (Suh, Whang, Kim, Bae, & Noh, 2003). Enzymatic hydrolysates derived

from soy protein extracts are known to promote several bioactive functions, such as antioxidative activity and cholesterol-lowering activity (Moure, Dominguez, & Parajo, 2006; Tsou, Kao, Tseng, & Chiang, 2010). A lipolysis-stimulating peptide VHVV derived from Flavourzyme-soy protein isolate (SPI) hydrolysate (F-SPIH) was found to potentially act as a functional anti-obesity diet ingredient (Tsou, Kao, Lu, Kao, & Chiang, 2013). However, the effect of VHVV against liver damage caused by high fat diet (HFD) is unclear. This study aimed to investigate the effects of VHVV on hepatic apoptosis and fibrosis in HFD induced obese mice. Male C57BL/6 mice that were fed with HFD for 8 weeks (HFD group) developed symptoms of NAFLD and its progression to apoptosis and fibrosis. However, HFD fed mouse treated with different doses of VHVV for 6 weeks showed a significant reduction in the symptoms of NAFLD related apoptosis and fibrosis. The TAG levels and low-density lipoprotein cholesterol (LDL-C) levels significantly declined in mice treated with low, medium and high levels of VHVV. However VHVV did not cause any effect on the HFD mediated elevation of total cholesterol (TC) levels. The administration of VHVV also

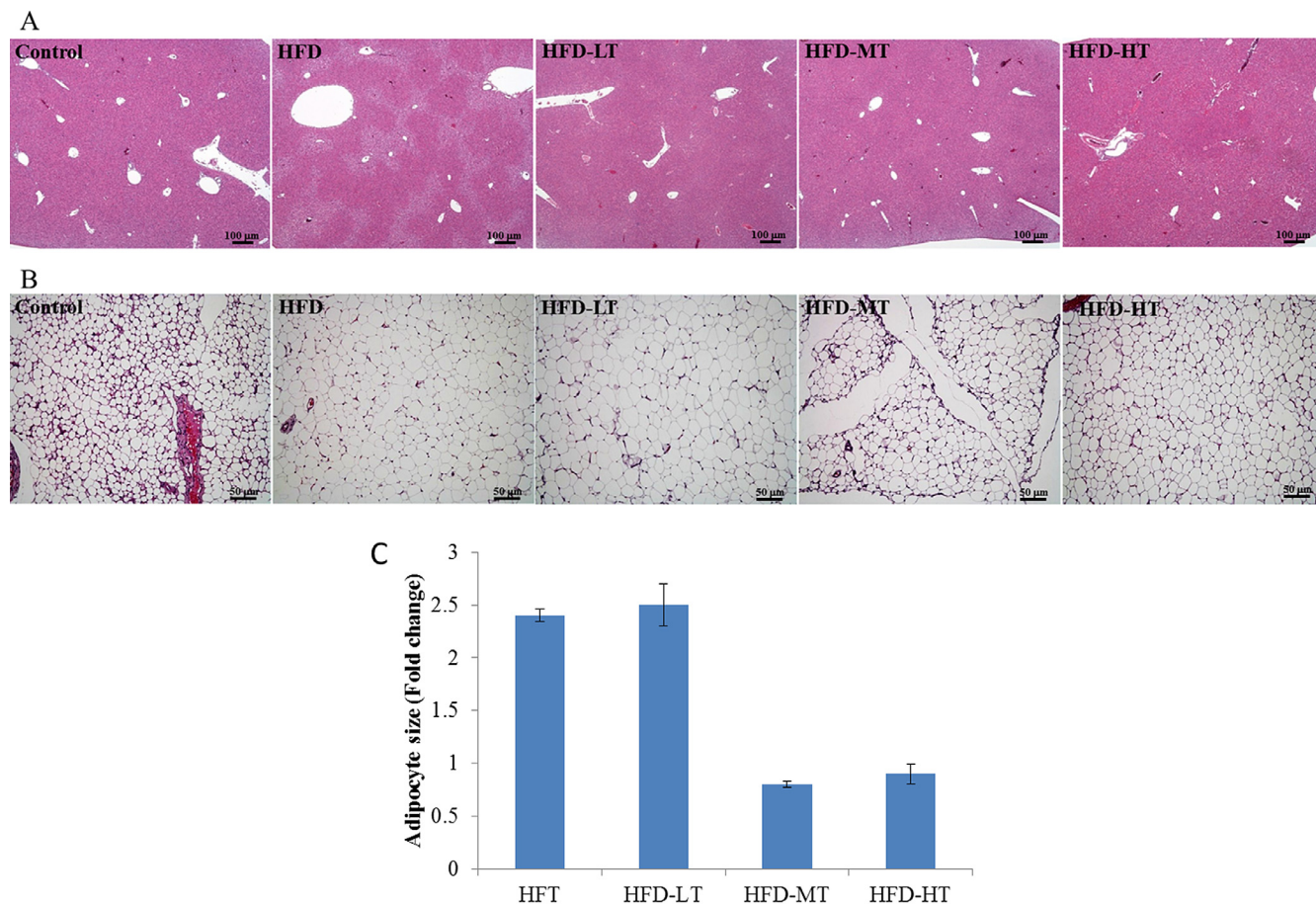


Fig. 2 – Hematoxylin and eosin (H &E) staining of heart sections show the liver tissue architecture (A) and micro- and macrovesicular steatosis (B). Histopathological analysis of hepatic sections of mice fed with regular diet (control), mice fed with high fat diet (HFD), mice fed with high fat diet with low dose VHVV (HFD+LT) treatment, mice fed with high fat diet with moderate dose VHVV (HFD+MT) treatment and mice fed with high fat diet with high dose VHVV (HFD+HT) treatment after 8 weeks of respective feeding. The results represent mean \pm SD of three independent experiments.

provided hepatic protection by reducing the accumulation of lipid droplets in the liver and adipose tissues. The levels of apoptosis and fibrosis related proteins were also significantly lower in VHVV treated groups than HFD group. Our results indicate that VHVV attenuates HFD induced lipid accumulation and also inhibits the subsequent apoptosis and fibrosis effects in mice livers. Administration of VHVV can therefore be considered as a potential therapeutic agent to ameliorate NAFLD effects.

2. Materials and methods

2.1. Animal experiments

This study was conducted following the IACUC-100-12 protocol and approved by the institutional animal care and use committee (IACUC) of China Medical University, Taiwan. One week prior to the experiments, the mice (6 week old male C57BL/6 weighing about 26 ± 1.3 g) were allowed to adapt to the environment and the diet. During the 1 week adaptation period, all the animals were individually housed in a room maintained at 24 ± 2 °C and $55 \pm 10\%$ humidity with a 12 h light cycle.

The mice were randomly divided into five groups ($n = 8$): sedentary control fed with standard chow, HFD induced obese sedentary control, HFD with low dose (5 mg/kg/day) VHVV treatment, HFD with moderate dose (15 mg/kg/day) VHVV treatment and HFD with high dose (25 mg/kg/day) VHVV treatment. The animals were fed with a standard laboratory diet (PMI Nutrition International, Brentwood, MO, USA) and were provided with reverse osmosis treated water *ad libitum*. For the next 8 weeks obesity was induced by giving HFD containing 60% of energy as fat. VHVV appropriately diluted in 0.9% saline was administered by intra-peritoneal (I.P.) injection in the last 6 weeks of HFD induction. Equal volumes of 0.9% saline were administered to groups of vehicle C and HFD through IP injection.

2.2. Hemotoxyline and eosin staining

The livers were excised; soaked in formalin; dehydrated by passing consecutively through 100%, 95% and 75% alcohol and were then embedded in paraffin wax. The embedded tissue blocks were then cut into 0.2 µm-thick sections and deparaffinized by soaking in xylene. For adipose tissue, adipose tissue that were removed from each mouse were fixed in 10%

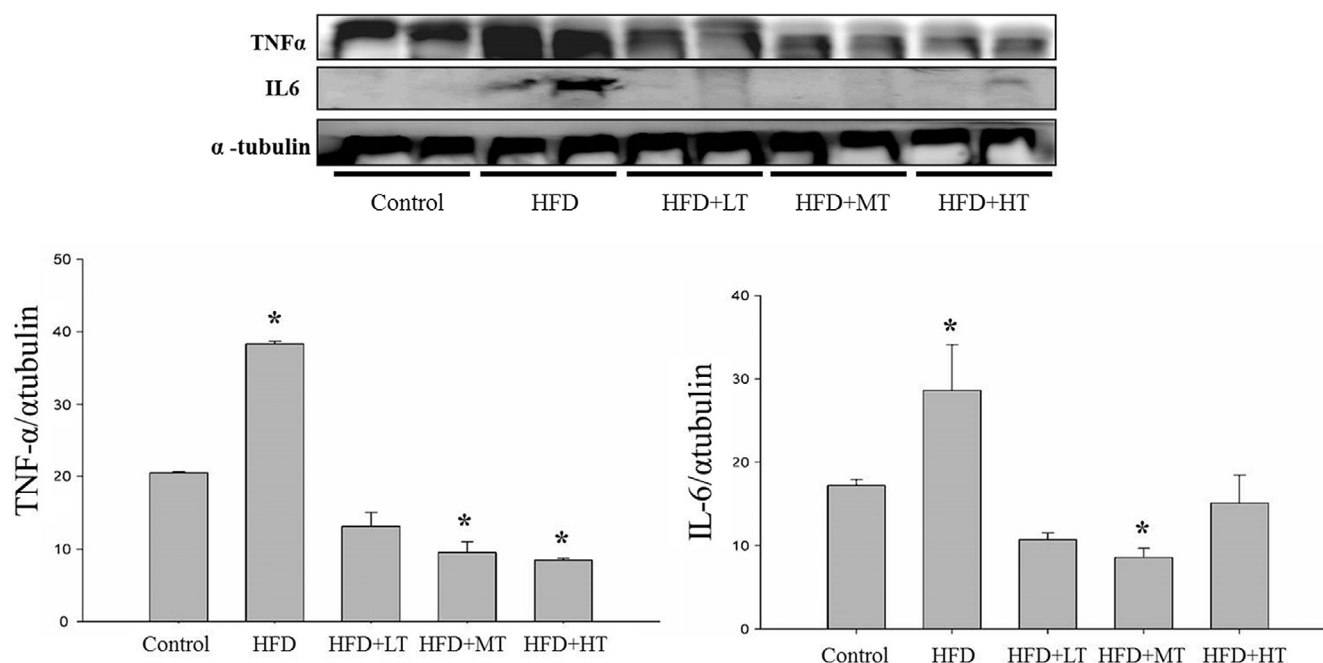


Fig. 3 – Effect of VHVV on inflammatory cytokines. Western blot analysis on the levels of interleukin-6 (IL 6) and tumor necrosis factor α (TNF α) shows the difference between their respective expression in mice fed with regular diet (control), mice fed with high fat diet (HFD), mice fed with high fat diet with low dose VHVV (HFD+LT) treatment, mice fed with high fat diet with moderate dose VHVV (HFD+MT) treatment and mice fed with high fat diet with high dose VHVV (HFD+HT) treatment after 8 weeks of respective feeding. The results represent mean \pm SD of three independent experiments. * $P < 0.05$ significant differences compared with the control group.

formaldehyde/PBS and dehydrated, embedded in tissue-freezing medium (Tissue-Tek OCT compound; Miles Inc., Elkhart, IN, USA) and frozen in dry ice and acetone. The adipose tissue was cut into 10- μ m sections (Yamauchi et al., 2001). The slices were stained by hematoxylin and eosin (H&E) and rinsed with water. Photomicrographs were obtained using Zeiss Axiophot microscopes (Carl Zeiss Microscopy, Thornwood, NY, USA). The cell area was determined by using ImageJ image processing program (National Institutes of Health, Bethesda, MD, USA).

2.3. Determination of lipid profile

Blood samples were collected from the test mice centrifuge tubes containing heparin (10 μ L, 1000 IU mL⁻¹). The blood plasma was separated by centrifugation at 10,000 rpm for 10 min; TAG, LDL-C and TC levels were measured by using commercially available assay kits (Beijing BHKT clinical reagent Ltd, Beijing, China).

2.4. Tissue protein extraction

Liver extract was obtained by homogenizing the liver tissue in lysis buffer (100 mg/mL). The homogenates were placed on ice and then centrifuged at 12,000 g for 40 min. The supernatants were collected and stored at -80 $^{\circ}$ C for further experiments.

2.5. Western blot

Protein concentration of the extract was determined by the Lowry's protein assay method. Protein samples were

separated in a 12% SDS polyacrylamide gel electrophoresis (SDS-PAGE) using 75 V of constant power supply. Proteins were then transferred to PVDF (GE Healthcare Life Sciences, Pittsburgh, PA, USA) membranes using 50 V current for 3 h. The membranes were incubated in 3% bovine serum albumin (BSA) in TBS buffer and the primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were added onto the membranes for conjugation with specific proteins. Horseradish peroxidase-labelled secondary antibodies were used for detection and pictures were finally taken with Fujifilm LAS-3000 (GE Healthcare Life Sciences). Quantifiable representation on the protein expression levels were made by normalizing their expression with that of α -tubulin internal control.

2.6. Statistical analysis

Quantitative data are shown as the mean \pm SD corresponding to three or more replicates. Analysis of statistical differences was done by one-way Student's t -test. A P -value of < 0.05 was considered statistically significant.

3. Results

3.1. VHVV administration alleviated HFD induced fatty liver

The HFD intake induced several notable changes in the test mice. The HFD feeding significantly increased the mice body weight and the VHVV treatment did not show any considerable change

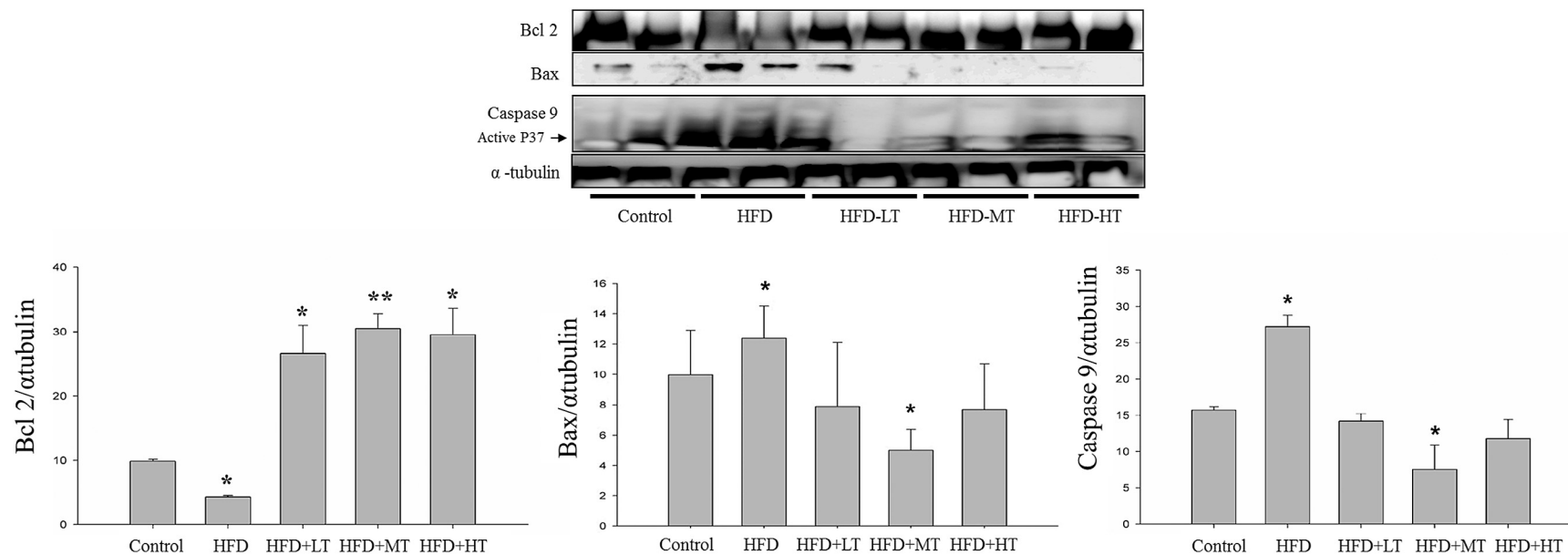


Fig. 4 – Effect of VHVV on the mediators of intrinsic apoptosis. The levels of apoptosis related proteins such as Bcl 2, Bax and cleaved Caspase 9 in the mice fed with high fat diet (HFD), high fat diet with moderate dose VHVV (HFD+MT), high fat diet with high dose VHVV (HFD+HT) after 8 weeks of respective feeding. The results represent mean \pm SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$, significant differences compared with the control group.

the body weight (Fig. 1A). The pathological susceptibility of mice liver to HFD and the imperviousness acquired due to VHVV treatment were evaluated by measuring the TAG and cholesterol levels. Long-term feeding on HFD induced fatty liver in mice displayed elevated levels of LDL-C, TC and TG (Fig. 1B-D). Moreover, massive fat accumulation in the livers of mice fed with HFD was also observed from H and E staining of the liver tissue (Fig. 2A). Adipocytes in the adipose tissue of the HFD fed mice were larger than those in the control mice. However, the respective adipocytes of mice treated with moderate and high dose VHVV were almost similar to that of the control mice (Fig. 2B and C). All doses of VHVV, particularly the moderate dose, significantly regulated the factors promoting fatty liver progression in the HFD fed mice without affecting their diet intake.

3.2. Effect of VHVV on the levels of inflammatory cytokines

Meanwhile in the HFD fed mouse group the levels of the tumor necrosis factor α (TNF α) and interleukin-6 (IL-6) increased with a corresponding increase in the levels of caspase 8 and active caspase 3. The levels of the corresponding proteins remained low in the VHVV administered mouse groups irrespective of the difference in the dosages (Fig. 3).

3.3. VHVV administration regulates apoptosis and survival related proteins

The protein expression in the HFD fed mice liver, when analyzed by western blotting, revealed that the development

of fatty liver was correlated with the suppression in the levels of apoptosis inhibitor B-cell lymphoma-2 (Bcl-2) and elevation in the levels of pro-apoptotic proteins such as Bcl-2 associated X protein (Bax), activated caspase 9, caspase 8 and caspase 3. However, in the mice groups administered with low, moderate and high levels of VHVV, the expression of Bax, and caspase 9 was found to be suppressed. The VHVV administration also enhanced the expression of apoptosis inhibitor protein Bcl-2 (Fig. 4). The apoptotic proteins of the extrinsic pathway such as the caspase 8 and caspase 3 were also found to be elevated by HFD and the levels were found to be suppressed on treatment with VHVV (Fig. 5). Meanwhile, VHVV administration enhanced the activation of cell survival proteins in the fatty livers of HFD mice. The levels of insulin-like growth factor 1 receptor (IGF-1R), phosphatidylinositol 3-kinase (pPI3K) and serine-threonine kinase (pAkt) increased when treated with low, moderate and high levels of VHVV (Fig. 6). However, moderate VHVV administration was relatively more effective in enhancing the survival proteins.

3.4. Effect of VHVV on liver fibrosis

Feeding with HFD also modulated fibrosis markers such as the matrix metalloproteinases (MMP) such as MMP 9 and MMP 2 in the mouse livers. The increased level of MMP 9 and MMP 2 expression in the HFD fed mice were found to be regulated after VHVV administration (Fig. 7).

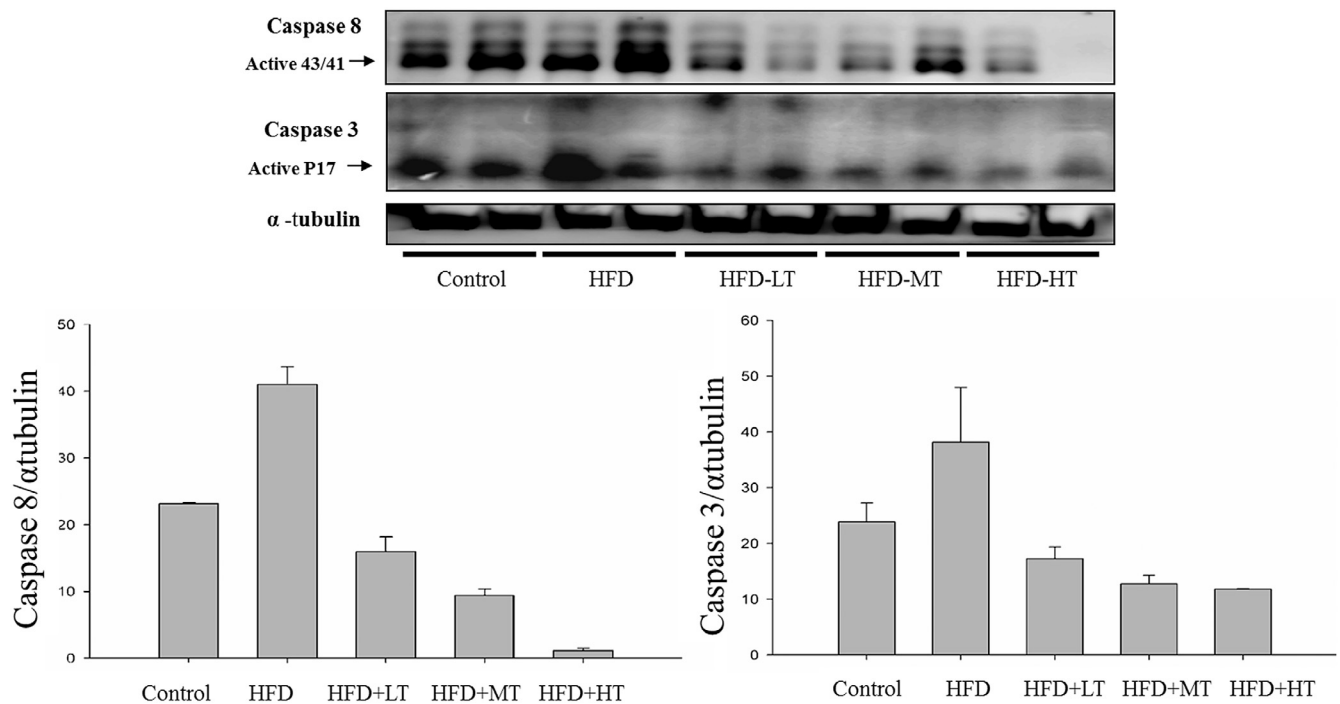


Fig. 5 – Effect of VHVV on apoptosis. The levels of proteins of cell apoptosis such as Caspase 8 and Caspase3 in the mice fed with high fat diet (HFD), high fat diet with moderate dose VHVV (HFD+MT), high fat diet with high dose VHVV (HFD+HT) after 8 weeks of respective feeding. The results represent mean \pm SD of three independent experiments. * $P < 0.05$ significant differences compared with the control group.

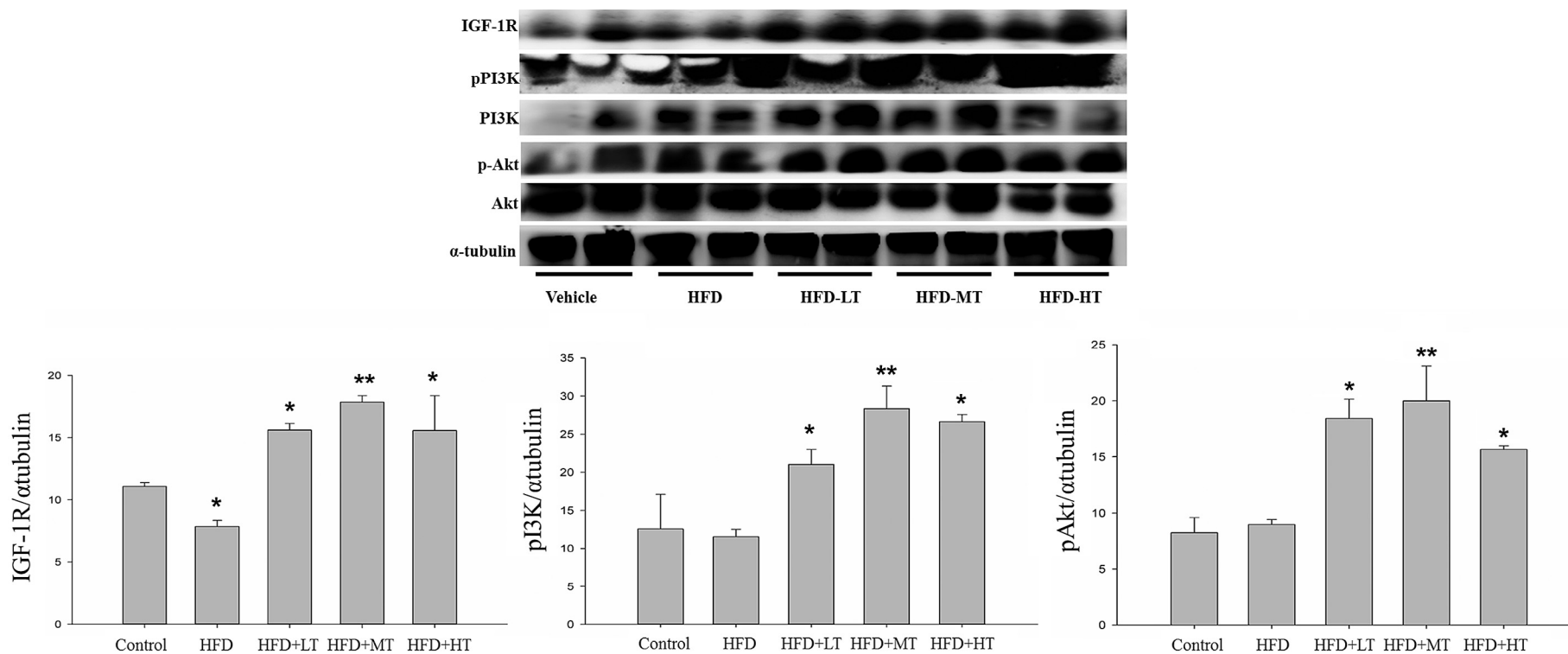


Fig. 6 – Effect of VHVV on survival proteins. The levels of proteins of cell survival such as IGF-1R, pPI3K, PI3K, p-Akt and Akt in the mice fed with high fat diet (HFD), high fat diet with moderate dose VHVV (HFD+MT), high fat diet with high dose VHVV (HFD+HT) after 8 weeks of respective feeding. The results represent mean \pm SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$, significant differences compared with the control group.

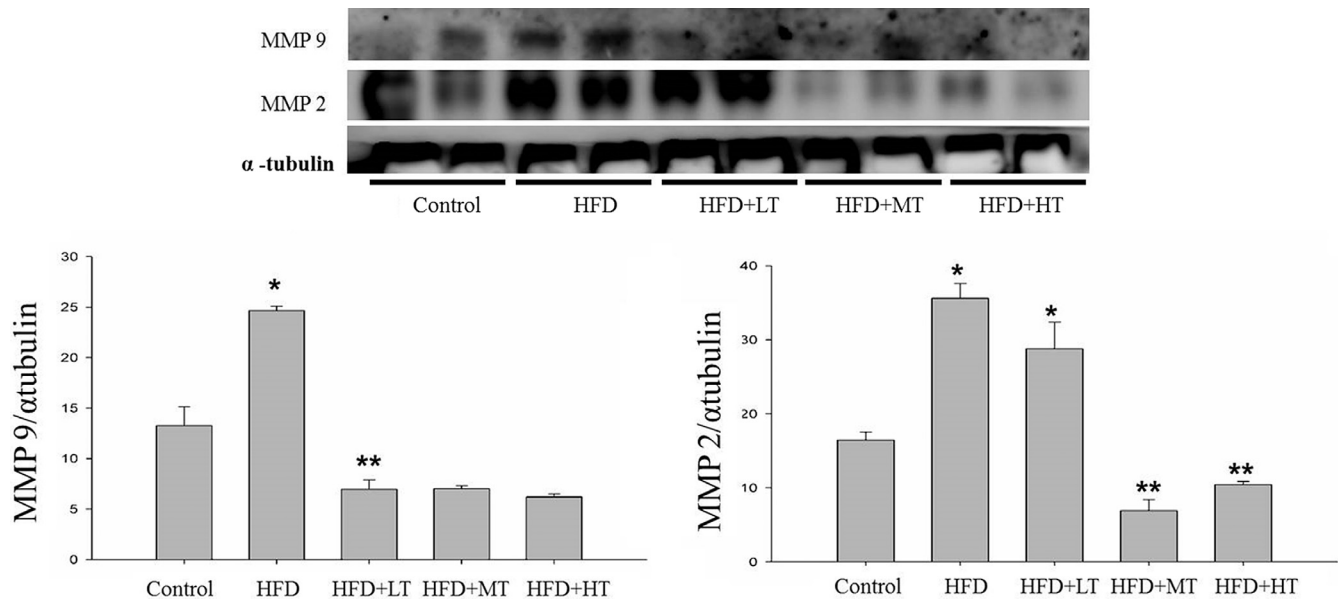


Fig. 7 – Effect of VHVV on fibrosis marker proteins. The levels of matrix metalloproteinases, as determined by western blot analysis, when compared with the respective expression levels in the mice fed with regular diet (control) shows modulations in the expression levels of proteins such as MMP9 and MMP2 in the mice fed with high fat diet (HFD), mice fed with high fat diet with low dose VHVV (HFD+LT) treatment, mice fed with high fat diet with moderate dose VHVV (HFD+MT) treatment and mice fed with high fat diet with high dose VHVV (HFD+HT) treatment after 8 weeks of respective feeding. The results represent mean \pm SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$, significant differences compared with the control group.

3.5. Effect of VHVV on regulatory nuclear receptor

To determine the effect of VHVV on the lipid metabolism the levels of the related nuclear receptor proteins such as peroxisome proliferator-activated receptor (PPAR) and Forkhead box protein O1 (FOXO-1a) were examined. The levels of PPAR- γ and PPAR- α in the HFD fed mice groups were found to be comparatively lower than that of the control group mice. However, expression levels of PPAR- γ and PPAR- α in the mice treated with various concentrations of VHVV remained higher as seen in the control mice. Meanwhile, the PGC-1 α , a co-activator of PPAR- γ was found to decrease in the HFD fed groups but remained at high levels in the VHVV administered group as in controls (Fig. 8).

4. Discussion

Imbalance between energy intake and energy expenditure causes abnormal or excessive fat accumulation and subsequently results in obesity. It poses a substantial health risk, as obesity is linked to several common diseases, such as type 2 diabetes, cardiovascular disease, NAFLD, and several types of cancer (Huang & Lee, 2012; Moore, 2010; Sundaram, Johnson, & Makowski, 2013). The HFD fed mice used in the experiments displayed symptoms of NAFLD as determined from liver histology. NAFLD is one of the leading causes of chronic liver disease worldwide and its pathogenesis is known to be multifactorial, involving obesity, insulin resistance, inflammation and oxidative stress. Targeting these pathways are consid-

ered as a potential treatment approach but the strategy has either shown limited efficacy or an unfavorable safety profile (Bouziana & Tziomalos, 2013).

In our experiments HFD fed mice developed hyperlipidemia associated obesity as determined by their increasing levels of LDL, TG, TC and total body weight (Mahamuni, Khose, Menaa, & Badole, 2012). Previous reports on experimental models have indicated that fatty liver may directly induce hepatic insulin resistance (Valenti et al., 2008). The histological examination of HFD fed mice livers also showed an increase in lipid accumulation and thus indicated the symptoms of NAFLD progression. However in the mice treated with VHVV, the levels remained considerably low revealing the positive effect of VHVV in controlling NAFLD in HFD fed mice.

Deregulation of the insulin signaling pathway is known to cause insulin resistance and NAFLD (Valenti et al., 2008). To find the molecular mechanism associated with the positive effect of VHVV on fatty acid metabolism, modulations in the expression of proteins such as PPAR α , PPAR γ , PGC-1 α and FOXO-1a were determined. PPAR α is a key factor for adjusting energy metabolism and fatty acid oxidation. PPAR γ enhances expression of proteins involved in fatty acid uptake, fatty acid transport and fatty acid synthesis. FOXO-1a is another transcription factor associated with gluconeogenesis that binds to PPAR γ and is required for the activation of gluconeogenesis by PGC-1 α (Valenti et al., 2008). The PPAR γ expression also reduces hepatic inflammation by decreasing expression of pro-inflammatory cytokines, such as TNF α and IL6 (Kallwitz, McLachlan, & Cotler, 2008). Our results show that the levels of PPAR α , PPAR α , PGC-1 α and FOXO-1a were suppressed in the livers of HFD fed mice. However, the levels were comparatively higher in mice treated

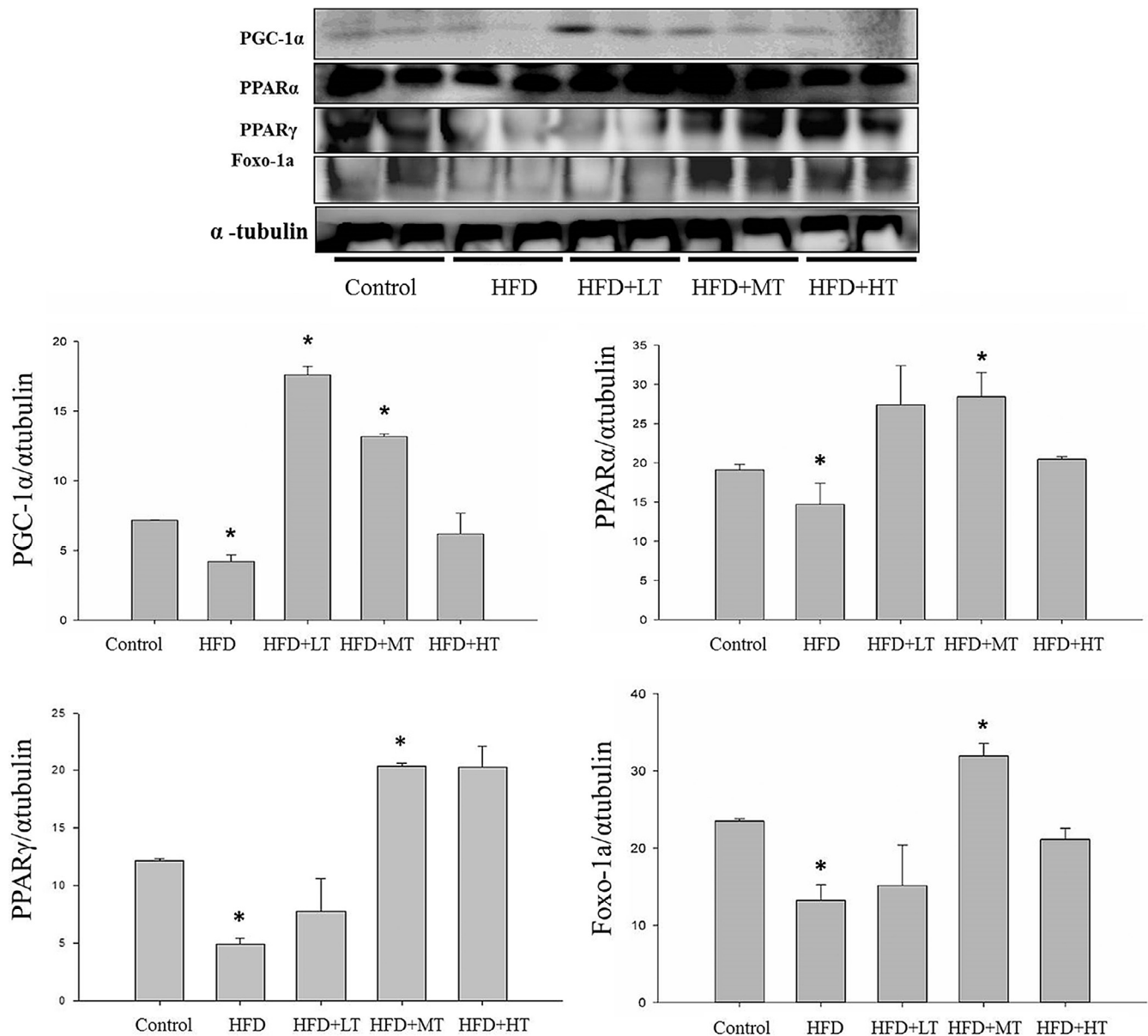


Fig. 8 – The levels of lipid metabolism related nuclear membrane proteins, as determined by western blot analysis, when compared with the respective expression levels in the mice fed with regular diet (control) shows modulations in the expression levels of proteins such as PGC-1α, PPARα, PPARγ and Foxo-1a in the mice fed with high fat diet (HFD), mice fed with high fat diet with low dose VHVV (HFD+LT) treatment, mice fed with high fat diet with moderate dose VHVV (HFD+MT) treatment and mice fed with high fat diet with high dose VHVV (HFD+HT) treatment after 8 weeks of respective feeding. The results represent mean ± SD of three independent experiments. * $P < 0.05$ significant differences compared with the control group.

with VHVV indicating a regulatory effect of VHVV administration on lipid metabolism. Therefore positive effect of VHVV against NAFLD is by elevating the lipid metabolism rate in the livers of mice fed with HFD.

Development of NAFLD usually correlates with unusual levels of serum TNF α and IL-6 (Mazza et al., 2012a). Hepatic inflammation resulting from adipose pro-inflammatory cytokines such as TNF-α and IL6 plays an important role in the development of NAFLD and progression of the fibrogenic process. Our results show that, treatment with low moderate and high doses of VHVV significantly inhibited TNF-α and IL6 expressions that were el-

evated by HFD. Therefore, VHVV potentially exhibits anti-inflammatory action to inhibit HFD-induced liver damage.

Inflammatory cytokines secreted by hepatocytes in response to liver injury, are involved in the pathogenesis of NAFLD as well as in its progression (Mazza et al., 2012b; Vuppalanchi & Chalasani, 2009). In correlation with the regulation in inflammatory cytokines, the results also indicate that the proteins of the intrinsic and the extrinsic apoptosis pathway that were elevated in HFD fed mice were regulated when treated with VHVV. The intrinsic pathway involves the disruption of mitochondrial membrane potential and release of

cytochrome c that triggers caspase 9. The extrinsic pathway involves the activation of death receptors such as the Fas receptor, recruitment of the adaptor molecule FADD and activation of caspase 8 (Green, 1998; Park et al., 2003). Both pathways are involved in the pathogenesis of NAFLD (Feldstein & Gores, 2005; Xiao et al., 2013). The members of the Bcl-2 family of proteins-Bcl-2 and Bax, that are respectively anti-apoptotic and pro-apoptotic factors, are crucial markers of the events in apoptosis (Park et al., 2003). The VHVV also down-regulated Bcl-2 while up-regulating Bax in the livers of HFD fed mice. Therefore, our results indicate that HFD increases of apoptosis in mouse livers but is significantly controlled when treated with VHVV.

Further the modulation in the cell survival such as the IGF1R, PI3K and AKT are determined to understand the level of recovery from HFD induced metabolic disorder. NAFLD and obesity are known to be associated with deregulation of the PI3K/AKT pathway (Matsuda, Kobayashi, & Kitagishi, 2013). The IGF1R mediated activation of PI3K and AKT prompts immune cell activation by regulation of the key inflammatory cytokines (Goto et al., 2009; Han et al., 2010). Administration with VHVV increased the levels of IGF1R, PI3K and AKT in the HFD fed mice indicating improvements in the conditions of hepatocyte survival. As a consequence of apoptosis associated liver injury, hepatic stellate cells are known to migrate to the site of apoptosis to engulf apoptotic bodies. In the process hepatic stellates are activated to promote the deposition of extracellular matrix and scar formation in the liver (Chakraborty, Oakley, & Walsh, 2012). This prompts the progression of liver fibrosis which is associated with corresponding increase in the levels of certain MMPs (Chakraborty et al., 2012; Han, 2006). In our results the hepatic MMP-2 and MMP-9 levels were found to increase in mice that were fed with HFD but their levels were found to be suppressed in VHVV treated mice groups.

In this study, the effect of VHVV to attenuate HFD related apoptosis and fibrosis effects was determined in HFD fed mouse liver. HFD induced changes in the mouse bodyweight; liver histology and lipid levels; expression of apoptosis and fibrosis related proteins were found to be regulated when treated with VHVV. Therefore low, moderate and high concentration of VHVV administration potentially rescues mouse livers from the effects of HFD.

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