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Antioxidant Capacity of Proteins and Hydrolysates from the Liver of Newborn Piglets, and Their Inhibitory Effects on Steatosis *in vitro*

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SUMMARY

Research background. Non-alcoholic steatohepatitis is a potentially progressive hepatic disorder that can lead to end-stage liver disease and hepatocellular carcinoma. The inhibitory effects of proteins and hydrolysates from the liver of newborn piglets on hepatic steatosis in oleic-acid-induced HepG2 cells were investigated *in vitro*.

Experimental approach. The extracted proteins from the liver of newborn piglets (NPLP) were hydrolysed with papain, pepsin, trypsin and Alcalase. Based on comparison of different enzyme digestion condition, a protein hydrolysis protocol was established to obtain hydrolysates with lipid-lowering effect.

Results and conclusions. NPLH-trypsin (trypsin-digested NPLP hydrolysate) exhibited strong antioxidant activity and possessed good inhibitory effects on lipogenesis and cholesterol accumulation in HepG2 cells at 150 μg/mL, with a triglyceride decrease of (43±3) % and cholesterol

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decrease of (31±5) %, comparing with 0.75 mM oleic acid induced model. The addition of NPLH-trypsin (300 μg/mL) decreased alanine aminotransferase and aspartate aminotransferase activities and increased superoxide dismutase activity.

Novelty and scientific contribution. This study demonstrated that NPLH-trypsin have a potential preventive effect on NAFLD in its early stage, and NPLH-trypsin has potential use as the modulator of lipid overaccumulation disease in food supplements.

Key words: newborn porcine liver, proteins hydrolysates, antioxidant activity, oleic acid; lipid accumulation

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is caused by the lipid accumulation in the liver without intaking excessive amounts of alcohol. NAFLD develop from simple steatosis accompanied by the absence of inflammation and non-alcoholic steatohepatitis and can progress to liver cirrhosis and malignant hepatic cancer (*1,2*). Non-alcoholic steatohepatitis is characterized by accumulation of fat, inflammation and injury, which is prevalent in the general population (*2*). A prevailing theory of NAFLD pathogenesis, known as the “double-hit” hypothesis, involves both inflammation and oxidative stress (*3*). Generally, lipid overaccumulation is caused by an imbalance between uptake (anabolism) and output (catabolism), which causes oxidative stress and triggers the overproduction of reactive oxygen species (ROS). Because as a significant pathway in activating the “second hit”, oxidative stress has an important role in hepatic cell damage and dysfunction. There is a subsequent vicious cycle of lipid overaccumulation in the fatty liver, resulting in hepatocyte injury and inflammation (*3,4*). In addition, liver injury may induce mitochondrial dysfunction and peroxidation damage, causing steatosis. In turn, oxidative stress decrease brings a balance between the production of ROS and antioxidant defences, enhancing lipid metabolism and lowering adipose accumulation, effectively (*4*). Thus, reduction of oxidative stress can indirectly regulate fatty acid synthesis and control fatty acid degradation occurring in NAFLD patients.

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Animal products such as bioactive proteins and protein hydrolysates have potent liver regeneration effects and may stimulate the regeneration of the liver after various types of liver injuries. These products include milk protein hydrolysate (*5*), tuna dark muscle protein (*6*), and pork-liver protein hydrolysate (*7*). Additionally, animal livers have long been recognized as a source of several nutritional proteins, bioactive polypeptides such as hepatocyte growth factor (HGF) and hepatocyte stimulator substance (HSS) (*8*). It is reported that HGF as a multifunctional cellular growth factor can be found in a wide variety of life cells and tissues, which plays a pivotal role in the liver regeneration (*9,10*). Moreover, hepatic stimulator substance and hepatic grows factor regulate the symptoms of NAFLD and convey resistance to hepatic injury by protecting mitochondrial function (*7,11*).

The objective of our work was to study the inhibitory effects of new-born porcine liver proteins and their hydrolysates on hepatic steatosis in oleic acid (OA)-induced HepG2 cells (treated with 0.75 mM OA in high-glucose Dulbecco's Modified Eagle Medium (DMEM)). In this study, to evaluate their lipid-lowering capacity, we investigated triglyceride (TG) and cholesterol (TC) accumulation; the activities of intracellular biochemical enzymes, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and superoxide dismutase (SOD); cellular antioxidant activity and the inhibition of lipid peroxidation.

MATERIALS AND METHODS

Material

TSK gel G2000 SWXL, Sephadex G50 (50-150 μm) were obtained from GE Healthcare Life Science, Pittsburgh, USA. Pepsin (300 000 U/g), papain (800 000 U/g), trypsin (1:250 U/g), cholesterol (TC), oleic acid (OA), and bicinchoninic acid (BCA) protein assay kit were obtained from Guangzhou Qiyun Biotech Co., Ltd., China. Alcalase 2.4 L (2.4 U/g) was from Sigma-Aldrich Corp., St. Louis, MO, USA. Dulbecco's modified eagle medium (DMEM), fetal bovine serum(FBS), bovine serum albumin (BSA), pravastatin, the ALT, AST and SOD testing kits were purchased from Nanjing Bio-engineering, Nanjing, China. All other reagents were of analytical grade.

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Preparation of newborn porcine liver proteins

The liver from a newborn pig (5 weeks old) was obtained from a market in Shenzhen City, Guangdong Province, China. The total protein content of the liver was 19.8 %. The liver was cut into pieces and washed under running tap water, then drained. First, the drained liver pieces were dissolved in water at a liver to water ratio of 1:2.8 (m/V). After homogenization, the liver solution was frozen at −20 °C for 6 h and ultrasonicated by 1200DT ultrasonic cell crusher (Biosafer Technologies Co., Ltd., Beijing, China) at a power of 300 W for 15 min. We repeated these processes five times. The soluble fraction (extracted newborn porcine liver proteins (NPLP) was centrifuged using Allegra™ 25R centrifuge (Beckman Coulter, Inc., Bremerhaven, Germany) at 8694×*g* for 10 min followed by filtration. The supernatants were lyophilized by FDU-1200 lyophilizer (Tokyo Rikakikai Co., Ltd., Tokyo, Japan) and stored at −20 °C.

Protein hydrolysis and optimization

The extracted NPLP were hydrolysed with four different proteases (pepsin, papain, trypsin and Alcalase). To achieve this, the NPLP were dissolved in water (*m*/*v*=1:3), and the pH, temperature and reaction time were adjusted to the optimal conditions for each protease. Following the hydrolysis, the enzymes were inactivated by heating at 100 °C for 15 min. The pepsin-digested NPLP hydrolysate (NPLH-pepsin), papain-digested NPLP hydrolysate (NPLH-papain), trypsin-digested NPLP hydrolysate (NPLH-trypsin) and Alcalase-digested NPLP hydrolysate (NPLH-Alcalase) were obtained by centrifugation at 869×*g* for 10 min, followed by filtration. The supernatants were lyophilized and stored at −20 °C. The TG inhibitory activity of each enzymatic hydrolysate (NPLH-pepsin, NPLH-papain, NPLH-trypsin and NPLH-Alcalase) on OA-induced HepG2 cells *in vitro* were measured.

The protein hydrolysis process with the highest activity (NPLH-trypsin) was selected for optimization. After the initial single-factor experiments, based on the Box-Behnken principle, a response surface design for three variables (time, temperature and pH) was used to optimize the NPLP hydrolysis processes, and TG inhibitory activity was used as the response variable (Table S1). The supernatants were obtained by centrifugation at 8694×*g* for 10 min, then lyophilized and stored

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at −20 °C.

Determination of antioxidant activities of NPLP and NPLHs

ORAC assay was demonstrated according to Qiu *et al.* (*12*). Each of NPLH-pepsin, NPLH-papain, NPLH-trypsin and NPLH-Alcalase was mixed with phosphate buffer (75 mM, pH=7.4) to 1 mg/mL. A 20 μL aliquot of each sample (NPLP and NPLH) was mixed with 120 μL fluorescein (300 nM) in each well. Plates were then incubated at 37 °C for about 30 min in a microtiter plate reader (R164720 Millipore CytoFluor, Perseptive Biosystems, Inc, Roanoke, USA). A solution of 2,2-azobis (2-amidinopropane) dihydrochloride (AAPH, 80 mM, 50 μL) (Sigma-Aldrich Corp., St. Louis, MO, USA) was added to each well; the plate was incubated under constant shaking, and the fluorescence was measured immediately in a microtiter plate reader (R164720 Millipore CytoFluor, Perseptive Biosystems, Inc, Roanoke, USA), at 1-minute intervals for 60 min. To evaluate the absorbance, the fluorescence was registered at 485 nm and 530 nm. The ORAC value was calculated using linear regression between the Trolox concentration (μM) in the range of 6.25-50 μM Trolox (*13*).

The radical-scavenging activity of DPPH was demonstrated according to a previously described method (*14*) with slight modification. Briefly, a solution of 0.16 mM DPPH (Nanjing Bio-engineering, Nanjing, China) was prepared in absolute methanol. Then, 180 μL of DPPH solution was mixed with 20 μL of the samples (NPLP and NPLH) in 96-well plates, which were incubated at room temperature for 30 min, then shaken at rpm thoroughly. The absorbance was read at 517 nm using a microplate spectrophotometer system (model 550, Bio-Rad, Hercules, USA). The control group plates contained the same volume DPPH without any extract.

Molecular mass distributions

The molecular mass distributions of NPLP and NPLHs were evaluated using HPLC (model LC-10ATvp pump and DGU-12A degasser, Thermo Fisher Scientific, Germering, Germany). During the experiment, samples were introduced to the gel column (TSK gel G2000 SWXL, TOSOH Biosciences, Grove, USA), and the absorbance were monitored under λ=220 nm. The flow phase was composed

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of phosphate buffer (pH=7.0), and the flow velocity was 0.5 mL/min.

Cytotoxicity of NPLP and NPLHs

HepG2 cells were purchased from the Animal Experimental Center of Sun Yat-Sen University, Guangzhou, China. These cells were seeded into a 96-well plate ($2 \cdot 10^4$ cells/well). The cells were incubated for 24 h in an incubator (model MCO-20AIC; Sanyo Electric Biomedical Co., Osaka, Japan) at 5 % CO_2, 37 °C. Then, the medium was removed, and each well was washed once with phosphate-buffered saline (PBS). The medium was replaced with serum-free DMEM containing different concentrations of NPLP and NPLHs, and the cultures were incubated for 24 h. The cells were washed twice with PBS. Then, 200 μL of 0.5 mg/mL MTT serum-free DMEM was added to each well and incubated for 4 h. Removed MTT solution and 150 mL dimethyl sulfoxide were added. The absorbance was read at 490 nm using a microplate reader (Thermo Fisher Scientific, Waltham, USA) and cell viability was calculated as:

$$\text{Viability} = \left(\frac{A_{\text{sample}}}{A_{\text{control}}}\right) \cdot 100 \qquad /1/$$

Inhibitory effect on oleic acid-induced fatty liver model in vitro

HepG2 cells were incubated for 24 h in a 6-well plate at a density of $2 \cdot 10^5$ cells/well. The regular medium was removed from the wells, and the wells were washed once with PBS. The cells were incubated with medium containing 0.75 mM bovine serum albumin (BSA) with oleic acid for another 24 h. NPLP and NPLH were respectively added to the 6-well plate with the inducer of OA–BSA and incubated for 24 h. Cells cultured in media with only 1 % BSA and 100 mg/L pravastatin were used as the control sample and positive sample, respectively.

Measurement of intracellular Triglyceride levels and oil red O staining

The intracellular TG concentration was measured using an enzymatic kit (Beijing BHKT Clinical Reagent Co., Ltd. Beijing, China.), and the total protein levels were measured with a BCA protein assay kit. Intracellular TG levels were expressed as micrograms of TG per milligram of cellular protein.

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Oil red O staining was applied to detect the degree of preadipocytes differentiation according to previous study (*15*).

Measurement of intracellular cholesterol levels

After 24 h of cultivation, the HepG2 cells were collected and broken, then a 2:1 chlorine/methanol solution was added. After being left for 12 h, the cholesterol contents of liquid were measured. We used qualitative–quantitative chromatographic analysis to analyse cholesterol levels. Intracellular compounds were investigated with an HPLC system with a diode array detector (model SPD-M10Avp) (Shimadzu, Kyoto, Japan). The solvent methanol was used for the mobile phase. The cytochylema from each group was injected into the column using a 20 μL loop valve. The flow rate was set to 1.0 mL/min, and absorbance was measured at 210 nm. Components were tentatively identified by comparing their retention times with the authentic standards at 210 nm and cholesterol content was calculated as:

$$w(\text{cholesterol}) = \left(\frac{A_{\text{t12.5}}}{A_{\text{control(t12.5)}}}\right) \cdot 100 \qquad /2/$$

where A_{t12} represents the peak area of the sample at 12.5 min.

Measurement of intracellular SOD, ALT and AST activities

SOD, ALT and AST activities were measured tested using enzymatic kits according to the manufacturer's instructions (Nanjing Bio-engineering, Nanjing, China). The total protein levels were tested with a BCA assay kit.

Statistical analysis

Data were presented as mean value ± standard deviation of the number of replicates. The significance of the differences ($p<0.05$, $p<0.01$) between the two groups were assessed using the paired t-test. All statistical analyses were performed using SPSS (*16*).

RESULTS AND DISCUSSION

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Cytotoxicity of NPLP and NPLHs and their effects on cell proliferation and TG accumulation in OA-induced hepatic steatosis

In this study, through a combination of freeze-thawing and ultrasonication, 91.9 % (*m*/*m*) of crude proteins were extracted from porcine livers. Different concentrations of NPLP, NPLH-pepsin, NPLH-papain, NPLH-trypsin and NPLH-Alcalase were incubated with HepG2 cells to determine their cytotoxicity (Fig. 1a). The results show that the five samples did not significantly reduce the cell viability over a period of 24 h at 300 μg/mL ($p>0.05$). Safe doses of 50-300 μg/mL were used to determine TG accumulation in the HepG2 cells. The data indicated that NPLH-trypsin was the most efficient suppressor of TG accumulation in the hepatocyte (Fig. 1b), with a maximum TG decrease of 40.3 % at 150 μg/mL. NPLH-pepsin and NPLP resulted in the maximum TG decrease of 30.7 % and 32.2 %, respectively (Fig. 1b). Although TG decrease rate of NPLH-trypsin was lower than that of the pravastatin group (100 mg/L, 51.2 %). According to existed literatures, the hepatic TG clearance capacity of NPLH-trypsin weaker than bioactive small molecules (polyphenols and flavonoids) (*14*). Liu *et al.* (*15*) suggested blueberry phenolic acid displays good efficiency in inhibiting TG accumulation in HepG2 cells, and maximal TG decrease was 58.6 %, when the concentration was 100 μg/mL. Alshammari *et al.* showed nimbolide from *Azadirachta indica* successfully reduces intracellular TC by 47.3% relative to the control (*17*). Nevertheless, relative to other protein or polypeptides, NPLH-trypsin has a strong capacity of decreasing hepatic TG accumulation.

Protein hydrolysis optimization

Trypsin hydrolysis was conducted on the extracted proteins. The single-factor experiment indicated that the optimum condition for trypsin hydrolysis included a pH of 8.5, a temperature of 50 °C and a reaction time of 7 h. Subsequently, a response surface design was performed on the three variables, temperature, pH and the reaction time, using TG concentration as the objective function (Fig. 2). Using regression analysis, we obtained the following response equation:

$$\text{TG (nmol/mg protein)} = 130.23 + 2.28 \cdot A + 0.99 \cdot B + 1.73 \cdot C + 0.40 \cdot A \cdot B + 0.29 \cdot A \cdot C + 1.20 \cdot B \cdot C + 7.53 \cdot$$

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$A^2 + 3.73 \cdot B^2 + 3.86 \cdot C^2$ /3/

According to the sum of square presented in Table 1, the contribution of the three factors to the degree of hydrolysis can be placed in the following order: temperature>pH>reaction time. The optimal hydrolysis conditions (minimum TG accumulation) were determined to be a pH of 8.4, temperature of 49.27 °C and reaction time of 6.91 h. Experimental verification under the conditions of a pH of 8.5, temperature of 49 °C, and a reaction time of 7 h determined the TG concentration to be 129.847 nmol/mg protein, close to the theoretical value of 128.847 nmol/mg protein. The lipid-lowering capability of the optimized hydrolysates was roughly improved by 3.2%, compared with unoptimized NPLH-trypsin (40.3 %, 150 μg/mL).

Molecular mass distributions of newborn porcine liver proteins and their hydrolysates

As shown in Table 2, the fraction of peptides in NPLH-trypsin with <3000 Da sizes were up to 85.07 %, which was the highest among the NPLP and NPLHs, with the peptides in the range <1000 Da and 1000-3000 Da being 38.14 % and 46.93 %, respectively. The peptides of NPLP in the range <3000 Da were 65.51 %, with the peptides of <1000 Da (39.29 %) and 1000-3000 Da (26.22 %), respectively. Additionally, the large peptides (>5000 Da) of NPLP were up to 20.7 %. Combined with previous results, low molecular weight peptides of NPLH-trypsin may contribute to its antioxidant and lipid lowering abilities. Moreover, it also was confirmed by other reports that small molecular peptide with peptide with good bioactivity been proposed a (*18*,*19*).

Antioxidant activities of NPLP, NPLH-pepsin, NPLH-trypsin, and NPLH-Alcalase

NPLH-trypsin exhibited higher radical scavenging activity compared with the other hydrolysates. For DPPH radical scavenging capacity, NPLH-trypsin (IC_{50}=7.2 mg/mL) exhibited higher antioxidant activity than NPLH-pepsin (IC_{50}=8.2 mg/mL) and NPLP (IC_{50}=9.2 mg/mL) (data not shown). In the investigation of the oxygen radical absorbance capacity, the samples (NPLP, NPLH-pepsin, and NPLH-trypsin) were demonstrated to have different capacities. NPLH-trypsin had the highest ORAC activity ((594±2) μM Trolox/g) among the NPLP and NPLHs. NPLH-pepsin produced the second-

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highest ORAC activity ((427±2) μM Trolox/g), whereas NPLP was determined to have the lowest ORAC value ((254±4) μM Trolox/g) ($p<0.05$). NPLH-trypsin showed significantly ($p<0.05$) higher reducing power than the other extracts (data not shown). These results suggested NPLH-trypsin have strong oxygen radical scavenging (ROS) activity, which might contribute to its lipid-lowering effect. Mayra *et al.* showed hepatocyte growth factor reduces free cholesterol- mediated lipotoxicity by inhibiting overproduction of ROS, comparing with normal cells (*20*). Xie *et al.* also pointed out dihydromyricetin reduces oleic acid-induced lipid accumulation in L02 and HepG2 cells by inhibiting lipogenesis and oxidative stress (*21*). Vidyashankar *et al.* reported quercetin effectively ameliorates NAFLD symptoms by decreased triacyl glycerol accumulation and increased cellular antioxidants in OA induced hepatic steatosis in HepG2 cells (*22*).

Inhibitory effects of NPLP, NPLH-pepsin, and NPLH-trypsin on fat accumulation in steatotic HepG2 cells

In HepG2 cells, NPLP, NPLH-pepsin, and NPLH-trypsin were evaluated for their effectiveness in inhibiting TG and fat overaccumulation (Fig. 3). The results showed that NPLP and NPLH-pepsin demonstrated the maximum TG clearance rate of roughly 30.7 % and 32.21 %, respectively (Figs. 3a and 3b). NPLH-trypsin had the good effectiveness in suppressing TG accumulation in steatotic cells, with a maximum TG decrease rate of 43.3 % at a concentration of 150 μg/mL, which was a weaker than blueberry phenolic acid (58.6 %, 100 μg/mL) (*15*). To investigate whether NPLP and NPLH affect OA-induced cellular steatosis, a simple qualitative method to analyse the amount of intracellular lipid droplets stored was performed using oil red O staining. The cells in the normal group exhibited irregular polygons, clear edges, and less red lipid droplets (Fig. 4). The normal cells exhibited irregular polygons, clear edges, and little red lipid droplets. After treated with 0.75 mM OA, HepG2 cells began to appear circular and intracellular lipid droplets became larger with a deeper red color. The number and size of the lipid droplets significantly reduced after NPLP and NPLH treatment of HepG2 cells, relative to those of the model group. At 150 μg/mL, the intracellular lipid droplets of cells treated with NPLH-trypsin became smaller with a slightly less intense red colour. In concurrence with the TG accumulation data, NPLH-trypsin exhibited the significantly most effective inhibition of TG ($p<0.05$) in

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HepG2 cells at 150 μg/mL.

Effects of NPLP, NPLH-pepsin, NPLH-trypsin, NPLH-Alcalase on cholesterol secretion in HepG2 cells

Cholesterol peaks were observed at absorbance of 210 nm at 12.5 min. As shown in Fig. 5, the amount of cholesterol significantly decreased in HepG2 cells treated with NPLP and NPLHs compared with the model group. NPLH-trypsin was the most efficient at suppressing TC accumulation in HepG2 cells compared with the model group, with a maximum TC clearance rate of 30.5 % at a concentration of 150 μg/mL.

Effects of NPLP, NPLH-pepsin, NPLH-trypsin, and NPLH-Alcalase on SOD in HepG2 cells

Superoxide dismutase (SOD) were measured in cell to investigate whether the lipid-decreasing effect of NPLP and NPLHs *in vitro* is related to their antioxidant capacity. It can be seen from Fig. 6 that the total SOD activity was inhibited in cells treated with 0.75 mM OA compared with the activity in the control group. Whereas SOD activity increased after treatment with NPLP and NPLHs. According to the obtained results, SOD activity significantly increased by 23.53 % ($p<0.05$) after treatment with 300 μg/mL NPLH-trypsin (Fig. 6c), compared with the model cells.

Effects of NPLP, NPLH-pepsin, NPLH-trypsin, NPLH-Alcalase on production of ALT and AST in HepG2 cells

In general, high ALT and AST levels in the blood are indicators of hepatitis. Specifically, ALT and AST are indicators of hepatic cell inflammation. Therefore, in the cell model, ALT and AST release assays were used to evaluate the progress of NAFLD. OA increase AST and ALT in steatotic hepatic cell significantly, compared with those in the control group ($p<0.05$), suggesting that HepG2 cells treated with OA exhibited oxidative stress. After treatment with NPLH-trypsin, ALT and AST activities reduce significantly (23 and 15.2%, respectively), relative to model (data not shown). There is no significant difference between the AST and ALT activities in the 300 μg/mL NPLH-trypsin treated groups and those in the control group. These results show that the biological properties of NPLH-

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trypsin increased the levels of AST and ALT activity at 150-300 μg/mL in HepG2 cells ($p<0.05$). Previous literatures reported bioactive molecules effectively reduce fat accumulation in hepatocytes by renewing AST and ALT activities (*7,8,9*). Yin *et al.* showed Hugan Qingzhi tablet exerts a preventive effect against hepatic steatosis, by enhancing AST and ALT enzyme activities (*23*). Shimiz*u et al.* showed that consumption of pork-liver protein hydrolysate decreases the body mass and inhibit hepatic lipogenesis in rats significantly (*7*). The mechanisms of the liver protein or protein hydrolysate inhibition of TG synthesis in hepatocytes and its relationship to their antioxidant properties remain to be elucidated.

To study the lipid-lowering effects and antioxidant activities of NPLP and NPLHs using *in vitro* experiments, HepG2 cells were incubated with a mixture of OA to induce cellular steatosis. The number of lipid droplets and the levels of TG and TC increased significantly in the 0.75 mM OA-treated cells, which indicates that the steatosis model *in vitro* had been established successfully. Obviously, 0.75 mM OA causes oxidative stress in hepatocytes, and it has been well documented that hepatic antioxidant systems are significantly decreased in several chronic liver diseases (*20*,*22*). NPLP and NPLHs reduced size of lipid droplets, the intracellular TG, and TC content significantly. The involvement of classical intracellular ROS scavengers, such as SOD, is of fundamental importance in the development of therapeutical approaches to oxidative-based liver pathologies. It is known that SOD plays an important role in hepatocyte defence against ROS, free radicals and electrophilic metabolites (*23-25*). Therefore, the present data showed that NPLP and NPLHs protect hepatocytes against oxidative stress and ameliorate intracellular TG, TC and lipid overaccumulation. Compared with OA-induced model, NPLH-trypsin decreased of 43.3 % TG in OA-induced HepG2 cells at 150 mg/mL, which was little lower than that of hypolipidemic drug pravastatin group (100 mg/L, 51.2 %). The TG decrease capacity of NPLH-trypsin was slight lower than of small bioactive molecules (polyphenols, flavonoids, and phenolic acids) (*14,15*), and higher than in other types of protein hydrolysate (*5,7*). These results indicate that NPLH-trypsin exhibits good inhibiting TG accumulation in HepG2 cells.

Literatures demonstrated NAFLD is a multifactorial disease resulting from a complex interaction

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of environment, enzymes and metabolism (*3,23,26*). The protective effects of bioactive molecules against oleic acid-induced hepatic steatosis by regulation of intracellular AST and ALT *in vitro* (*23*). Moreover, the experimental results show that AST and ALT activities significantly decreased in the NPLH-trypsin group. The data indicated that 300 μg/mL NPLH-trypsin possesses a strong hepatoprotective effect because aminotransferase is generally considered to be an indicator of hepatocyte injury. Hence, NPLH-trypsin reduced the progression of hepatic steatosis because of its antioxidant properties, which can protect mitochondria from peroxidation damage, inhibit TG overaccumulation, and recover normal metabolism.

Oleic acid can increase lipid, TG and TC accumulation and induce mitochondrial dysfunction, which increase steatosis in cells. NPLP and NPLH reduced these symptoms, exerting inhibitory effects on the overaccumulation of TG in hepatic cells. In addition, NPLP and NPLH effectively weaken intracellular oxidative stress at high concentrations, while ineffectively increasing TG overaccumulation (*26, 27*). NPLP and NPLHs are a mixture of HGF, HSS and various free amino acids, which confer resistance to hepatic injury and oxidative stress by improving mitochondrial functions. Therefore, the data directed towards counteracting oxidative stress might contribute for NPLP and NPLH improving symptoms of NAFLD effectively.

However, the lipid-decreasing mechanisms of NPLH-trypsin require further investigation in the future. We aim to focus on the cellular energy regulation in NAFLD and signalling pathways on the relative lipogenic enzymes. Experiments using model animals are needed to confirm the present findings, especially the lipid-decreasing effects of NPLH-trypsin in the alimentary tract and liver.

CONCLUSIONS

The results of the study clearly suggested that newborn porcine liver proteins and hydrolysates have the potential to scavenge free radicals and prevent hepatic steatosis. The triglyceride inhibitory capabilities were different in the protein and their hydrolysates. Generally, the proteins and all of the hydrolysates showed good inhibitory activity on triglyceride and cholesterol accumulation, and NPLH-trypsin was the most effective lipid accumulation inhibitor with a maximum TG reduction of 43.3 %.

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Therefore, newborn porcine liver proteins and their hydrolysates exert good protective effects to reduce the risk of non-alcoholic fatty liver disease.

CONFLICT OF INTEREST

The authors report no conflicts of interest.

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AUTHORS' CONTRIBUTION

X. Zhang was responsible for the design of this project and article published. R. Zhang contributed to project experiments and acquisition of data or analysis. L. Yin and J. Chen drafted and revised the article.

SUPPLEMENTARY MATERIALS

All supplementary materials are available at: www.ftb.com.hr.

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Table 1. Analysis of variance (ANOVA) of the liver proteins from newborn piglets hydrolysed with trypsin

Items	Sum of squares	df	Mean square	F value	P
Model	475.9101	9	52.8789	43.66345	< 0.0001
Temperature/°C (A)	41.67845	1	41.67845	34.41495	0.0006
t/h (B)	7.860613	1	7.860613	6.490707	0.0382
pH (C)	23.83951	1	23.83951	19.68489	0.0030
AB	0.6561	1	0.6561	0.541758	0.4856
AC	0.3364	1	0.3364	0.277774	0.6144
BC	5.784025	1	5.784025	4.776016	0.0651
A^2	238.5346	1	238.5346	196.9641	< 0.0001
B^2	58.7142	1	58.7142	48.48181	0.0002
C^2	62.71078	1	62.71078	51.78188	0.0002
Residual	8.477395	7	1.211056		
Lack of fit	6.328275	3	2.109425	3.926119	0.1098
Pure error	2.14912	4	0.53728		
Cor total	484.3875	16			

Significant, $p<0.05$; Very significant, $p<0.01$

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Table 2. Molecular mass distributions of NPLP, NPLH-pepsin and NPLH-trypsin

Range of *M*/Da	Peak area/(%)		
	NPLH-pepsin	NPLH-trypsin	NPLP
>10000	3.953	1.162	13.352
10000-5000	4.945	1.503	7.343
5000-3000	11.379	12.26	13.479
3000-1000	42.292	46.93	39.29
<1000	37.02	38.14	26.22

NPLP= liver proteins from newborn piglets, NPLH-pepsin=pepsin-digested NPLP hydrolysate, NPLH-trypsin=trypsin-digested NPLP hydrolysate

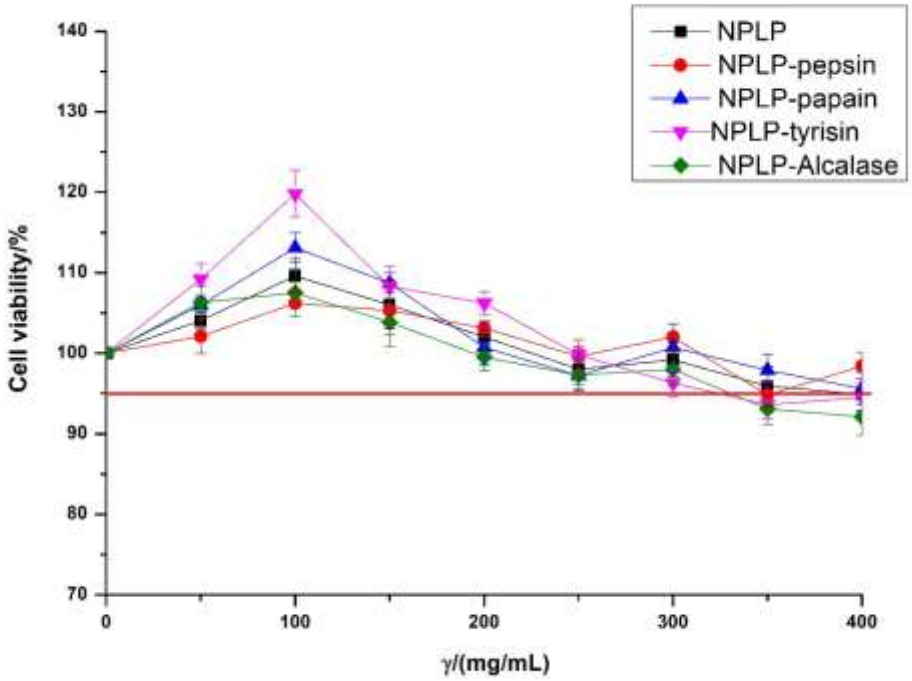

Fig. 1a

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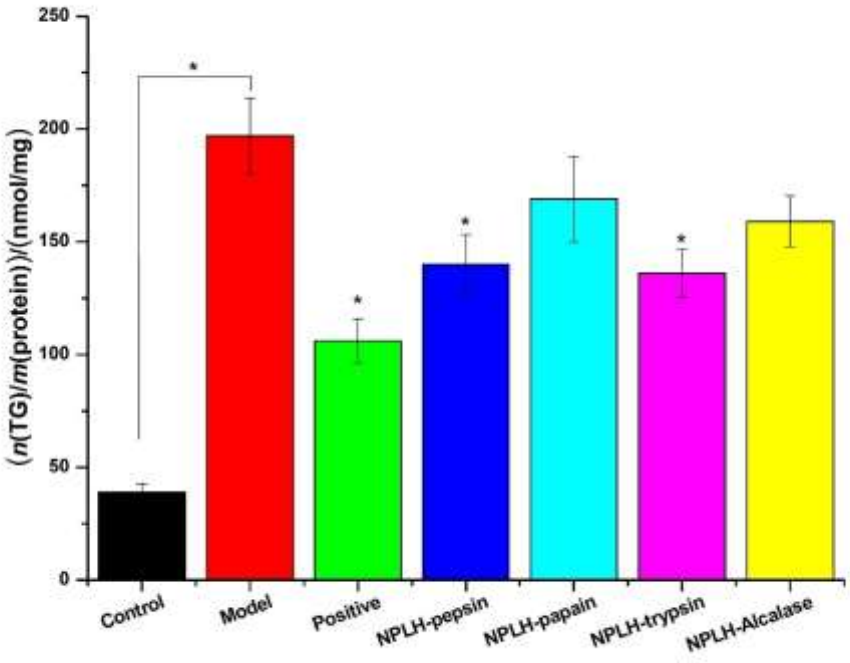

Fig. 1b

Fig. 1. Cytotoxic effect of (a) NPLP, NPLH-pepsin, NPLH-papain, NPLH-trypsin and NPLH-Alcalase on HepG2 cells. Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay and is expressed as a percentage of control cells with sample-free DMEM medium. (b) Effect of exposure to 0.75 mmol/L OA on intracellular TG accumulation. Inhibitory effect of NPLP, NPLH-pepsin, NPLH-papain, NPLH-trypsin and NPLH-Alcalase at 300 μg/mL on TG overaccumulation in HepG2 cells. ##$p < 0.01$ relative to the control(1 % BSA without oleic acid); *$p < 0.05$ and **$p < 0.01$ relative to the model (0.75 mM oleic acid induced cell). All tests were performed in triplicate, and values are presented as mean values ± S.D. NPLP=newborn porcine liver proteins, NPLH-pepsin=pepsin-digested NPLP hydrolysate, NPLH-trypsin=trypsin-digested NPLP hydrolysate, NPLH-Alcalase=Alcalase-digested NPLP hydrolysate

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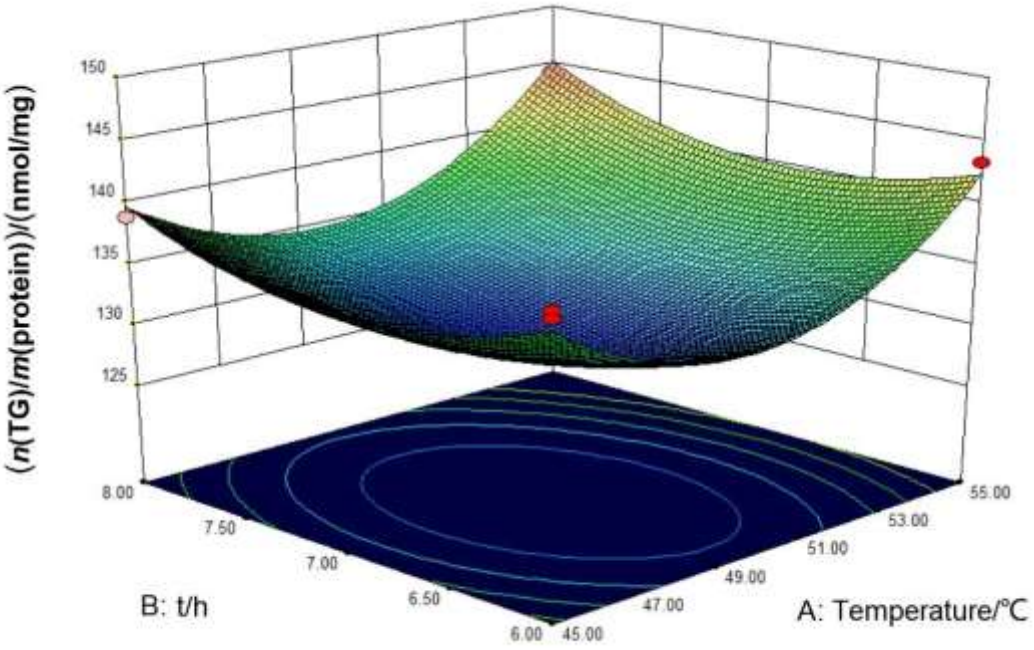

Fig. 2a

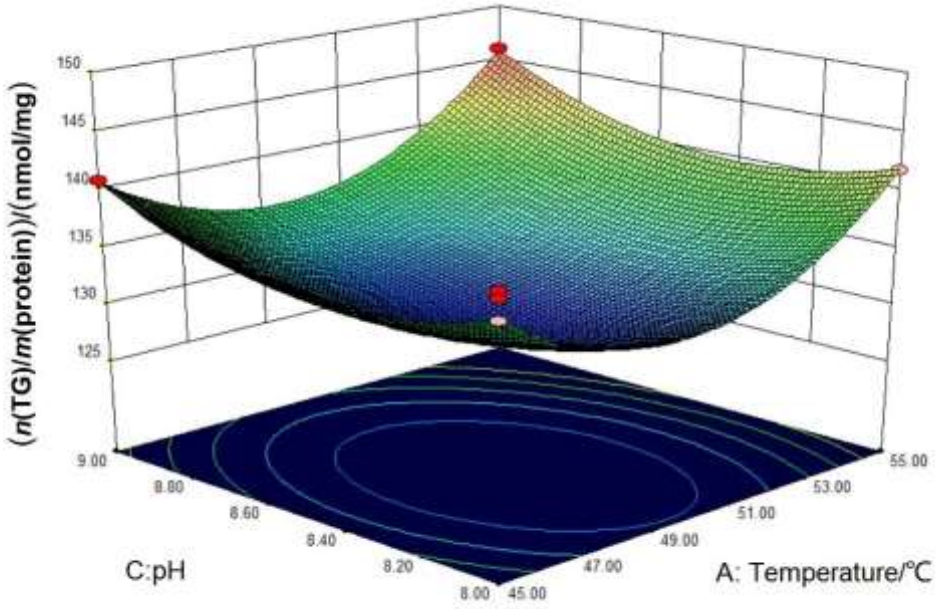

Fig. 2b

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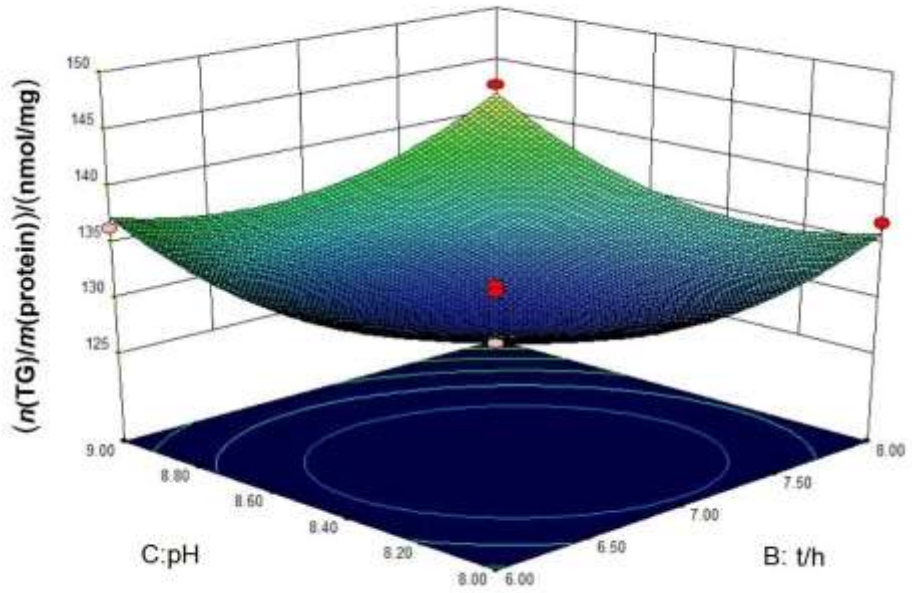

Fig. 2c

Fig. 2. Effects of cross-interaction among three parameters on degree of hydrolysis for trypsin: a) pH values and temperatures, b) pH values and enzyme-to-substrate ratio, and c) temperature and enzyme-to-substrate ratio

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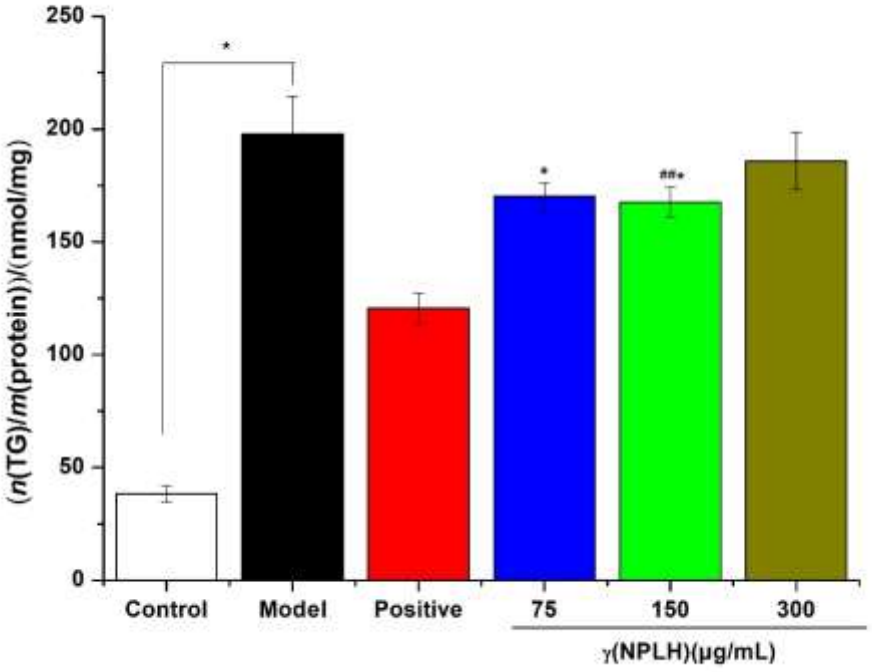

Fig. 3a

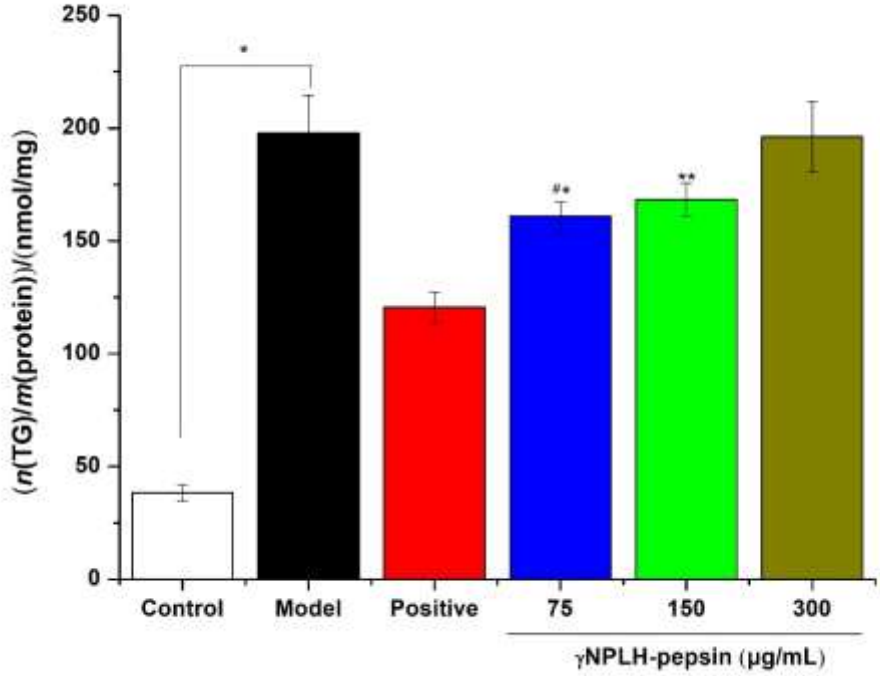

Fig. 3b

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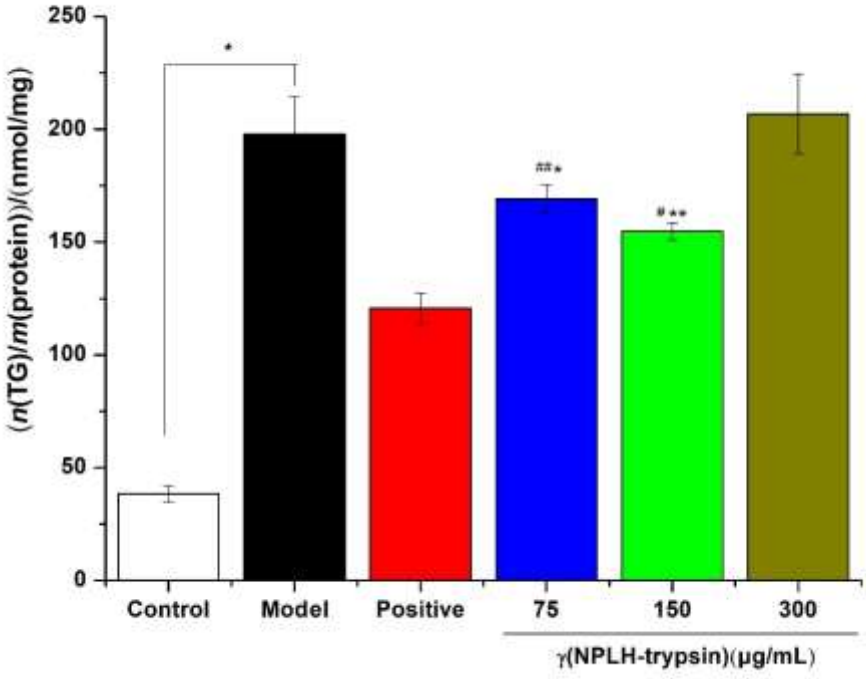

Fig. 3c

Fig. 3. Effect of exposure to 0.75 mmol/L OA on intracellular TG accumulation. Inhibitory effect of: a) NPLP, b) NPLH-pepsin and c) NPLH-trypsin at 75, 150 and 300 μg/mL on TG overaccumulation in HepG2 cells; ##p<0.01 relative to the control sample; *p < 0.05 and **p<0.01 relative to the model sample. Abbreviations as in Fig. 1.

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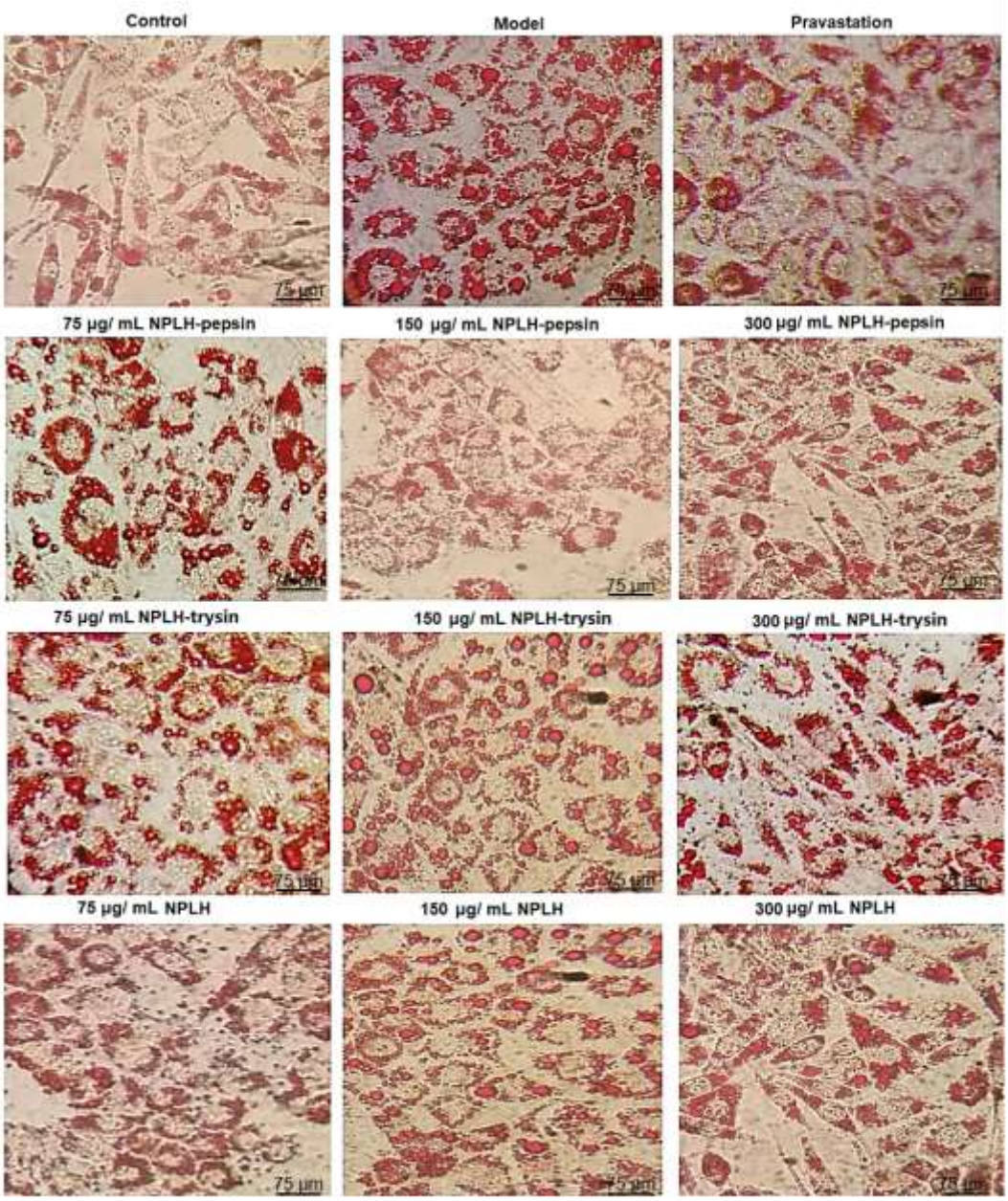

Fig. 4. Oil red O staining of TG overaccumulation in HepG2 cells. Abbreviations as in Fig. 1. Oil red O staining of corresponding fractions (NPLP, NPLH-pepsin and NPLH-trypsin) at 75 μg/ mL,150 μg/mL, and 300 μg/mL. Original magnification was 200×, scale bars represent 75 μm.

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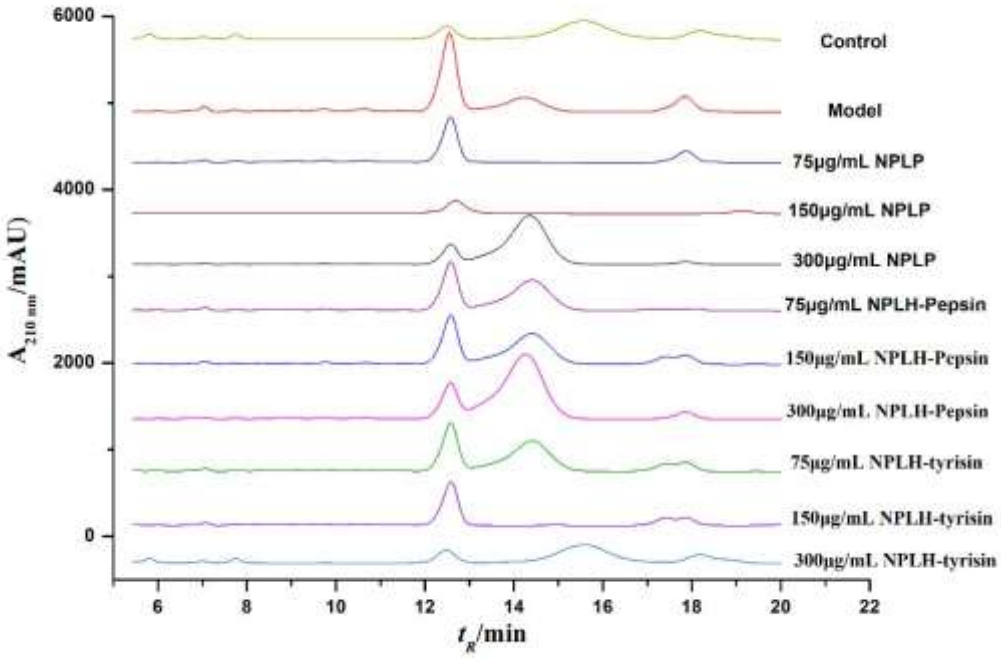

Fig. 5. HPLC analysis of the effect of exposure to 0.75 mmol/L OA on intracellular cholesterol accumulation and treatment with NPLP, NPLH-pepsin and NPLH-trypsin. Pure cholesterol was used as standard showed an obvious peak appear at approximate 12.5 min demonstrated at 210 nm. Abbreviations as in Fig. 1

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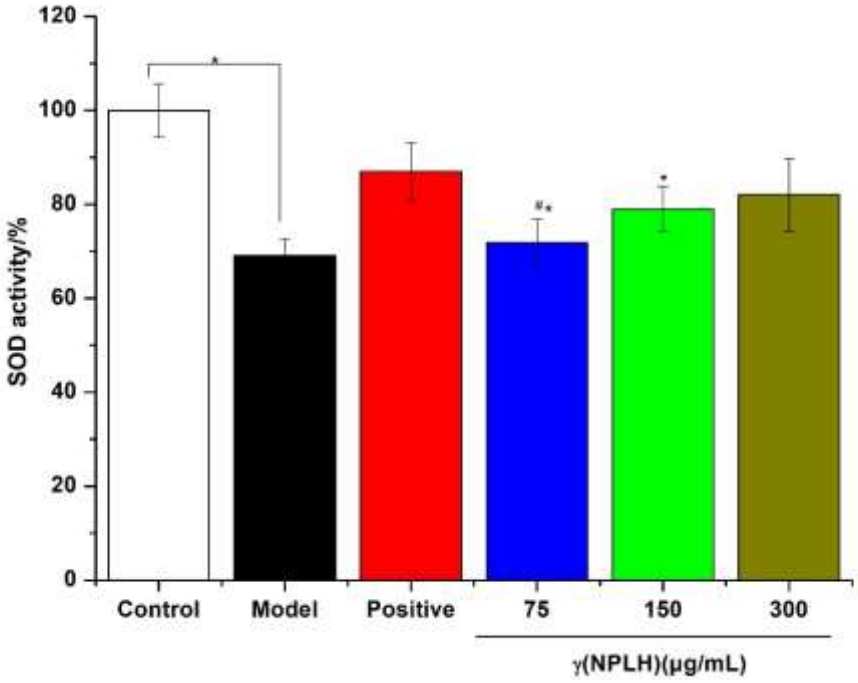

Fig. 6a

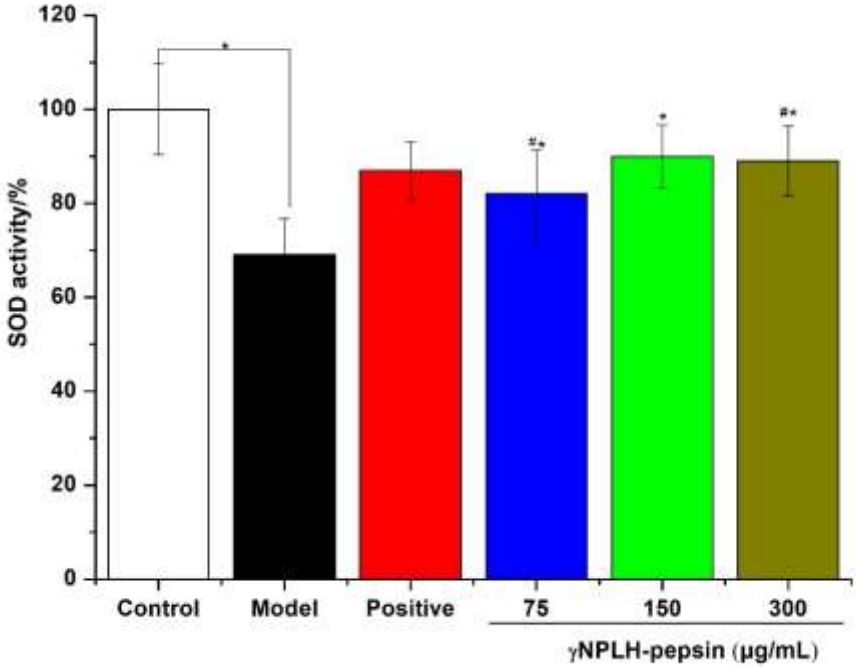

Fig. 6b

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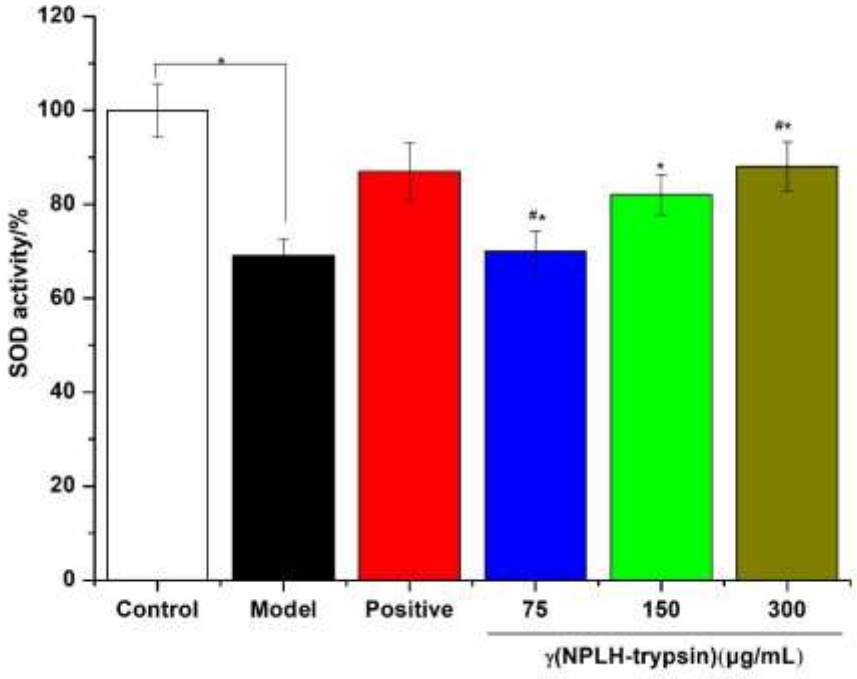

Fig. 6c

Fig. 6. Effect of exposure to 0.75 mmol/L OA on the intracellular antioxidant enzyme superoxide dismutase (SOD) in fatty cells and inhibitory effect of: a) NPLP, b) NPLH-pepsin and c) NPLH-trypsin on SOD activities in HepG2 cells; ##$p<0.01$ relative to the control sample; *$p<0.05$ and **$p<0.01$ relative to the model sample. Abbreviations as in Fig. 1

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Table S1. Response surface design of variance for newborn porcine liver proteins hydrolysed with trypsin

Group	Temperature/°C (A)	*t*/h (B)	pH (C)	(*n*(TG)/*m*(protein))/(nmol/mg)
1	45	7	9	140.86
2	45	7	8	137.84
3	50	7	8.5	129.4
4	50	7	8.5	131.3
5	55	7	8	141.8
6	45	6	8.5	139.5
7	55	6	8.5	143.28
8	45	8	8.5	138.9
9	55	7	9	145.98
10	55	8	8.5	144.3
11	50	8	8	136.85
12	50	7	8.5	130.52
13	50	6	9	136.4
14	50	6	8	135.5
15	50	8	9	142.56
16	50	7	8.5	129.75
17	50	7	8.5	130.2