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PROTEOMIC ANALYSIS OF LIVER ON HIGH FAT DIET MICE TREATED WITH *TOONA SINENSIS* (MELIACEAE) LEAF EXTRACT

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ABSTRACT

The major pathogenesis of metabolic syndrome is the development of insulin resistance, which promotes the elevation of blood pressure, dyslipidemia, and dysregulation of glucose metabolism. Liver is the principal regulator of glucose and lipid metabolism by controlling hepatic glucose production, glycogen storage and lipogenesis. *Toona sinensis* (TS) has been reported to be beneficial on health metabolic syndrome, however the effects of TS on liver metabolism is not clear. In this study, effects of TS on liver metabolism of high fat diet mice were investigated for the underlying mechanism. Mice were fed with high fat diet for 8 weeks followed by treatment of TSL-E (100 mg/kg body weight) for 4 weeks. Two-dimensional gel electrophoresis followed by mass spectrometry was carried out to identify differential expression of liver proteins. In addition, western blot were performed for validation of proteomic analysis. Up-regulated expression of genes involved in β-oxidation (PPARα and ACO) was observed in liver of TSL-E treated mice. In contract, down-regulated expression of genes was involved in lipogenesis (ACC), lipids transport (L-FABP) and decreased expression of proteins was involved in gluconeogenesis (Transaldolase, Enolase, PCK2 and Nudix). In conclusion, TS mediates its anti-metabolic syndrome potential through improvement of dyslipidemia and blood glucose by promoting β-oxidation and inhibiting gluconeogenesis and lipogenesis.

Keywords: Toona sinensis, β-oxidation, gluconeogenesis, lipogenesis, glycogen synthesis, 2D electrophoresis, PPARα/γ, liver, hepatocytes.

1. INTRODUCTION

Toona sinensis is well known as antioxidant sources. It has long been used as a traditional Chinese medicine for a wide variety of conditions, including rheumatoid arthritis, cervicitis, urethritis, tympanitis, gastric ulcers, enteritis, dysentery, itchiness, and cancer. While the underlying pharmacological mechanisms of TS remain unclear, various biological activities of TS leaf extracts have been reported. Recent scientific investigations demonstrated that aqueous

leaf extracts of TS possess a variety of biological activities, including antioxidant, anticancer, anti-inflammation, ant diabetes. In the present study, effects of TS on liver metabolism of high fat diet mice were investigated for the underlying mechanism. Proteomic analysis provides a better understanding of dynamic and overall views of the cell machinery under various conditions. Thus, the differentially expressed proteins in liver of mice fed with TSL-E were identified by proteomic analysis and confirmed by Western blot.

2. MATERIALS AND METHODS

2.1. Reagents and Materials

The 2-DE reagent including acrylamide solution (25 %) thiourea, immobiline drystrips, immobilized pH gradients (IPG) buffer (pH = 3–10), IPG cover mineral oil, iodoacetamide (IAA), TEMED, trifluoroacetic acid (TFA), 2-DE clean-up kit, 2-DE Quantkit, and silver staining kit were purchased from GEhealthcare (Piscataway, NJ, USA), 3-[(3-cholamidoropyl) dimethylammonio]-1propane sulfonate (CHAPS), dithiothreitol (DTT), EDTA, NaF, NaCl, NP-40, PMSF, sodiumdodecyl sulfate (SDS), tween 20, urea, Na_3VO_4, methanol (HPLC grade,> 99.9 %), and sodium deoxycholate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Protein marker and polyvinylidene fluoride (PVDF) membrane were purchased from Invitrogen (Carlsbad, CA, USA). Trisbase and trichloroacetic acid (TCA) were purchased from J. T. Baker (Phillipsburg, NJ, USA). The primary and secondary antibodies for Western blotting were purchased from Santa Cruz (Santa Cruz, CA, USA).

2.2. Animals and Treatments

The C57/B6 mice were purchased from the laboratory animal center of National Cheng Kung University, Tainan, Taiwan. All mice were housed alone in standard cages for one week at least before the experiments began. Animals were housed in a well-ventilated room maintained at 22 ± 2 °C, and 12 h light-dark cycle. After one week of adaptation, obesity animals were induced by feeding with high fat diet for next 8 weeks. The high fat diet consisted of food with 60 % of the calories coming from fat (5.24 kcal/g, 60 % kcal from lard/soybean 9.8:1, D12492; Research Diets, New Brunswick NJ). Animals were randomly divided into five groups (n=8) for the study: Control: mice were fed with normal diet; HFD: mice were fed with the HFD; PIO: mice were fed with the HFD plus pioglitazones; TSL-E: mice were fed with HFD plus TSL-E and PIO+TSL-E: mice were fed with the HFD plus pioglitazone and TSL-E. All animals were fed with 5 g of food chow (LabDiet, Cat. No. 5001) mixed with or without the TSL extracts for 8 weeks. Additional normal food chow and sterilized distilled water were supplied ad libitum while the TSL extract contained food chow was consumed completely. Animals were sacrificed with CO_2 at the end-point of this experiment, liver were isolated immediately and stored at -30 to -80 ^{0}C for further analysis.

Body weight changes were measured each week from the start point of the experiments to the end point of experiments. The levels of serum cholesterol (Cho), triglycerides (TG) were determined using an automatic blood chemistry analyzer Dry-Chem 4000i (Fujifilm, Saitama, Japan). Blood glucose concentrations were determined by a blood glucose meter (Accu-Check Advantage, Roche).

2.3. Preparation of TSL extracts

The lyophilized extracts of TSL (1 g) purchased from the Taiwan Toona Biotech Corp (Kaohsiung, Taiwan) were dissolved in 10 mL of ethanol (95 %) and shaken at room temperature for 12 h. Crude extracts were collected from the supernatant and then concentrated by using a vacuum freeze-dryer. The TSL-E powder was collected and stored in the dark at room temperature for the following assays.

2.4. Cell culture

FL83B hepatocyte is a gift of Professor Lee-Yan-Sheen in National Taiwan University. The cells were incubated in F12K medium containing FBS 10 % and penicillin and streptomycin 1 % in 10 cm petri dishes at 37 ^{0}C and CO_2 5 %. Experiments were performed on cells that were 80–90 % confluent.

2.5. Protein extraction and qualification

Cells were washed by PBS and collected scraping in lysis buffer (Sucrose 0,32M, KH_2PO_4 10 mM, Na_3VO_4 1 mM; $PMSF_2$ mM; EDTA 1 mM; NaF 1 mM, pH 7.4). After incubation on ice for 30 minutes, lysates were collected by centrifugation at 10,000 rpm for 30 minutes at 4 ^{0}C. Protein concentration was estimated by Bradford method. The protein assay dye reagent concentrate (BioRad) was diluted 5 fold with distilled water. BSA protein standard was diluted to 800, 400, 200, 100, 50, 25, 0 µg/mL with 50 mM phosphate buffer saline (PBS). The concentrations PBS diluted protein samples were controlled in the standard curve range. Diluted protein samples 10 µl and BSA protein standard were added to protein assay dye reagent, transferred to the ELISA plate. The OD 595 nm was read by Tecan Sunrise machine.

2.6. Electrophoresis and blotting

Equal amounts 20 µg of total proteins were electrophorylated on SDS-PAGE 10 % and transferred to polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with nonfat dried milk 5 % in TBST buffer (tris-HCl 20 mM, pH 7.4, NaCl 150 mM, tween 20 0.1 %) for 1 hour then hybridized with specific primary antibody overnight at 4 ^{0}C. Subsequently, the membrane was washed with TBST buffer and incubated with secondary antibodies for 1 hour. Protein bands were visualized by enhanced chemiluminescence kits (ECL Plus, Amersham). The band quantification was performed using LAS-3000 (Fujifilm, Tokyo, Japan) and Multi Gauge software v 3.0 (Fujifilm).

2.7. Two-dimensions electrophoresis

For preparation of total protein extract, frozen liver tissue 100 mg were homogenized in lysis buffer 0.5 ml consisting of urea 7 M, thiourea 2 M, and CHAPS 4 %,Tris–HCl 25 mM, DTT 50 mM, and protease inhibitor cocktail 1 % v/v (Sigma–Aldrich) using homogenizer. Samples were centrifuged at 13,000 rpm at 4 ^{0}C for 20 minutes. The supernatants containing the total liver proteins were removed the contaminants by using a 2 DE Clean-Up Kit (GE Healthcare, United Kingdom).The protein concentrations of the extracts were determined by Protein Assay Kit (Bio-Rad Laboratories). 2-DE was performed in triplicate for three mice in each experimental group. First, using the protocol recommended by the manufacturer, IPG IEF of samples was carried out on pH 3 ~ 10, 13 cm IPG DryStrips. IPG strips were passively rehydrated overnight in strip holders in rehydration solution 250 µL containing 50 µg of the liver protein sample

2.8. Measurement of MMP Level

FL83B cells were collected in PBS. A Beckman-Coulter Epics XL-MCL Analyzer (Beckman-Coulter, Hialeah, FL) equipped with a single 488 nm excitation source was used for all flow cytometric analyses. $DiOC_6$, a lipophilic, cationic, fluorescent probe, can bind to

mitochondrial inner membrane and be used for quantitative measurement of MMP. Fl83B cells were incubated with 4 nmol/L $DiOC_6$ at 37 ^{0}C for 15 min. The level of MMP was measured as an increase in FL1 on a log scale for 5000–10,000 events.

2. RESULTS

3.1. Effect of TSL-E on body weight, triglyceride content, serum cholesterol and serum glucose in HFD mice

At the end of the treatment period, the HFD mice were overweight compared with the control. The HFD+TSL-E mice, on the other hand, were lighter than the HFD animals, reaching a body weight not different from that of the control animals (Figure 1). Serum glucose level was higher in HFD mice than in control mice, whereas in HFD+TSL-E animals it was reduced at the end of the treatment period (Figure 2A), respectively. However, HFD mice exhibited significantly elevated levels of the plasma triglyceride and serum cholesterol and HFD+TSL-E was not significantly different from that of HFD mice (Figure 2B, 2C).

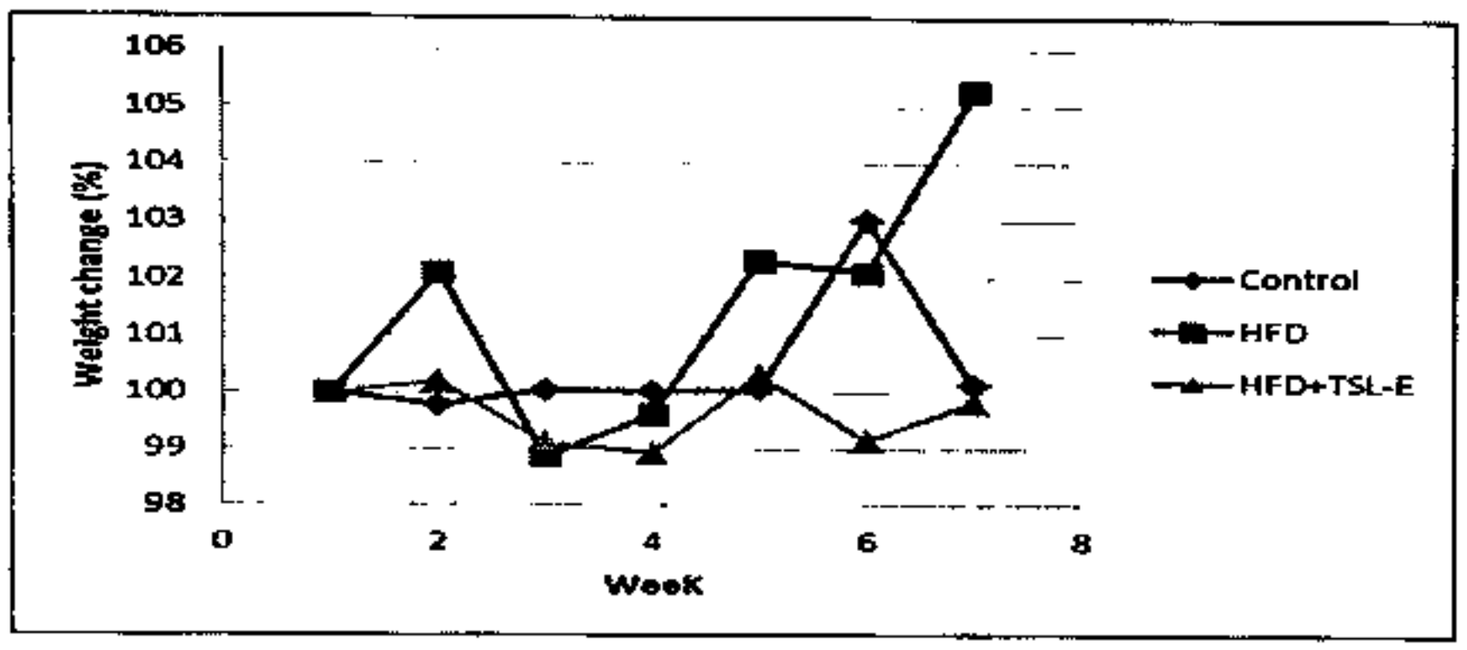


Figure 1. Effect of TSL-E on body weight of HFD mice.

C57BL/6 mice were fed with HFD for 8 weeks to induce obese then treated with TSL-E for 6 weeks. The body weight was determined each week. Body weight was set 100 % as the body weight at the first week. At the end of treatment period, body weight of HFD mice were raised significantly, up to 5 % compared with the control. TSL-E feeding HFD mice restored the body weight to normal level of control mice.

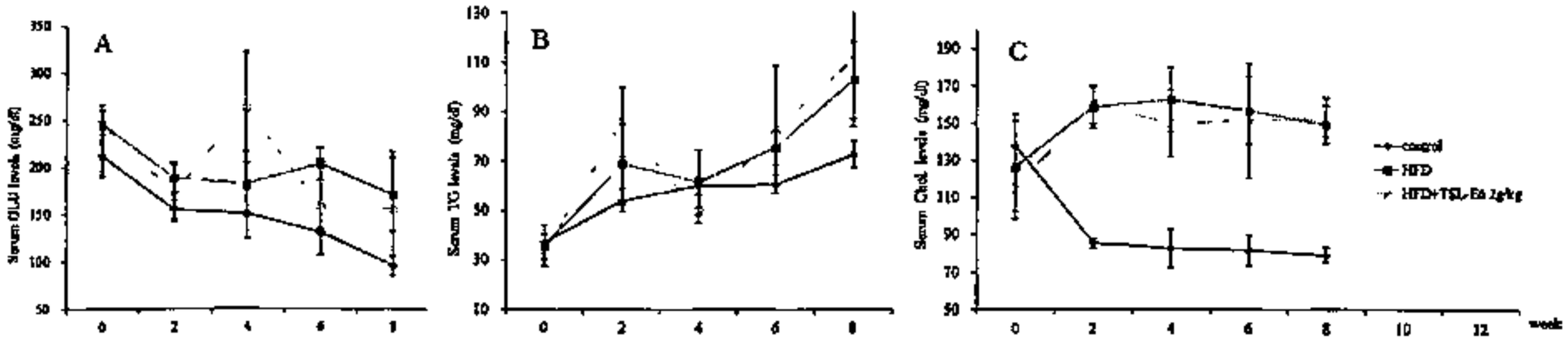


Figure 2. Effect of TSL-E treatment on serum glucose, triglyceride and cholesterol level of HFD mice.

C57BL/6 mice were fed with HFD for 8 weeks to induce obese then treated with TSL-E for 6 weeks. The serum glucose (GLU) (A), serum triglyceride (TG) (B) and serum cholesterol

(Chol) (C) were determined each week. HFD mice increased triglyceride, cholesterol and glucose level compared with control mice.

3.2. Proteomic profiling of TSL-E regulated protein on liver of HFD mice

To gain insight into TSL-E effects on liver metabolism of HFD mice, we subjected liver of HFD; HFD treated with TSL-E and control mice to 2D-E and MS proteomics because liver is the major site for glucose and lipid metabolism. TSL-E groups showed the highest number of protein spots, respectively (1195 spots), while control and HFD groups just showed 876 and 815 protein spots (Figure 3). Compared control groups with TSL-E groups there were 179 spots were up regulation and 185 spots were down regulated. Similarly, compared TSL-E groups with the HFD groups, 174 spots were up regulated and 190 spots were down regulated. Compared with control groups, HFD groups showed the highest spots up regulation (186 spots) but lowest spots down regulation (178 spots). Differentially expressed proteins in livers of control, HFD, and HFD+TSL-E mice were identified by mass spectrometry and reported in Table 1. Additional experimental details for mass spectrometry identifications are reported in Table 2.

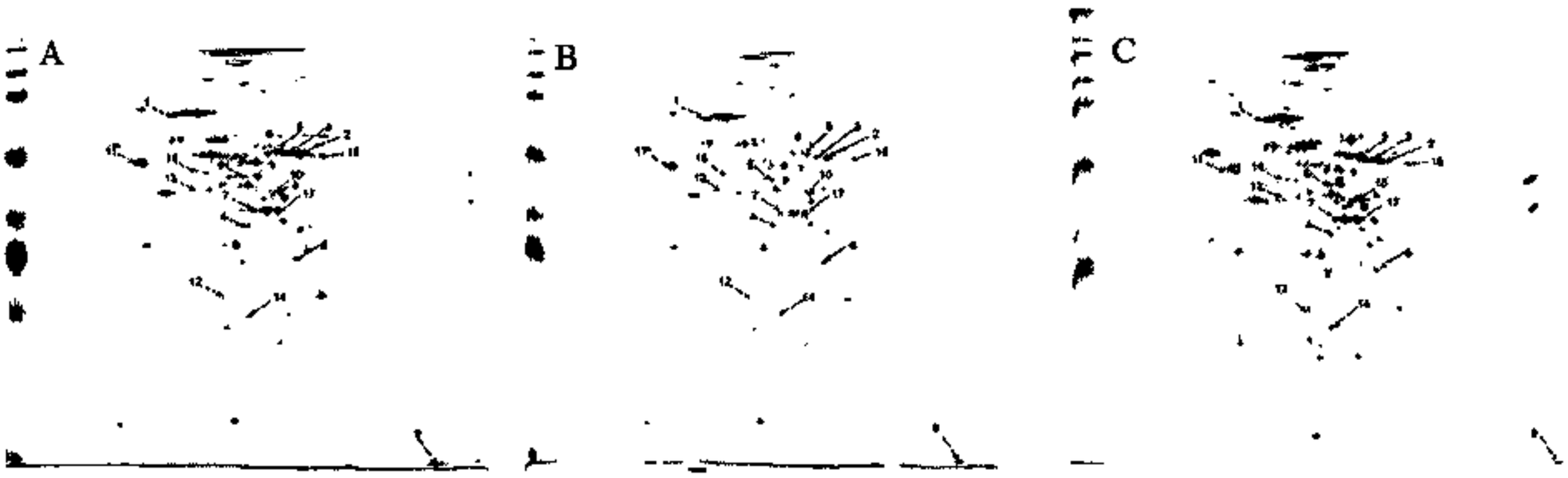
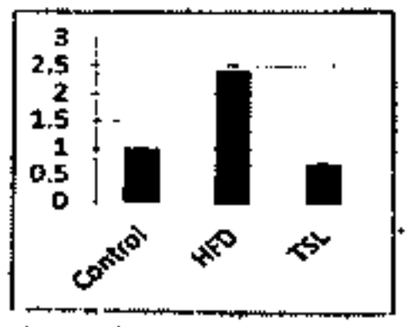


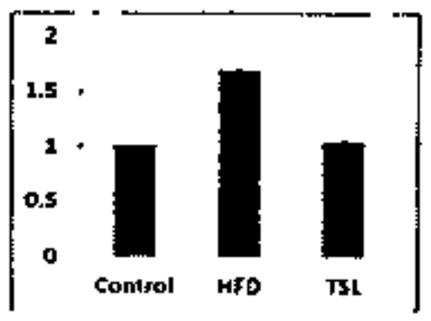
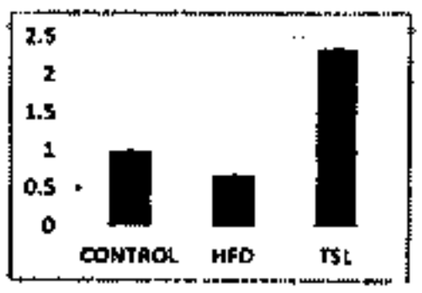
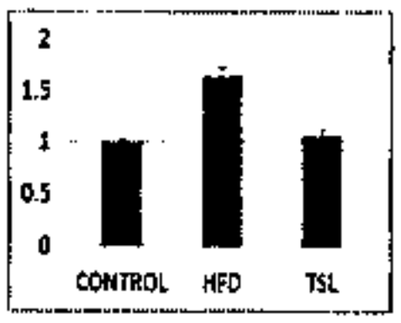
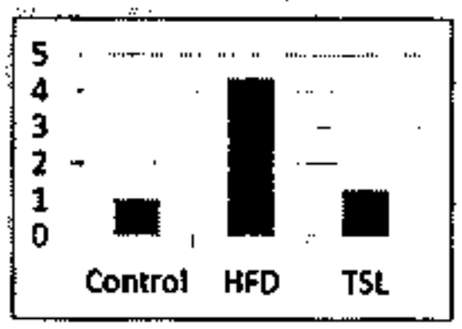
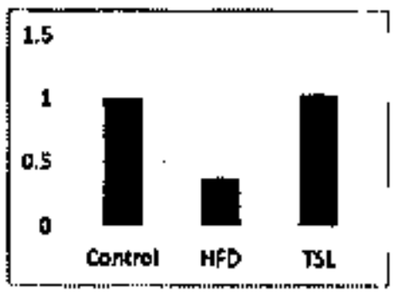
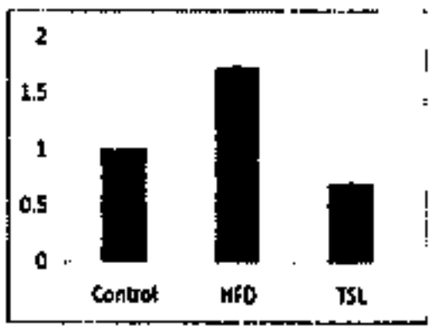
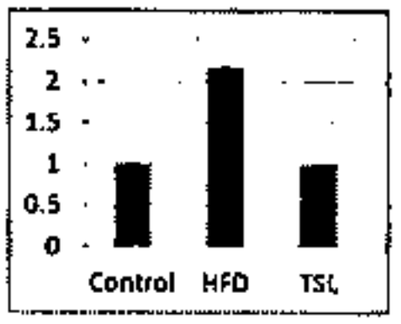
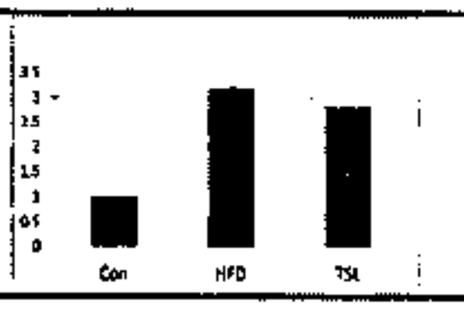
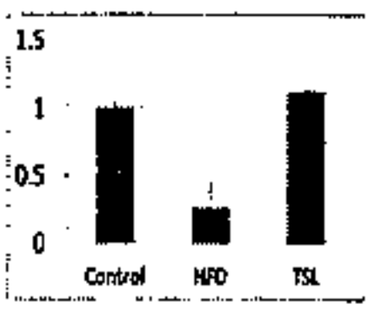
Figure 3. The 2D-E images obtained from the liver of control, HFD mice and HFD + TSL-E groups.

Representative 2D-E images obtained from liver protein of mice in control group (A), HFD group (B) and HFD+TSL-E(C). 2D-E was performed using a nonlinear pH range of 3–10 in the first dimension (13cm strips) and SDS-PAGE (12 %) in the second. Protein loading was 60 μg, and the gels were stained using silver. Identified protein spots among those with a density that differed significantly between experimental groups ($P < 0.05$) are marked and numbered.

Table 1. Mass spectrometry identified of the significant difference protein in liver.

No spot	Swissprot acession	Protein name	P value (Between groups)	Relative expression
1	gi|6678499	UDP-glucose 6-dehydrogenase	P<0.001	

Proteomic analysis of liver on high fat diet mice treated with toona sinensis (meliaceae) leaf extract.

2	gi|387144	Muscle-specific enolase beta subunit	0.026	
3	gi|48686112	Transaldolase 1	0.098	
4	gi|1009706	Sorbitol dehydrogenase precursor	0.085	
5	gi|21706762	Nudix (nucleoside diphosphate linked moiety X)-type motif 9 [Mus musculus]	0.017	
6	gi3452686	Protein kinase C lambda [Mus musculus]	0.025	
7	gi|31982522	Short-chain specific acyl-CoA dehydrogenase	P<0.001	
8	gi|13097375	Electron transferring flavoprotein, alpha polypeptide	0.009	
9	gi|8393343	Fatty acid-binding protein	0.003	
10	gi|191804	Aldehyde dehydrogenase II	0.023	

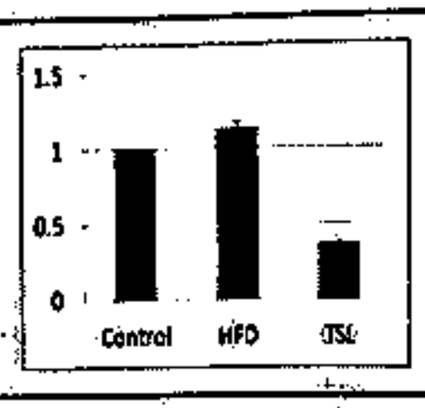
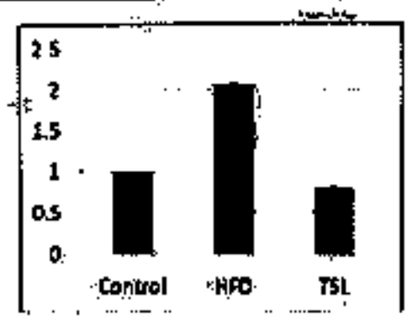
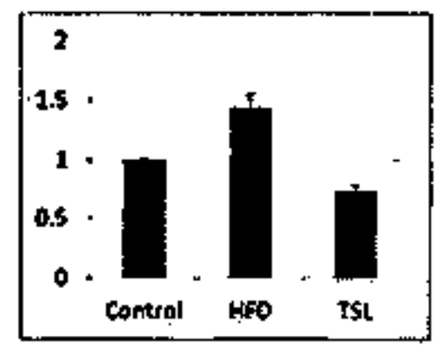
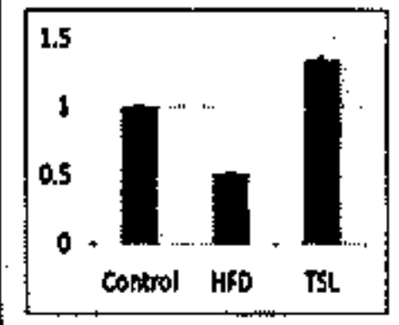
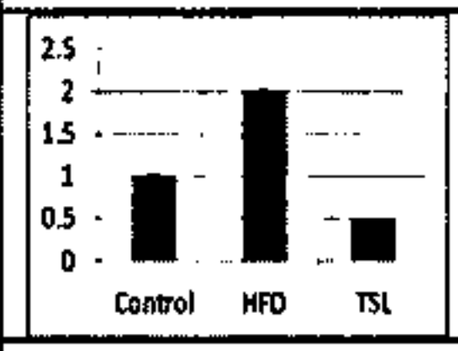
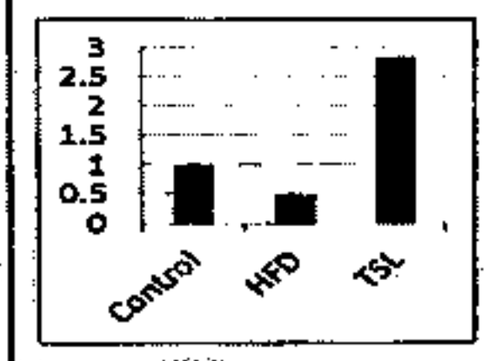
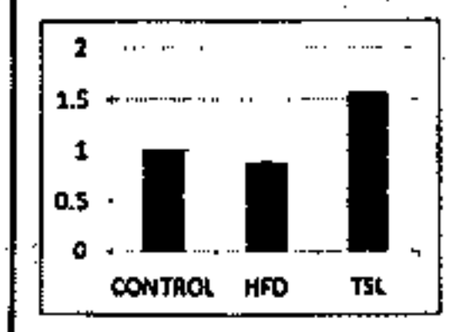
11	gi|17391295	Hspd1 protein	0.005	
12	gi|55925018	Catalase	0.001	
13	gi|31560686	Heat shock-related 70 kDa protein 2	0.036	
14	gi|6671549	Peroxiredoxin-6 [Mus musculus]	0.048	
15	gi|148692928	Glutamate dehydrogenase 1	P<0.001	
16	gi|1841443	Glutamine synthase	0.002	
17	gi|19526790	S-adenosylmethionine synthase isoform type-1	0.019	

Table 2. Mascot - identified differentially expressed protein in liver of mice.

No	Protein name	Mass	Score	Matches	Sequences	emPAI	pI
1	Heat shock-related 70 kDa protein 2	69599	778	40(22)	9(5)	0.52	5.51
2	UDP-glucose 6-dehydrogenase	54797	104	3(2)	3(2)	0.14	7.49
3	Catalase	4375	90	3(0)	2(0)	1.04	9.42
4	Transaldolase 1	19251	28	1(0)	1(0)	0.20	9.18

5	Glutamate dehydrogenase 1	54185	255	29(10)	9(4)	0.95	7.66
6	Muscle-specific enolase β subunit	40680	112	1(1)	1(1)	0.09	6.45
7	Short-chain specific acyl-CoA dehydrogenase	44861	95	2(1)	2(1)	0.08	8.68
8	Electron transfer flavor protein	35018	746	27(19)	8(4)	1.27	8.62
9	Fatty acid-binding protein	14236	294	13(9)	7(4)	8.11	8.59
10	Protein kinase C lambda	67156	35	1(0)	1(0)	0.06	5.53
11	Glutamine Synthase	42162	141	6(3)	5(2)	0.29	6.57
12	Hspd1 protein	26998	137	10 (4)	3(2)	0.69	4.76
13	Nudix (nucleoside diphosphate linked moiety X)-type motif 9	38599	29	1(1)	1(1)	0.1	6.39
14	Peroxiredoxin-6	24811	153	12(8)	6(3)	1.63	5.98
15	Aldehyde dehydrogenase II	54447	109	7(3)	4(2)	0.22	7.89
16	S-adenosylmethionine synthase isoform type-1	43481	297	9(6)	3(1)	0.09	5.51
17	Sorbitol dehydrogenase precursor	40066	151	19(5)	7(3)	0.43	6.60

Mass spectrometry identified the differentially expressed proteins in livers of control, HFD, and HFD+TSL-E mice. Accession number, protein name and cellular role are reported according to Swiss-Prot database. Differential expression ($P < 0.05$) were identified by anova-test. Values were calculated by comparing mean relative intensity (RI) values between spots within the same experiment (representative analysis set, n = 4). Mascot identified differentially expressed proteins in livers of control, HFD, and HFD+TSL-E mice. Protein name, the predicted molecular mass, pI are reported according to Mascot search result.

When our interest was limited to differential expressions of at least 2-fold and a statistical significance of at least 95 % ($P < 0.05$), the counts were 17 protein spots, respectively. These proteins can be separated into 4 groups: anti-oxidative stress, lipid metabolism, glucose homeostatic, nitrogen and amino acid metabolism. TSL-E showed the anti-oxidation effects by revealing the protein expressions of heat shock-related 70 kDa protein 2 (Hsp70), Hspd1, peroxiredoxin 6 (Prdx 6), CAT, nudix (nucleoside diphosphate linked moiety X)-type motif 9 (NUDT9) and aldehyde dehydrogenase (ALDH). Glucose metabolism related proteins included UDP-glucose 6-dehydrogenase (UGDH), muscle-specific enolase β subunit (MSE), sorbitol dehydrogenase precursor (SDH), transaldolase (TAL). TSL-E affected lipid metabolism by altering the protein expression of short-chain specific acyl-CoA dehydrogenase (SCAD), electron transfer flavor protein (ETF), fatty acid-binding protein (FABP). The expressions of s-adenosylmethionine synthase (SAM), glutamate dehydrogenase (GLDH) and glutamine synthase (GS) were alternated by TSL-E indicating that TSL-E also effect nitrogen metabolism in liver.

3.3. Effect of TSL-E on PPARα, PPARγ, PCK2 and HMG-CoA protein expression in liver of mice

PPARα and PPARγ are members of the PPAR family of nuclear transcription receptors and play a central role in glucose metabolism, lipid biosynthesis and insulin sensitivity. Phosphoenolpyruvate carboxykinase 2 (PCK2) is a key enzyme on gluconeogenesis pathway. 3-hydroxy-3-methylglutaryl coenzyme A synthase (HMG-CS) contains an important catalytic cysteine residue that acts as a nucleophile in the first step of reaction: the acetylation of enzyme

by acetyl-CoA to release the reduced coenzyme A. HMG-CS is an intermediate in both cholesterol synthesis and ketogenesis. To investigate the effects of TSL-E on glucose and lipid metabolism, the Western blot was used to identify the expression of PPARα, PPARα, PCK2 and HMG-CS in liver of mice.

As shown in Figure 4A and 4B, PPARα and PPARγ protein expression were up regulated in HFD mice. Interestingly, the PPARα and PPARγ expression were higher in TSL-E treatment compared with that of HFD mice. PIO, a full PPARγ agonist significantly increased the PPARγ expression. The PPARα expression in TSL-E treatment was higher than that in PIO treatment while the PPARγ expression in TSL-E was lower than that in PIO treatment. Combined TSL-E and PIO did not change the PPARγ expression but significantly increased PPARα expression. HFD and HFD+PIO elevated the PCK2 protein expression in liver (Figure 4C). TSL-E treatment decreased the expression of PCK2 compared with HFD and HFD+PIO, respectively. TSL-E combined PIO also decreased PCK2 expression compared with PIO only. HFD up regulated the HMG-CS expression (Figure 4D). The expression of HMG-CS in HFD fed TSL-E were elevated and up to a level higher than that in HFD mice. TSL-E combined PIO decreased HMG-CS protein expression in liver of mice compared with TSL-E only.

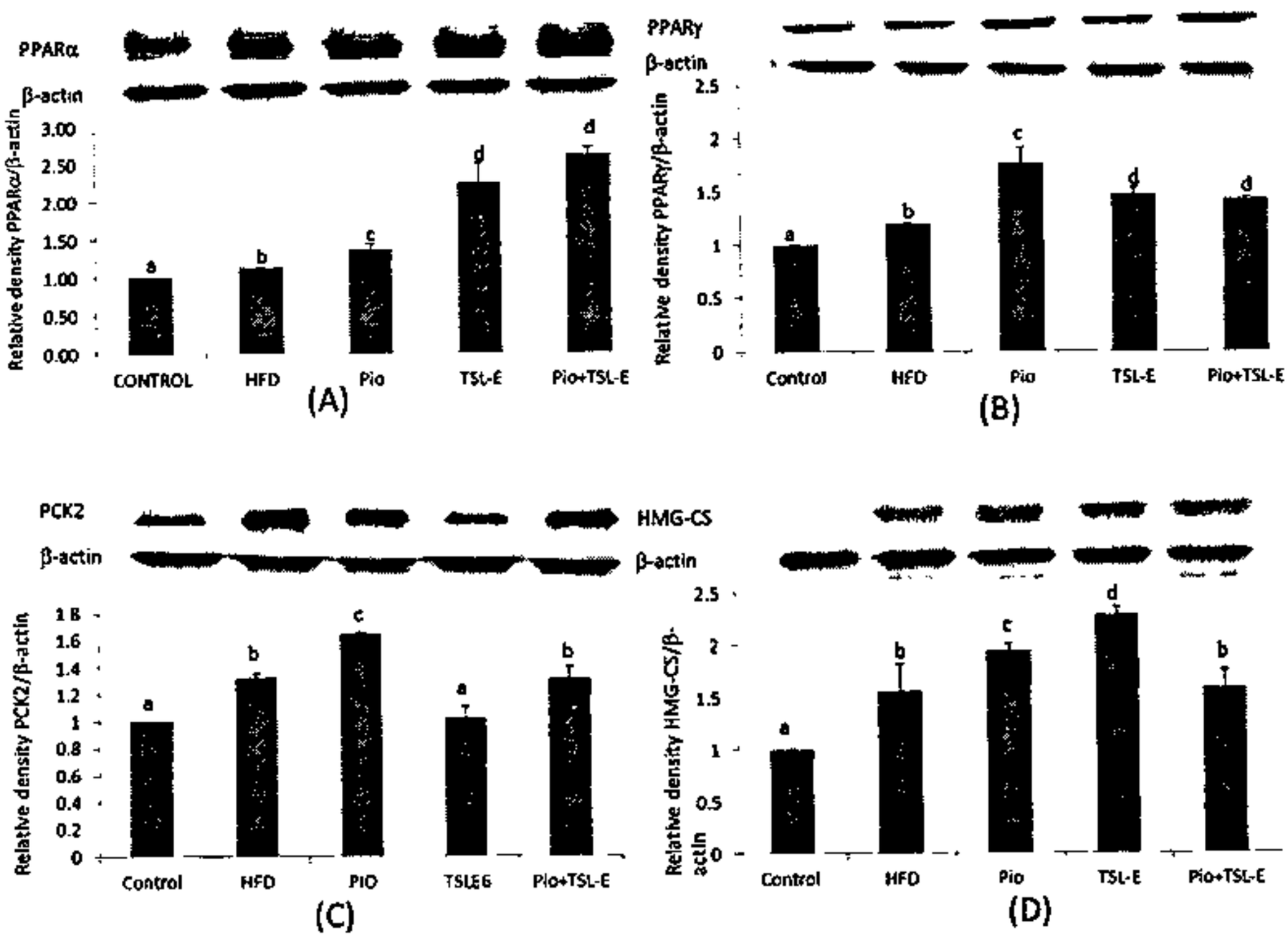


Figure 4. The protein expression of PPARα, PPARγ, PCK2 and HMG-CoA in liver.

Control: mice were fed with normal diet; HFD: mice were fed with the HFD; PIO: mice were fed with the HFD plus pioglitazones (5mg/kg body weight); TSL-E: mice were fed with HFD plus TSL-E (0.5g/kg body weight) and PIO+TSL-E: mice were fed with the high-fat diet plus pioglitazone and TSL-E. TSL-E increased the protein expression of PPARα and PPARγ. Data are expressed as means plus standard deviations of three similar experiments. The data are given as means ± SE. a, b, c, d: bars with superscripts without a common letter differ significantly (student *t* test, $P < 0.05$).

4. DISCUSSIONS

4.1. TSL-E improved liver metabolism by suppressed the elevated of gluconeogenesis and increased lipolysis in liver of HFD mice

4.1.1. TSL-E suppressed the elevated of gluconeogenesis in liver of HFD mice

HFD have been used as model for obesity, dyslipidaemia and IR in rodents for many decades [1]. Our model with HFD was pronounced obesity which increased body weight at the end of treatment period (Figure 1). We found that numerous enzymes associated with gluconeogenesis were increased in liver of HFD mice and normalized in HFD+TSL-E mice including UGDH, ETF and SDH. UGDH provides cursors for an array of extracellular matrix glycosaminoglycans such as heparin and hyaluronic acid, and also catalyzes the crucial structural modification of xenobiotic toxins required for their eventual eliminations [2]. Disturbed metabolism of glycosaminoglycans (GAGs) has been proposed to play an important role in the pathogenesis of diabetic complications [3]. Via UDP-glucose, glucose flux from gluconeogenesis was converted to glycosaminoglycans or glycogen. In the present study, HFD increased UGDH, and HFD+TSL-E restored UGDH to normal level. SDH is a member of the super family of medium chain dehydrogenases/reductases. SDH, the second enzyme in the polyol pathway, oxidizes sorbitol to fructose in the presence of NAD. Fructose is converted to glucose by the way of gluconeogenesis through condensation of triose- phosphate to fructose 1, 6 biphosphate. Recent studies have shown that flux via polyol pathway enzymes aldose reductase and sorbitol dehydrogenase is required to mediate hypertension complications of diabetes [4]. Oxidation of sorbitol to fructose by SDH causes oxidative stress because its co-factor NAD+ is converted to NADH and NADH is the substrate for NAD(P)H oxidase to generate ROS [5]. Caffeic acid (CA) and ellagic acid (EA) at 5 % mediated its anti-diabetes effects by decreased the levels of sorbitol dehydrogenase [6]. In this study, HFD increased SDH and HFD+TSL-E restored to normal level of SDH suggested that TSL-E might inhibit the activation of polyol pathway in HFD condition.

β-oxidation represents an important source of energy for the body during times of fasting and metabolic stress, providing carbon substrates for gluconeogenesis and contributing with electrons to the respiratory chain for energy production. Increased transfer of electrons from reducing equivalents generated by β-oxidation could contribute to increased mitochondrial ROS generation [7]. HFD mice increased EFT expression that contributed to increase oxidation and gluconeogenesis and HFD+TSL-E decreased EFT expression in liver. Moreover, HFD decreased TAL and HFD+TSL-E increased the expression of TAL. Our results indicated that TSL-E decreased gluconeogenesis via increased TAL expression in HFD mice. With respect to gluconeogenesis there was a large increase in a key steps, PCK2 have been confirmed by using Western blot. The PCK2 expression in liver of HFD mice was significantly increased (Figure 4) and HDF+TSL-E decreased the expression of PCK2 compared with HFD and HFD+PIO groups.

Hepatic gluconeogenesis contributes to elevation of fasting glucose. Hyperglycemia in type 2 diabetes is characterized by enhanced glucose production in the liver. In the presence of IR, enhanced glucose output by liver contributes to hyperglycemia. Inhibition of hepatic glucose production contributes to glycemic control in the diabetic patients by insulin sensitizers. Several reports have documented that inhibited or decreased gluconeogenesis has benefit in patients with type 2 diabetes and IR suggesting that TSL-E can be used as a therapy for type 2 diabetes patients.

4.1.2. TSL-E increased lipolysis in liver of HFD mice

The protein expression of FABP was increased in both HFD and HFD+TSL-E mice (Table 1). FABP transfer fatty acids between extra- and intracellular membranes. Expression of FABP is regulated by dietary long chain fatty acid (LCFA) as well as by stimulating mitochondrial and peroxisomal oxidation of LCFAs [8]. FABP physically interacts with PPARα and functionally interacts with both PPARα and PPARγ [9].

SCAD plays a pivotal role in energy metabolism providing fuel after prolonged starvation or during periods of increased energy requirements [10]. Although HFD+TSL-E decreased the protein expression of SCAD compared with HFD, the β-oxidation was up regulated in HFD+TSL-E feeding mice. Indeed, PPARα regulates mRNA expression of genes involved in FA oxidation, and synthetic PPARα agonists decrease circulating lipid levels and are commonly used to treat hyperlipidemia and other dyslipidemic states [11]. The PPARα expression in HFD+TSL-E was higher in HFD mice. Similarly, HFD+TSL-E significantly increased the expression of HMG-CSprotein in liver of mice. Due to FABP and HMG-CS are target genes of PPARα, the activation of PPARα leads to up regulation of FABP and HMG-CS. FABP enhances cellular LCFA uptake, enhances intracellular transport/diffusion through the cytoplasm and targets LCFA to peroxisomes for β-oxidation generate acetyl-CoA. Acetyl-CoA is the product of β-oxidation and is passed into of the tricarboxylic acid (TCA) cycle for the production of additional adenosine triphosphate (ATP) or is used for the production of ketones under the expression of HMG-CS.

IR in HFD has recorded increasing de novo lipogenesis [12]. However, de novo hepatic lipogenesis was normally contributed by a minor portion to total hepatic triglyceride synthesis. Increased plasma FFA levels may result in intracellular accumulation of lipid metabolites in the liver, leading to fatty liver, liver IR and type 2 diabetes [13]. Therefore, one potential strategy for improving metabolic syndrome is not only to reduce the level of plasma FFA, but also to oxidize instead of accumulates intracellular TG in the liver. TSL-E does not decrease lipogenesis but enhance lipolysis in liver of HFD suggesting that TSL-E may have benefit in metabolic syndrome.

4.2. TSL-E decreased oxidative stress in liver of HFD diet mice

Under the oxidative stress of HFD, the mice increased the expression of anti-oxidation protein included Hsp70, CAT, Prdx 6 and NUDT9.

CAT is enzyme that catalyzes the decomposition of hydrogen peroxide to water and oxygen, protecting cells. Feeding mice with HFD caused a significant increase in the activity of CAT. HFD increase oxidation of fatty acids through the peroxisomal oxidation pathway that is associated with increased generation of hydrogen peroxide. Therefore, the increased activity of CAT would suggest a compensatory response of hepatic defense system under condition of enhanced H_2O_2 generation.

Hsp70 are central components of the cellular network of molecular chaperones and folding catalysts. Hsp70 assist a wide range of folding processes, including the folding and assembly of newly synthesized proteins, refolding of misfolded and aggregated proteins, membrane translocation of organelle and secretory proteins, and control of the activity of regulatory proteins [14]. A few studies involving overexpression of Hsp70 in cell lines have shown protection against heat shock and oxidative stress. Hsp70 levels in the mononuclear cells are

elevated reflecting increased oxidative stress in patients with diabetes [15]. Hsp70 is thought to be beneficial in defending against oxidative injury. But if overexpression of Hsp70 continues, the ability to respond to subsequent stressful conditions such as aging, exposure to oxidizing chemicals or reduced antioxidants may be impaired. In diabetes, continuous hyperglycemia, or attenuated activities of anti-oxidative enzymes may cause chronic overexpression of Hsp70 and subsequent reduction of the ability to cope with stressful conditions. In our results, HFD increased Hsp70 protein expression and HFD+TSL-E normalized to the level of control groups suggested that the stress condition in HFD were decreased under TSL-E treatment.

The peroxiredoxins, belong to the rapidly growing family of the thiol-specific antioxidant proteins that are highly conserved from bacteria to mammals. Prdx6 uses glutathione and ascorbate as electron donors and is the only member of the Prdx family that has the ability to remove H_2O_2 and phospholipid hydroperoxide and is, therefore, able to reduce the accumulation of phospholipid hydroperoxides in plasma membranes [16]. Previous study has been reported that HFD induced the decreased expression of genes associated with antioxidant defense, such as superoxide dismutase-3, metallothionein-1, glutathione peroxidase-5, and Prdx 6 [17]. Consistently, in our study HFD decreased the expression of Prdx 6 and HFD+TSL-E significant increased Prdx 6 expression in liver of HFD mice.

Moreover, S-adenosylmethionine synthesis is the first step in methionine metabolism in a reaction catalyzed by methionine adenosyl transferase. SAM is the principal biologic methyl donor, the precursor for polyamine bio synthesis and a precursor of glutathione (GSH) by means of the transulfuration pathway. The increase of SAM can lead to increase homocyteine, therefore increase the generation of glutathione. The beneficial effect of SAM in preservation solutions could therefore include direct radical scavenging as well as acting as a precursor for intracellular GSH [18]. Recently, Fonseca V. et al. were able to demonstrate that the PPAR agonist troglitazone had significant increases in hepatic concentrations of S-adenosylhomocysteine (SAH) and S-adenosylmethionine (SAM) plus SAH. Additionally, there was a significant decline in the SAM/SAH ratio. In our result, TSL-E treatment increased the SAM level compared with both control and HFD group indicated that the anti-oxidative stress activity of TSL-E might through the increase the GSH level in liver.

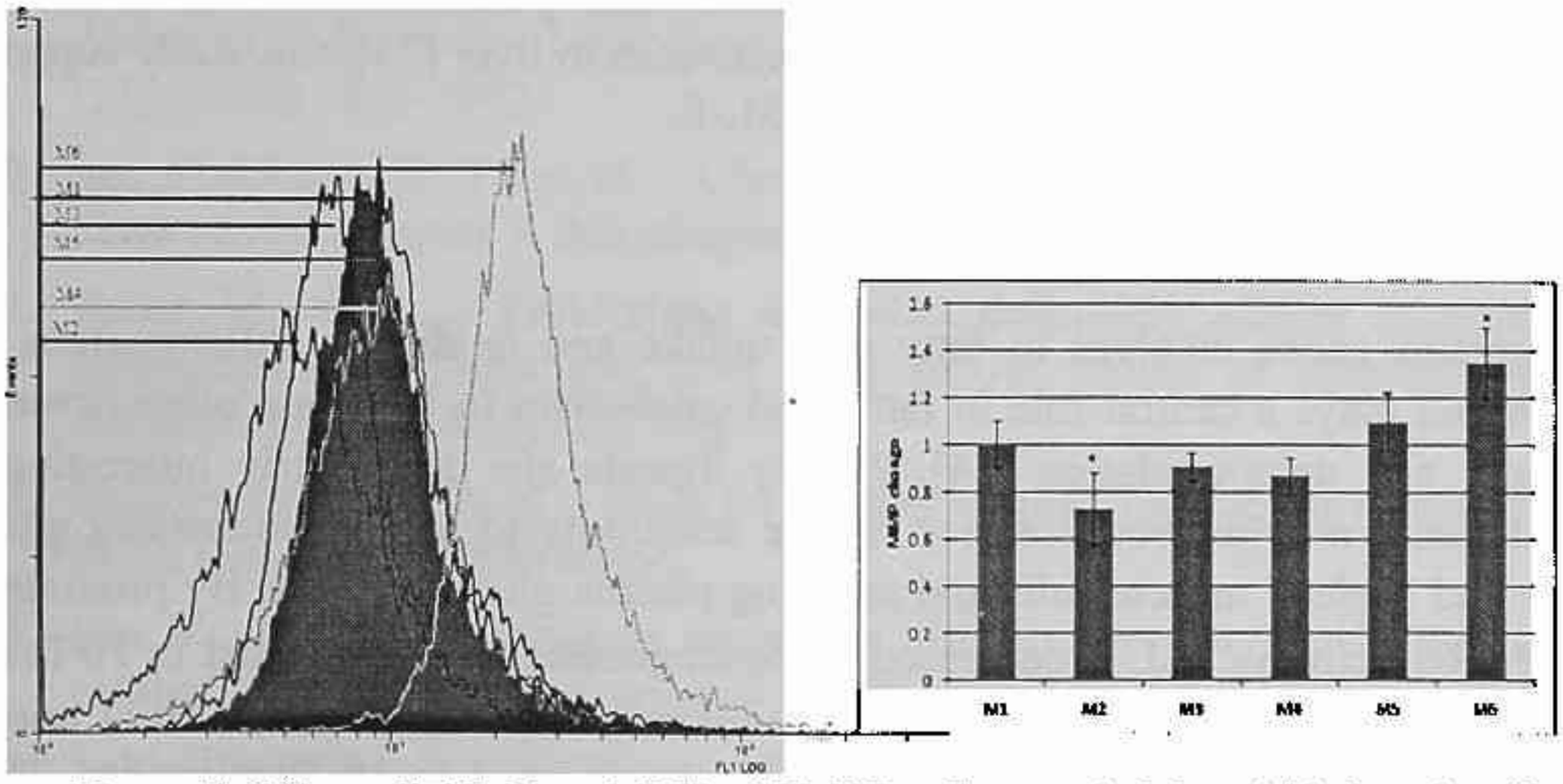


Figure 5. Effect of TSL-E on MMP of FL83B cell treated oleic acid induced oxidative stress.

The FL83B hepatocytes were cultured in normal condition (M1), treated with 200 μM OA (M2) treated with TSL after added OA (M3, M4) or pretreated with TSL-E before added OA (M5, M60combine with the TSL-E treatment (pretreat and treatment).. * $P < 0.05$, Student's *t* test.

Mitochondrial aldehyde dehydrogenase is responsible for metabolizing of ethanol and their activities contribute to the rate of ethanol elimination from the blood [19]. Ethanol metabolism also produces reactive species, including acetaldehyde and free radicals, which can directly attack proteins, lipids, and many other cellular components. Diabetic rats exhibited significantly increased ROS, accompanied by decreasing in ALDH2 activity and expression [20]. In our study, HFD decreased the ALDH2 expression and after fed with TSL-E, ALDH2 was significantly increased, restored to normal level. This result also confirmed the anti-oxidation effect of TSL-E on the liver of high fat mice.

Mitochondria are considered as the main source of ROS in the cell. The ability of ROS-mediated oxidative stress has been associated with lipid peroxidation and loss of MMP [21]. Moreover, the damage to the mitochondrial compartment might be the toxic effect of accumulation of compounds that are normally degraded by peroxisomal β-oxidation, particularly by fatty acids. To confirm the anti-oxidation effect of TSL-E, we investigated the effect of TSL-E on MMP in HepG2 using flow cytometry with $DiCO_6$ dye. While OA caused loss of MMP, the TSL-E treatment restored it indicated that under anti-oxidative activity of TSL-E, the ROS induced by OA were decreased associated with meliorated the MMP level (Figure 5).

Taken together, TSL-E showed the anti-oxidation activity by increasing the expression of Prdx6 and its glutathione supply S-adenosine methionine. The increase of Prdx6 suppressed the elevated of H_2O_2 generation in HFD mice, since restored to normal level of CAT, Hsp70 proteins expression. This results is consistent with previous documentation that protein oxidation and oxidative damage have been previously associated with diabetes [22] and TSL-E have anti-oxidation activity.

Since alterations in oxidative capacity and mitochondrial functions may have an important role to play in the pathophysiology of diets induced obesity and its complications. HFD is redirected into harmful pathways of non-oxidative metabolism with intracellular accumulations of toxic metabolites. The present results provide further evidence that TSL-E supplement, while improve dyslipidemia effects, may furnish protection against diet-induced liver damage, possibly by reducing oxidative stress. In previous study in our lab, we also found that *Toona sinensis* extracted exhibited the ant oxidative effect which increased the enzyme activities of CAT, cupper/zinc superoxide dismutase (Cu/Zn SOD), glutathione peroxidase (GPx), glutathione reductase (GR), and Glutathione S transferase (GST) activities in liver [23], this study supported another evident to confirm anti-oxidation activity of TSL-E.

4.3. Proposed pathway of TSL-E effect on HFD

PPARα regulates genes involves in fatty acid uptake and oxidation, inflammation, and vascular function and plays a central role in fatty acid catabolism in liver and other tissues by up-regulating beta- and delta-oxidation [24]. PPARγ ligands are particularly interesting for treating type 2 diabetes mellitus because they restore sensitivity to insulin, increasing glucose uptake into liver and skeletal muscle cells and reducing plasma glucose levels. By promoted β-oxidation via PPARα activity, TSL-E decreased gluconeogenesis which elevated in HFD mice caused hypertension and IR. PPARα activation also controls hyperlipidemia in HFD mice by decreased lipogenesis. While PCK2 protein expression is elevated in HFD mice, the TSL-E treatment decreased PCK2 compared with HFD groups. These results suggest that TSL-E is more efficient activity on PPARα relative to PPARγ. The interaction between PPARα and PPARγ agonist activity of TSL-E can alleviate side effect, such as increased body weight

associated with PPARγ agonists. The classic full PPARγ agonists have a variety of side effect, chiefly weight gain due to edema and increased fat mass [25]. However, the side effect associated with PPARγ activation may be circumvented through the combined activation of PPARα and PPARγ, which is known to result in a complementary and synergistic increase in lipid metabolism and insulin sensitivity [26]. In summary, TSL-E might manipulate the lipolysis and energy homeostasis in liver through the regulation of PPARα/γ downstream signaling pathway.

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TÓM TẮT

PHÂN TÍCH PROTEOMICS MÔ GAN CHUỘT NHẮT CHO ĂN VỚI HÀM LƯỢNG CHẤT BÉO CAO TRONG SỰ ĐÁP ỨNG VỚI DỊCH CHIẾT BẰNG CỒN CỦA LÁ CÂY TÔNG DÙ (*TOONA SINENSIS* ROEM)

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Các bệnh sinh chủ yếu của hội chứng chuyển hóa là sự phát triển kháng insulin, thúc đẩy sự tăng huyết áp, rối loạn mỡ máu và rối loạn chuyển hóa đường. Gan là cơ quan chính của chuyển hóa đường và chất béo bằng cách kiểm soát sản xuất glucose, lưu trữ glycogen và sinh tổng hợp chất béo. Nhiều nghiên cứu đã cho thấy rằng cây tông dù tác dụng tốt lên bệnh nhân mắc bệnh rối loại chuyển hóa, tuy nhiên ảnh hưởng của cây tông dù lên hoạt động của gan chưa được nghiên cứu rõ ràng. Nghiên cứu này nhằm làm rõ ảnh hưởng dịch chiết cây tông dù lên quá trình sinh tổng hợp của gan chuột nhắt được cho ăn với hàm lượng chất béo cao. Chuột nhắt được cho ăn với lượng chất béo cao trong 8 tuần để gây rối loạn chuyển hóa, sau đó chuột được cho ăn thức ăn có bổ sung dịch chiết tông dù (100 mg/kg) trong 4 tuần. Chúng tôi sử dụng kỹ thuật phân tích điện di 2 chiều kết hợp với sắc kí khối phổ để xác định sự biểu hiện của các protein mô gan chuột. Ngoài ra, kỹ thuật lai Western cũng được thực hiện để kiểm tra kết quả của phân tích proteomics. Kết quả cho thấy, có sự gia tăng biểu hiện của các gen liên quan quá trình oxi hóa beta (PPARα và ACO) và ngược lại, giảm biểu hiện của các gen liên quan quá trình sinh tổng hợp chất béo (ACC), vận chuyển chất béo (l-FABP) và quá trình sinh tổng hợp đường (Transaldolase, Enolase, PCK2 and Nudix). Có thể kết luận rằng, dịch chiết bằng cồn của lá cây tông dù có tiềm năng kháng lại hội chứng chuyển hóa thông qua sự cải thiện rối loạn chuyển hóa mỡ và đường máu bằng cách tăng cường quá trình oxi hóa beta và làm giảm sinh tổng hợp đường và chất béo.

Từ khóa: cây tông dù, oxi hóa beta, sinh tổng hợp đường, sinh tổng hợp chất béo, sinh tổng hợp glycogen, PPARα/γ, gan, tế bào gan.