ORIGINAL ARTICLE

Basic Study

Altered distribution of regulatory lymphocytes by oral administration of soy-extracts exerts a hepatoprotective effect alleviating immune mediated liver injury, non-alcoholic steatohepatitis and insulin resistance

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Abstract

AIM: To determine the immune-modulatory and the hepatoprotective effects of oral administration of two soy extracts in immune mediated liver injury and non-alcoholic steatohepatitis (NASH).

METHODS: Two soy extracts, M1 and OS, were orally administered to mice with concanavalin A (ConA) immune-mediated hepatitis, to high-fat diet (HFD) mice and to methionine and choline reduced diet combined with HFD mice. Animals were followed for disease and immune biomarkers.

RESULTS: Oral administration of OS and M1 had an additive effect in alleviating ConA hepatitis manifested by a decrease in alanine aminotransferase and aspartate aminotransferase serum levels. Oral administration of the OS and M1 soy derived fractions, ameliorated liver injury in the high fat diet model of NASH, manifested by a decrease in hepatic triglyceride levels, improvement in liver histology, decreased serum cholesterol and triglycerides and improved insulin resistance. In the methionine and choline reduced diet combined with the high fat diet model, we noted a decrease in hepatic triglycerides and improvement in blood glucose levels and liver histology. The effects were associated

with reduced serum tumor necrosis factor alpha and alteration of regulatory T cell distribution.

CONCLUSION: Oral administration of the combination of OS and M1 soy derived extracts exerted an adjuvant effect in the gut-immune system, altering the distribution of regulatory T cells, and alleviating immune mediated liver injury, hyperlipidemia and insulin resistance.

Key words: Non-alcoholic steatohepatitis; Fatty liver; Regulatory T cells; Soy; Type 2 diabetes; ConA hepatitis

Core tip: Oral administration of the combination of OS and M1 soy derived extracts exerted an adjuvant effect in the gut-immune system, altering the distribution of regulatory T cells, and alleviating immune mediated liver injury, hyperlipidemia and insulin resistance. Oral administration of these extracts had an additive effect in alleviating concanavalin A hepatitis and ameliorated liver injury in the high fat diet model of non-alcoholic steatohepatitis, and in the methionine and choline reduced diet combined with the high fat diet model. The effects were associated with reduced serum tumor necrosis factor alpha and alteration of regulatory T cell distribution.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is increasingly becoming a major health burden[1,2]. Non-alcoholic steatohepatitis (NASH) is characterized by fatty infiltration of the liver with hepatocytes ballooning and lobular inflammation, and is associated with increased risk of cirrhosis and hepatocellular carcinoma[3]. Both NAFLD and NASH are associated with obesity and diabetes[4].

Liver steatosis is associated with an increase in free circulating fatty acids[4-7]. Lipids normally undergo metabolism in the liver by β-oxidation. The metabolism of excess lipids via their incorporation into the very low density lipoprotein mechanism is down regulated in patients with NASH[4-7]. Accumulation of lipids in the liver is also associated with increased susceptibility to multiple oxidative factors. In addition, a reactive inflammatory process secondary to faulty lipid metabolism may lead to the secretion of cytokines

and adipokines including tumor necrosis factor alpha (TNF-α). TNF-α further decreases the anti-oxidant capacity of the liver, promoting mitochondrial damage. Activation of the immune system and of hepatic stellate cells lead to liver inflammation and liver fibrosis in hepatic steatosis[4-7]. The increase in visceral adipose tissue is also associated with an increased release of free fatty acids, inducing a low grade inflammation state by cytokine release. This state is associated induced insulin resistance by down regulating the insulin receptor substrate 1 (IRS-1)[8].

In patients with NASH an increased secretion of pro-inflammatory cytokines from adipose tissue (*e.g.*, TNF-α) was described[9,10]. An increased level of macrophages which further exacerbate the pro-inflammatory state was shown. Regulatory T cells (Tregs) can suppress several immune mediated disorders via alteration of effector T cells and the cytokine paradigm. In animal models of NASH, promotion of Tregs was associated with alteration of macrophage function and alteration of the cytokine paradigm from a pro- to an anti-inflammatory one[11,12]. A similar phenomenon was suggested in humans[13]. These effects were associated with alleviation of liver steatosis and insulin resistance.

Soy is part of the regular diet in many countries. Soybean is a rich source of vegetable protein, complex carbohydrates, polyunsaturated fat, soluble sugars and phytoestrogens[14-17]. Some soy proteins are also associated with fatty acids, saponins, isoflavones and phospholipids. Soy derived products are safe and were suggested beneficial in immune mediated disorders[14-17]. Soy products were reported to decrease the oxidative stress and the subsequent pro-inflammatory cytokine secretion and lipid peroxidation in the MCD diet model of NASH[18]. Specific components of soy, including isoflavones, can increase the anti-oxidant capacity of hepatocytes and decrease oxidative stress[17]. They also have beneficial effect in the treatment of gastric cancer[19]. One of the components of soy, β-glucosylceramide (GC), was shown to exert an immune modulatory effect in various models[20-22], further supporting the potential to use soy derived extracts as immune modulators. Epidemiological studies have shown that soy products might decrease the morbidity of NASH[23] as well as lower serum lipids in the blood and liver, reduce liver uptake of lipids, improve anti-oxidant ability and insulin resistance[24].

The aim of the present study was to determine the immune-mediated hepatoprotective effect of soy extracts. We have tested several soy extracts in three mice models: the concanavalin A (ConA) immune-mediated hepatitis model, and in two models of NASH. Oral administration of the combination of OS and M1 soy derived extracts exerted an adjuvant effect in the gut-immune system, altered the distribution of Tregs, alleviated immune mediated liver injury, hyperlipidemia, insulin resistance and liver damage

in the high fat diet (HFD), and in the methionine and choline reduced (HFD/MCR) model.

MATERIALS AND METHODS

Animals
All mice were maintained in the animal core of the Hebrew University Hadassah Medical School (Jerusalem, Israel). The mice were given the diets described below (HFD, HFD/MCR) and had free access to water and were maintained in a 12-h light-dark cycle. All experiments were performed in accordance with the guidelines of the Hebrew University-Hadassah Institutional Committee for Care and Use of Laboratory Animals with the approval of the Ethics Committee.

Oral administration of soy extracts
Two soy extracts were received from Solbar Israel (CHS), and studied in the below described animal models. OS-fraction, derived from the solvent extraction of soybeans into oil, and contains mainly tri- and di-glycerides, free fatty acids and phosphatides; MI- fraction which is derived from aqueous-ethanol extraction left after the solvent extraction, and contains mainly isoflavones, sugars (oligo-, di-, mono-), and lipids (including phosphatides, phytosterols, saponins).

Induction of ConA hepatitis
ConA (MP Biomedicals, United States) was dissolved in 50 mmol/L Tris pH 7, 150 mmol/L NaCl, 4 mmol/L CaCl$_2$ and was injected into the tail vein at a dose of 500 μg/mouse (15 mg/kg).

Animal models of NASH
Two models of NASH were studied. Model A: Harlan, TD88137, in this diet 42% of the caloric intake is derived from fat andit resembles a severe form of the human fatty diet. The mice given this diet exhibited features of fatty liver with characteristics of NASH, weight gain, hyperlipidemia and insulin resistance. Model B: HFD with methionine and choline reduced diet (Harlan, TD10810), this diet outpointed the inflammatory elements of NASH and oxidative stress. It's advantageous over the methionine and choline deficient diet due to less weight reduction[25].

Assessment of the effect of oral soy extracts on liver damage in ConA hepatitis model
Experimental groups: Eight groups were examined, 11-12 wk old male mice were purchased from Harlan Laboratories (Jerusalem, Israel). Four to six mice were treated in each group. To induce autoimmune hepatitis, all mice were injected iv (tail vein) with ConA. All mice were treated orally for three days with OS and M-1 soy compounds prior to ConA injections. Mice were sacrificed 14 h after the ConA injection. Mice in control Group A were treated with PBS. Group B was treated with GC (25 microgram/mouse), a

natural β-glycosphingolipid, (Avanti Polar lipids, AL, United States). Group C was treated with 0.35 μg of dexamethasone. Group D was administered with 3 μg of M1 soy fraction. Group E with 3 μg OS of soy fraction. Groups F, G and H were treated with different combinations of OS and M1 soy-derived extracts in 0.3, 3, and 30 μg doses, for each fraction respectively.

Histological examination of the liver: Paraffin-embedded liver sections were prepared from each mouse. The livers were cut into 4-5 μm thin slices and stained with hematoxylin-eosin (HE). Slides were scored to assess the extent of liver damage as described[26].

Liver enzymes: Serum was obtained from individual mice. The serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were determined with an automatic analyzer.

Assessment of the effect of oral soy extracts on liver damage in high fat diet model of NASH
Experimental groups: Six groups with five mice each, of 6-7 wk old male mice were obtained from Harlan Laboratories (Jerusalem, Israel). Mice received HFD diet in which 42% of calories are derived from fat, administered orally 3 times a week for 3 mo. Group A was treated with 30 μL of PBS. Group B with 3 μg of OS soy fraction. Group C with 25 μg GC. Group D with 3 μg of M1 soy fraction. Groups E and F were administered with combinations of soy products in doses of 3 μg and 0.3 μg for each component respectively.

Assessment of the effect of oral soy extracts on liver damage in the HFD/MCR model of NASH
For the HFD/MCR model, four groups were included; each group contained 6 mice purchased from Harlan Laboratories (Jerusalem, Israel). Mice under this diet regimen were administered orally 3 times a week for 3 mo. Group A was treated with 30 μL of PBS. Group B given 0.3 μg of OS and 0.6 μg of M1. Group C was treated with 3 μg OS and 6 μg M1. Group D was treated with 9 μg OS and 18 μg M1.

Measurement of lipid accumulation in the liver and plasma triglycerides
Plasma triglycerides (TGs) and total cholesterol were measured by the Reflovet Plus clinical chemistry analyzer (Roche Diagnostics, GmbH, Mannheim, Germany). TGs were extracted from aliquots of snap-frozen livers using a modification of the Folch method. Hepatic TG content was assayed spectrophotometrically using a GPO-Trinder kit (Sigma, Rehovot, Israel) and was normalized to the protein content in the homogenate. The HFD mice were weighed every 2 wk. Serum ALT, TGs and cholesterol were measured every 2 wk by the Reflovet device

while HFD/MCR mice were monitored for body weight, serum ALT, and triglycerides, every 4 wk.

Evaluation of insulin action and sensitivity

Insulin sensitivity was determined by oral glucose tolerance test. Mice underwent a glucose tolerance test after overnight fasting. Glucose was administered orally (1.25 g/kg body weight). Serum glucose measurements were performed every 15 min for 3 h (180 min). Glucose levels were measured by a standard glucometer. Fasting blood glucose (FBG) was monitored every two weeks. Serum insulin levels were determined using a commercially available ELISA kit (Mercodia AB; Uppsala, Sweden), according to the manufacturer's instructions.

Histological analysis of hepatic tissues

For histopathology, livers from individual mice were fixed in 10% formaldehyde solution and kept at room temperature until use. The tissue blocks were then embedded in paraffin, sectioned and stained with HE for morphological examination. Specimens were examined under a light microscope.

Assessment of the effect of soy extracts on the systemic immune system

The immune modulatory effect of soy extracts was determined by FACS analysis and serum cytokines.

Isolation of splenocytes and hepatic lymphocytes

Spleens and livers were kept in RPMI-1640 supplemented with FCS. Spleens were crushed through a 70 μm nylon cell strainer[27] and centrifuged (1250 rpm for 7 min) to remove debris. Red blood cells were lysed with 1 mL of cold 155 mM ammonium chloride lysis buffer and immediately centrifuged (1250 rpm for 3 min). Splenocytes were then washed and resuspended in 1 mL RPMI + FCS. Any remains of connective tissue were removed. The viability, as assessed using trypan blue staining, was above 90%. For the isolation of hepatic lymphocytes, livers were first crushed through a stainless mesh (size 60, Sigma), and the cell suspension was placed in a 50 mL tube for 5 min so that cell debris could pellet. The cell suspension was slowly underlaid with 10 mL of lymphoprep (Ficoll, Axis-Shield PoC AS, Oslo, Norway) in a 50 mL tube. The tubes were then centrifuged at 1800 rpm for 18 min. Cells at the interface were collected and transferred to a new tube that was centrifuged again at 1800 rpm for 10 min. The resulting pellet of hepatocyte-depleted lymphocytes was resuspended in a final volume of 250 μL. Approximately 1×10^6 intrahepatic lymphocytes were recovered per mouse liver.

Flow cytometry

Flow cytometry was performed on splenocytes and hepatic lymphocytes, which were resuspended in 1 mL of FACS buffer (PBS + 1% BSA + 0.1% sodium azide). Cells were stained with the diluted anti-LAP antibody (50 μL /sample), FITC-conjugated anti-CD4/CD8 (0.5 μL per sample), PE-conjugated anti-CD25/NK1.1 Pacific Blue–conjugated anti-CD3 (3 μL per sample), and PerCP-conjugated anti-CD45 (2 μL per sample). All stains were performed after blocking the Fc receptor with anti-mouse CD16/CD32 (BD Fc Block). Flow cytometry was performed using a FACScan flow cytometer and Cellquest software.

Cytokine measurement

Serum IFN-γ levels were measured in each animal using a commercially available "sandwich" ELISA kit (Quantikine, R&D Systems, MN, United States).

Statistical analysis

All analysis was performed using Excel 2003 (Microsoft, Redmond, WA, United States). The variables were expressed as mean ± SD. The comparison of two independent groups was performed using Student's *t*-test. All tests applied were two-tailed. *P* value of 0.05 or less was considered to be statistically significant.

RESULTS

Oral administration of soy-extracts (OS, M1) alleviated ConA-mediated immune hepatitis

Figure 1A shows the effect of oral administration of the soy extracts (M1, OS) in immune-mediated hepatitis. All treated groups showed statistically significant improvement in the levels of liver enzymes, ALT and AST (*P* < 0.05). No dose dependency was noted. Administration of GC or dexamethasone also significantly alleviated immune mediated liver damage (*P* < 0.01, *P* < 0.004, respectively). Figure 1B shows that the hepatoprotective effect of the soy extracts was associated with a reduction in serum levels of pro-inflammatory cytokine IFN-γ. This effect was most notable in mice treated with the combination of OS and M1 (*P* < 0.005), for mice treated with 30 μg of OS and M1 in lowering (*P* = 0.001). Figure 1C shows the effect of oral administration of soy extracts on TNF-α serum levels. The 30 micrograms dose of OS and M, lowered the pro-inflammatory cytokines TNF-α and INF-γ (*P* = 0.0001 *vs P* = 0.00001 and *P* < 0.005 *vs P* = 0.001 respectively).

Oral administration of soy extracts alleviated metabolic syndrome in HFD model

Oral M1 and OS soy extracts alleviated liver damage and insulin resistance. No significant changes were noted in body weight between all groups.

Figure 2A shows the effect of oral administration of soy extracts on serum cholesterol levels that were measured every two weeks. Administration of 0.3 μg OS + 0.3 μg M1 was associated with a significant decrease in serum levels noted as early as week 2,
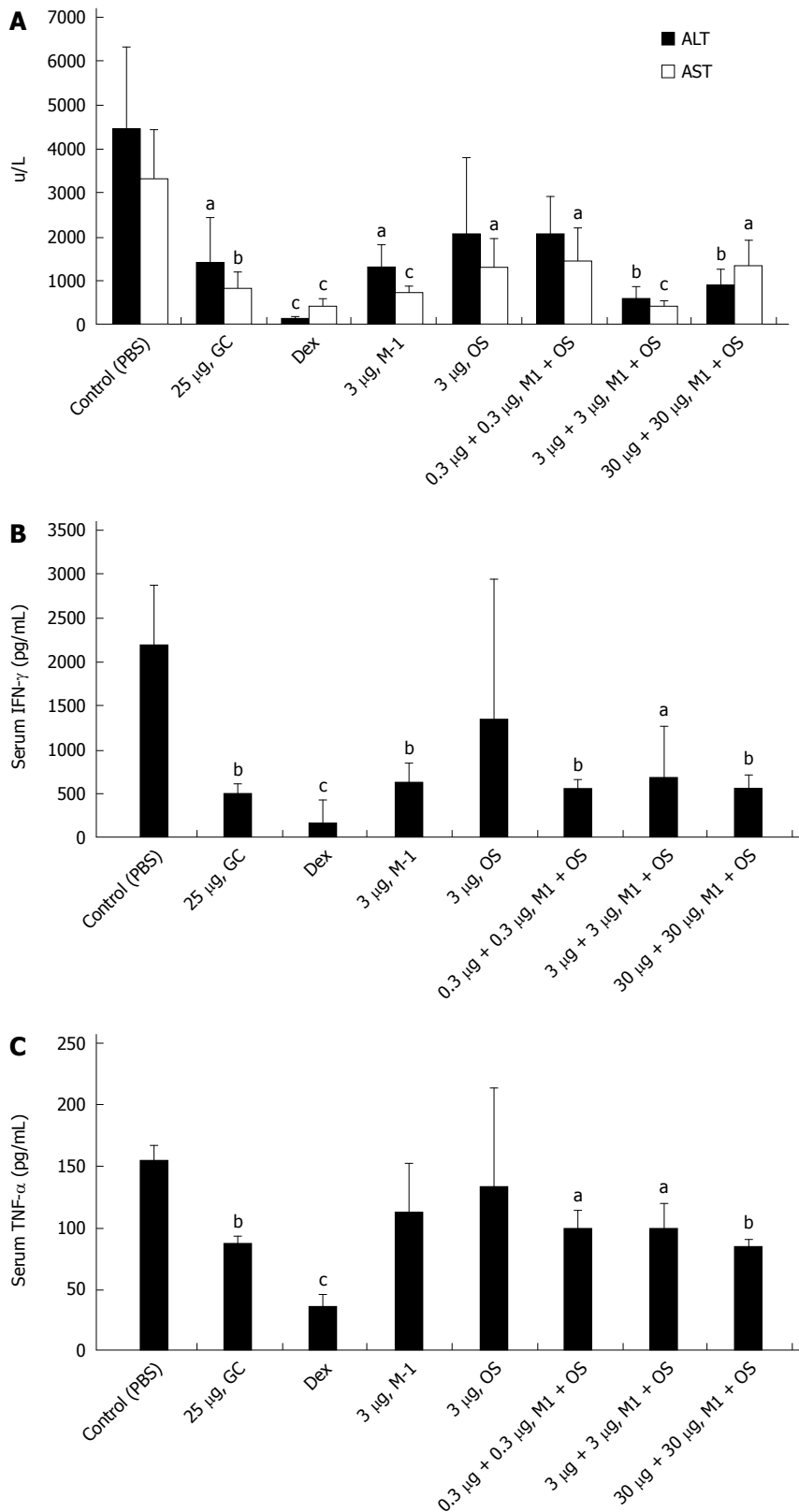
Figure 1 Effect of soy extracts on concanavalin A immune mediated liver injury. A: Combination of soy extracts in doses of 3 μg and 30 μg of both OS and M1, administered to mice injected with concanavalin A (ConA), significantly decrease aspartate aminotransferase and alanine aminotransferase serum levels ([a]$P < 0.04$, [b]$P < 0.01$, [c]$P < 0.004$ *vs* control); B: The com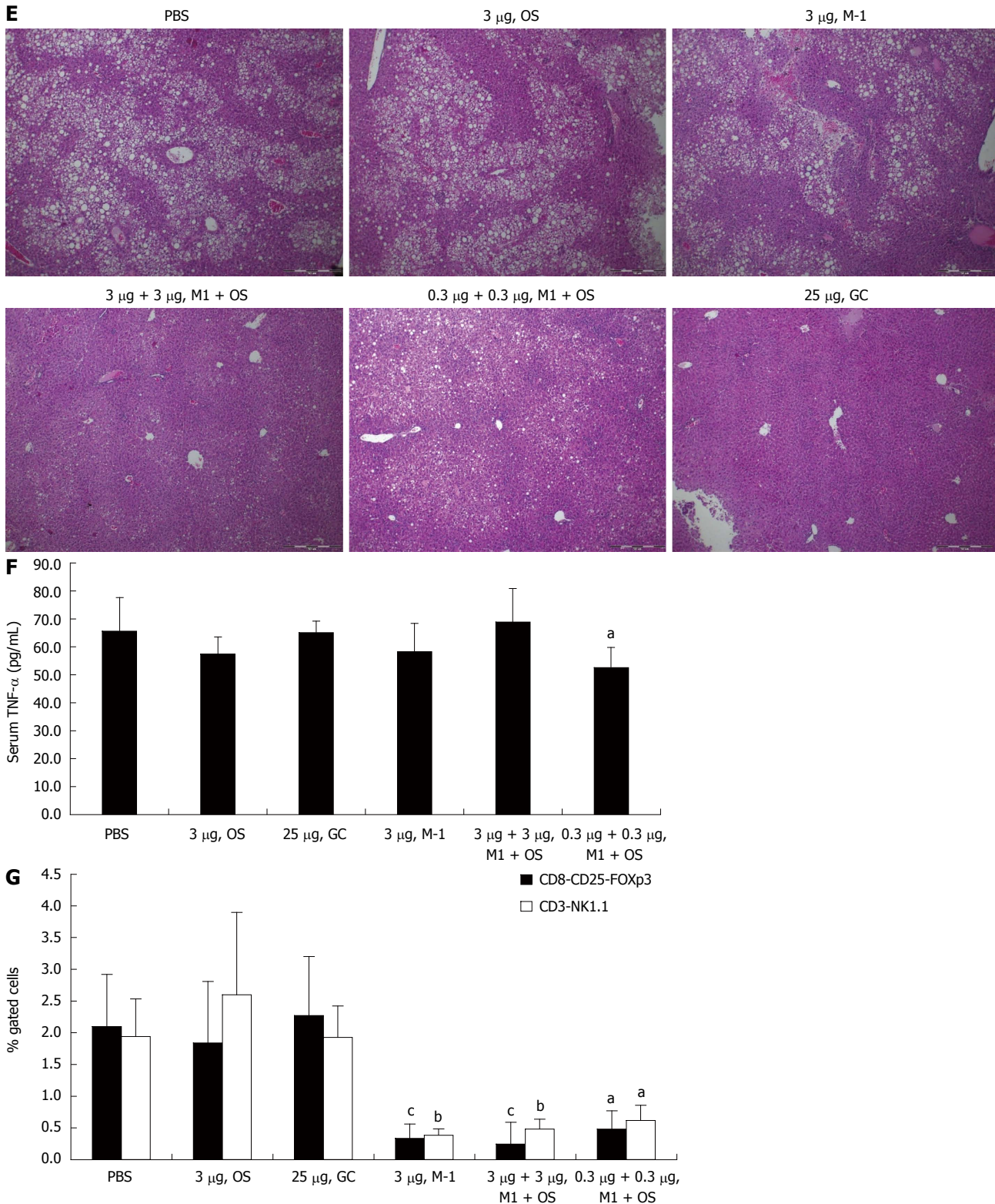bination of OS and M1 soy extracts in all doses and 3 μg M1 were associated with significant reduction of serum interferon gamma levels measured by ELISA at the end of the treatment period ([a]$P < 0.02$, [b]$P < 0.005$, [c]$P = 0.001$ *vs* control); C: All combinations of soy extracts were associated with a significant decrease in serum TNF-α serum levels measured by ELISA at the end of the study ([a]$P < 0.005$, [b]$P = 0.0001$, [c]$P = 0.00001$ *vs* control). AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.
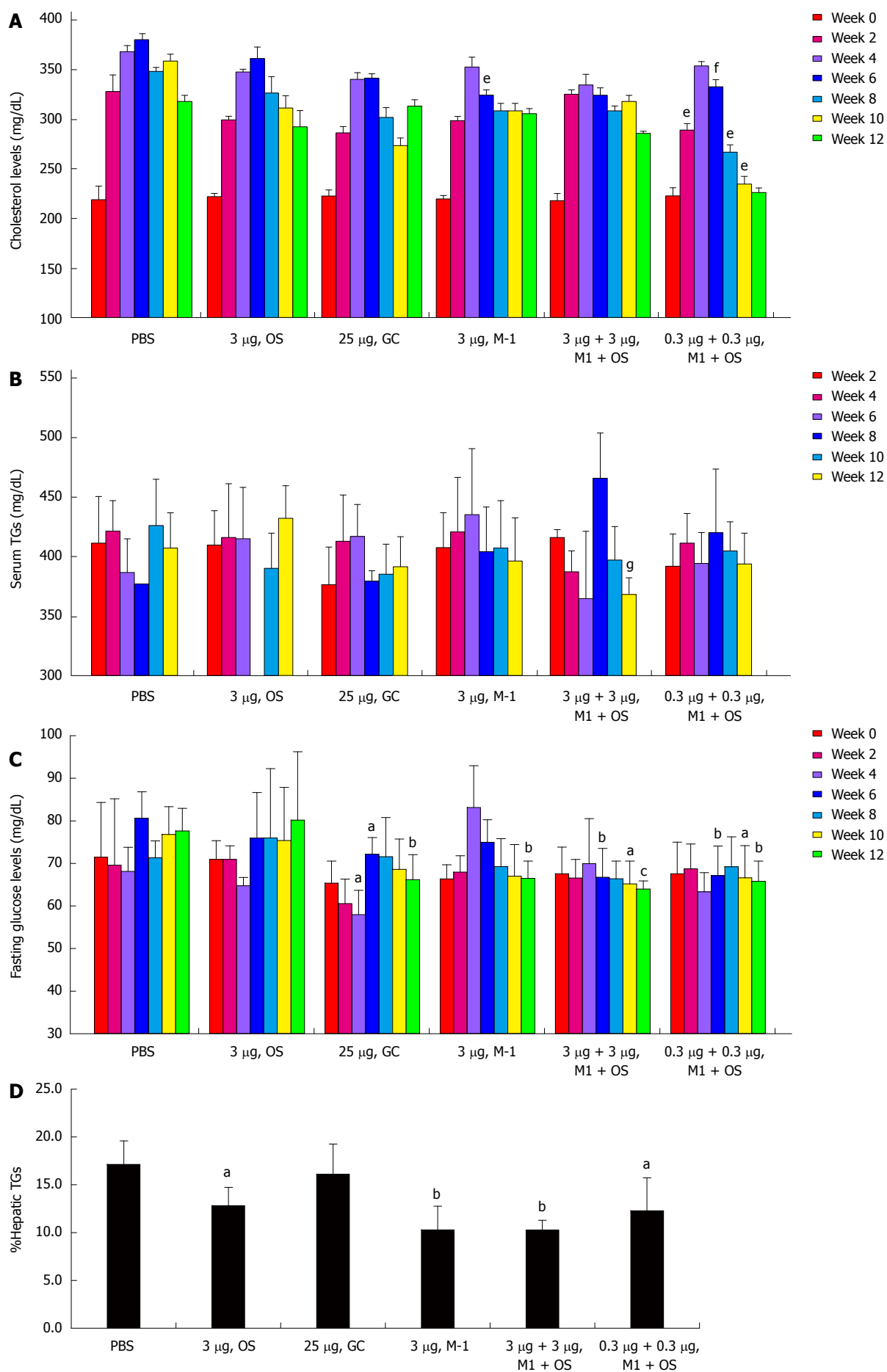
Figure 2 Effect of soy extracts on a high fat diet mouse model of non-alcoholic steatohepatitis. A: Serum cholesterol levels were measured every two weeks in all groups. The combination of 0.3 μg of OS and M1 was associated with significant a decrease of serum cholesterol level compared to the control group ([e]P < 0.05, [f]P < 0.01 *vs* control); B: Serum triglyceride levels were measured every two weeks in all groups. The combination of 3 μg of each OS and M1 serum levels on week 12 ([g]P < 0.03 *vs* control); C: Fasting serum glucose levels were measured every two weeks in all groups. The combination of OS and M1 improved fasting glucose levels as evident from week number 6. A dose of 3 μg of M1 significantly improved fasting glucose level at week 12 ([a]P < 0.04, [b]P < 0.008, [c]P < 0.0005 vs control); D: Hepatic triglyceride content was measured in all mice at the end of the study. The combination of OS and M1, and M1 alone, were associated with significant reduction of hepatic fat content ([a]P < 0.05, [b]P < 0.003 *vs* control); E: Representative H&E (magnification × 10) sections from liver biopsies performed in all groups at the end of the treatment period; F: Serum TNF-α levels were measured by ELISA at the end of the study. A significant reduction was noted in mice treated with the combination of 3 μg M1 and 3 μg OS ([a]P < 0.05 *vs* control); G: FACS analysis was performed on splenocytes for NKT and CD8+CD25+Foxp3+ regulatory T lymphocytes. Both the M1 + OS combination and the M1 alone treated group showed a significant reduction in these subsets ([a]P < 0.002, [b]P < 0.0009, [c]P = 0.0003 *vs* control).

in comparison with the control group ($P < 0.05$). Similarly, Figure 2B shows the effect of oral administration of soy extracts on serum triglyceride levels. A significant effect on serum TGs was observed in week 12 in the 3 μg OS + 3 μg M1 treated group ($P < 0.03$). Oral administration of the soy extracts improved insulin resistance. Figure 2C shows the effect of oral administration of soy extracts on fasting glucose levels. The fasting glucose levels were significantly decreased by the combination of M1 and OS in two doses (0.3 and 3 μg for each extract, $P < 0.008$, $P < 0.0005$, respectively). The glucose tolerance test improved in all treatment groups by weeks 4 and 12 (data not shown). Serum insulin was reduced in two of the treated groups (3 μg OS and 3 μg of each OS and M1) but did not reach statistical significance.

Oral administration of the soy extracts improved liver damage in this model. Figure 2D shows the effect of oral administration of soy extracts on liver triglyceride content. A significant reduction in hepatic TGs was observed in all soy extracts, as the combination of 3 μg M1 and 3 μg OS, and the 3 μg M1, were the most effective ones compared with the control group ($P < 0.003$). Figure 2E shows representative sections from liver biopsies performed at the end of the treatment period in all mice in all groups. Alleviation of hepatic steatosis and improved liver cell architecture were noted in mice treated with the combination of 3 μg M1 and 3 μg OS.

The beneficial effects noted in this model were associated with alteration of the immune response. Figure 2F shows the effect of oral administration of soy extracts on TNF-α serum levels. Serum TNF-α was significantly reduced in mice treated with the combination of 3 μg M1 and 3 μg OS ($P < 0.05$). Figure 2G shows the effect of oral administration of soy extracts on NKT and CD8+CD25+Foxp3+ regulatory T lymphocytes. Both combinations of M1 + OS, and the M1 alone treated group showed a significant reduction of these subsets in the spleens.

Oral administration of soy extracts alleviated fatty liver in mice given HFD/MCR diet, in a NASH model
Similar to the effect on HFD mice, oral M1 and OS soy extracts alleviated liver damage and insulin resistance in the HFD/MCD model without a significant change in body weight. Treatment with 3 μg M1 and 6 μg OS extracts showed a trend in reduction of ALT serum levels (data not shown, $P = 0.6$). Oral administration of soy extracts was associated with a beneficial effect on serum TGs and a significant decrease was noted in 3 μg M1 and 6 μg OS treated mice and for 0.6 μg M1 treated mice at week 12 (data not shown, $P < 0.02$). Figure 3A shows the effect of oral administration of soy extracts on fasting serum glucose levels measured on weeks 0, 7 and 13. Fasting glucose levels were significantly improved in all treated groups compared with the controls ($P < 0.05$). The effect was more

profound in the 3 μg M1 and 6 μg OS, and 9 μg M1 and 18 μg OS treated groups ($P = 0.003$ *vs* $P = 0.008$, respectively). No significant different was noted for fasting serum insulin levels.

Figure 3B shows the effect of oral administration of soy extracts on liver triglyceride content. Treatment with the soy extract was associated with a significant reduction of hepatic tissue triglycerides at the end of the study for the 9 μg M1 and 18 μg OS treated group ($P < 0.05$). Figure 3C shows representative sections from liver biopsies performed at the end of the treatment period in all mice in all groups. Alleviation of hepatic steatosis and improved liver cell architecture were noted in mice in all groups, and was mostly profound for mice treated with the combination of 9 μg M1 and 18 μg OS. Figure 3D shows the significant improvement in the pathological NAS score noted in all treated groups.

The beneficial effects noted in this model were associated with alteration of the immune response. Serum TNF-α levels were significantly decreased in the 3 μg OS + 3 μg M1 treated group ($P < 0.05$, not shown). Figure 3E shows the effect of oral administration of soy extracts on regulatory lymphocytes. CD4+CD25+Foxp3+ and NKT lymphocyte subsets were significantly increased in the spleen of mice treated with the combination of 9 μg M1 and 18 μg OS ($P < 0.05$).

DISCUSSION

Oral administration of the combination of OS and M1 soy-derived extracts exerted a systemic immune effect *via* an effect on the gut-immune system, alleviating immune-mediated liver injury in the ConA hepatitis model, hyperlipidemia, insulin resistance and liver damage, in HFD and HFD/MCD mouse models of NASH.

In the ConA hepatitis model, liver damage results from a cytokine storm mediated by kupffer cells and NKT cells. Both IFN-γ and TNF-α play a role in mediating the damage[28]. Oral administration of the combination of OS and M1 alleviated the immune-mediated liver damage that was manifested by a decrease in liver enzymes. The combination of OS and M1 was superior to either fraction alone in reducing liver enzymes in a dose-dependent manner. The hepatoprotective effect of the two soy extracts examined in the present study was associated with reduction of pro-inflammatory cytokines IFN-γ and TNF-α. Similar to the effect on liver enzymes, the anti-inflammatory effect was mostly noted in mice treated with the combination of the two extracts in a dose dependent manner.

Alteration of the immune system, increase in oxidative stress, and pro-inflammatory cytokines are associated with insulin resistance and liver inflammatory response in NASH[29-31]. Both the HFD, and the HFD/MCR, models of NASH are characterized by severe depletion of hepatic anti-oxidants[32]. The HFD

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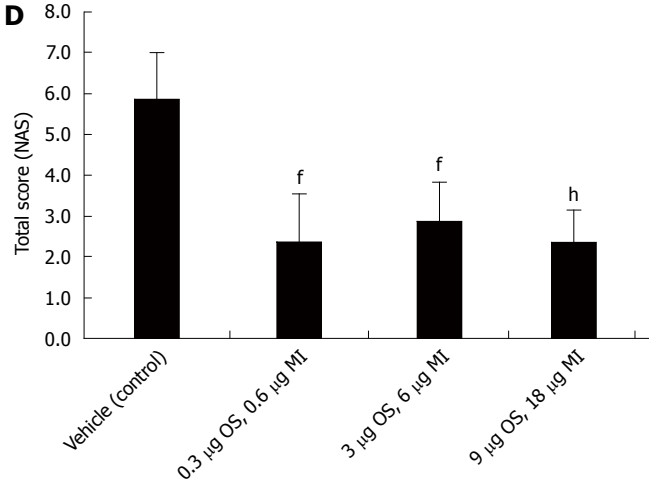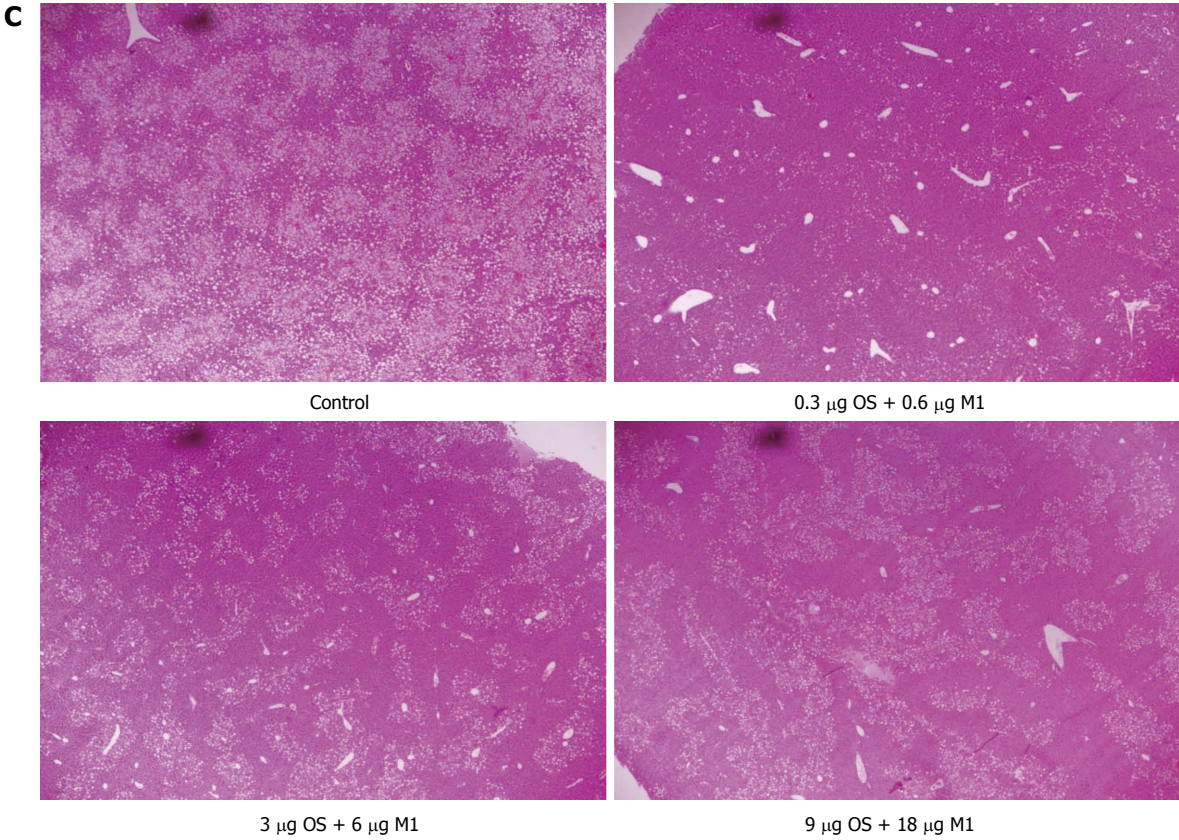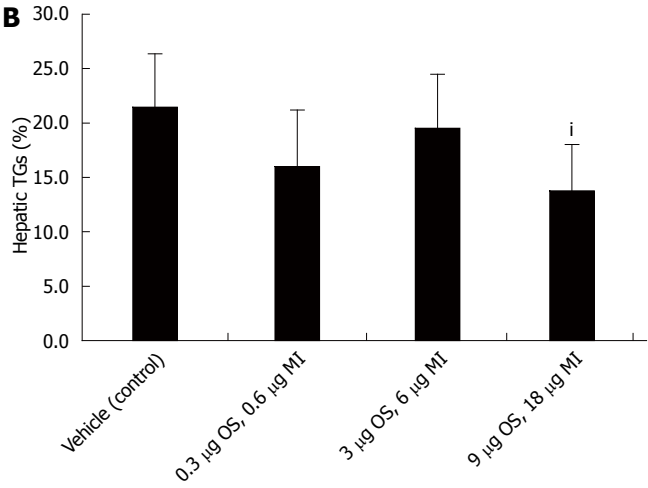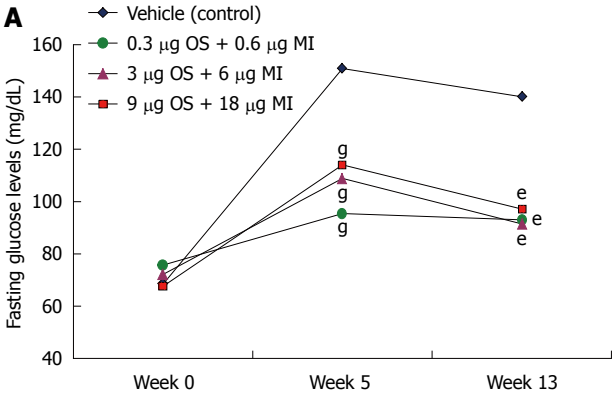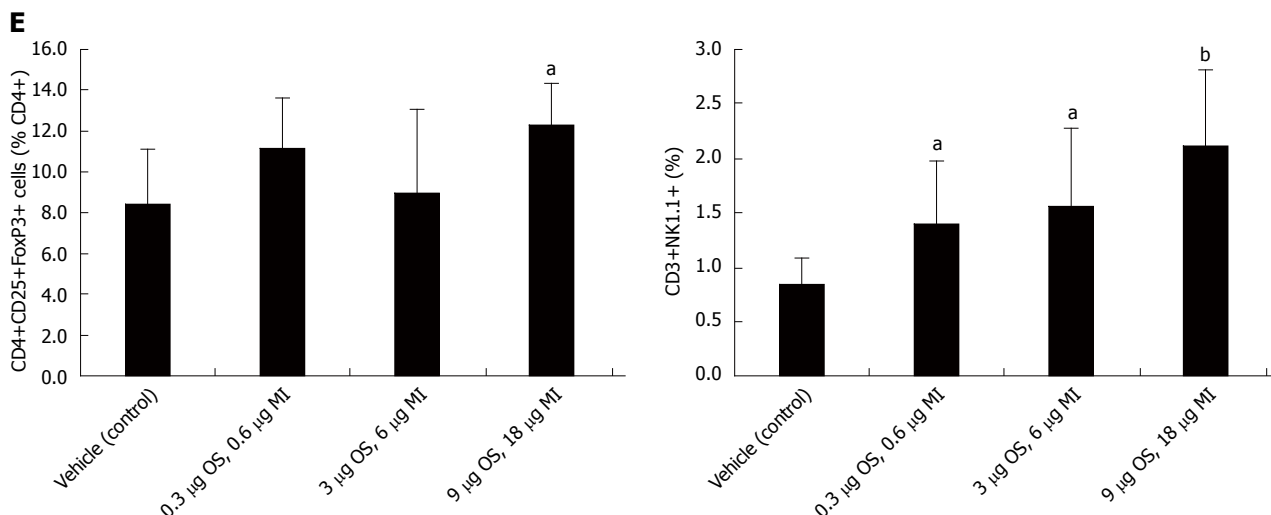
Figure 3 Effect of soy extracts on the high-fat diet/MCD mouse model of non-alcoholic steatohepatitis. A: The effect of oral administration of soy extracts on fasting serum glucose levels measured on weeks 0, 7 and 13. Glucose levels improved in all treated groups compared to the controls, and the effect was more profound in the 3 μg M1 and 6 μg OS, and 9 μg M1 and 18 μg OS treated groups ([e]P < 0.01, [g]P < 0.001 *vs* control); B: Hepatic triglyceride content was measured in all mice at the end of the study. The combination of 9 μg M1 and 18 μg OS treated groups was associated with significant reduction of hepatic fat content ([i]P < 0.05 *vs* control); C: Representative HE (magnification × 10) sections from liver biopsies performed from all groups at the end of the treatment period; D: The pathological NAS score was performed on liver sections from all groups at the end of treatment. A significant improvement was noted in mice in all treated groups ([f]P < 0.001, [h]P = 0.0001 *vs* control); E: FACS analysis was performed on splenocytes for NKT and CD4+CD25+Foxp3+ regulatory T lymphocytes. Both regulatory lymphocyte subsets significantly increased in the spleen of mice treated with the combination of 9 μg M1 and 18 μg OS ([a]P < 0.05, [b]P < 0.005 *vs* control).

model is associated with insulin resistance but not always with histological steatohepatitis[33,34]. The MCD diet model is associated with histological features of steatohepatitis, but usually is not associated with insulin resistance and obesity[35]. The HFD/MCD model provides a model for NASH in which mice manifest both insulin resistance and histological features of NASH[25]. TNF-α plays a key role in the development of insulin resistance and NASH in both models, similar to its role in humans with metabolic syndrome[36,37]. The data of the present study supports the notion of a systemic immune modulatory anti-inflammatory effect of the two tested soy-extracts, which may target the inflammatory pathways activated in these models.

In the both models of NASH, oral administration of the combination of OS and M1 was associated with alleviation of liver damage and a decrease in plasma levels of cholesterol and/or triglycerides, decrease of hepatic content of triglycerides, improved liver histology and alleviation of insulin resistance. These effects were associated with skewing the immune profile towards an anti-inflammatory paradigm as manifested by a decrease in TNF-α serum levels and alteration in the distribution of different subsets of regulatory lymphocytes.

Tregs are important for the control of the immune response in the setting of immune mediated disorders[38,39]. They play a role in protection from liver damage in immune-mediated hepatitis, NAFLD, NASH, insulin resistance and metabolic syndrome[40-43]. The results of the present study suggests that the two tested soy extracts altered the distribution of Tregs, further supporting the notion that immune modulatory

therapy may be beneficial in NASH[11].

Previous studies showed that animals fed with soy manifested a reduction in total cholesterol, triglycerides, and blood pressure[43]. Soy protein lowered plasma cholesterol concentrations, and body fat accumulation in the animal model of NASH[24]. Soy protein intake decreased the hepatic lipid depots of triacylglycerols and cholesterol, and decreased the concentrations of lipid peroxides[24]. Rats fed a soy protein diet showed improved anti-oxidative potential due to increases in superoxide dismutase and catalase activities, and a decrease in the protein expression of cytochrome P450 2E1[24]. Soy derived products decreased the amount of subcutaneous and mesenteric fat[24]. Plasma leptin levels were down-regulated and the HFD-induced increase in liver weight and lipid accumulation in the liver were suppressed[44]. Genistein, a soy isoflavone, improves insulin sensitivity[45]. Administration of genistein to fructose-fed rats significantly reduced biochemical and histological abnormalities, activating the antioxidant profile, decreasing IL-6 and TNF-α concentrations, ameliorating liver steatosis and insulin-resistance[45]. Soy protein reduced liver steatosis in the HFD model *via* induction of PPARalpha-regulated genes[46]. An association between the anti-inflammatory effect of isoflavones and the regulation of their immune function was described[47]. The effects of soy on *de novo* lipogenic carbohydrate responsive element binding protein (ChREBP) and anti-adipogenic Wnt signaling were recently described[48]. Isoflavones suppress ChREBP signaling *via* protein kinase A (PKA) and/or 5'-AMP activated protein kinase (AMPK)-

dependent phosphorylation, which prevents ChREBP from binding to the promoter regions of lipogenic enzymes. Isoflavones stimulate Wnt signaling *via* estrogen receptor-dependent pathways, which in turn inactivate glycogen synthase kinase-3 beta (GSK-3beta), transactivate T-cell factor/lymphoid-enhancer factor (TCF/LEF), degrade adipogenic peroxisome proliferator-activated receptor gamma (PPARgamma), and augment p300/CBP, the transcriptional co-activators of TCF/LEF[48].

Soy derived products are safe and beneficial in humans. Soy is part of the regular diet in many countries. Soybean is a rich source of vegetable protein, complex carbohydrates, polyunsaturated fat, soluble sugars and phytoestrogens[14-17]. Some soy proteins are also associated with fatty acids, saponins, isoflavones and phospholipids. Epidemiological studies showed that soy products might decrease the morbidity of NASH[23], lower serum lipids, reduce liver uptake of lipids, improve the anti-oxidant ability, and decrease insulin resistance[24]. Soy milk consumption was associated with better blood pressure control among diabetic patients with nephropathy[49]. Soy phytoestrogen supplementation reduced insulin resistance, hsCRP, and blood pressure[50]. Pasta enriched with soy improved endothelial function and had beneficial effects on cardiovascular risk markers in patients with type 2 diabetes[51]. Consumption of soy protein improved serum lipids in adults with type 2 diabetes[52]. Soy-protein consumption reduced proteinuria in type 2 diabetes with nephropathy[53]. A daily supplement of soy protein prevents the increase in subcutaneous and total abdominal fat in postmenopausal women[54]. A significantly greater improvement was observed in CVD risk factors in postmenopausal women on a program incorporating 30 g of soy protein and 4 g of phytosterols per day than with standard therapy[55]. In postmenopausal hypercholesterolemic women soy protein improves endothelial function regardless of changes in plasma lipoproteins[56]. Consumption of soy protein has been shown to improve blood lipid levels in nondiabetic subjects[16].

Oral immune therapy is an active process that uses the inherent ability of the GI tract's immune system to control unwanted systemic immune responses, by inducing Tregs that suppress the chronic inflammatory state associated with metabolic syndrome[57]. This may involve alteration of NKT-DC cell interaction in the gut. The interaction between DC and NKT cells in gut associated lymphoid tissue may contribute to the promotion of Tregs, thereby alleviating immune mediated target organ damage[12,58]. One possible mechanism for the effect of these extracts may be *via* the soy-derived glycosphingolipid-mediated alteration of NKT-dendritic cell (DC) cross talk in the gut immune system[57,59]. NKT cell function is dependent on the activity of glycosphingolipids[60]. β-glucosylceramide, an active ingredient in soy, was shown to exert an

immune modulatory effect in various models and was also tested in clinical trials[21,26,42,61-63]. The combination of several soy derived-β-glycosphingolipids had a synergistic effect in comparison to each other alone[64,65].

The data in the present study suggests that a combination of the two tested soy extracts exerts a synergistic effect on most of the clinical and immunological parameters tested. While the active molecules in each of the tested extracts remains to be determined, it is postulated that different compositions of the extracts activate different pathways of the immune system that may underline the outcome.

In conclusion, our study suggests that the oral administration of soy derived molecules exerts a hepatoprotective effect alleviating immune-mediated liver injury, hyperlipidemia, insulin resistance, and liver damage, in several models. Being soy-derived safe products, the OS and M1 extracts may become a treatment option for humans with NASH and metabolic syndrome.

COMMENTS

Background
Non-alcoholic steatohepatitis (NASH) is a growing health problem and no approved therapy is available. NASH is associated with a pro-inflammatory state. Soy derived molecules were suggested to exert an adjuvant effect *via* activation of innate immune cells in the gut wall, thereby alleviating immune mediated disorders. The aim of the present study was to determine the immune-modulatory and the hepatoprotective effects of oral administration of two soy extracts in immune mediated liver injury and NASH.

Research frontiers
Two soy extracts, M1 and OS, were orally administered to mice with Concanavalin A (ConA) immune-mediated hepatitis, to high-fat diet (HFD) mice and to methionine and choline reduced diet combined with HFD mice. Oral administration of OS and M1 had an additive effect in alleviating ConA hepatitis manifested by a decrease in ALT and AST serum levels. Oral administration of the OS and M1 soy derived fractions, ameliorated liver injury in the high fat diet model of NASH, manifested by a decrease in hepatic triglyceride levels, improvement in liver histology, decreased serum cholesterol and triglycerides and improved insulin resistance. In the methionine and choline reduced diet combined with the high fat diet model, the authors noted a decrease in hepatic triglycerides and improvement in blood glucose levels and liver histology. The effects were associated with reduced serum TNF-α and alteration of regulatory T cell distribution.

Innovations and breakthroughs
Oral administration of the combination of OS and M1 soy derived extracts exerted an adjuvant effect in the gut-immune system, altering the distribution of regulatory T cells, and alleviating immune mediated liver injury, hyperlipidemia and insulin resistance.

Applications
The results of the study suggest that these extracts can be further developed to be used for the treatment of non-alcoholic fatty liver disease and NASH.

Peer-review
The authors tried to determine the immune-modulatory and the hepatoprotective effects of oral administration of two soy extracts in immune mediated liver injury and NASH. Oral administration of the combination of OS and M1 soy derived extracts exerted an adjuvant effect in the gut-immune system, altering the distribution of regulatory T cells, and alleviating immune mediated liver injury, hyperlipidemia and insulin resistance. They present evidence for the idea that soy extracts are hepatoprotective and immuno-modulatory in several different models for both livers damage (ConA, HFD, MCR) and altered immune response. Overall, the evidence that there is something in the two extracts that ameliorates liver damage and alters the immune response is strong.

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