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The alcohol extract of North American ginseng (*Panax quinquefolius*) reduces fatty liver, dyslipidemia, and other complications of metabolic syndrome in a mouse model

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Running title: American ginseng root extract alleviates metabolic syndrome

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Abstract

We investigated if the North American ginseng could reduce development of the metabolic syndrome phenotype in a mouse model (ETKO) of the disease. Young ETKO has no disease but similarly to humans starts to develop the fatty liver, hypertriglyceridemia, obesity, and insulin resistance at 25-30 weeks of age, and the disease continues to progress by ageing. ETKO mice were given orally an ethanol extract of ginseng roots at 4 weeks and 32 weeks of age. Treatments with ginseng eliminated the ETKO fatty liver, reduced hepatic and intestinal lipoprotein secretion and the level of circulating lipids. Improvements by ginseng treatments were manifested in reducing the expression of genes involved in the regulation of fatty acid and triglyceride (fat) synthesis and secretion by the lipoproteins on one hand, and by stimulating the fatty acid oxidation and triglyceride degradation by lipolysis on the other hand. These processes altogether improved the glucose and fatty acid/triglyceride metabolism, reduced the liver fat load, and reversed the progression of the metabolic syndrome. These data established that treatments with the North American ginseng could alleviate metabolic syndrome by maintaining a better balance between glucose and fatty acid metabolism, lipoprotein secretion and energy homeostasis in disease-prone states.

Key words: North American ginseng extract; root of *Panax quinquefolius*, fatty liver, intestine, lipoprotein secretion, hypertriglyceridemia

Introduction

The abnormalities in lipid metabolism are central to obesity and development of metabolic syndrome. The excess of circulating free fatty acids (FFAs) could be generated by multiple factors including genetic predispositions, poor dieting, alcohol abuse, and even medical treatments (Musso et al. 2016; Liu et al, 2016). Chronically elevated FFAs interfere with glucose and lipid metabolism and action of insulin, inhibit mitochondrial energy production and induce inflammation (Ertunc and Hotamisligil 2016). The liver's essential role is to maintain the blood glucose levels within a narrow physiological range. The liver converts excess of glucose into FFAs and diacylglycerol (DAG) by the process of lipogenesis and stores them as triglycerides (TG) in the lipid droplets (Blasiolo et al. 2007; Liu et al. 2016). Stored TG is secreted from the liver by very-low density lipoproteins (VLDLs). In the circulation, VLDL TG content is progressively reduced by the action of lipoprotein lipase and hepatic lipase (Blasiolo et al. 2007; Singh et al. 2012; Geldenhuys et al. 2016). After TG removal, VLDL particles enriched in cholesterol are remodeled into low-density lipoproteins (LDL). LDL is returned to the liver for final degradation and if the liver is not efficient in absorbing and processing the LDL particles, they could accumulate in the circulation and cause atherosclerosis (Siri-Tarino and Krauss 2016).

Previous studies have shown that disturbances in lipid absorption within intestinal cells could also contribute development of dyslipidemia and fatty liver diseases (FLD) (Janarden et al. 2006; Singh et al. 2012). Dietary TG is incorporated into intestinal lipoprotein particles, chylomicrons, and secreted into the circulation (Julve et al. 2016). Chylomicron TG is processed by lipoprotein lipase and released FFAs are delivered to peripheral tissues. Similarly to LDL, the remaining chylomicron remnants are removed by the liver. Therefore, the fate of dietary lipids in the intestinal cells and circulation is tightly connected with the liver function, and if distorted it could contribute to development of metabolic syndrome (Singh et al. 2012; Visschers et al. 2013).

There has been a shift to natural health products and herbal medicines in the attempt to prevent symptoms that are associated with development of metabolic syndrome. Asian ginseng (*Panax ginseng* C.A. Meyer, Araliaceae) has demonstrated benefits against hyperglycemia (Yun et al. 2004), cancer (Helmes et al. 2004), and obesity (Mollah et al. 2009). Ginseng active components include a mixture of various types of saponins (ginsenosides), polysaccharides, fatty acids, peptides, and essential oils (Qiu et al. 2008). Ginsenosides of *Panax ginseng* contribute to anti-

depressant effects (Yamada et al. 2011) and cardiovascular protection (Joh et al 2011) and ginsenoside Rg3 is a potent anti-tumor agent (Liu et al. 2011). These and other previous studies suggested that ginseng may also have potential for the treatment of metabolic syndrome because of strong anti-inflammatory and antioxidant properties of common ginseng saponins (Thung et al 2012). The exact mode of action of ginseng saponins on lipid metabolism however has not been firmly established. This led us to investigate the beneficial effects of alcoholic extract of North American ginseng (*Panax quinquefolius*, Araliaceae) in Pcyt2 gene deficient mice (ETKO/Pcyt2^{+/-}), that similarly to humans develop obesity, hypertriglyceridemia and insulin resistance (Fullerton and Bakovic 2010; Fullerton et al. 2009; Fullerton et al. 2007).

The Pcyt2 gene encodes CTP:phosphoethanolamine cytidylyltransferase, the main regulatory enzyme in the ethanolamine phospholipid biosynthesis. The mouse heterozygous for Pcyt2 (ETKO) is genetically adapted to store the surplus of FFAs and DAG in the form of TG. This adaptation results in continual accumulation of lipids, leading to development of adult-onset obesity and metabolic syndrome. Therefore the objective of this study was to establish the potential of a well characterized ginseng ethanol extract on lipid metabolism and other complications of metabolic syndrome. This work includes novel effects of ginseng on lipoprotein secretion, postprandial lipid load and fatty acid oxidation which were not previously investigated (Thung et al 2012).

Materials and methods

Preparation of the North American ginseng extract (NAGE)

North American Ginseng- *P. quinquefolius* was obtained from the Ontario Ginseng Growers Association. Four-year old plants were collected in 2007 from five different farms in Ontario. Ginseng root extracts from each farm were prepared individually and combined to produce composite extracts which were used for simultaneous phytochemical and pharmacological studies under the Ontario Ginseng Innovation and Research Consortium (OGIRC). The experiments were performed during the same period when the first characterization of the NAGE extract appeared in the publication (Sen et al 2011 and Azike et al. 2011).

Ginseng root samples were extracted by Naturex (USA). Four kg of ground roots were soaked three times during 5h in 16L of 75% ethanol at 40°C. After extraction, the solutions were filtered and the solvent evaporated under vacuum at 45°C; the three pools were combined and concentrated until the total solids on a dry basis were around 60%. These concentrates were lyophilized with a freeze dryer (Labconco, USA) at -50°C under reduced pressure to produce alcoholic ginseng extracts (NAGE) in a powder form. The yields of the final NAGE from the initial ground root was 35.30±5%.

The composition of NAGE ginsenosides (100 mg/ml methanol) was performed with a Waters 1525 HPLC System with a binary pump and UV detector and a reversed-phase C18 column (Dikma Technologies, USA). Gradient elution consisted of [A] water and [B] acetonitrile at a flow of 1.3 mL/min as follows: 0 min, 80-20%; 0-60 min, 58-42%; 60-70 min, 10-90%; 70-80 min, 80-20%. Absorbance of the eluates was monitored at 203 nm. Ginsenosides (purity > 98%) used as standards for HPLC analysis were from Indofine Chemical Company. Stock solutions were in methanol at a concentration of 1000 µg/mL. NAGE contained 28.25% of total ginsenosides (Rb₁, Re, Rc, Rd, Rg₁ and Rb₂) with Rb₁ and Re as the two most predominant ginsenosides. No detectable levels of Rh₁ were measured (Azike et al. 2011). The NAGE composition used in this and three other independent studies (Sen et al. 2011, 2012 and 2013) is described in Table 1.

Animals and genotyping

ETKO mice were generated and genotyped as described previously (Fullerton et al. 2007). All of the procedures conducted were approved by the University of Guelph Animal Care Committee and were in accordance with guidelines of the Canadian Council on Animal Care. The mice were exposed to a 12h light/dark cycle beginning with light at 7:00 a.m. Male mice aging from 6-50 weeks of age were fed *ad libitum* a standardized diet (Harlan Teklad S-2335) and had free access to water.

Oral treatments with alcohol extract of American Ginseng:

We performed two trials, a prevention trial before obesity develops in the young ETKO mice (Trial 1) and an intervention trial (Trial 2) with older obese ETKO. The objective in Trial 1 was to prevent a rise in plasma glucose with ginseng treatments, and the objective in Trial 2 was to reduce already elevated lipids and glucose with ginseng treatments.

Trial 1: Prevention trial using non-obese mice: Groups included one-month old male and female mice (n=6-10 per group): a) control group untreated (U-Ctrl); b) control group treated with NAGE (T-Ctrl); c) ETKO untreated (U-ETKO) and d) ETKO treated with NAGE (U-ETKO). Untreated groups (U) were administered orally 100µL saline daily and treated groups (T) received 200 mg/kg/100 µL of NAGE daily. Oral gavage for all groups lasted 24 weeks when mice were 7-month old. The primary outcome was the serum glucose response to long-term NAGE.

Trial 2: Intervention trial using obese mice: Four groups included 8 month old male or female mice (n=6 per group): a) lean wild-type control group untreated (U-Ctrl); b) wild-type treated with NAGE (T-Ctrl); c) ETKO obese group untreated (U-ETKO) and d) ETKO obese group treated with NAGE (T-ETKO). Untreated groups (U) were administered orally 100µL saline daily and treated groups (T) received 200 mg/kg/100 µL of NAGE daily. Treatments for all groups lasted 1 month. This setup is an exact model of the approach employed by Lee et al. (2009) in determining the beneficial effects of wild ginseng in a rat model of diabetes when after a dose response tests, 200 mg/kg was chosen as the daily amount of extracts supplied to the rats. The same dose has been tested twice with the NAGE extract in our previous mouse studies (Sen at al. 2012 and Sen at al. 2013). The primary outcome was the serum lipid lowering and glucose response to short-term NAGE treatment.

Liver histology and lipid content

To examine liver histology, fresh liver were dissected, fixed in 10% formalin + phosphate-buffered saline and embedded in paraffin; 10- μ m liver sections were stained with hematoxylin and eosin and visualized by light microscopy. Tissue lipids were analyzed as previously (Fullerton et al. 2007). Plasma and tissue TG content was measured using a colorimetric assay (TR0100) from Sigma.

Lipase assays and liver lipoprotein secretion

Mice were injected via the retro-orbital plexus with 0.1 unit/g of heparin, and hepatic lipase (HL) and lipoprotein lipase (LPL) determined using 2.5 μ Ci/ml [3 H]trioleate as described (Singh et al. 2012). Differences in the rates of TG appearance in the plasma were a quantitative measure of the liver lipoprotein secretion. Mice were injected with 10% of poloxamer to inhibit plasma lipolysis (Singh et al. 2012). Blood was collected via the saphenous vein from 0-4h and plasma TG determined by the TG reagent kit (Sigma). Plasma TG content at different time points was expressed per total body mass (μ mol/kg).

Immunoblotting

AMPK, ACC, pACC and PKC α were determined by immunoblotting using 10% SDS-PAGE. Membranes were blocked with 5% milk in 1X PBST and incubated overnight at 4°C with anti-AMPK (Abcam), anti-ACC or anti-pACC (Abcam) and anti-PKC α (Santa Cruz) antibodies. Membranes were then incubated with the HRP-conjugated anti-rabbit IgG and visualized by chemiluminescence (Sigma-Aldrich).

Glucose tolerance test

Plasma glucose tolerance test (PGTT) was measured in mice fasted for 6h. Mice were injected intraperitoneally with 2g/kg of glucose and glucose measured in the venous blood immediately before injection and 15, 30, 60 and 120 min after the injection using an automatic glucose monitor. The obtained glucose time curves and the area under the curves (AUC) for the total differences between the NAGE treated and untreated mice were compared.

Oral lipid-load test and intestinal secretion

Differences in the intestinal chylomicron secretion were performed as previously (Singh et al. 2012). For TG turnover experiments, fasted mice were given an intragastric load of 200 μ l of olive oil and TG secreted in the blood circulation were determined. TG turnover was determined by the integration of TG content during the entire post-load period by comparison of the total differences of the area under the curves (AUC).

Plasma clearance and tissue uptake of lipid particles

Differences in the amounts of the blood lipids in NAGE treated and untreated mice were measured after intravenously injecting [3 H]TO radiolabeled lipid particles as described previously (Singh et al. 2012). The rate of plasma disappearance of [3 H]TO particles was measured at 0, 5, 10, 15 and 30 min after injection. Various tissues (liver, heart, muscle, adipose, kidney) from NAGE treated and untreated mice were also collected at the end of the treatment. The [3 H] activity incorporated in various tissues was determined in identical amounts of the homogenized tissues. The incorporated activity (dpm/mg tissue) was adjusted for the total radioactivity in the blood 30 min after the particle injection and compared among NAGE treated and untreated groups

Gene expression

The expression of liver and intestinal genes was determined as initially described (Fullerton et al. 2009, Singh et al. 2012). The liver genes tested included the lipogenic genes SREBP1, FAS, DGAT 1 and DGAT2 and the mitochondrial oxidation genes PGC1 α and PPAR α . The intestinal genes included the genes responsible for the lipid absorption and chylomicron formation, CD36, MGAT1, FATP4, and MTP. The primers and conditions were initially described (Fullerton et al. 2009, Singh et al. 2012). The MTP activity was measured using a fluorometric activity assay kit from Roar Biomedical (Sing et al 2012).

Statistical analysis

The measured values were expressed as means $\pm$ S.D. For glucose tolerance tests, data were analyzed with 1-way ANOVA, with the factor being NAGE treatment. The rest of the data (body weight, liver weight, plasma triglycerides, lipoprotein secretion, enzyme activities, lipid turnover) were analyzed by 2-way ANOVA, where the factors were NAGE and genotype (wild

type control mice and ETKO mice). The post hoc analysis was carried out by a least significant difference test ($p < 0.05$). There was no need for data to be log-transformed. For PCR and western blotting, differences between treatments were expressed as differences in band densities. The band densities were analyzed by ImageJ software. Statistical analysis for PCR and western blotting was performed by 2-way ANOVA. Graph Pad Prism software was used for all statistical analysis and graph preparations.

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Results

NAGE prevents and improves hyperglycemia

Since ETKO obesity progressively worsens over time (Fullerton et al. 2009) we first probed if an earlier supplementation of NAGE could prevent increase in blood glucose and development of hyperglycemia. The experiments started with 1 month old mice and lasted 24 weeks. As shown in Fig. 1A, 12 weeks of NAGE treatment has no effect on blood glucose. At that time the untreated ETKO glucose was not elevated. ETKO develops hyperglycemia in the following weeks but the continuation of NAGE treatment prevented its development. At 24 weeks of NAGE intake ETKO blood glucose was as in control mice (Fig. 1B). To establish if NAGE can also reduce already developed hyperglycemia we performed a second, 4 week trial using 8 month old ETKO (Fig. 1 C,D). NAGE significantly reduced ETKO fasting glucose and at the end of the trial the levels were comparable to the control mice. Thus, the results from both preventative (long term) and intervention (short term) trials indicated that NAGE displayed a strong glucose lowering effect as shown in human studies (Mucalo et al. 2012). In the next sections, we focused on the mechanisms of the NAGE lipid lowering effects.

NAGE reduces liver mass and plasma triglyceride content

We measured the effect of NAGE on body weight in the 4 week trial (Fig. 2A-C). NAGE mildly reduced ETKO body weight and did not change the weight of lean controls. Treated ETKO did not lose the visceral mass but had significantly reduced liver mass (Fig. 2B,C). The loss in the liver mass was apparent from the histological data (Fig. 2D, E). An abundant presence of lipid droplets in ETKO liver was diminished by the NAGE treatment. Furthermore, NAGE also reduced ETKO plasma TG that was otherwise elevated (Fig. 2F). These results showed that the main positive effects of NAGE treatments were on ETKO liver and plasma lipids.

NAGE normalizes triglyceride content and secretion from the fatty liver

The effects of NAGE on the accumulation of liver lipids and lipoprotein secretion are shown in Fig. 3. Liver microsomal transfer protein (MTP) is a well-known regulator of the lipoprotein assembly and secretion and as shown in Fig. 3A, ETKO MTP activity was 2-fold above the control levels. After 1-month of NAGE treatment the MTP activity was significantly reduced in

ETKO liver (Fig. 3A) and that coincided with a reduction in the liver TG and DAG content. Both TG and DAG were well above control levels in untreated ETKO liver (Fig. 3B). Finally, the reductions in the MTP activity and TG content coincided with the reduced VLDL secretion from the liver. The rates and the total amount of TG that appeared in the circulation indicated that NAGE lowered the lipoprotein secretion from ETKO liver but it did not modify the secretion from controls (Fig. 3C,D).

NAGE attenuates expression of lipogenic genes

As a further indication of NAGE positive effect on ETKO lipid lowering, we tested a set of the genes important for fatty acid synthesis and oxidation. The transcription factor SREBP1 and its target gene fatty acid synthase (FAS) were overexpressed in ETKO liver (Fullerton et al. 2009; Fullerton and Bakovic 2010) and NAGE significantly reduced their expression (Fig. 4A,B). There was only a minor effect of the NAGE treatment on DGAT1 and DGAT2 (Fig. 4C,F). The mitochondrial fatty acid oxidation gene PGC1 α was not affected while PPAR α that was upregulated in ETKO was reduced by NAGE (Fig. 4D,E).

NAGE reduces acetyl-CoA carboxylase and PKC α

The major function of acetyl-CoA carboxylase (ACC) is the formation of malonyl-CoA for fatty acid synthesis. The regulation of ACC is very complex and includes the allosteric activation with glutamate and citrate, transcriptional up regulation with SREBP1 and insulin, posttranslational activation by phosphatases and inhibition by phosphorylation with AMP activated kinase (AMPK) (Kraegen et.al. 2005). NAGE elevated the AMPK protein levels in both ETKO and control livers (Fig. 5A,B). ACC protein content and phosphorylation were highly elevated only in ETKO liver and they both were reduced to control levels after treatments with NAGE (Fig. 5 C,D). Interestingly, NAGE treatments stimulated the AMPK protein in both ETKO and control livers, without affecting ACC phosphorylation, suggesting a more general positive effect of NAGE on AMPK. Finally, PKC α negatively regulates the liver lipid metabolism and insulin signaling (Griffin et.al. 1999) and it was up regulated in ETKO liver (Fig. 5E,F). NAGE treatments reduced the PKC α protein to control levels, and that was a further indication that ETKO liver function improved after the NAGE treatments.

NAGE reduces intestinal lipid gene expression

ETKO has facilitated absorption of dietary lipids (Singh et al. 2012). As a test of NAGE effects on intestinal function, we assayed the expression of the genes for intestinal lipid absorption, synthesis and transport (Fig. 6). The lipogenic genes FAS and SREBP1 (Fig. 6A, F) and the fatty acid transporters CD36 and FATP4 (Fig. 6D,E) were particularly elevated in the ETKO intestine. NAGE treatments significantly reduced the expression of these genes in the ETKO intestine and did not modify the expression in the control intestine. Interestingly, the MGAT1 and DGAT1 expression were not considerably different between ETKO and controls and were not affected by the NAGE treatments (Fig. 6B,C).

NAGE reduces intestinal secretion and postprandial triglyceride levels

The positive aspects of postprandial NAGE activities were further investigated at the functional level by measuring intestinal lipid secretion and turnover (Fig. 7). We determined the plasma TG turnover after an intragastric load of olive oil. Untreated ETKO exhibited faster secretion of TG and TG remained in the plasma at higher levels than in untreated controls (Fig. 7A). NAGE did not affect plasma TG in control animals however significantly stimulated TG clearance and reduced the TG content in ETKO plasma (Fig. 7A,B).

To investigate the specific effect of NAGE on chylomicron secretion, the rate of secretion was monitored by the appearance of [^3H]trioleate (TO) in the plasma at various times after the oral lipid load. As shown in Fig. 7C NAGE treated ETKO had reduced secretion of intestinal [^3H]TO relative to untreated ETKO. The intestinal secretion of [^3H]TO remained unchanged in the control mice treated with NAGE. Finally, degree of reduction in the plasma TG in Fig. 7A-C agreed with the level of inhibition of the intestinal MTP activity and mRNA levels (Fig. 8A,B), altogether showing that NAGE strongly inhibited postprandial lipid absorption and secretion from ETKO intestine.

NAGE improves plasma clearance of triglyceride-rich lipoproteins

Plasma TG clearance was monitored by degradation of [^3H]TO labeled particles injected into the circulation (Fig. 8C). TG degradation was markedly improved in NAGE treated ETKO and remained unchanged in the control mice. To see if the facilitated [^3H]TO degradation resulted in an improved lipid uptake in peripheral tissues, the incorporated [^3H]TO activity was also monitored in various tissues (Fig. 8D). At the end of the [^3H]TO injection (30 min), NAGE

treated ETKO showed more efficient tissue uptake of the circulating [^3H] lipids, with major improvements observed in the liver and adipose tissue. These tissues are the most important for FA uptake and were the most impaired in the untreated ETKO.

To investigate whether the NAGE improved TG lipolysis, HL and LPL activities were measured in the post-heparin plasma (Fig.9A) and as a total lipolytic activity (TG hydrolase) in the heart and adipose tissue (Fig.9B). TG hydrolase activity was determined after removal of the surface LPL activity by heparin. As before (Singh et al. 2012), the ETKO plasma had reduced HL and LPL activities, and the plasma and organ activities significantly improved after the NAGE treatments (Fig. 9A,B).

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Discussion

ETKO hyperglycemia is accompanied with an elevated secretion of the liver (VLDL) and intestinal (chylomicrons) lipoproteins and an inefficient lipoprotein processing, causing hyperlipidemia. Remarkably, those pathological abnormalities were diminished only after one month of oral treatments with NAGE. The lipid droplets disappeared from ETKO liver, the lipoprotein secretion was reduced and the circulating triglyceride content normalized. NAGE also reduced the expression and activity of the liver microsomal triglyceride transfer protein (MTP), critical for VLDL and chylomicron synthesis and secretion. Finally, NAGE treatments increased the amount of AMP activated kinase (AMPK), the enzyme responsible for ATP production and fatty acid oxidation in mitochondria. Untreated ETKO had a diminished capacity for mitochondrial fatty acid oxidation and reduced levels of AMPK. Therefore the major target of NAGE includes the critical liver metabolic pathways, providing a better balancing of the glucose and fatty acid metabolism with the requirements for the energy production.

In addition to improving the liver post-absorptive metabolism, the NAGE also reduced the intestinal absorption and processing of dietary lipids. ETKO has an increased capacity to absorb dietary lipids. The NAGE treatments normalized those problems and contributed to the elimination of ETKO hyperlipidemia by decreasing the expression of intestinal genes responsible for the chylomicron synthesis and secretion. By measuring postprandial TG turnover we showed that in addition to reducing chylomicron secretion NAGE enhanced plasma TG degradation, the process regulated by plasma lipases. Separate measurements of lipase activities confirmed the turnover data, and established that stimulated plasma lipolysis was a significant contributing factor to the lipid-lowering effects of NAGE. The facilitated lipolysis was further assisted with faster lipid uptake and utilization by peripheral tissues, demonstrating general improvements in lipid handling. Finally, the better lipid handling also had positive effects on plasma glucose and insulin levels and reduced most complications of ETKO metabolic syndrome.

We previously showed that the dosage of NAGE used in this study (200 mg/kg body weight) prevented endothelial dysfunction (Sen et al. 2011) and diabetic nephropathy (Sen et al. 2012) by its glucose lowering and antioxidant activities. Furthermore, NAGE significantly prevented upregulation of extracellular matrix proteins and vasoactive factors in the heart and retina of the diabetic mice (Sen et al. 2013). Similarly to NAGE, a recent study confirmed the therapeutic

potential of tissue culture raised mountain ginseng adventitious root (TCMGARs) extract enriched with ginsenosides in treating hyperglycemia and diabetes. TCMGARs treatments with 250 and 500 mg/kg body weight significantly lowered the blood glucose, cholesterol and triglyceride content in streptozotocin-induced diabetic rats (Murthy et al. 2014).

The predominant NAGE ginsenosides are Re, Rb1, and Rc (31.6, 58.1 and 4.45 mg/g-dry weight respectively) and NAGE was efficient in lowering glucose and lipid levels, due to richness in those specific ginsenosides. Individually, Re is known to exhibit potent antidiabetic effects (Attele et al. 2002), Rc is antiadipogenic (Kim et al. 2014) and Rb1 improves insulin signaling and fatty liver by activating AMPK (Shen et al. 2013, Shen et al. 2015) and by down-regulating intestinal transport (Wang et al 2015), the effects similar to those observed in the present study. TCMGAR contains significantly less Re, Rb1, and Rc (0.2, 4.1 and 2 mg/g-dry weight respectively) and have Rg3 (11.2 mg/g dry weigh) and Rh2 (3.8 mg/g dry weigh), which may explain TCMGARs impact on lowering triglyceride and glucose (Murthy et al. 2014, Lee et al. 2015) but at somewhat higher doses than NAGE.

Recent systematic reviews assessing the efficacy and safety of ginseng varieties in any type of disease or in healthy individuals reported that they are generally safe and particularly promising for improving glucose metabolism and immune function, with implications for several diseases including type 2 diabetes (Shergis et al. 2013, Shishtar et al. 2014). Vuksan's group conducted a systematic review and meta-analysis of randomized controlled trials focussing on the effect of variety of ginseng species on fasting blood glucose in people with and without type 2 diabetes. They showed that ginseng supplementation could modestly, but significantly improve fasting blood glucose in those individuals (Shishtar et al. 2014). The same group also performed systemic analysis for the effects of ginseng on lowering high-blood pressure, however results of this analysis were less conclusive (Komishon et al. 2016). A meta-analysis focusing on ginseng effects on FLD and lipoprotein metabolism is urgently needed. The newest recommendations from the North American Society of Pediatric Gastroenterology Hematology and Nutrition (NASPGHAN) is that obese children should be screened for non-alcoholic FLD (Vos et al. 2017) and treated by preventive nutritional and life-style strategies. In overweight adult patients with non-alcoholic FLD, the liver function and adipokine levels could be improved after 3 weeks of red ginseng treatment (Hong et al. 2016). Alcoholic FLD (Gyampy and Wan 2010) could also

be significantly reduced by red ginseng treatment (Bang et al. 2014). Right now there are no effective drugs on the market for the treatment of FLD and some available treatments could in fact exacerbate not reduce the FLD. In addition, there are conditions where drug treatments themselves could trigger development of FLD, such as antipsychotic drugs and anti-HIV drugs. In case of type 2 diabetes, ironically the best glucose lowering drugs increase not reduce FLD and that further exacerbate the disease (Lund and Knop 2012). In type 2 diabetes, there is a vicious cycle between the poor diet, FLD and drug treatments. Our preclinical data suggests that NAGE supplementation could be beneficial not only for glucose lowering but also for prevention of hypertriglyceridemia and FLD. We showed that NAGE could prevent those complications by reducing lipid (fat) formation from glucose, by reverting liver and intestinal lipoprotein secretion and by improving general lipid distribution and metabolism. Our data indicate that NAGE treatments could provide a mechanism to prevent the vicious glucose-lipid cycle and reverse the metabolic disease. Further clinical studies are however needed to explore ginseng's potential as an effective treatment for human conditions caused by poor dieting, alcohol consumption or drug therapies.

Abbreviations: diacylglycerol (DAG), triglycerides (TG), free fatty acids (FFA), very-low density lipoproteins (VLDLs), fatty liver diseases (FLD), Pcyt2 gene deficient mice (ETKO/Pcyt2^{+/-}), North American ginseng extract (NAGE), hepatic lipase (HL), lipoprotein lipase (LPL), plasma insulin tolerance test (PITT), plasma glucose tolerance test (PGTT), area under the curves (AUC), trioleate (TO).

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Conflict of Interest

There are no conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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Figure legends

Figure 1. Improvements in ETKO fasting blood glucose by NAGE treatments. (A,B) Prevention trial using non-obese mice: (A) Glucose tolerance test showing that a long-term usage of NAGE for 12 weeks has no effect on blood glucose in 4 month old ETKO. At that age untreated ETKO (U-ETKO) have normal blood glucose as untreated controls(U-Ctrl). (B) Continuous treatment with NAGE for 24 weeks showed positive effect on blood glucose in 7 month old ETKO (T-ETKO vs U-ETKO). At that age untreated ETKO has elevated blood glucose ((U-ETKO vs. U-Ctrl). Groups included one-month old male and female mice (n=6-10 per group); Untreated groups (U) were administered orally 100 μ L saline daily and treated groups (T) received 200 mg/kg/100 μ L of NAGE daily. Oral gavage for all groups lasted 24 weeks when mice were 7-month old. (C) Intervention trial using obese mice: Glucose tolerance test showing that a short-term (4 weeks) usage of NAGE has a glucose lowering effect in 8 month old ETKO (T-ETKO). At that age untreated ETKO (U-ETKO) is obese and has elevated blood glucose relative to 8 month old control mice (U-Ctrl). Untreated (U) mice were administered orally 100 μ L saline daily and treated groups (T) with 200 mg/kg/100 μ L of NAGE daily. At the end of all trials (A, B and C), mice were injected intraperitoneally with 2g/kg of glucose and change in blood glucose measured 0-120 min. Results are expressed as an average change in glucose concentration (mg/dl $\pm$ S.D.) in time (min). Total changes in plasma glucose clearance are compared from the integrated areas under the curves (AUC) in 4-week (D) and 24-week NAGE trials (E). Differences in blood glucose and the AUCs from multiple measurements were analyzed by one-way ANOVA. The obtained P values are shown and groups different from all other groups indicated as (*).

Figure 2. Reductions in body-weight, fatty liver and plasma lipids. (A) Change in the ETKO body weight after treatments with NAGE. (B) The liver weight was reduced in the NAGE treated ETKO (T-ETKO) but not in treated controls (T-Ctrl). (C) The mass of the visceral fat was only mildly affected by the treatments. ETKO fatty liver was reduced when stained with hematoxylin-eosin before (D) and after (E) treatments. (F.) Untreated U-ETKO had elevated triglyceride (TG) content in the plasma. After treatments (T-ETKO) the blood TG were reduced to the control levels (U-Ctrl). Comparison were performed by two-way ANOVA; (*) indicates significant differences between treated (T) and untreated (U) groups and genotypes (Ctrl and ETKO). P values for two-way ANOVA are indicated. For Fig.2C, significant differences existed only between the genotypes with the t-test value $p < 0.005$.

Figure 3. Reductions in the VLDL secretion, MTP activity and liver lipid content. (A) U-ETKO liver has upregulated MTP activity and treatments significantly inhibited the MTP activity in the T-ETKO liver. (B) At the same time, T-ETKO triglycerides (TG) and diacylglycerides (DAG) were significantly reduced relative to U-ETKO and U-Ctrl. Differences in A and B were determined by two-way ANOVA (*) and t-test (#) and corresponding P and p values are indicated. (C) Plasma TG content was determined at 0-4h after inhibition of degradation with the lipoprotein lipase inhibitor poloxamer. Changes in the rates of the plasma TG appearance reflect

the level of the VLDL secretion before (U) and after treatments (T). (D) The average area under curve (AUC) shows that the ETKO VLDL secretion was reduced and not different from controls after NAGE treatments. Data in C and D were analysed by two-way ANOVA with $P=0.0003$.

Figure 4. Improvements in the liver gene expression. The lipogenic genes (A) FAS, (B) SREBP-1c were upregulated in U-ETKO relative to U-Ctrl and treatments significantly reduced their expression. (C) DGAT-1 and (D) DGAT-2 expressions were not significantly different for any group of animals. The expression of the genes for fatty acid oxidation showed no change for PGC-1 α (E) or a minor reduction for PPAR α (F) in treated ETKO. Statistical significance between the NAGE treated (T) and NAGE untreated (U) ETKO and T and U Ctrl groups was determined by two-way ANOVA. P values are indicated for each gene separately.

Figure 5. Immunoblots showing improvements in ETKO AMPK, ACC and PKC α . (A) Liver AMPK protein in T-Ctrl and U-Ctrl, T-ETKO and U-ETKO groups and (B) densitometric analysis both showing that NAGE augmented the AMPK protein content in all treated groups. (C) Total ACC protein and (D) its phosphorylation (pACC) were abnormally high in U-ETKO and normalized with NAGE; there was no effect of NAGE on Ctrl. (E, F) PKC α was significantly elevated in U-ETKO and NAGE reduced PKC α in T-ETKO to Ctrl groups. The statistical analysis was performed using two-way ANOVA and the obtained P values are indicated.

Figure 6. Reductions in ETKO intestinal gene expression. (A) The fatty acid synthase (FAS), (D) fatty acid transporter protein 4 (FATP4), (E) fatty acid transporter CD36, and (F) sterol regulatory binding protein 1 (SREBP-1) mRNA were all significantly reduced in T-ETKO and not different from Ctrl levels. (B) diacylglycerol transferase 1 (DGAT-1) and (C) monoacylglycerol acyltransferase (MGAT1) were not significantly different among the four groups. Data analysis was performed using two-way ANOVA and the obtained P values are shown separately for each gene.

Figure 7. Improvements in postprandial lipid turnover and secretion. (A) T- and U-ETKO and T- and U-Ctrl groups were given an intragastric bolus of 200 μ l of olive oil and blood lipid measured at different time points (0-6 h) after the lipid load. Changes in blood TG content (μ mol/kg) $\pm$ SD (n=6) were plotted against the time, and (B) differences in plasma TG turnover compared from the integrated areas under the curve (AUC). (C) After an intragastric load of [3H]TO in 200 μ l of olive oil, the [3H] was counted at 0-180 min and plasma counts (dpm/ml $\pm$ SD for six mice in each group) were plotted against the time. Data analysis was performed using two-way ANOVA and the obtained P values are indicated (*).

Figure 8. Intestinal MTP activity and plasma lipid clearance. (A) The MTP activity is expressed as fluorescence (F) units/min/ μ g of protein. U-ETKO intestine had upregulated MTP activity and NAGE treatments significantly inhibited the MTP activity. (B) Intestinal MTP mRNA expression was significantly reduced in both, T-ETKO and T-Ctrl groups. (C) The clearance rate

of lipid particles from the circulation was slower in U-ETKO relative to U-Ctrl and was significantly improved by NAGE. The initial [3H]TO dose was 100x10³ dpm. The radiolabelled particles were counted at different times (2-30 min) post-injection. (D). Delivery of plasma [3H]TO particles to the peripheral tissues (liver, heart, muscle, adipose, and kidney) was determined 30 min after injection. The total radioactivity is expressed as a dpm/g tissues for 4 animals in each group. The postprandial lipid uptake was significantly improved in T-ETKO liver, heart, adipocytes and kidney, as indicated by two-way ANOVA and P values in each tissue.

Figure 9. Improvements in ETKO lipolysis. (A) NAGE treated ETKO shows improved TG lipolysis. The plasma hepatic lipase (HL) and lipoprotein lipase (LPL) activities were determined using radiolabeled substrate (2.5 µCi/ml [3H]TO). ETKO has reduced HL and LPL activities; the NAGE treatments stimulated their activities in ETKO but not in Ctrl mice. The activities were expressed as [FFA] µmol/h/g tissue ± SD (n= 6) at P<0.001 for HL and P<0.0004 for LPL. (B) NAGE stimulated the tissue lipases in T-ETKO but not in T-Ctrl ; the activity was measured as [FFA] µmol/h/g tissue ± SD for n= 4 and t-test values *p <0.05 and **p<0.001.

Draft

Table 1. Ginsenoside content in the American ginseng alcohol extract and dry roots

Content /Ginsenoside	Rg1	Re	Rb1	Rc	Rb2	Rd	TOTAL
mg/g Extract	2.9	89.6	164.5	12.6	1.8	11.1	282.5
% Extract dry weight	0.29	8.96	16.45	1.26	0.18	1.11	28.25
mg/g Ginseng root	1.02	31.63	58.07	4.45	0.64	3.92	99.72
% Root dry weight	0.1	3.16	5.81	0.45	0.06	0.39	9.972

Draft

A. 12 weeks of NAGE; young ETKO **B. 24 weeks of NAGE; young ETKO** **C. 4 weeks of NAGE ; old ETKO** **D. 4 weeks of NAGE; old ETKO** **E. 24 weeks of NAGE; young ETKO** **Figure 1**

A. Total Body weight

B. Liver weight

D. Untreated fatty liver

C. Visceral fat weight

F. Plasma lipids

E. NAGE treated fatty liver

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Figure 2

A. Liver MTP activity **B. Liver triglycerides and diglycerides** **C. VLDL secretion** **D. Total change in VLDL secretion** **Figure 3**

A. Fatty acid synthase

B. SREBP1

C. DGAT1

D. PGC1- α

E. PPAR- α

F. DGAT2

Figure 4

A. AMPK protein **E. PKCα protein** **C. ACC protein** **D. ACC phosphorylation** **F.** **Figure 5.**

Figure 6.

A. Postprandial lipid turnover **C. Chylomicron secretion** **B. Total change in postprandial lipids** **Figure 7.**

A. Intestinal MTP activity

B. Intestinal MTP expression

C. Degradation of postprandial lipids

D. Organ uptake of postprandial lipids

Figure 8.

A. Plasma lipases **B. Tissue TG hydrolases** **Figure . 9**