Chapter 4

Linking biological activity with herbal constituents by systems biology-based approaches: effects of *Panax ginseng* in type 2 diabetic Goto-Kakizaki rats

Abstract

Although a number of animal experiments and clinical trials have investigated the effects of ginseng roots on diabetes, the relationship between their therapeutic effects on diabetes and the quality and the growth age of this herb have not yet been reported. This study systematically investigated the effects of 3- to 6-year-old ginseng roots on glycemic and plasma lipid control in a rat model of type 2 diabetes. Six groups of male Goto-Kakizaki (GK) rats received either metformin, 3- to 6-year-old ginseng roots, or no treatment. The treatments were administered twice daily for 9 weeks. A combined approach was used that involved applying liquid chromatography–mass spectrometry-based lipidomics, measuring biochemical parameters and profiling the components of ginseng roots of different ages. Compared to the untreated controls, treatment with 4- and 6-year-old ginseng roots significantly improved glucose disposal, and 5-year-old ginseng treatment significantly increased high density lipoprotein cholesterol. Treatment with 6-year-old ginseng significantly decreased total plasma triacylglyceride (TG) and very-low-density lipoprotein cholesterol and improved plasma glycated hemoglobin (HbA1c). In addition, treatment with 4- to 6-year-old ginseng influenced plasma lipidomics in diabetic GK rats by reducing TG lipid species. Metformin significantly reduced fasting blood glucose by 41% and reduced HbA1c by 11%, but showed no effects on the plasma lipid parameters. The present study demonstrates that ginseng roots show growth age-dependent therapeutic effects on hyperlipidemia and hyperglycemia in diabetic GK rats. These age-dependent effects may be linked with the variation in both the ratios and concentrations of specific bioactive ginsenosides in ginseng roots of different growth ages. This study introduced novel systems biology-based approaches for linking biological activities with potential active components in herbal mixtures.

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Introduction

The incidence and prevalence of diabetes, particularly type 2 diabetes (T2D), are increasing rapidly worldwide.[1] T2D is a chronic metabolic disorder characterized by increased insulin resistance, impaired insulin secretion, and progressive β-cell dysfunction leading to hyperglycemia.[2,3] The majority (50–70%) of T2D patients have an atherogenic profile, manifested as an elevation in plasma triacylglyceride (TG) and apolipoprotein B levels, a decrease in high-density lipoprotein cholesterol (HDL-C) levels, and a preponderance of small, dense, low-density and very-low-density lipoprotein (LDL and VLDL) particles.[2-4] Individuals with T2D have a substantial risk of morbidity and mortality from major coronary events, such as cardiovascular, cerebrovascular, and peripheral vascular diseases.[5-7] To this end, the prevention and treatment of diabetes is crucial to reduce the risk factors for diabetic complications.

Clinical trials have provided adequate evidence that hyperglycemia and hyperlipidemia are fundamental risk factors for the vascular complications associated with diabetes.[8-9] To reduce these two risk factors, proper diet and regular exercise have been emphasized as a high priority. Lipid-lowering and glycemia-lowering medications are currently used to treat diabetes and its associated complications in mainstream medical approaches.[10] However, the current drug treatments for diabetes have three major limitations: 1) the high frequencies of side effects, 2) the high rates of secondary failure, and 3) the rapidly increasing costs of new diabetic drug development. Due to these concerns, researchers have been searching for more effective and multi-targets therapies. This strategy differs from the ‘one drug fits all’ treatment concept and may reduce side effects.

Natural products, such as herbs, have a much longer history of use in the treatment of diabetes, than modern pharmaceuticals. For example, in China, as early as the period from 1368 to 1644, the root of *Panax ginseng* C.A. Mey (often simply referred to as ginseng in this text) was reported to treat diabetic symptoms in an ancient medicinal textbook titled *Compendium of Materia Medica* (*Ben Cao Gang Mu*) by Shizhen Li (1518–1593). Since then, the popularity of treating illnesses like diabetes with ginseng has continued to grow worldwide. Reports obtained from *in vivo* animal experiments and clinical trials have demonstrated that radix ginseng, the dried root of *Panax ginseng* C.A. Meyer, has a wide range of therapeutic effects on the central nervous system, the cardiovascular system, and the immune system.[11-13] Pharmacological studies have indicated that radix ginseng can ameliorate diabetes by improving glucose homeostasis and insulin sensitivity and alleviate diabetes-induced oxidative stress by inhibiting lipid peroxidation.[14-16] However, the lack of standardization in quality control and quality assurance in producing ginseng and ginseng products often leads to inconclusive results when they are used to treat diabetes.[17] In part, this lack of standardization is due to the complex chemical composition of ginseng roots, which exhibit regional variation and age-dependent variation in growth that influence the compositional ratios of certain ginsenosides.[18] Sengupta *et al*.[17] observed that reconstituting a ginseng extract by adding two ginsenosides, Rg1 and Rb1, in a defined ratio could alter the angiogenic outcome: the dominance of Rg1 led to angiogenesis, whereas the dominance of Rb1 exerted an opposing effect. These observations strongly

suggest that quality control and quality assurance must be addressed when making ginseng products.

Ginseng is a perennial herb that can grow for dozens of years. The major components in ginseng are ginsenosides, which are responsible for most of ginseng's biological and pharmacological activities. Ginseng cultivators have long observed that the ginseng growth period is directly related to its therapeutic quality. Ginseng grown for ≥ 4-year-old is regarded to be of the appropriate quality for medicinal use.[19] Although ginseng has been demonstrated to have beneficial effects on diabetes management, no research has yet been performed to determine how the ginseng's age and its related quality affect its therapeutic efficacy.

With these considerations, we used systems biology based metabolomics approaches to evaluate the therapeutic effects of ginseng roots grown for 3–6 years on the regulation of hyperglycemia and dyslipidemia in a Goto-Kakizaki (GK) rat model with spontaneous T2D. The GK rat model displays fasting hyperglycemia and has a non-obese phenotype.[20] Metformin, a widely used oral medicine in T2D treatment to reduce hepatic gluconeogenesis and improve glucose uptake,[21-22] was administered as a positive control. We aimed to evaluate possible ginseng-induced effects on lowering glucose and improving glucose tolerance. We hypothesized that ginseng roots show growth age-dependent effects on improving glycemia and lipid metabolism in diabetic GK rats. We hypothesized that these age-dependent effects may be due to the fact that the ratios and concentrations of specific ginsenosides in the ginseng roots change during growth.

Experimental

Chemicals and reagents

Liquid chromatography grade dichloromethane (CH_2Cl_2), methanol (MeOH), isopropanol (IPA), and acetonitrile (ACN) were purchased from Tedia (Fairfield, USA). Distilled water was purified using a Milli-Q system (Millipore, Bedford, MA, USA). Analytical grade ammonium formate (AmFm) was obtained from Sigma-Aldrich (St. Louis, USA). Analytical grade glucose was from Chengdu Kelong Chemical Reagent Plant (Chengdu, China). Chemical grade sodium carboxymethylcellulose was purchased from Tianjin Guangfu Fine Chemical Research Institute (Tianjin, China). Ginseng samples, harvested after 3–6 years of growth, were purchased as dried roots from Fusong Chengan Ecology Ginseng Co., Ltd. (Jilin, China). Nine ginsenoside standards, including Rb1, Rb2, Rb3, Rc, Rd, Re, Rf, Rg1, and Rg2, were purchased from Chengdu Cogon Bio-tech Co., Ltd. (Chengdu, China). Metformin was purchased from Shandong Linuo Kefeng Pharmaceutical Company Co., Ltd. (Shandong, China). Synthetic lipid standards were purchased from Avanti Polar Lipids, Inc. (Alabaster, Alabama, USA) and Sigma-Aldrich (Munich, Germany). Digoxin and leucine-enkephalin was obtained from Sigma-Aldrich (Munich, Germany).

Ginseng plant cultivation

Radix Panax ginseng C.A. Mey was used in this study. All of the ginseng plants were cultivated at one farm located GAP station in a village (Fusong Chengan Ecology Ginseng Co., Ltd) in Jilin Province (Northern China). Ginseng plants of different ages were cultivated at the same location and harvested at the same time (*i.e.* in autumn). After harvesting the plants, the ginseng roots were air dried in the sun. According to Chinese Pharmacopeia, the water content is not more than 12.0% and total ash is not more than 5.0% and acid-insoluble ash is not more than 1.0%. The main ginseng roots containing secondary roots without root hair were used in the present study.

Animal protocols and ginseng or metformin administration

All animal experiments were approved by an institutional ethical committee on animal care and experimentation under authorization number 113 (West China School of Pharmacy, Sichuan University, Chengdu, China). Four-week-old, male GK rats, weighing 260–320 g, were purchased from Shanghai Slac Laboratory Animal Co., Ltd. (Shanghai, China). All animals were housed in a temperature-controlled room with a 12-h light/dark cycle. Food and water was freely available, except during fasting periods before some experiments.

After a one-week adaptive feeding period, 37 GK rats were randomly divided into six groups for a 9-week treatment intervention: group 1, non-treated control (n = 5); group 2, positive control (n = 8) receiving intraperitoneal administration of metformin at a dosage of 75 mg/kg body weight/day, groups 3, 4, 5, and 6 (n = 4, 5, 5, and 5, respectively), receiving intraperitoneal administration of 3-, 4-, 5-, or 6-year-old ginseng roots, respectively, at a dosage of 1.875 g/kg body weight/day, twice daily. Both ginseng and metformin were ground into powder and mixed with 0.5% sodium carboxymethylcellulose before administration. The non-treated animals were injected with 0.5% sodium carboxymethylcellulose at 2× 1 mL/100 g body weight/day without any herbal medicine or drug. No adverse effects were observed in animals after treatments with ginseng roots, metformin, or the vehicle.

Notably, plasma biochemical parameters including total cholesterol (TC), TG, HDL-C, low-density lipoprotein cholesterol (LDL-C), very-low-density lipoprotein cholesterol (VLDL-C), and plasma glycated hemoglobin (HbA1c) were measured at the end of the experiment. Fasting blood glucose (FBG) was measured at week 0 and week 9. The intraperitoneal glucose tolerance test (IPGTT) was also performed at week 9. In addition, body weight and food intake were measured weekly throughout the experiment.

Table 1 Study design and time points at which both clinical parameters and lipidomic profiling were done

Animal	Treatments	Group size	
Diabetic GK rat	non-treated control	5	
	metformin (positive control)	8	
	3 yr old ginseng	4	
	4 yr old ginseng	5	
	5 yr old ginseng	5	
	6 yr old ginseng	5	
Randomization after adaptively fed for one week			sampling
Body weight and food intake			wk0 ~ wk9
Fasting blood glucose			wk0, wk9
Glucose tolerance test			wk9
Plasma cholesterol and triglycerides			wk9
HDL-C			wk9
LDL-C			wk9
VLDL-C			wk9
HbA1c			wk9
Sacrifice with plasma collection for lipidomics			wk9

Sacrifice and sample collection

Animals were sacrificed with rapid asphyxiation with CO2 and opened longitudinally after the 9-week experiment (endpoint). Blood was collected via saphenous vein puncture before the start of the treatment (*i.e.* t = week 0) and via heart puncture after sacrifice (*i.e.* t = week 9) in CB 300 LH microvettes (Sarstedt, Nümbrecht, Germany), containing lithium heparin, and the samples were placed on ice immediately after collection. Plasma samples were obtained after centrifugation at 3000g for 5 min at 4 °C. Aliquots of plasma were frozen and stored at –80 °C until use.

Plasma lipid biochemical parameters

Plasma TC and TG were measured by the fully automatic enzymatic method using enzymatic kits obtained from Shanghai Fosun Long March Medical Science Co., Ltd. (Shanghai, China). Plasma HDL-C, LDL-C, and VLDL-C were measured using enzymatic kits purchased from Wenzhou Dongou Jinma Bio-tech Co., Ltd. (Wenzhou, China). All measurements were carried out on plasma samples taken after animals were fasted for 4 h.

Plasma glycemic metabolic parameter

HbA1c was measured using an immune-based assay. After the animals were fasted for 4 h, FBG concentrations were measured before and after treatment. Blood glucose levels were

determined in blood samples taken from the tail vein with a glucose assay kit using the GOD-PAP method (Sichuan Maker Bio-tech Co., Ltd., Chengdu, China). The IPGTT was performed on the final day of treatment on the 9th week using an intraperitoneal administration of glucose (1.25 g/kg body weight) after the animals were fasted for 4 h. Blood glucose levels were also determined in blood samples from the tail vein at 0 min (prior to glucose administration), and 30, 60, 120, 180 and 240 min after the glucose administration.

Liquid chromatography–mass spectrometry (LC-MS) lipid profiling

Lipid extraction. Lipid extracts were obtained via a modified Bligh/Dyer extraction procedure, as described previously.[23] Briefly, 30 μL of internal standard (I.S.) mixture (see Supplementary Table S1 for details) were added to 30 μL of plasma followed by the addition of 540 μL of 2:1 CH_2Cl_2/MeOH. The mixture was thoroughly vortexed, and then 120 μL of water was added to form a two-phase system. The lipids were dissolved in the bottom organic phase. After being centrifuged at 6000*g* for 10 min at 10 °C, 100 μL of the lipid extracts from the bottom layer were transferred and diluted 5 times with ACN/IPA/water (65:30:5, v/v/v). Then 10 μL of the lipid extracts were loaded for LC-MS lipid profiling analysis. Notably, each sample was prepared in duplicate and each prepared sample was injected once.

LC-MS analysis. LC-MS lipidomics analysis was performed on a hybrid ion-trap time-of-flight mass spectrometer (IT-TOF-MS; Shimadzu, Kyoto, Japan). The mass spectrometer was coupled with an ultra fast liquid chromatography (LC) system (Shimadzu, Kyoto, Japan). An Ascentis® Express C_8 column (2.7 μm particle size, 90 Å, 2.1 × 150 mm; Sigma-Aldrich, Munich, Germany) was used for the LC separation. The LC separation conditions used were identical to the previously published methods.[23] The plates with diluted lipid extracts were maintained at 12 °C.

Plasma lipid profiling was carried out on the Shimadzu IT-TOF-MS equipped with an electrospray ion source. MS survey scans were acquired in the positive ion mode. The voltages of the interface and the detector of the TOF analyzer were set to 4.5 kV and 1.6 kV, respectively. The temperatures of the curved desorption line and heat block were both set to 200 °C. The flow rate of the nebulizing gas was 1.5 L/min. The dry gas pressure was 0.2 MPa. The flight tube temperature was stable at 40 °C, and the ion trap pressure was maintained at 1.6×10^{-2} Pa. Ultra-high purity argon was used for collision and ion cooling. The data were collected at a mass range of *m/z* 400–1500 with an ion scan duration of 20 ms using LCMS solution software (Shimadzu, Kyoto, Japan).

All study samples were randomly analyzed. Quality control (QC) samples, prepared by pooling all of the plasma samples, were regularly measured in the sequence to monitor the response of the LC–MS system and assess the lipid profiling platform.

LC–MS ginsenoside profiling

Ginseng extraction. Thirty milligrams of powdered ginseng root were weighed in a new 2 mL Eppendorf vial followed by the addition of 1 mL MeOH/water (4:1, v/v) containing 0.32 µg/mL digoxin, which was used as the IS for ginsenoside analysis. After thoroughly vortexing the mixture for 3 min, the resulted suspension was placed in a shaker and incessantly shaken for 1 h at room temperature. The suspension was then placed in an ultrasonic bath at 4 °C for 1 h. Next, the suspension was centrifuged at 10000*g* for 10 min at 20 °C. Subsequently, 600 µL of supernatant was transferred and filtered using a Whatman polypropylene syringe filter with a 0.2 µm pore size and a 25 mm diameter (Whatman, Netherlands B.V.). The filtered ginseng extracts were diluted 5 times with MeOH/water (1:1, v/v) before being analyzed by LC-MS.

Ginsenoside analysis. The diluted ginseng root extracts were analyzed on an Acquity™ ultra-performance liquid chromatography/quadrupole time-of-flight mass spectrometry (UPLC/Q-TOF MS, Synapt™ G1 HDMS system, Water Corp., Milford, USA) equipped with an MS pump, an autosampler (Waters Corp., Milford, USA), and a Waters UPLC T_3 column (2.1 mm × 100 mm, id 1.8 µm, Waters Corp., Milford, USA). The separation was carried out with a binary solvent consisting of water (15 mM AmFm) and ACN. The binary gradient started with 20% B until 0.5 min, increased to 21% B from 0.5 to 7 min, to 30% B from 7 to 10 min, to 40% B from 10 to 18 min, to 80% B from 18 to 20 min, and was maintained at 80% B from 20–23 min. Then from 23–23.1 min, solvent B was decreased to 20%, and it was then maintained for 4.9 min for column re-equilibration. The flow rate was 0.50 mL/min. The column oven temperature was set to 35 °C, and the temperature of the autosampler tray was maintained at 10 °C. The following parameters were used for mass spectrometry: capillary voltage, 2.5 kV; reference cone voltage, 50 V; sampling cone voltage, 35 V; extraction cone voltage, 4.0 V; source temperature, 80 °C; desolvation temperature, 400 °C; desolvation gas flow, 700 L/h; cone gas flow, 20 L/h; reference scan frequency, 10 s; scan time, 1.0 s; interscan time, 0.02 s; and lock mass, 554.2615 (leucine-enkephalin). The ginsenoside profiling was acquired under negative ionization mode. The ginsenoside profiling data were recorded by using MassLynx™ (MassLynx V4.1 SCN 639).

Before carrying out the ginsenoside profiling of the ginseng root samples, a compact validation of the method was performed, and the analytical characteristics were evaluated in terms of linearity, repeatability, recovery, and limit of detection (LOD) for nine, representative ginsenoside standards. Digoxin was used as the I.S. The results of the compact method validation were satisfactory for the profiling analysis of complex herb mixtures.[24]

Statistical analysis

All measured biological parameters are presented as means ± SD. Univariate statistics were performed with SPSS (Statistical Product and Service Solutions) using one-way analysis of variance (ANOVA) with the two-sided Dunnett post hoc test for multiple

comparisons to investigate the differences between the control and treatment groups. Differences among groups were considered to be statistically significant if $p < 0.05$.

During the analysis of the lipid metabolism parameters, the appropriate lipid I.S. was used to correct the signal intensities of the lipids in each ionization mode (see Supplementary Table S1 for details). Thereafter, peak areas were calculated for each lipid species relative to the standards to account for the variability in MS signals among the different groups of samples. Principle component analysis (PCA) was chosen to visualize the clustering pattern related to the samples and the conditions.

Results

Treatment effects on biochemical parameters

Throughout the 9-week treatment period, neither food intake nor body weight was significantly changed in any of the treated rats when compared to the untreated controls. Within each group, food intake was comparable, and the body weight of the animals showed a continuous increase from week 1 to week 9 (data not shown).

The plasma biochemical parameters (*i.e.* TC, TG, HDL-C, LDL-C, and VLDL-C) were measured after 4 h fasting in all the GK rats at week 9. These results indicated that there was a significant decrease in the levels of TG (1.27 ± 0.33 *vs*. 0.85 ± 0.28 mmol/L, $p < 0.05$) and VLDL-C (0.58 ± 0.15 *vs*. 0.39 ± 0.13 mmol/L, $p < 0.05$) in the GK rats that received the 6-year-old ginseng root treatment compared to the untreated control. There was a significant increase in HDL-C (1.27 ± 0.13 *vs*. 1.48 ± 0.09 mmol/L, $p < 0.05$) in the GK rats that received the 5-year-old ginseng root treatment compared to the untreated controls. Additionally, there was a trend of improvement (*i.e.* $0.05 < p < 0.1$) in TG (1.27 ± 0.33 *vs*. 0.90 ± 0.27 mmol/L), HDL-C (1.27 ± 0.13 *vs*. 1.39 ± 0.15 mmol/L), and VLDL-C (0.58 ± 0.15 *vs*. 0.41 ± 0.12 mmol/L) levels in GK rats that received the 4-year-old ginseng root treatment *vs*. the untreated control. Interestingly, the metformin-treated animals did not show the above trends in the lipid biochemical parameters. The mean baseline (*i.e.* t = week 0) FBG in all of the treatment groups was comparable to that in the untreated control group (data not shown). At the end of the experiment, a significant reduction in FBG (9.36 ± 4.27 *vs*. 5.52 ± 1.26 mmol/L, $p = 0.03$) was observed only in the metformin treated group *vs*. untreated controls, but no reduction in FBG was observed in the ginseng treated groups.

No reduction in HbA1c was observed in animals following the treatments with 3- to 5-year-old ginseng roots at the end of the study; however, animals receiving treatment with either 6-year-old ginseng root or metformin displayed a decrease tendency of –11% ($p = 0.094$) and –11% ($p = 0.067$), respectively (vs. untreated controls) (Figure 1).

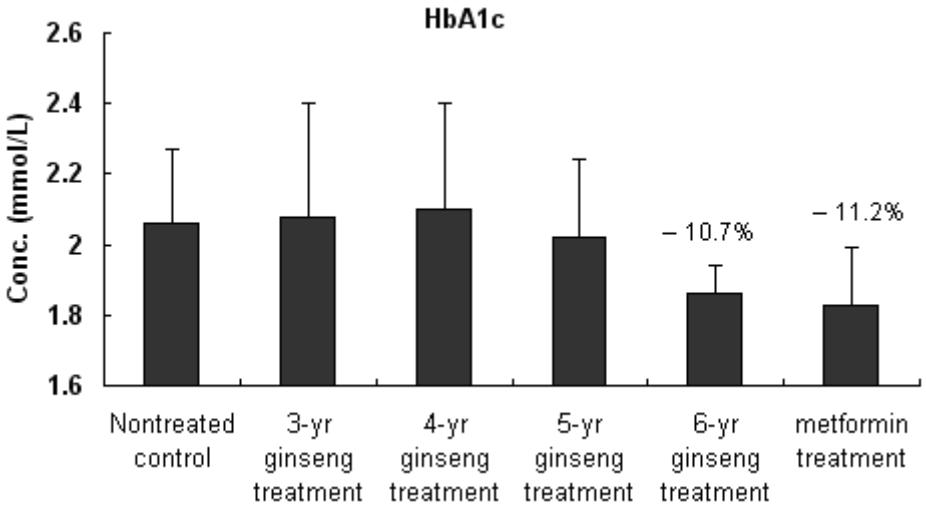

Figure 1. HbA1c levels in GK rats that received treatment with 3- to 6-year-old ginseng root or metformin, or nontreatment. The HbA1c levels exhibited a tendency to decrease ($p < 0.1$, for both) in the 6-year-old ginseng root and metformin treatment groups *vs*. untreated controls, respectively.

In addition, glucose tolerance was evaluated by IPGTT at week 9. Compared to the nontreated control rats, significant glucose disposal was observed in GK rats responding to 4- and 6-year-old ginseng root treatments at 180 min (*i.e.* 19.91 ± 4.73 *vs*. 9.83 ± 3.83 and 19.91 ± 4.73 *vs*. 11.28 ± 3.18, respectively; both $p < 0.05$) and at 240 min (*i.e.* 17.75 ± 3.96 *vs*. 6.88 ± 2.18 and 17.75 ± 3.96 *vs*. 8.63 ± 2.01, respectively; both $p < 0.01$) (Figure 2).

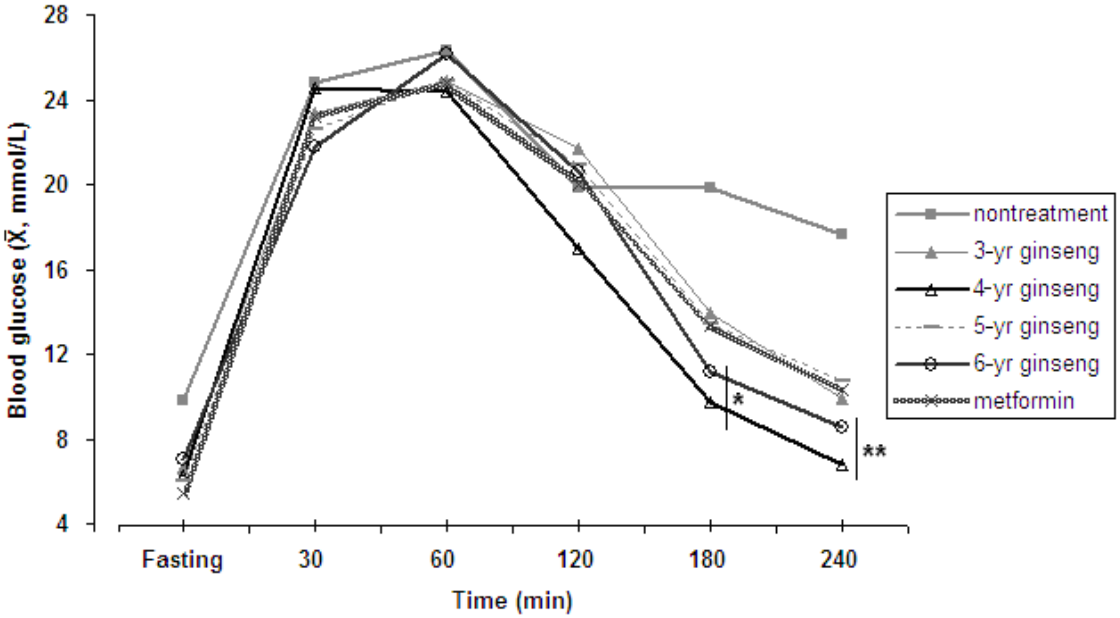

Figure 2. Intraperitoneal glucose tolerance test results. The intraperitoneal glucose tolerance test was performed at the end of the experiment in untreated GK rats and in animals treated with 3- to 6-year-old ginseng roots or metformin. Significantly higher rates of glucose disposal at 180 min and 240 min were observed in GK rats after treatment with 4- and 6-year-old ginseng roots. *$P <$ 0.05, **$p < 0.01$ compared to untreated rats.

In order to further evaluate the overall glucose exposure, the area under the curve (AUC) of glucose disposal was calculated. The glucose AUC corresponding to the 4- and 6-year-old ginseng treatments decreased by 15% and 8%, respectively, in comparison to the untreated rats. Meanwhile, the glucose AUC of the metformin-treated animals was comparable with the 3- and 5-year-old ginseng treatment groups, and there was no significant decrease when compared to that of the untreated rats. Collectively, the ginseng treatments displayed a similar glucose response curve to the metformin treatment. The treatments with 4- and 6-year-old ginseng induced better glucose tolerance than metformin.

Relationship between efficacy and the growth ages of the ginseng roots

A total of 96 individual lipids including lyso-phosphatidylcholine (LPC), phosphatidylcholine (PC), phosphoethanolamine (PE), sphingomyelin (SPM), diacylglyceride (DG), TG and cholesterol ester (ChE) were identified and quantified in the lipidomics study. In order to identify general clusters in the data from the nontreated controls and the rats undergoing the different treatments and to examine which lipids contributed most to the clusters, we carried out PCA on the plasma lipidomics data from all of the study samples, including the 3- to 6-year-old ginseng treatment, the metformin treatment, and the untreated control groups. Figure 3 displays the PCA score plot based on a PC2 *vs*. PC1 model. The score plot (consisting of the symbols '▲,' '★,' '+,' and '○') showed a clear separation between the 6-year-old ginseng treated group (symbols connected with a bold dashed line) and the nontreated control animals (symbols connected with a bold solid line). The first two principal components accounted for 55.1% of the variance, *i.e.* more than half of the total variance in the model. In addition, there was a clear trend of separation between the 4-year-old ginseng treated group (symbols connected with a gray bold solid line in Figure 3) and the nontreated controls. No separation was found between the untreated controls and 3- or 5-year-old ginseng root treatment groups. Furthermore, the PCA score plot showed that metformin-treated animals (symbols connected with a brown dotted dash line in Figure 3) largely overlapped with that of the nontreated animals. The PCA biplot indicated that the TG lipids dominated the clustering patterns of the 6-year-old ginseng treatment group *vs*. the nontreated controls and of the 4-year-old ginseng treatment *vs*. nontreated controls. Interestingly, TG lipids were found to be even more abundant in metformin-treated rats than in the untreated rats according to the biplot, suggesting that metformin has very limited effects on plasma lipid metabolism in diabetic GK rats. In summary, this initial PCA suggested striking differences between the patterns depending on the growth age of the ginseng roots, in spite of the fact that the ginseng had the same genetic background, were planted in the same region, and were under identical harvesting and processing conditions.

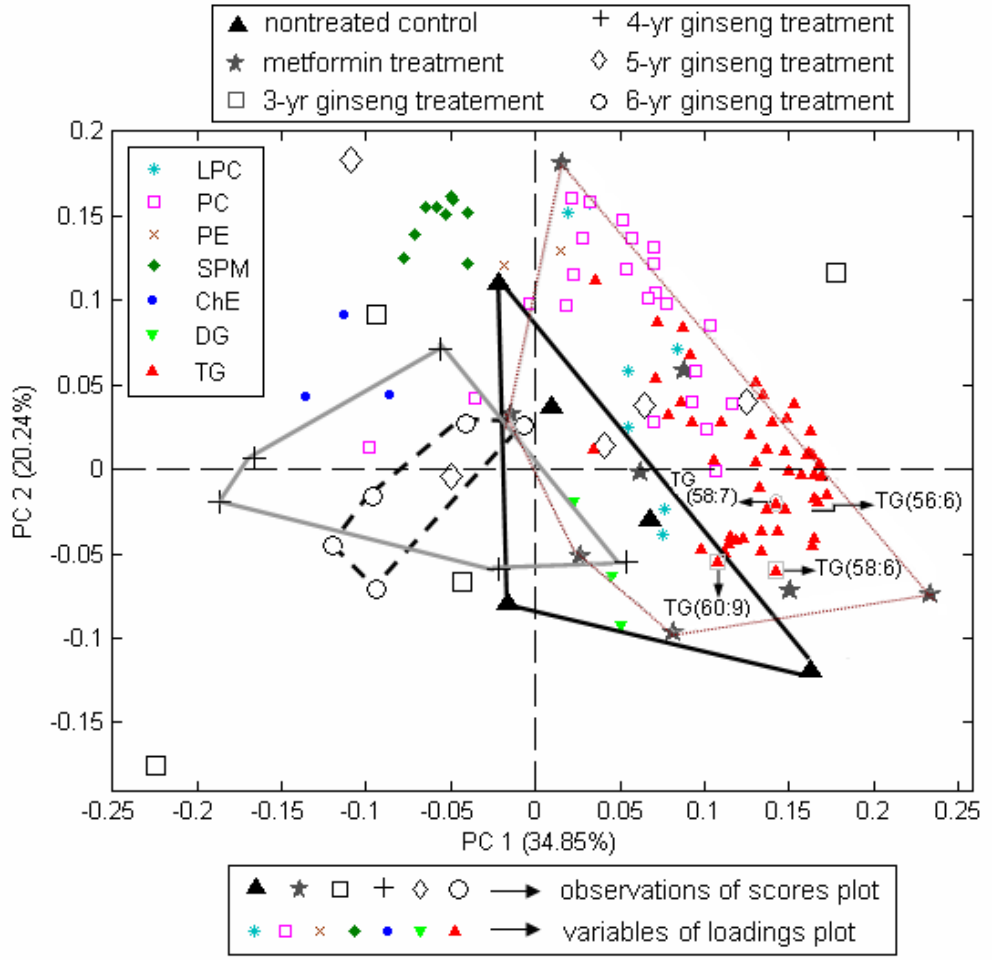

Figure 3 Score and loading biplots of PCA of plasma lipidomics data from all study samples to reveal general clusters of rats of nontreatment and receiving different treatments (scores) and to examine which lipids contributed most to the clusters (loadings). Symbols of '▲' connected with a black bold solid line stand for nontreated control group, symbols of '★' connected with a brown dotted dash line stand for metformin treated group, symbols of '+' connected with a gray bold solid line stand for 4-year-old ginseng treated group, symbols of '○' connected with a black bold dashed line stand for 6-year-old ginseng treated group. Symbols of '*,' '□,' '×,' '◆,' '●,' '▼' and '▲'represent lipid species of LPC, PC, PE, SPM, ChE, DG and TG, respectively.

To further identify any potential underlying patterns causing the observed differences in the PCA model, we analyzed the content of individual lipid molecular species in the groups treated with 3- to 6-year-old ginseng *vs*. the untreated controls using one-way ANOVA with the 2-sided Dunnett post hoc test. In total, only TG (58:6) and TG (58:7) showed significant reductions (p = 0.021 and 0.022, respectively), and TG (56:6) and TG (60:9) showed a decreasing trend (p = 0.085 and 0.097, respectively) in the 6-year-old ginseng treatment group *vs*. the nontreated controls. Although rarely statistical significance was observed, most of the TG lipids showed a decreasing trend in groups treated with 4- to 6-year-old ginseng roots (data not shown) compared to the nontreated controls, which suggests that the effects of ginseng root treatment on plasma lipid metabolism in diabetic GK rats are growth age-dependent.

Relationships between the Biologically Active Components in Ginseng Roots and the Biological Responses

In order to find correlations between the biologically active components in ginseng roots and biological responses, we first investigated whether there were growth age-dependent differences in the ginsenosides of ginseng roots. PCA was performed on the mean-centered plus unit variance-scaled LC–MS data from all the ginseng root samples with 3–6 years of growth. Figure 4A presents the PCA score plot (consisting of the symbols '▼,' '□,' '×,' and '+') on the basis of the PC3 *vs*. PC1. The variation in PC3 may reflect the metabolites responsible for the age-dependent differences. The 3- to 6-year-old ginseng roots are discriminated in PC3, and the 6-year-old ginseng samples are located close to the 4-year-old ginseng samples. Meanwhile, the 5- and 3-year-old ginseng samples, which partially overlap with each other, are located relatively far away from the 6-year-old samples, but close to the 4-year-old ginseng samples. The age-dependent differences were also related to the sizes of ginseng roots, as shown in Figure 4B.

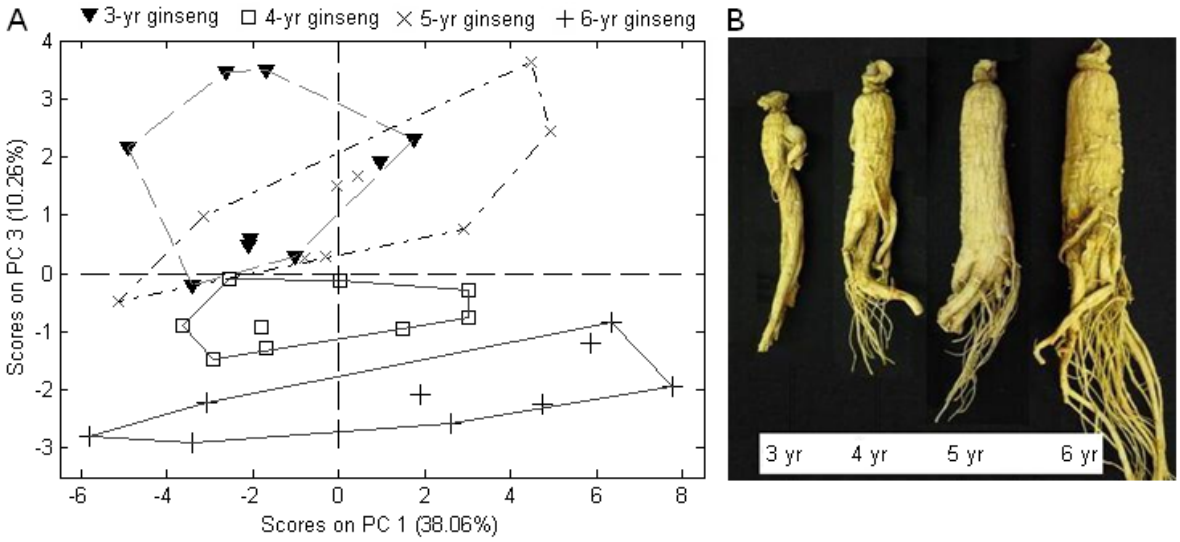

Figure 4. PC3 *vs*. PC1 score plot of extracted *Panax* ginseng roots and dry morphology of ginseng roots. (A) PC3 *vs*. PC1 score plot of all MeOH/H_2O extracted *Panax* ginseng roots '▼,' '□,' '×,' and '+' represent 3-, 4-, 5- and 6-year-old ginseng roots, respectively. The PCA score plot reveals that 3- to 6-year-old ginseng roots are discriminated in PC3. The 6-year-old ginseng samples are located close to the 4-year-old ginseng samples, while the 5- and 3-year-old ginseng samples partially overlap with each other, but are located relatively far away from the 6-year-old ginseng samples and close to the 4-year-old ginseng samples. (B) Dry morphology of 3- to 6-year-old ginseng roots.

In the next step, we analyzed the variation in the concentrations of the individual components in ginseng roots of different growth ages on a univariate basis. Figure 5 displayed the concentration variation (in %) of 8 specific ginsenosides in the 3- to 6-year-old ginseng roots. In the 4-year-old ginseng roots, the most abundant four compounds were Rb2, notoginsenoside R1 (Noto-R1), malonly-Rb2 (mal-Rb2), and malonly-Rb3 (mal-Rb3). In the 5-year-old ginseng roots, the most abundant two compounds were Rb3 and Rd. In the 6-year-old ginseng roots, two compounds, namely Re and gypenoside XVII, were most

abundant. Interestingly, Rb2, Noto-R1, mal-Rb2, and mal-Rb3 continued decreasing in abundance with age in the 4- to 6-year-old ginseng roots, and Rb3 and Rd continued increasing with age in the 3- to 5-year-old ginseng roots. The variation in the concentrations of these eight compounds with growth age may be account for the different therapeutic effects of 4- to 6-year-old ginseng roots. The concentrations and ratios of these compounds might serve as valuable markers for therapeutic quality control.

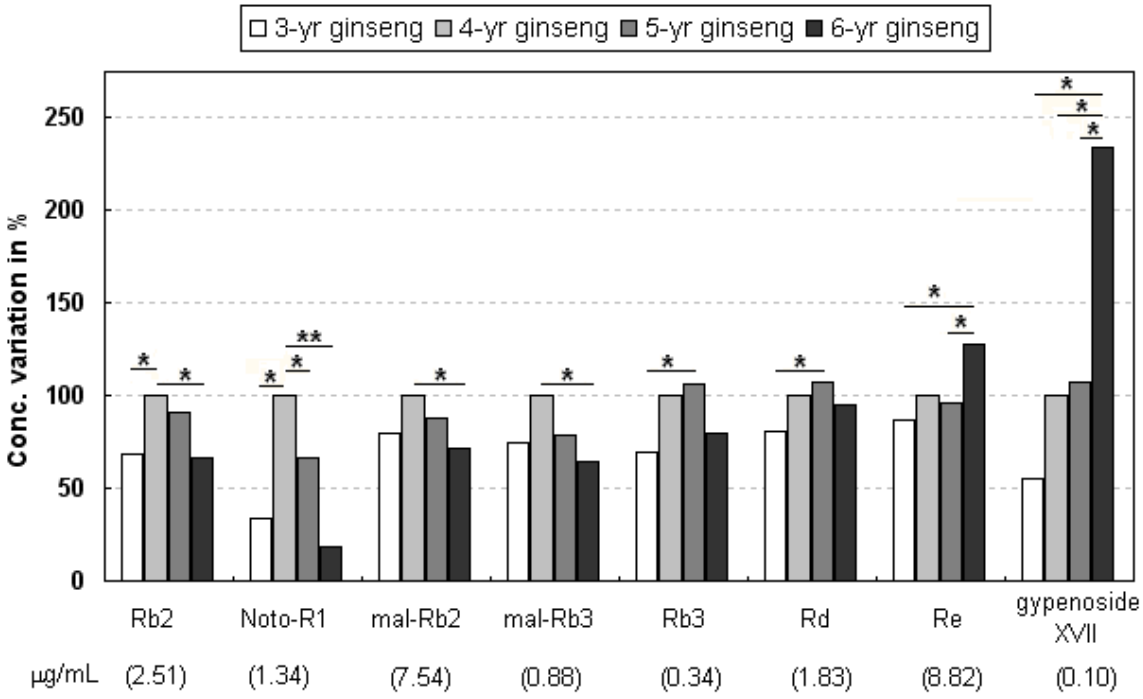

Figure 5. Concentration variation (in %) of eight specific ginsenosides in the 3- to 6-year-old ginseng roots. The concentrations of the ginsenosides in the 4-year-old ginseng roots were set equal to 1. The concentrations values (μg/mL) of the ginsenosides in the 4-year-old ginseng root are given along the X-axis below the name of eight ginsenosides. The ginsenosides Rb2, noto-R1, mal-Rb2 and mal-Rb3 were most abundant in 4-year-old ginseng roots, ginsenosides Rb3 and Rd were most abundant in the 5-year-old ginseng roots, and ginsenosides Re and gypenoside XVII were most abundant in the 6-year-old ginseng roots. These eight ginsenosides are responsible for the age-dependent differentiation among the ginsenoside profiles (*$p < 0.05$, **$p < 0.01$).

Discussion

Panax ginseng C.A. Mey root and its products have long been used for herbal treatments and as dietary supplements with documented health benefits, including antioxidation, anti-hyperglycemia, anti-atherosclerosis, and anti-cancer effects.[25-29] For diabetes management, ginseng root extracts have been demonstrated to lower blood glucose,[30] increase insulin sensitivity,[31] and regulate lipid metabolism.[32-33]

Although the research investigating the effects of ginseng roots in diabetes is rapidly growing, researchers have not yet examined how the therapeutic effects of this ginseng are related to its quality and the growth age. The aims of this study were: (1) to evaluate the effects of ginseng roots grown for 3–6 years on regulating hyperglycemia and hyperlipidemia in a T2D GK rat model, and (2) to link the biologically active components

in ginseng roots to their biological effects. To this end, we first tested lipid- and glycemic-related biological parameters, and then incorporated two advanced technologies as part of a systems biology approach. These two technologies involved: (a) the application of lipidomics to a study of 37 GK rats with T2D that received 9-week, parallel interventions, which included a nontreatment control group, and (b) the application of profiling ginsenosides to identify the differences among ginseng roots of different growth ages. Our study presents three major findings.

Firstly, 4- to 6-year-old ginseng roots were observed to improve hyperlipidemia and hyperglycemia in diabetic GK rats. Six-year-old ginseng had the most beneficial effect on hyperlipidemia, followed by 5-year-old and then 4-year-old ginseng, based on the observation that 6-year-old ginseng significantly improved plasma TG and VLDL-C and 5-year-old ginseng significantly improved HDL-C in comparison to the untreated controls. Treatment with 4-year-old ginseng led to a trend of improvement in TG, HDL-C, and VLDL-C, although these improvements were not statistically significant. In contrast to the ginseng treatments, metformin showed very limited effects on these lipid-related biochemical parameters. However, in terms of glycemic control, metformin significantly improved FBG (reduced by 41%, $p = 0.030$ *vs*. control) and produced a substantial improvement tendency in HbA1c (reduced by 11.2% *vs*. control). Our results are consistent with those reported in a previous review, which concluded that metformin has a beneficial effect on glucose-lowering but rather limited effect on plasma lipid profile in T2D.[34] Meanwhile, 4- to 6-year-old ginseng roots produced positive effects on glycemic control by improving glucose tolerance (4- and 6-year-old ginsengs in IPGTT) and a decreasing tendency of HbA1c (6-year-old ginseng). In our current study, the glycemic regulation effects produced by ginseng were equal to or better than those produced by metformin treatment. Accordingly, under the current experimental conditions, the treatments with 4- to 6-year-old ginseng roots, especially 6-year-old ginseng, were superior to metformin treatment in terms of producing beneficial effects on lipid regulation and glucose tolerance. Moreover, metformin is associated with adverse gastrointestinal effects, including taste disturbance, appetite loss, nausea or vomiting, abdominal pain, and diarrhea,[21] which cause patients to become intolerant to metformin. Ginseng is commonly used as a dietary supplement and herbal medicine in China, and its adverse effects have been scarcely reported. Therefore, ginseng may be an effective and safe initial intervention to improve glucose- and lipid-related parameters in people with T2D.

Secondly, we identified distinct clusters in the lipid profiles, which demonstrate differences between the levels of lipid metabolites in the intervention groups after the 9-week parallel treatments. The PCA of the plasma lipidomics data revealed a clear difference between the 6-year-old ginseng-treated group and the nontreated controls. The TG lipids contributed the most to this difference. Four TG species were identified as discriminating lipids (*i.e.* TG 56:6, 58:6, 58:7, and 60:9) in the total lipid pattern. In addition, a large number of TG molecules showed non-significant decreasing trends in the GK rats receiving 4- to 6-year-old ginseng treatments in comparison to the untreated controls, indicating that the TG-lowering effect of ginseng is growth-age dependent. The

growth age of the ginseng roots can account for the variation in the contents of specific biologically active components.

Thirdly, we identified general growth and age-related trends by profiling components in ginseng roots, and we precisely defined changes in individual components. Through further integration with biological parameters, we predicted linkages between bioactive components in ginseng roots and the bioactivities related to growth ages. Ginsenosides (*i.e.* ginseng saponins) have been well recognized as major bioactive components, which are responsible for the biological and pharmacological activities of ginseng roots and tissues. In the present study, we examined the identified 27 ginsenosides found in all ginseng root extracts. We combined our biochemical and lipidomic results in the animal model together with the variations in the concentrations of specific ginsenosides in the 3- to 6-year-old ginseng roots. This method allowed us to link the improvements in lipid and glycemic metabolism due to treatment with 4-year-old ginseng root to Noto-R1, Rb2, mal-Rb2, and mal-Rb3. Meanwhile, the improvements due to treatment with 5-year-old ginseng roots were linked to Rb3 and Rd, and the beneficial effects of 6-year-old ginseng root treatment were associated with Re and gypenoside XVII. However, it is possible that the efficacy of 6-year-old ginseng root treatment could be due to a combination of many different ginsenosides in a specific concentration ratio (*e.g.* high concentrations of gypenoside XVII and Re, and a low concentration of Noto-R1, see Figure 5). We observed that four ginsenosides were related to the 4-year-old ginseng root treatment effects, which is partially supported by the results of a previous study on the medicinal use of pure ginseng components to treat diabetes. Yang *et al*. provided experimental evidence on the clinical application of six representative notoginsenosides, including notoginsenoside R1, in the diabetic, obese KK-Ay mouse model and demonstrated that notoginsenosides improved insulin and leptin sensitivity.[35] Liu *et al*. reported that mal-ginsenosides, including mal-Rb1, mal-Rb2, mal-Rc, and mal-Rd, significantly lowered plasma glucose without changing the hepatic glycogen and cholesterol levels in streptozotocin-induced diabetic mice.[36] Moreover, ginsenoside Rb2 is reported to be capable of lowering TG levels in 3T3-L1 adipocytes cultured under high energy conditions by stimulating the expression of SREBP and leptin mRNA.[37] Although the present study could not precisely determine the beneficial actions of these four ginsenosides on glycemic and lipid control in diabetes, it provided experimental evidence on the hyperglycemic and hyperlipidemia regulation effects of Asian ginseng roots and identified potential bioactive components.

Although no relevant research on diabetes has been reported in literature for the two specific ginsenosides identified in the 5-year-old ginseng root (*i.e.* Rb3 and Rd), several studies indicated that these components play an important role in health promotion and disease prevention. For example, a recent study demonstrated that ginsenoside Rb3 can significantly ameliorate myocardial injury and heart function impairment induced by Isuprel in rats by increasing the activities of myocardial antioxidant enzymes and inhibiting myocardial lipid peroxidation in myocardial ischemia.[38] Ginsenoside Rd was considered to be a potential compound for cancer prevention due to its inhibitory action on 26S proteasome activity and its low toxicity.[39]

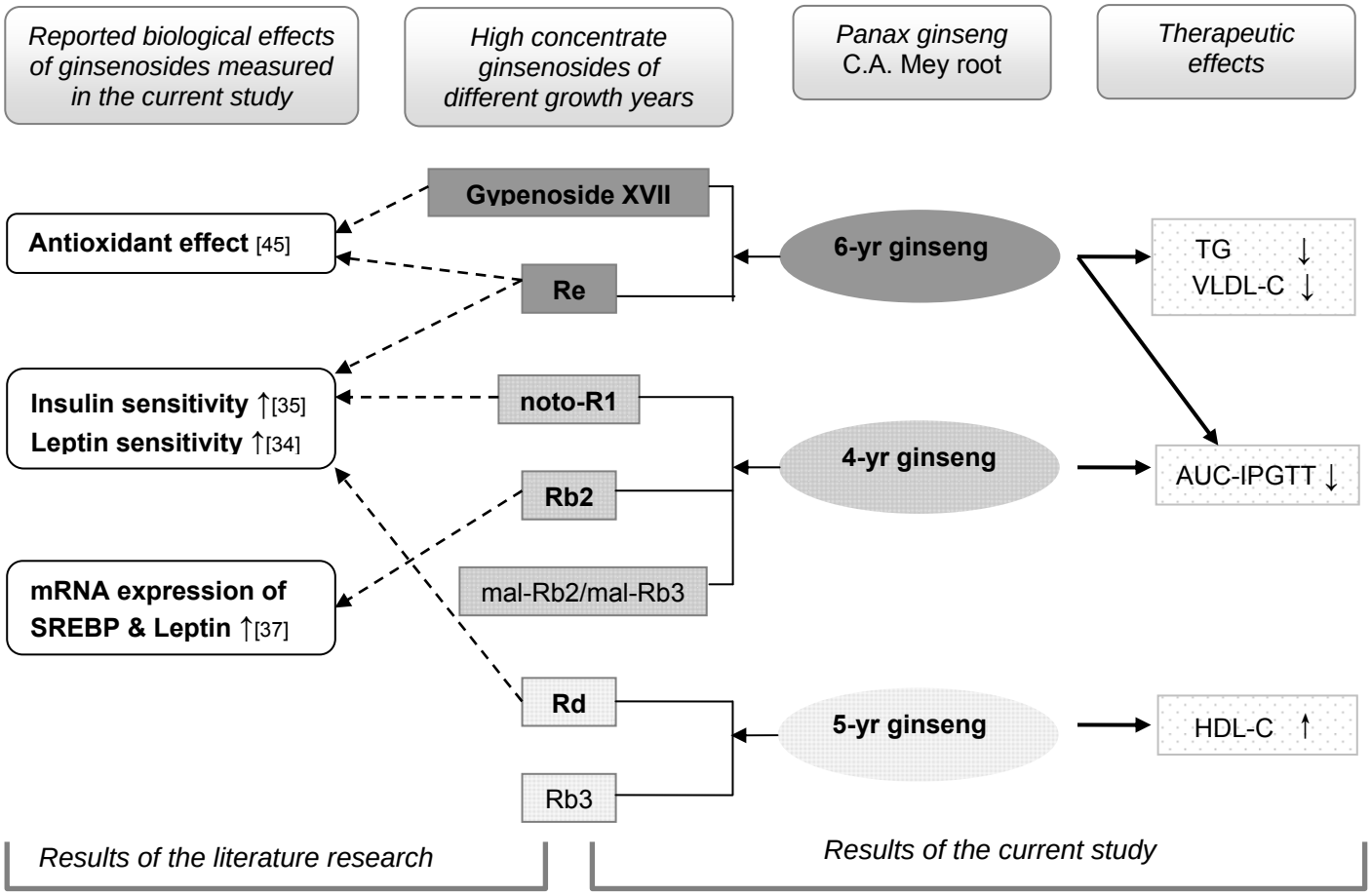

Figure 6. Illustration of observed ginseng therapeutic effects and the correlated ginsenoside biological effects. The columns on the left summarize the literature reports of the biological effects of ginsenosides that were measured in the current study. The references are displayed with the relevant biological effects. The remaining three columns display the results found in the current study. The 4- to 6-year-old ginseng roots shared some similar effects.

For the two ginsenosides identified in the 6-year-old ginseng root (*i.e.* Re and gypenoside XVII), there has been substantial research related to their biological effects in diabetic/diseased animal models and humans.[40-42] For instance, ginsenoside Re was demonstrated not only to exhibit a dose-dependent anti-hyperglycemic effect in diabetic *ob/ob* mice,[26,43] but it also had a significant anti-hyperlipidemic efficacy in streptozotocin-induced diabetic rats.[44] Gypenoside XVII has been reported to have a protective effect against oxidative stress in phagocytes, vascular endothelial cells, and liver microsomes, which indicates that it may play an important role in the prevention and treatment of atherosclerosis, liver disease, and inflammation.[45] Based on our findings and the reported effects of the above-mentioned ginsenosides in the literature, we summarized the observed ginseng therapeutic effects and the correlated ginsenoside biological effects in Figure 6. We considered only the ginsenosides with the highest concentrations for the ginseng of each growth ages. However, it is likely that combinations of different ginsenosides in various ratios will be the key to explain the observed multi-dimensional pharmacologic effects. By using the Matrigel implant model and reconstituting the extracts using distinct ratios of Rg1 and Rb1, Sengupta *et al.*[17] observed that different defined ratios led to opposing biological effects, which could alter angiogenic outcomes. In the future, additional pre-clinical and clinical experiments on the medicinal use of purified individual compounds or combination of specific ginsenosides will be necessary to confirm their efficacy on T2D. These studies may provide an opportunity to develop a new class of agents for diabetes care. Furthermore, this line of research can also lead to the development of new bioactive markets for quality control of herbal extracts.

Conclusions

In conclusion, by applying LC–MS-based lipidomics, measuring biochemical parameters, and profiling the components of ginseng roots of different ages, we demonstrate that ginseng roots show growth age-dependent therapeutic effects on hyperlipidemia and hyperglycemia in diabetic GK rats. These effects may be linked with the age-dependent variations in concentrations of specific bioactive ginsenosides in the ginseng roots. The present study provides novel and valuable experimental evidence on the age-dependent biological actions of ginseng in the treatment of diabetes. The results demonstrate that 4- to 6-year-old (*i.e.* ≥ 4-year-old) ginseng roots contain bioactive ginsenosides that may prove to be valuable in the development of drugs or dietary supplements to regulate lipid levels and increase glucose tolerance. Our results suggest that future investigations should examine the biological effects of combinations of specific ginsenosides from ginseng roots.

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