# Licorice Flavonoids Suppress Abdominal Fat Accumulation and Increase in Blood Glucose Level in Obese Diabetic KK-A$^y$ Mice

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**Licorice, the root of the *Glycyrrhiza* species, is one of the most frequently employed botanicals in traditional medicines. In this study, we investigated the effects of hydrophobic flavonoids from *Glycyrrhiza glabra* LINNE on abdominal fat accumulation and blood glucose level in obese diabetic KK-A$^y$ mice. In order to enrich a fraction of hydrophobic flavonoids, licorice flavonoid oil (LFO) was prepared by further extracting licorice ethanolic extract with medium-chain triglycerides (MCT), and adjusting the concentration of glabridin, the major flavonoid of licorice, to 1.2% in oil. KK-A$^y$ mice aged 6 weeks were assigned to 5 groups ($n$=6 each), and fed a high-fat diet containing 0 (control), 0.5%, 1%, or 2% LFO, or 0.5% conjugated linoleic acid (CLA) for 4 weeks. Compared with the control, body weight gain and weights of abdominal adipose tissues were suppressed ($p$<0.05) by feeding the diet containing 2% LFO, and blood glucose levels after 2 and 4 weeks were suppressed by all of the diets containing LFO. Although CLA feeding suppressed ($p$<0.05) body weight gain, it increased ($p$<0.05) blood glucose level after 2 weeks compared with the control level. Furthermore, LFO and licorice ethanolic extract stimulated human adipocyte differentiation *in vitro*. These results indicate that licorice hydrophobic flavonoids have abdominal fat-lowering and hypoglycemic effects, possibly mediated *via* activation of peroxisome proliferator-activated receptor-$\gamma$ (PPAR-$\gamma$).**

**Key words** licorice flavonoid; diabetes; abdominal obesity; the metabolic syndrome

Licorice, the root of the leguminous *Glycyrrhiza* plant species, has been used for over 4000 years since ancient Egyptian times, and is one of the most frequently employed botanicals in traditional medicines.[1)] There are several species of licorice, including *Glycyrrhiza uralensis* FISCHER, *G. glabra* LINNE and *G. inflata* BATALIN, and they include species-specific flavonoids.[1,2)] Our previous study showed that nonaqueous fractions of *G. uralensis* have peroxisome proliferator-activated receptor-$\gamma$ (PPAR-$\gamma$) ligand-binding activity, and that the active compounds in the nonaqueous fractions are prenylflavonoids such as glycycoumarin, glycyrin, dehydroglyasperin C and dehydroglyasperin D.[3,4)] A licorice ethanolic extract from *G. uralensis* has been found to be effective in preventing and/or ameliorating diabetes, abdominal obesity and hypertension in three animal models.[3)] Glabridin in *G. glabra*, one of the most frequently studied flavonoids of licorice, has antinephritic and radical scavenging activities,[5)] exhibits inhibition of serotonin re-uptake,[6)] has anti-*Helicobacter pylori* activity,[7)] estrogen-like activity,[8)] and antioxidative activity,[9—14)] and inhibits melanogenesis and inflammation.[15)]

The metabolic syndrome is a cluster of several metabolic and cardiovascular disease risk factors including central obesity, dyslipidemia, glucose intolerance, insulin resistance and hypertension,[16)] and is becoming recognized as a major public health problem. Central obesity, so-called abdominal obesity or visceral fat obesity, is believed to elicit insulin resistance, which is a major cause of the metabolic syndrome. Thiazolidinedione drugs such as troglitazone, pioglitazone and rosiglitazone, which have been approved for type 2 diabetes,[17,18)] have potent PPAR-$\gamma$ agonistic activity and stimulate adipocyte differentiation through PPAR-$\gamma$ activation.[19,20)] Many studies of these drugs indicate that PPAR-$\gamma$ agonists have significant effects on the metabolic syndrome.[21—23)]

In this study, we demonstrated the effects of licorice hydrophobic flavonoids from *G. glabra* on abdominal fat accumulation and blood glucose level in obese diabetic KK-A$^y$ mice, and elucidated their mechanisms of action by showing that they induce differentiation of human adipocytes.

## MATERIALS AND METHODS

**Preparation of Licorice Flavonoid Oil (LFO)** The roots of licorice, *Glycyrrhiza glabra* LINNE, harvested in Afganistan were extracted with 5 volumes of 95% ethanol twice, and licorice ethanolic extract was obtained by filtration and concentration. The licorice ethanolic extract was dissolved in medium-chain triglycerides (MCT; C8 : C10 = 99 : 1; Riken Vitamin Co., Ltd., Tokyo, Japan), mixed, evaporated to remove ethanol, and filtered. The obtained oil including licorice flavonoids was used as LFO, after adjusting glabridin concentration to 1.2% (w/w) in oil. A standard for glabridin, a major compound of licorice flavonoid, was purchased from Wako Pure Chemical Inductries, Ltd. (Osaka, Japan).

**Animal Experiment** Female genetically type 2 diabetic KK-A$^y$/Ta mice (Clea Japan, Inc., Tokyo, Japan) were housed in an animal laboratory with a controlled environment at 20—24 °C temperature, 45—65% humidity and 12-h (7:30—19:30) light/dark cycle, and reared with their respective groups in cages. The mice were at 6 weeks of age divided into 5 groups of 6 mice each by body weight and blood glucose level, and fed a high-fat diet (Table 1; Oriental Yeast, Co., Ltd., Tokyo Japan) as a basal experimental diet. In treatment groups, mice were fed diets containing 0.5%, 1% or 2% LFO or 0.5% conjugated linoleic acid (CLA, Rinoru Oil Mills Co., Ltd., Tokyo, Japan). For all groups, the dietary

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MCT concentration was 2% in diet. Mice consumed these diets and water *ad libitum* for 4 weeks. Blood glucose levels measured from the tail vein were determined using a blood glucose level monitor Glutest Ace (Sanwa Kagaku Kenkyusho Co., Ltd., Nagoya, Japan) initially and after 2 and 4 weeks of feeding. Then the mice were killed under diethyl ether anesthesia to collect mesenteric, perirenal and periuterine adipose tissues.

**Human Adipocyte Differentiation Assay** The effects of LFO and licorice ethanolic extract on human adipocyte differentiation were examined at Zen-Bio, Inc. (Research Triangle Park, NC, U.S.A.). Primary human subcutaneous preadipocytes suspended in preadipocyte medium were plated at $1.35\times10^4$ cells/well and allowed to adhere on 96-well plates at day 1. Medium was changed to initiation medium at day 2, and to adipocyte medium at day 5, 9 and 12. Basal medium consisted of DMEM/Ham's F-12 (1 : 1, v/v), 15 mM HEPES buffer and antibiotics. The preadipocyte medium contained 10% (v/v) fetal bovine serum (FBS) in the basal medium, and the initiation medium contained 3% (v/v) FBS, 33 $\mu$M biotin, 17 $\mu$M pantothenate, 100 nM human insulin, 1 $\mu$M dexamethasone and 0.25 mM 3-isobutyl-1-methylxanthine (IBMX). The adipocyte medium was of the same composition as the initiation medium but without IBMX. Sample dissolved in dimethyl sulfoxide (DMSO) was added to the medium at a final concentration of 0.1% (v/v) DMSO. Rosiglitazone maleate was used at 1 $\mu$M as a positive control. At day 15, the cells were washed twice with phosphate buffered saline (PBS) and lysed. Accumulated triglyceride was measured as glycerol concentration using Infinity reagent (Sigma Chemical Co., St. Louis, MO, U.S.A.).

**Statistical Analysis** Data were analyzed using the SAS/STAT software computerized statistical analysis program (SAS Institute Inc., Cary, NC, U.S.A.). When statistical significance was detected by one-way analysis of variance (ANOVA), Tukey's multiple comparison test was applied to identify significant differences. Differences with $p<0.05$ were considered significant. Values in the text are means±S.E.M.

## RESULTS

The licorice ethanolic extract from *Glycyrrhiza glabra* LINNE is rich in flavonoids and poor in glycyrrhizin. Glabridin was a major licorice flavonoid in this fraction, with a concentration of 8—9%. We used MCT to dissolve water-insoluble flavonoids of licorice ethanolic extract. LFO was prepared by further extracting a licorice ethanolic extract and adjusting glabridin concentration to 1.2% in oil. Consequently, LFO contained MCT at 88% and a solid fraction derived from a licorice ethanolic extract at 12%. In addition, the polyphenol content of LFO was determined to be approximately 10% by the Folin–Denis method using glabridin as a standard compound.

For female KK-A$^y$ mice aged 6 weeks fed a high-fat diet as a control group, body weight increased from 27.1±0.41 g to 51.7±0.77 g over 4 weeks, and body weight gain was 24.6±0.55 g/4-week (Table 2). Food intakes were nearly the same in all groups, even when diets contained LFO or CLA. From food intakes and body weight, daily doses of LFO at 0.5%, 1% and 2% were calculated to be 523, 1045 and 2093 mg/kg body weight, respectively, and the daily dose of CLA at 0.5% was 579 mg/kg body weight. Body weight gain in the group fed 2% LFO was significantly ($p<0.01$) lower than that in the control group (Table 2). In addition, weights of abdominal adipose tissues, especially mesenteric and periuterine adipose tissues, in the group fed 2% LFO were significantly ($p<0.05$) lower than those in the control group. However, 0.5% and 1% LFO affected neither body weight gain nor weights of abdominal adipose tissues compared with the control group. Thus, feeding 2% LFO suppressed the body weight gain and abdominal fat accumulation induced by a high-fat diet.

Blood glucose level in the control group was significantly ($p<0.01$) increased during the 4 weeks period (Fig. 1). No significant increase in blood glucose level was observed in

Table 1. Composition of Basal Experimental Diet

| | g/kg diet |
|---|---|
| Casein | 250 |
| Corn starch | 148.69 |
| Sucrose | 200 |
| Soybean oil | 20 |
| Lard | 140 |
| Beef tallow | 140 |
| Cellulose powder | 50 |
| AIN-93 mineral mixture | 35 |
| AIN-93 vitamin mixture | 10 |
| Choline bitartrate | 2.5 |
| *tert*-Butylhydroquinone | 0.06 |
| L-Cystine | 3.75 |

The energy ratio was fat 53%, carbohydrate 27%, protein 20%, and total energy 21 MJ/kg-diet. AIN-93 mineral mixture and AIN-93 vitamin mixture referred to the ref. 24.

Table 2. Body Weight Gain and Adipose Tissue Weights of KK-A$^y$ Mice Fed High-Fat Diet Containing LFO or CLA for 4 Weeks

| | Control | LFO | | | CLA |
|---|---|---|---|---|---|
| | | 0.5% | 1% | 2% | 0.5% |
| Body weight gain (g/4-week) | 24.6±0.55$^{b)}$ | 24.4±0.69$^{b)}$ | 24.6±0.43$^{b)}$ | 18.9±0.75$^{a)}$ | 20.8±0.48$^{a)}$ |
| Adipose tissue weight (g) | | | | | |
| Intra-abdominal | 9.65±0.63$^{b,c)}$ | 9.88±0.24$^{c)}$ | 10.22±0.41$^{c)}$ | 7.56±0.19$^{a)}$ | 7.97±0.45$^{a,b)}$ |
| Mesenteric | 1.76±0.09$^{b)}$ | 1.79±0.06$^{b)}$ | 1.90±0.07$^{b)}$ | 1.37±0.04$^{a)}$ | 1.63±0.15$^{a,b)}$ |
| Perirenal | 2.06±0.13$^{a,b)}$ | 2.10±0.10$^{a,b)}$ | 2.37±0.19$^{b)}$ | 1.67±0.12$^{a)}$ | 1.66±0.17$^{a)}$ |
| Periuterine | 5.83±0.55$^{b,c)}$ | 5.99±0.18$^{c)}$ | 5.95±0.18$^{c)}$ | 4.51±0.14$^{a)}$ | 4.67±0.26$^{a,b)}$ |

Values are means±S.E.M. of 6 mice. *a*)—*c*) Means in a row without a common letter differ, $p<0.05$. Weight of intra-abdominal adipose tissue was the sum of that of mesenteric, perirenal and periuterine adipose tissues.

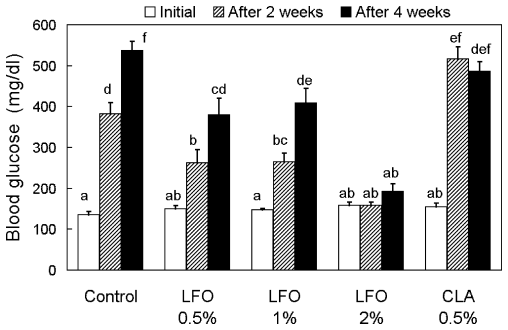


Fig. 1. Blood Glucose Level in KK-A$^y$ Mice Fed High-Fat Diet Containing 0 (Control), 0.5%, 1% or 2% LFO or 0.5% CLA Initially and after 2 and 4 Weeks of Feeding

Values are mean±S.E.M. of 6 mice. a—f: Means with different letters differ, $p<0.05$.

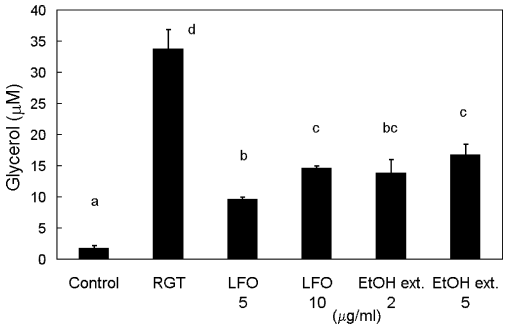


Fig. 2. Effects of LFO (5, 10 $\mu$g/ml) and Licorice Ethanolic Extract (EtOH Ext., 2, 5 $\mu$g/ml) on Human Adipocyte Differentiation

Rosiglitazone (RGT) at 1 $\mu$M was used as a positive control. Accumulated triglyceride in adipocytes was measured as glycerol. Values are mean±S.D. of $n=3$. a—d: Means with different letters differ, $p<0.05$.

the group fed 2% LFO. In the groups fed 0.5% and 1% LFO, although significant increase in blood glucose level was observed, the levels after 2 and 4 weeks were significantly ($p<0.05$) lower than in the control group. This result indicated that feeding 0.5—2% LFO suppressed the increase in blood glucose level during development of hyperglycemia in KK-A$^y$ mice. In our other experiment with the same condition, the increase in blood glucose level in KK-A$^y$ mice was completely suppressed by feeding 0.2% troglitazone or 0.02% pioglitazone (data not shown).

Compared with the control, feeding of CLA decreased ($p<0.01$) body weight gain but not weights of abdominal adipose tissues (Table 2). Blood glucose level after 2 weeks was significantly ($p<0.01$) higher in the CLA group than in the control group, although the levels after 4 weeks were similar to that in the control group (Fig. 1). CLA thus promoted development of hyperglycemia in KK-A$^y$ mice, despite the fact that it reduced body weight gain.

To elucidate the mechanism of abdominal fat-lowering and hypoglycemic effects of LFO, we tested the effects of LFO and licorice ethanolic extract in an *in vitro* human adipocyte differentiation assay. In this assay, degree of differentiation was evaluated by measuring concentration of glycerol amount of triglyceride accumulated in cells. LFO and licorice ethanolic extract significantly ($p<0.01$) increased glycerol concentration compared with control (Fig. 2), indicating that both licorice flavonoid preparations stimulated differentiation from preadipocytes to mature adipocytes. However, the potency of LFO and the licorice ethanolic extract was weaker than that of rosiglitazone, a potent PPAR-$\gamma$ agonist.

## DISCUSSION

The licorice ethanolic extract from *Glycyrrhiza glabra* LINNE contains hydrophobic flavonoids in high proportion but glycyrrhizin in trace amount. This fraction has very strong antioxidant activity, and therefore, has been used as a food additive as an antioxidant in Japan. On the other hand, LFO is a unique food ingredient prepared by further extraction of licorice ethanolic extract with MCT to obtain more bioactive hydrophobic flavonoids, and it expected to exhibit greater bioavailability and to be widely applicable to foods.

The objective of this study was to evaluate the effect of LFO, which was prepared as a hydrophobic flavonoid-enriched fraction of *Glycyrrhiza glabra*, on abdominal fat accumulation and blood glucose level in obese diabetic KK-A$^y$ mice fed a high-fat diet. Female KK-A$^y$ mice are used for evaluation of antiobesity and antidiabetic effects of compounds and extracts,[25—27] since body weight over 8 weeks old is higher in female than in male. For this reason, we used female KK-A$^y$ mice in this study. Our animal study showed that LFO consumption significantly suppressed body weight gain, weights of abdominal adipose tissues and increase in blood glucose level compared with control diet. This result indicated that LFO was effective in preventing diabetes and obesity, particularly visceral fat accumulation. In addition, we demonstrated that LFO and licorice ethanolic extract, which exhibited PPAR-$\gamma$ ligand binding activity (data not shown), stimulated adipocyte differentiation similar to a PPAR-$\gamma$ agonist, although the potency of both licorice flavonoid preparations was weaker than that of rosiglitazone. These findings suggest that the abdominal fat-lowering and hypoglycemic effects of licorice hydrophobic flavonoids in LFO are exerted through moderate PPAR-$\gamma$ agonistic activity.

Glabridin is a major flavonoid in *G. glabra*, and its antioxidative properties have been well studied.[5,9—14] In addition, it has been reported that a licorice ethanolic extract as well as glabridin protects LDL oxidation in atherosclerotic apolipoprotein E-deficient mice,[13] and exhibits antiatherosclerotic effects in hypercholesterolemic patients.[28] On the other hand, PPAR-$\gamma$ activation is a key factor in treatment of type 2 diabetes, insulin resistance and atherosclerosis.[29,30] Consequently, not only the antioxidant activity of licorice hydrophobic flavonoids including glabridin but also their PPAR-$\gamma$ agonistic activity may contribute to antiatherosclerotic effects.

Many studies in animals and humans have shown that MCT consumption increases energy expenditure and decreases fat depot size and body weight gain compared with long-chain triglycerides consumption.[31,32] However, the effect of addition of MCT to licorice hydrophobic flavonoids in this animal study may have been negligible since the diets fed to all the groups including the control group were adjusted to contain the same amount of MCT (2% in diet).

It has been reported that, in addition to modulating obesity and diabetes,[33—35] CLA affects PPAR-$\gamma$ activity.[33,36,37] We therefore used CLA as a reference compound. Although

CLA consumption by KK-A$^y$ mice fed a high-fat diet reduced body weight gain, beneficial effects of CLA on abdominal fat accumulation and blood glucose level were not observed in this animal model. On the contrary, CLA promoted development of hyperglycemia in this model. As Brown and McIntosh noted, the *trans*-10, *cis*-12 isomer of CLA as well as mixed isomers induce insulin resistance.[33] Our results may support their observation, and indicate species and/or strain differences in response to CLA between Zucker diabetic fatty rats[38,39] and KK-A$^y$ mice.

In conclusion, we demonstrated that licorice hydrophobic flavonoids had abdominal fat-lowering and hypoglycemic effects in an obese diabetic model animal. Furthermore, our *in vitro* studies suggested that these effects were mediated through PPAR-$\gamma$ activation. A recent study with a transgenic model of visceral obesity has suggested that excess accumulation of visceral fat is an important molecular basis of the metabolic syndrome.[40] In this regard, KK-A$^y$ mice can be considered a model of the metabolic syndrome, since type 2 diabetes/hyperglycemia and obesity/abdominal obesity developed simultaneously. Licorice hydrophobic flavonoids in LFO may be useful for improving visceral fat obesity in preventing the metabolic syndrome including type 2 diabetes.

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