Combined Effects of Fangchinoline from Stephania Tetrandra Radix and Formononetin and Calycosin from *Astragalus membranaceus* Radix on Hyperglycemia and Hypoinsulinemia in Streptozotocin-Diabetic Mice

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The anti-hyperglycemic action of Stephania tetrandra Radix (Stephania) is potentiated by *Astragalus membranaceus* BUNGE Radix (Astragali) in streptozotocin (STZ)-diabetic ddY mice (Tsutsumi *et al.*, *Biol. Pharm. Bull.*, 26, 313 (2003)). Fangchinoline (0.3—3 mg/kg), a main constituent of Stephania, decreased the high level of blood glucose and increased the low level of blood insulin in STZ-diabetic mice. Here, we investigated the combined effects of fangchinoline with isoflavone or isoflavonoid components (formononetin, calycosin and ononin) of Astragali on the hyperglycemia and hypoinsulinemia of STZ-diabetic mice. Formononetin, calycosin and ononin (0.03—0.1 mg/kg) alone did not affect the blood glucose or blood insulin level of the diabetic mice. Formononetin and calycosin (0.03—0.1 mg/kg) potentiated the anti-hyperglycemic action of fangchinoline (0.3 mg/kg), but ononin did not. Formononetin (0.1 mg/kg) facilitated the fangchinoline-induced insulin release, and calycosin (0.1 mg/kg) also facilitated it, though without statistical significance. In conclusion, the combined effect of fangchinoline with formononetin and calycosin on hyperglycemia in the diabetic mice accounted well for the therapeutic effect of the combination of Stephania with Astragali in Boi-ogi-to. The anti-hyperglycemic action of formononetin appeared to be due to its potentiating action on insulin release. Our strategy for studying combinations of crude drugs and their components in Kampo medicine has uncovered new potentiating effects of formononetin and calycosin on the anti-hyperglycemic action of fangchinoline in STZ-diabetic mice.

Key words fangchinoline; formononetin; anti-hyperglycemic effect; combined effect; streptozotocin-diabetic mouse

Boi-ogi-to (Fang-ji-huang-qi-tang) is a traditional prescription in Kampo medicine and consists of Stephania tetrandra Radix (Stephania), *Astragalus membranaceus* BUNGE Radix (Astragali), Atractylodes Lancea Rhizoma, Glycyrrhizae Radix, Zingiberis Rhizoma and Zizyphi Fructus. Boi-ogi-to has long been used clinically in the treatment of arthritis and edema in China and Japan. It also improves abnormal glucose and lipid metabolism in obese diabetic patients.[1] We have reported that Boi-ogi-to increases blood insulin and decreases blood glucose levels in streptozotocin (STZ)-diabetic mice. The anti-hyperglycemic and anti-hypoinsulinemic actions of Boi-ogi-to depend on the combination of Stephania and Astragali.[2] Astragali does not have a direct anti-hyperglycemic effect, but potentiates the actions of Stephania on blood levels of both glucose and insulin in STZ-induced diabetes.[3,4] In addition, Stephania suppresses abnormal choroidal and retinal neovascularization in STZ-induced diabetes *in vitro* and *in vivo*.[5]

Stephania contains many bis-benzylisoquinoline-type constituents, such as tetrandrine and fangchinoline.[6,7] Fangchinoline significantly improves hyperglycemia of STZ-diabetic mice.[4] Tetrandrine inhibits precapillary formation of vascular endothelial cells and neovascularization of cultured choroidal explants in STZ-diabetic rats,[8,9] but does not affect hyperglycemia in diabetic mice.[4] Fangchinoline and tetrandrine show different anti-inflammatory actions *via* inhibition of cyclooxygenase and interleukin-5 activities.[10] In other studies, fangchinoline and tetrandrine were found to have similar inhibitory activities on both angiotensin I converting enzyme,[6,7] and induction of proinflammatory cytokines, interleukin-1 and tumor necrosis factor-alpha by *Staphylococcus aureus* Cowan 1-stimulated human peripheral blood mononuclear cells.[11]

Astragali contains many isoflavones and isoflavonoids, such as formononetin, calycosin and ononin, and many saponins, such as astragaloside IV, astragaloside II, astragaloside I, and acetylastragaloside I.[12] Formononetin significantly reduces arachidonic acid release and production of nitric oxide in lipopolysaccharide activated RAW 264.7 macrophages.[13] It also shows estrogen receptor agonistic activity in human breast cell line MCF-7.[14,15] Formononetin and calycosin activate the peroxisome proliferator-activated receptors (PPAR) alpha and gamma. The action of formononetin is more potent than that of calycosin.[16] Calycosin is a vasorelaxant and is a noncompetitive Ca^{2+} channel blocker, whose action is endothelium-independent and unrelated to intracellular Ca^{2+} release. The effect of calycosin on Ca^{2+} channel blockade may be different from that of dihydropyridines.[17] In addition, formononetin, calycosin and ononin all inhibit glutamate-induced cell damage by increasing endogenous antioxidant and stabilizing cell membrane structures.[18]

In the present study, the combined effects of fangchinoline with formononetin, calycosin and ononin on the blood levels of glucose and insulin in STZ-diabetic mice were investigated to throw light on the combined effect of Stephania and Astragali in Boi-ogi-to.

MATERIALS AND METHODS

Preparation of Streptozotocin-Diabetic Mice Fed male mice (ddY strain; 4 weeks of age; 16—20 g; Sankyo Labo Service Co., Inc., Tokyo, Japan) were injected with a single dose (150 mg/kg) of STZ (Sigma, St. Louis, MO, U.S.A.) in

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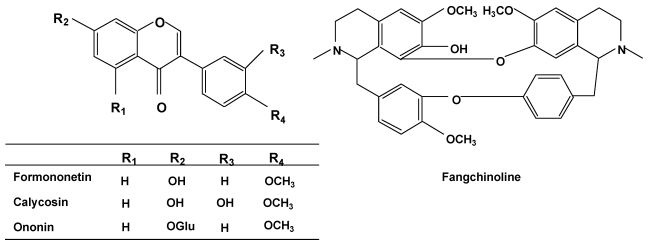

	R_1	R_2	R_3	R_4
Formononetin	H	OH	H	OCH_3
Calycosin	H	OH	OH	OCH_3
Ononin	H	OGlu	H	OCH_3

Chart 1. Chemical Structures of Formononetin, Calycosin, Ononin and Fangchinoline

saline into the tail vein. STZ-induced diabetic mice (7—8 weeks of age; blood glucose above 300 mg/dl) were used for experiments 3—4 weeks after the injection. Age-matched normal male mice (ddY strain; 7—8 weeks of age) were used in the control experiments. These mice were given PMI Laboratory Diet (Japan SLC, Shizuoka, Japan) and water *ad libitum* and kept at 25—26 °C with lights on from 7 a.m. to 7 p.m. Drugs were administered to mice that had been fasted for 3 h. The Ethics Review Committee for Animal Experimentation of Hokuriku University approved the experimental protocol.

Administration of Drugs Fangchinoline and formononetin were obtained from Tsumura Co., Ltd., Tokyo. Calycosin and ononin were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Their chemical structures are shown in Chart 1. Astragali (*Astragalus membranaceus* BUNGE) was collected from Neimenggu, China, during autumn. Astragali was extracted in 6 volumes of distilled water at 96—98 °C for 40 min with an automatic extractor "Torobi" (Tochimoto, Osaka). A water extract of the drug was filtered through a mesh (No. 42, Sanpo, Tokyo), lyophilized with a freeze-drier (DF-03G, ULVAC, Tokyo), and stored at 4 °C. The dry weight yield of extracts was 15.0% (w/w).[3] The components were suspended homogeneously in saline and administered intraperitoneally (i.p.: 0.1 ml/10 g body weight) into mice that had been fasted for 3 h.

Measurement of Blood Glucose and Blood Insulin Levels Blood samples were collected from the neck vein plexus of mice and centrifuged at 8000 rpm at 25 °C for 5 min. Blood glucose levels of the supernatant were measured by the glucose oxidase method with a blood glucose monitor set (Terumo Ltd., Tokyo). Blood glucose levels were measured in fasted mice before, and 6 h after the administration of drugs or saline, respectively. The fall % of blood glucose (BG) was calculated as [BG (before drug treatment)−BG (after drug treatment)]/[BG (before drug treatment)−85]×100. The average BG of 3 h-fasted normal mice is 85.[2,3] Blood insulin levels of 3 h-fasted mice were measured with a mouse ELISA kit for insulin (Morinaga, Yokohama, Japan) 2 and 6 h after the administration of drugs or saline, respectively.

Statistical Analyses All values were expressed as means±S.E.M. Differences between group data were evaluated by *F*-test and unpaired *t*-test at $p=0.05$ or 0.01. A value of $p<0.05$ was considered statistically significant.

RESULTS

Effect of Astragali on the Anti-hyperglycemic Action of Fangchinoline in STZ-Diabetic Mice Fangchinoline alone had no effect at 0.3 mg/kg, but showed anti-hyperglycemic action in STZ-diabetic mice at higher doses in a dose-dependent manner (Fig. 1A). The extract of Astragali (0.48—160 mg/kg) alone did not affect the blood glucose level of diabetic mice. However, Astragali (3—100 mg/kg) significantly reduced the high blood glucose of the diabetic mice in the presence of fangchinoline (0.3 mg/kg) in a dose-dependent manner (Fig. 1B). Since 0.3 mg/kg fangchinoline alone had no effect, Astragali potentiated the anti-hyperglycemic action of fangchinoline in the diabetic mice.

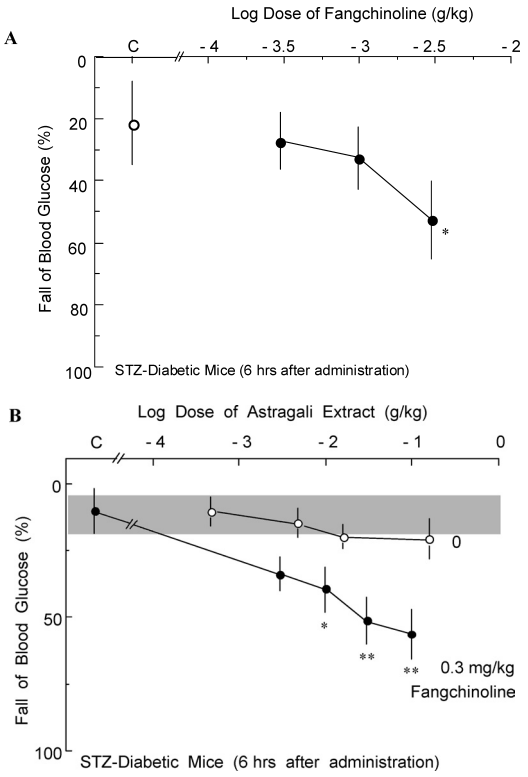

Fig. 1. Effect of Fangchinoline (A) and Astragali Combined with Fangchinoline (B) on the Blood Glucose Level in STZ-Diabetic Mice

A; Blood glucose levels were measured before and 6 h after intraperitoneal administration of fangchinoline in 3 h-fasted STZ-diabetic mice. The decrease % of blood glucose was calculated as described in Materials and Methods. The values are expressed as means±S.E.M. of 6—15 data. $*p<0.05$: Significantly different from the value in the saline group without drug (C). B; Blood glucose levels were measured before and 6 h after simultaneous intraperitoneal administration of Astragali extract and 0.3 mg/kg fangchinoline (●) or Astragali extract alone (○) in 3-h-fasted STZ-diabetic mice. The values are expressed as means±S.E.M. of 6—15 data. C: Fangchinoline (0.3 mg/kg) without Astragali. Shadowed area shows the data for the saline group. $*p<0.05$, $**p<0.01$: Significantly different from fangchinoline alone (C).

Effects of Formononetin, Calycosin and Ononin on the Anti-hyperglycemic Action of Fangchinoline in STZ-Diabetic Mice The effects of isoflavonoids from Astragali, *i.e.*, formononetin, calycosin and ononin, on the anti-hyperglycemic action of fangchinoline 6 h after administration were investigated (Figs. 2—4). Formononetin (0.03—0.1 mg/kg) alone did not affect the high blood glucose in the diabetic mice. However, similar doses of formononetin significantly reduced the high blood glucose in the presence of 0.3 mg/kg fangchinoline in a dose-dependent manner (Fig. 2). Calycosin (0.03—0.1 mg/kg) alone did not affect the high blood glucose in the diabetic mice, but similar doses of calycosin significantly reduced the high blood glucose in the presence of 0.3 mg/kg fangchinoline in a dose-dependent manner (Fig. 3). The anti-hyperglycemic action of calycosin

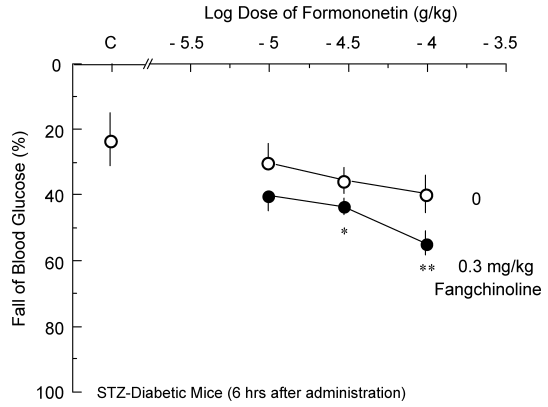

Fig. 2. Combined Effects of Formononetin with Fangchinoline on Blood Glucose Level in STZ-Diabetic Mice

Blood glucose levels were measured before and 6 h after simultaneous administration of formononetin and 0.3 mg/kg fangchinoline (●), formononetin alone (○) or saline (C: ○) into 3-h-fasted diabetic mice. The values are expressed as means±S.E.M. of 6—15 data. $*p<0.05$, $**p<0.01$: Significantly different from the saline group without drug (C).

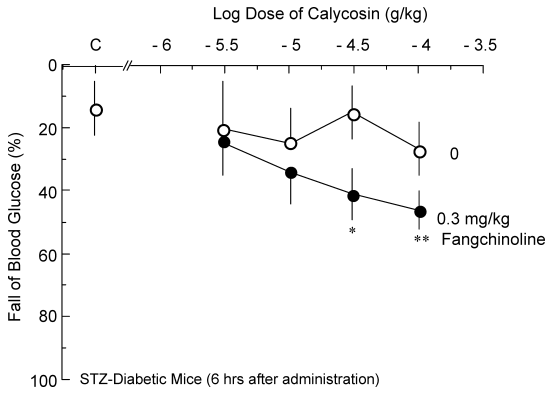

Fig. 3. Combined Effects of Calycosin and Fangchinoline on Blood Glucose Level in STZ-Diabetic Mice

Blood glucose levels were measured before and 6 h after simultaneous administration of calycosin and 0.3 mg/kg fangchinoline (●), calycosin alone (○) or saline (C: ○) into 3-h-fasted diabetic mice. The values are expressed as means±S.E.M. of 6—15 data. $*p<0.05$, $**p<0.01$: Significantly different from the saline group without drug (C).

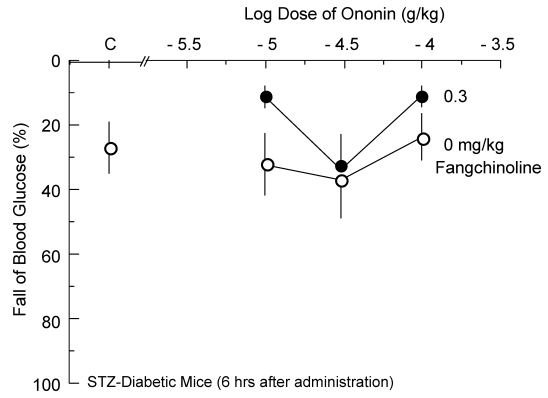

Fig. 4. Effects of Ononin Combined with Fangchinoline on Blood Glucose Level in STZ-Diabetic Mice

Blood glucose levels were measured before and 6 h after simultaneous administration of ononin and 0.3 mg/kg fangchinoline (●), ononin alone (○) or saline (C: ○) into 3-h-fasted diabetic mice. The values are expressed as means±S.E.M. of 6—15 data.

was similar to that of formononetin in the diabetic mice. Ononin (0.03—0.1 mg/kg) did not affect blood glucose in the diabetic mice either in the presence or absence of 0.3 mg/kg

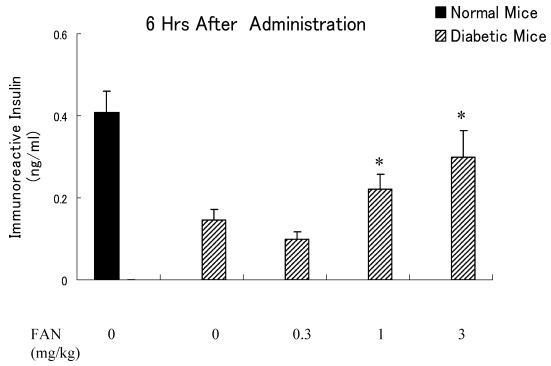

Fig. 5. Effect of Fangchinoline (FAN) on Blood Insulin Level in STZ-Diabetic Mice

Immunoreactive insulin levels were measured at 6 h after administration of FAN into 3-h-fasted diabetic and normal mice. The values are presented as means±S.E.M. of 6—15 data. $*p<0.05$: Significantly different from the saline group without drug.

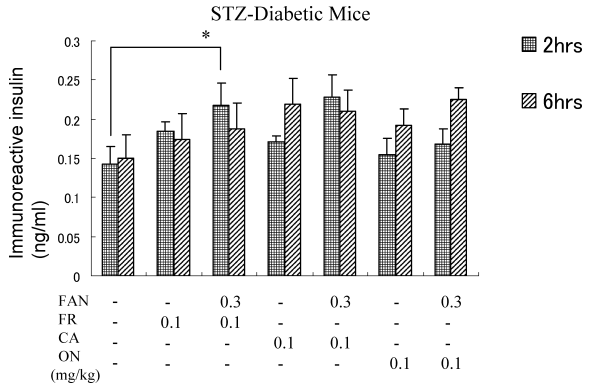

Fig. 6. Combined Effects of Formononetin (FR), Calycosin (CA) and Ononin (ON) with Fangchinoline (FAN) on Blood Insulin Level in STZ-Diabetic Mice

The immunoreactive insulin levels were measured 2 and 6 h after administration of these components in the presence of 0.3 mg/kg FAN into 3-h-fasted diabetic mice. The values are presented as means±S.E.M. of 6—10 data. $*p<0.05$: Significantly different from the saline group without drug.

fangchinoline (Fig. 4). These results demonstrate that formononetin and calycosin potentiate the anti-hyperglycemic action of fangchinoline.

Effects of Formononetin and Calycosin on Fangchinoline-Induced Insulin Release in STZ-Diabetic Mice Blood insulin level in 3-h-fasted diabetic mice 21 d after STZ treatment was decreased to 36% of that in the age-matched normal mice. Fangchinoline had no effect at 0.3 mg/kg but showed increasing anti-hypoinsulinemic action in the diabetic mice at 1—3 mg/kg in a dose-dependent manner (Fig. 5). Formononetin (0.1 mg/kg) alone did not affect insulin release, but significantly increased blood insulin level in the diabetic mice in the presence of fangchinoline (0.3 mg/kg) 2 h after their combined administration in the STZ-diabetic mice compared with the saline group (Fig. 6). The same dose of calycosin also increased the blood insulin level in the diabetic mice in the presence of 0.3 mg/kg fangchinoline 2 h after administration though without statistical significance. However, the effect of formononetin on the insulin release was lost within 6 h. The same dose of ononin tended to increase insulin release in the presence of fangchinoline 6 h after combined administration (Fig. 6). These results indicate that formononetin acts synergistically with fangchinoline to

improve hypoinsulinemia. Their action on hypoinsulinemia occurred earlier than their action on hyperglycemia in the STZ-diabetic mice.

DISCUSSION

The Kampo medicine Stephania plays a key role in the anti-hyperglycemic action of Boi-ogi-to in STZ-induced diabetic mice. One of the mechanism of its anti-hyperglycemic action is an release in blood insulin level in animals with the STZ-induced diabetes.[2,3] The anti-hyperglycemic action of Stephania is mediated by fangchinoline, a major bis-benzylisoquinoline component of Stephania, but not by tetrandrine, another major component.[4] The mechanism of the anti-hyperglycemic action of fangchinoline involves an increase in blood insulin level in animals with STZ-induced diabetes. Stephania also inhibits neovascularization of choroidal and retinal capillaries, which is a complication in STZ-diabetic rats *in vivo* and *in vitro*.[5,19] Tetrandrine inhibits choroidal and retinal neovascularization in the STZ-diabetic rat.[8,9] Fangchinoline and tetrandrine appear to have different roles in relation to the actions of Stephania on STZ-diabetic complication.

Elevated blood levels of glucose accelerate the formation and deposition of advanced glycation end products (AGEs).[20] AGEs stimulate release of angiogenic factors from macrophages and thus increase neovascularization.[21—23] N^{ε}-(Carboxymethyl)lysine (CML) adduct, one of major structures of AGEs in the posterior ocular region, stimulates release of tumor necrosis factor (TNF) α, vascular endothelial growth factors (VEGF) and platelet-derived growth factor (PDGF)-B. These angiogenic factors thus cause over-production of microvessels in retinal and choroidal capillaries in STZ-induced diabetes.[24—26] Stephania not only decreases the formation of AGE through its anti-hyperglycemic action but also inhibits growth factor-induced neovascularization in STZ-diabetes. Fangchinoline and tetrandrine have different roles in indirect and direct neovascularization as a diabetic complication.

Astragali in Boi-ogi-to potentiates the anti-hyperglycemic action of Stephania in diabetic mice. Astragali contains many isoflavonoids such as formononetin, calycosin and ononin, and many saponins.[12] Formononetin and calycosin did not have anti-hyperglycemic action singly, but potentiated the anti-hyperglycemic action of fangchinoline in diabetic mice. Formononetin potentiated fangchinoline's anti-hypoinsulinemic action in diabetic mice earlier after its administration than the anti-hyperglycemic action (Figs. 2, 6), indicating that formononetin reduces blood glucose in the diabetic mice through the potentiation of fangchinoline-induced insulin release. Calycosin also had a potent anti-hyperglycemic action, but did not significantly influence fangchinoline-induced insulin release (Figs. 3, 6). Ononin did not have such a potentiating action in terms of blood level of glucose, but tended to enhance fangchinoline-induced insulin release (Figs. 4, 6). Formononetin significantly reduces arachidonic acid release and production of nitric oxide in lipopolysaccharide activated RAW 264.7 macrophages.[13] Formononetin and calycosin activated the peroxisome proliferator-activated receptors (PPAR) alpha and gamma. The action of formononetin on PPAR was more potent than that of calycosin.[16] In addition, formononetin, calycosin and ononin inhibited glutamate-induced cell damage by increasing endogenous antioxidant and stabilizing cell membrane structures.[18] Our results suggest that the production of endogenous antioxidant is not one of the mechanism of the potentiating actions of formononetin and calycosin. However, it is not clear why calycosin has a weaker potency for fangchinoline-induced insulin release than formononetin, or what is the mechanism of its action.

Formononetin significantly potentiated the action of fangchinoline on insulin release at 2 h after their combined administration, but not at 6 h (Fig. 6). In addition, formononetin did not affect the action of fangchinoline on hyperglycemia 2 h after combined administration (data not shown) but significantly potentiated the anti-hyperglycemic action of fangchinoline at 6 h (Fig. 2). These results demonstrate that there is a time lag of approximately 4 h between the induction of anti-hypoinsulinemic action and the induction of anti-hyperglycemic action in the STZ-diabetic mice *in vivo*. Ononin, a glycoside of formononetin, tended to increase blood insulin level in the present of fangchinoline at 6 h after combined administration, but did not affect the anti-hyperglycemic action during the same time period. These results suggest that it may take approximately 4 h to produce the aglycons, formononetin through the hydrolysis of ononin in STZ-diabetic mice *in vivo*. Ononin might exhibit anti-hyperglycemic action 10 h after administration in the STZ-diabetic mice. Astragali potentiates the action of Stephania on both insulin release and glucose uptake 6 h after combined administration.[3] Some components such as ononin in Astragali may have a role in maintaining the action of formononetin on the fangchinoline-induced increase in insulin release and decrease in blood glucose in STZ-diabetic mice. Further study on this point is needed.

The contents of formononetin and calycosin were estimated to be 0.05% and 0.06%, respectively in Astragali extract,[27] indicating that 100 mg/kg Astragali extract contains approximately 0.05 mg/kg formononetin and 0.06 mg/kg calycosin. We, therefore, chose the dose range of 0.01—0.1 mg/kg for these components in the present experiments. The potencies of formononetin and calycosin for anti-hyperglycemic actions were approximately 2000-fold and 1600-fold greater than that of extract of Astragali, respectively. From these data, it could be calculated that the combined activities of formononetin and calycosin in Astragali extract account almost entirely for the total anti-hyperglycemic activity of Astragali extract, suggesting that formononetin and calycosin are the major active components. Ononin may supply formononetin in STZ-diabetic mice. Thus, we have found a new pharmacological activity of the known components, formononetin and calycosin in Astragali.

The relationship of chemical structure to potentiating action on insulin release was investigated using formononetin, calycosin and ononin isolated from Astragali (Fig. 6). Formononetin, calycosin and ononin are 7-hydroxy, 4′-methoxyisoflavone, 3′,7-dihydroxy, 4′-methoxy isoflavone, and 7-*O*-glucosyl, 4′-methoxy isoflavone, respectively. The 7-hydroxyl and 4′-methoxy groups of isoflavone seem to be important for the potentiating action towards fangchinoline in STZ-diabetic mice. Ononine, a glycoside of formononetin did not affect the action of fangchinoline on insulin release or glucose uptake in STZ-diabetic mice at 6 h after adminis-

tration.

In conclusion, formononetin and calycosin potentiated fangchinoline-induced anti-hyperglycemic action in the diabetic mice. The action of formononetin depended on fangchinoline-induced insulin release.

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