Chinese Medicine, 2011, 2, 130-137
doi:10.4236/cm.2011.24022 Published Online December 2011 (http://www.SciRP.org/journal/cm)

Effects of Bofutsushosan and Gardeniae Fructus on Diabetic Serum Parameters in Streptozotocin-Induced Diabetic Mice

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Received July 9, 2011; *revised August* 14, 2011; *accepted August* 30, 2011

Abstract

Streptozotocin (STZ)-induced diabetic mice increased levels of serum glucose, triglyceride and cholesterol, and decreased level of serum insulin. Effects of Bofutsushosan (BOF: *Pulvis ledebouriellae compositae*: 防風通聖散) and its composed crude drug, gardeniae fructus (GF: 山梔子) were investigated on levels of these diabetic parameters (serum glucose, insulin, triglyceride and cholesterol) in STZ-diabetic mice. BOF and GF were extracted in 10 volumes of distilled water with an automatic extractor "Torobi". STZ-induced diabetic mice with serum glucose level of over 600 mg/dl at 3 - 4 weeks after intravenous injection of 150 mg/kg STZ were used for experiments. BOF extract, GF extract, geniposide (a main constituent of GF), and glibenclamide were administered intraperitoneally into 3-hour-fasted STZ-diabetic mice. At 6 hours after administration, BOF extract (100 - 300 mg/kg) decreased high levels of serum glucose, triglyceride and cholesterol, and also increased low level of serum insulin in STZ-diabetic mice in a dose-dependent manner, respectively. Anti-diabetic drug glibenclamide (0.3 - 1 mg/kg) as positive control significantly decreased serum glucose and cholesterol levels, and increased serum insulin level in the diabetic mice. GF extract (30 - 300 mg/kg) decreased serum glucose, triglyceride and cholesterol levels but did not affect serum insulin level in the diabetic mice. Geniposide (10 - 100 mg/kg), decreased serum glucose level but did not affect serum insulin and triglyceride levels in the diabetic mice. These results demonstrated that intraperitoneally administrated BOF extract improved abnormal levels of serum glucose, insulin, triglyceride and cholesterol in the STZ-diabetic mice as being similar to glibenclamide. GF extract has an important role in a part of improving actions of BOF in the diabetic mice. The action of GF extract on serum glucose was parallel with the action of geniposide in the diabetic mice, supporting roles of geniposide in anti-hyperglycemic action of GF.

Keywords: Bofutsushosan (*Pulvis Ledebouriellae Compositae*: 防風通聖散), Gardeniae Fructus (山梔子), Geniposide, Streptozotocin-Induced Diabetic Mice, Anti-Hyperglycemic Action, Anti-Hyperlipidemic Action

1. Introduction

Diabetes mellitus is a chronic heterogeneous disease characterized by hyperglycemia resulting from both insulin resistance and insulin deficiency secondary to pancreatic cell failure. Diabetes mellitus through hyperglycemia is widely known to be a major factor that leads to microvascular and neural complication [1]. Hyperglycemia-

induced end organ damage in diabetes mellitus is associated with 1) accumulation of advanced glycation end products [2,3]; 2) increased oxidative stress [4,5]; 3) polyol metabolic pathway [6,7]; and 4) activation of protein kinase C pathway [8,9]. Insulin deficiency in diabetes mellitus stimulates lipolysis in the adipose tissue, and gives rise to hyperlipidemia and fatty liver [10]. Although diabetes mellitus is characterized as a disease of carbohydrate metabolism, abnormalities of lipid and lipoprotein metabolism are commonly observed. Hyperlipidemia [11] and atherosclerosis [12] are frequently associated with diabetes mellitus, indicating alterations of cholesterol metabolism in this diabetes [13]. In addition, there is triglyceride enrichment of both high-density lipoprotein (HDL) and low-density lipoprotein (LDL). Therefore, the lipid profile in type 2 diabetic subjects generally consists of elevated triglycerides and LDL cholesterol, and reduced HDL cholesterol [14,15]. Diabetes-related dyslipidaemia has also been shown to be an important contributor to vascular dysfunction. Atherosclerosis is, however, associated with macrovascular disease and thus the role of dyslipidaemia versus hyperglycemia in the pathogenesis of microvascular disease remains unclear and continues to be debated [16].

Streptozotocin (STZ) is N-nitroso derivative of glucosamine and has been commonly used to induce not only animal models of insulin-dependent diabetes mellitus (IDDM, type 1) but also non-insulin-dependent diabetes mellitus (NIDDM, type 2) with hypoinsulinemia by STZ administration to neonates (1 or 2 day old mice) [17-19]. It has been reported that STZ is capable of producing mild to severe types of diabetes according to the dosages used when it is given to animals by either single intravenous or intraperitoneal (*i.p.*) injection [20].

Using this experimental model, we sought to investigate the efficacy of traditional Chinese medicine, bofutsushosan (BOF: *Pulvis ledebouriellae compositae*) on the prevention of abnormal levels of diabetic parameters, serum glucose, insulin, triglyceride and cholesterol. BOF is consisted of 18 crude drugs as described in **Table 1** [21]. BOF is indicated for the relief of the following symptoms of those patients with hypertension, insulin resistance and thick subcutaneous fat in the abdomen and tendency to constipation [21]. BOF has been successfully used in patients with hyper functional constitution who exhibit risk factors for cerebrovascular events. Gardeniae fructus (GF) has traditionally classified as an antipyretic agent in BOF. Chemical isolated from GF has been suggested to increase glucose-stimulated insulin release from pancreatic cells of type 2 diabetes mellitus model [22]. In the present study, effects of extracts of BOF and GF were compared on levels of the diabetic parameters in STZ-diabetic mice to study a role of GF in action of BOF in the STZ-diabetic mice.

2. Materials and Methods

2.1. Preparation of Streptozotocin-Diabetic Mice

Fed male mice (ddY strain; 4 weeks of age; 16 - 20 g; Japan SLC, Shizuoka, Japan) were injected with a single dose (150 mg/kg) of STZ (Sigma, St. Louis, MO, U.S.A.) in saline into the tail vein. STZ-induced diabetic mice (7-8 weeks of age; blood glucose over 600 mg/dl) were used for experiments at 3 - 4 weeks after the injection of STZ. Age-matched normal male mice (ddY strain; 7 - 8 weeks of age) were used in the control experiments. These mice were given CRF-1 (Oriental Yeast Co., Tokyo, Japan) and water ad libitum and kept at 25˚C - 26˚C with lights on from 7 a.m. to 7 p.m. Drugs were administered intraperitoneally to mice that had been fasted for 3 hours. The Ethics Review Committee for Animal Experimentation of Hokuriku University approved the experimental protocol.

2.2. Preparation and Administration of Drugs

BOF consists of Ephedrae Herba, Saposhnikoviae Radix, Zingiberis Rhizoma, Schizonepetae Spica, Rhei Rhizoma, Natrium Sulfuricum, Glycyrrhizae Radix, Forsythiae

Table 1. Crude drugs composed in bofutsushosan.

	Crude drugs	content (g)[a]
1	Ephedrae Herba	1.2
2	Saposhnikoviae Radix	1.2
3	Zingiberis Rhizoma	0.4
4	Schizonepetae Spica	1.2
5	Rhei Rhizoma	1.5
6	Natrium Sulfuricum	0.6
7	Glycyrrhizae Radix	2.0
8	Forsythiae Fructus	1.2
9	Platycodi Radix	2.0
10	Cnidii Rhizoma	1.2
11	Scutellariae Radix	2.0
12	Gardeniae Fructus	1.2
13	Gypsum Fibrosum	2.0
14	Talcum	3.0
15	Angelicae Radix	1.2
16	Paeoniae Radix	1.2
17	Atractylodis Lanceae Rhizoma	2.0
18	Menthae Herba	1.2

[a]Each value (g) was represented as dry weight.

Fructus, Platycodi Radix, Cnidii Rhizoma, Scutellariae Radix, GF, Gypsum Fibrosum, Talcum, Angelicae Radix, Paeoniae Radix, Atractylodis Lanceae Rhizoma, and Menthae Herba. BOF and GF were purchased from Tsumura Co. (Tokyo) and extracted in 10 volumes of distilled water with an automatic extractor "Torobi" (Tochimoto, Osaka, Japan) for 60 min. 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 [23,24]. The dry weight yields of BOF and GF extracts were 27.5% and 67.7% (w/w), respectively. BOF extract, GF extract, geniposide (Wako, Osaka), a main constituent of GF, and glibenclamide (Wako) were administered intraperitoneally (0.1 ml/10 g body weight) into 3-hour-fasted STZ-diabetic mice.

2.3. Measurement of Glucose, Insulin, Triglyceride and Cholesterol Levels in Serum

Blood was collected from the neck vein plexus of diabetic mice at 6 hours after the administration of drugs, and centrifuged at 8000 rpm at 25˚C for 5 min. Serum glucose level of the supernatant was measured by the glucose oxidase method with a serum glucose monitor set (MEDISAFE MINI, Terumo, Tokyo). Serum glucose levels were measured in fasted mice before, and 6 hours after the administration of drugs or saline, respectively. The fall % of serum glucose (SG) was calculated as [SG (before drug treatment) – SG (after drug treatment)]/[SG (before drug treatment) – 85] × 100. The average of SG of 3-hour-fasted normal mice is 85 mg/dl [23,24]. Serum insulin levels of 3-hour-fasted mice were measured with a mouse ELISA kit for insulin (Morinaga, Yokohama, Japan) 6 hours after the administration of drugs or saline. Serum total triglyceride and cholesterol levels were measured with ELISA kits for triglyceride and cholesterol (Wako), respectively.

2.4. Statistical Analyses

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

3. Results

3.1. STZ-Induced Changes of Levels of Body Weight, Serum Glucose, Insulin, Triglyceride and Cholesterol in ddY Mice

Levels of body weight, serum glucose, insulin, triglyceride and cholesterol in ddY mice during 3 weeks after injection of STZ were compared with those in age-matched normal mice in **Table 2**. The body weights of STZ-treated mice were significantly decreased to 93% of those of age-matched normal mice. Levels of diabetic parameters in sera of 3-hour-fasted ddY mice with STZ treatment were compared with those of 3-hour-fasted normal mice. Serum glucose levels of STZ-treated mice were 996.8 mg/dl and significantly 5.4-fold greater than those of normal mice. Serum insulin levels of STZ-treated mice were 183.7 pg/ml and lowered by 82% of normal insulin level. Levels of serum triglyceride and serum cholesterol of STZ-treated mice were 187.3 and 183.2 mg/dl, respectively and increased to 87% and 35% of normal levels. Therefore, we used STZ-treated mice as the diabetic model.

3.2. Effects of Bofutsushosan (BOF) and Glibenclamide on Levels of Serum Glucose and Insulin in STZ-Diabetic Mice

Effects of BOF extract (30 - 300 mg/kg) on level of blood glucose were compared with that of anti-diabetic drug glibenclamide as positive control in *i.p.* treatment during 6 hours. After the drug injection, BOF extract (30 - 300 mg/kg) decreased high level of serum glucose of STZ-diabetic mice in a dose-dependent manner (**Figure 1**). Glibenclamide (Glc; 0.3 - 1 mg/kg) also significantly decreased high serum glucose level of STZ-diabetic mice. Glibenclamide showed anti-hyperglycemic action by its *i.p.* administration as well as BOF extract did. BOF extract (30 - 100 mg/kg) was significantly elevated serum insulin level in a dose-dependent manner. Glibenclamide (0.3 - 1 mg/kg) also significantly increased serum insulin level (**Figure 2**) in the STZ-diabetic mice. Effects of BOF extract and glibenclamide on elevation of serum insulin levels were parallel with those anti-hyperglycemic effects in STZ-diabetic mice.

Table 2. Body weight, serum glucose, insulin, triglyceride (TG) and cholesterol (CH) of STZ-diabetic mice.

	Body weight (g)	Glucose (mg/dl)	Insulin (pg/ml)	TG (mg/dl)	CH (mg/dl)
Normal mice	37.5 ± 0.3	183.3 ± 3.7	1024 ± 64.2	100.2 ± 5.2	135.4 ± 3.7
STZ mice	34.9 ± 0.3**	996.8 ± 43.3**	183.7 ± 25.0**	187.3 ± 10.3**	183.2 ± 4.8**

Values represent means ± S.E.M. **$p < 0.01$: Significantly different from normal mice.

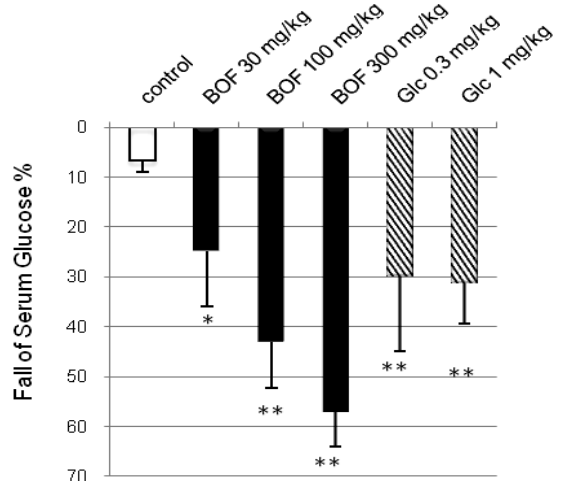

Figure 1. Effects of bofutsushosan (BOF) extract and glibenclamide (Glc) on serum glucose level in STZ-diabetic mice. Serum glucose levels were measured before and 6 hours after *i.p.* administration of BOF extract or Glc into 3-hour-fasted diabetic mice. The values are expressed as means ± S.E.M. of 5 - 25 data. **p* < 0.05, ***p* < 0.01: Significantly different from control water group.

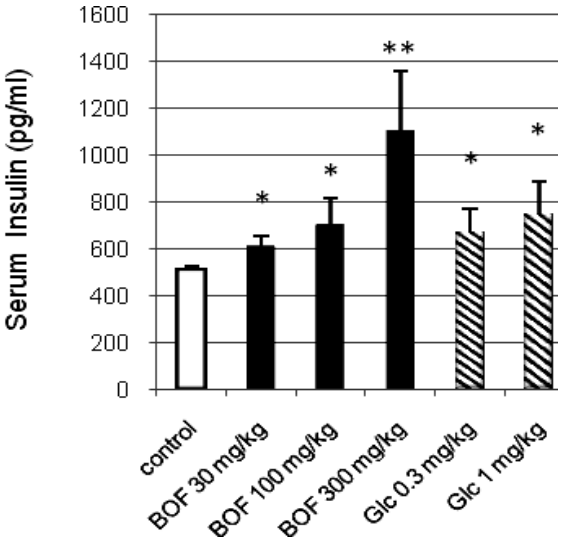

Figure 2. Effects of bofutsushosan (BOF) extract and glibenclamide (Glc) on serum insulin level in STZ-diabetic mice. Serum insulin level was measured at 6 hours after *i.p.* administration of BOF extract or Glc into 3-hour-fasted diabetic mice. The values are expressed as means ± S.E.M. of 5 - 25 data. **p* < 0.05, ***p* < 0.01: Significantly different from control water group.

3.3. Effects of Bofutsushosan (BOF) and Glibenclamide on Levels of Serum Triglyceride and Serum Cholesterol in STZ-Diabetic Mice

BOF extract (30 - 300 mg/kg) lowered serum triglyceride level of STZ-diabetic mice in a dose-dependent manner. However, glibenclamide (0.3 - 1 mg/kg) did not affect the serum triglyceride level (**Figure 3**). BOF extract (30 - 300 mg/kg) also significantly lowered serum cholesterol level of STZ-diabetic mice. Glibenclamide had a weak action for lowering serum cholesterol level when administered only a high dose of 1 mg/kg (**Figure 4**).

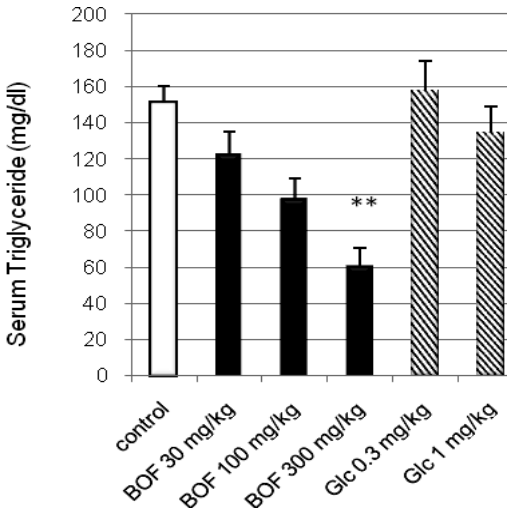

Figure 3. Effects of bofutsushosan (BOF) extract and glibenclamide (Glc) on serum triglyceride level in STZ-diabetic mice. Serum triglyceride level was measured at 6 hours after *i.p.* administration with BOF extract or Glc into 3-hour-fasted diabetic mice. The values are expressed as means ± S.E.M. of 5-25 data. **p* < 0.05, ***p* < 0.01: Significantly different from the control water group.

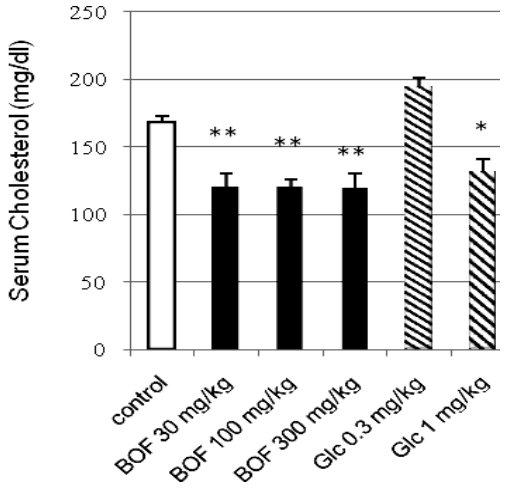

Figure 4. Effect of bofutsushosan (BOF) extract and glibenclamide (Glc) on serum cholesterol in STZ-diabetic mice. Serum cholesterol level was measured at 6 hours after *i.p.* administration with BOF extract or Glc into 3-hour-fasted diabetic mice. The values are expressed as means ± S.E.M. of 5 - 25 data. **p* < 0.05, ***p* < 0.01: Significantly different from the control water group.

3.4. Effects of Gardeniae Fructus (GF) and Geniposide on Serum Diabetic Parameters in STZ-Diabetic Mice

Effects of extract of GF composed in BOF were investigated on levels of the diabetic parameters in STZ-diabetic mice. GF extract (30 - 300 mg/kg) in an *i.p.* administration lowered high serum glucose level in a dose-dependent manner **(Figure 5**). Geniposide (Gep; 10 - 100 mg/kg), a main compound of GF, also lowered high blood glucose level in a dose-dependent manner (**Figure 5**). Anti-hyperglycemic action of GF extract was parallel with that of geniposide. GF extract (30 - 300 mg/kg) in an *i.p.* administration did not affect serum insulin level of STZ-diabetic mice. Geniposide (10 - 100 mg/kg) also did not affect serum insulin level of the diabetic mice (**Figure 6**). GF extract (30 - 300 mg/kg) lowered serum triglyceride level (**Figure 7**) and serum cholesterol level (**Figure 8**) in a dose-dependent manner. However, geniposide (10 - 100 mg/kg) did not affect serum triglyceride level (**Figure 7**) and increased serum cholesterol level conversely (**Figure 8**).

4. Discussion

Diabetes mellitus is characterized as a disease of carbohydrate metabolism and abnormalities of lipid and lipoprotein metabolism. Insulin deficiency stimulates lipolysis in the adipose tissue, and gives rise to hyperlipidemia and fatty liver in diabetes mellitus [10]. Hyperlipidemia

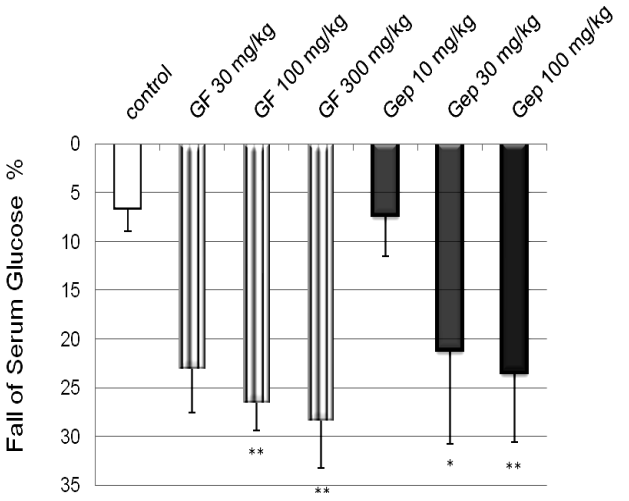

Figure 5. Effects of gardeniae fructus (GF) extract and geniposide (Gep) on the serum glucose level in STZ-diabetic mice. Serum glucose levels were measured before and 6 hours after *i.p.* administration with GF extract or Gep into 3-hour-fasted diabetic mice. The values are expressed as means ± S.E.M. of 5 - 15 data. **p* < 0.05, ***p* < 0.01: Significantly different from the control water group.

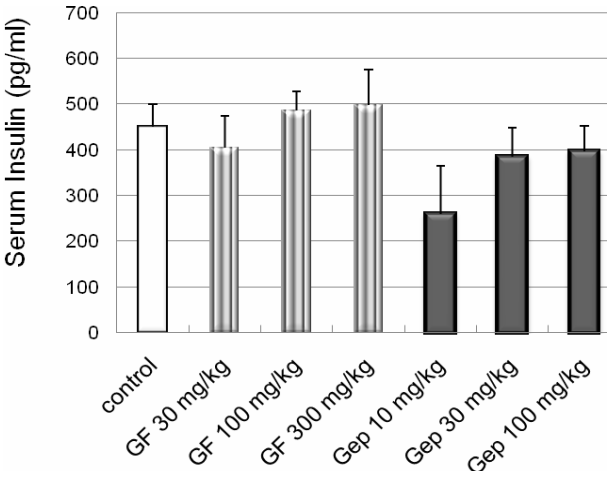

Figure 6. Effects of gardeniae fructus (GF) extract and geniposide (Gep) on serum insulin level in STZ-diabetic mice. The immunoreactive insulin level was measured at 6 hours after *i.p.* administration with GF extract or Gep into 3-hour-fasted diabetic mice. The values are expressed as means ± S.E.M. of 5 - 15 data.

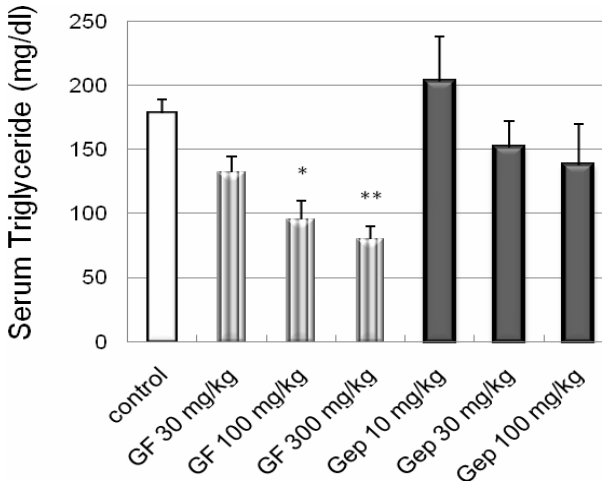

Figure 7. Effects of gardeniae fructus (GF) extract and geniposide (Gep) on serum triglyceride level in STZ-diabetic mice. Serum triglyceride level was measured at 6 hours after *i.p.* administration with GF extract or Gep into 3-hour-fasted diabetic mice. The values are expressed as means ± S.E.M. of 5 - 15 data. **p* < 0.05, ***p* < 0.01: Significantly different from the control water group.

and atherosclerosis are frequently associated with diabetes mellitus, indicating alterations of cholesterol metabolism [11-13]. The lipid profile in type 2 diabetic subjects generally consists of elevated triglycerides and LDL cholesterol, and reduced HDL cholesterol. In the present study, STZ-diabetic mice were used as diabetic model and showed significant increase of serum glucose, total triglyceride and total cholesterol levels, and decrease of serum insulin level (**Table 2**).

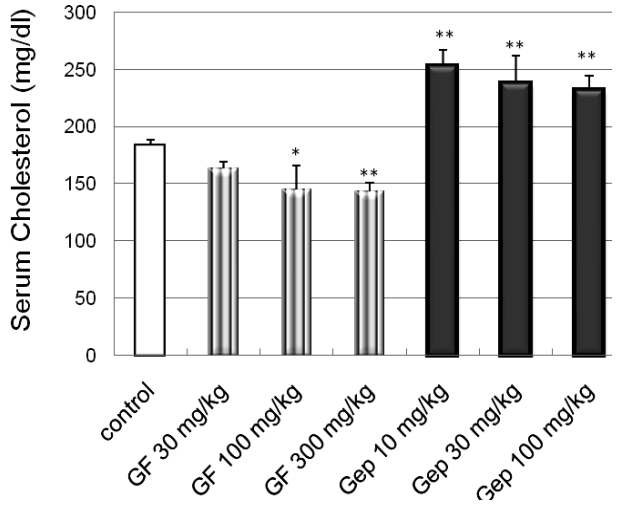

Figure 8. Effects of gardeniae fructus (GF) extract and geniposide (Gep) on the serum cholesterol level in STZ-diabetic mice. Serum cholesterol level was measured at 6 hours after *i.p.* administration with GF extract or Gep into 3-hour-fasted diabetic mice. The values are expressed as means ± S.E.M. of 5 - 15 data. *$p < 0.05$, **$p < 0.01$: Significantly different from the control water group.

BOF is a traditional Chinese medicine consisted of 18 crude drugs as described in **Table 1** [21]. BOF extract and glibenclamide, an oral anti-diabetic agent, were given a single *i.p.* administration to the STZ-diabetic mice. BOF extract lowered high serum glucose level in a dose-dependent manner as parallel to elevate low serum insulin level. The improving actions of BOF extract on serum levels of glucose and insulin were similar to those of glibenclamide in STZ-diabetic mice (**Figures 1** and **2**). These results indicate that our STZ-diabetic mice can be released insulin from pancreatic β cells by both glibenclamide and BOF extract. STZ has been reported to produce mild to severe types of diabetes mellitus according to the dosages used and experimental conditions [20]. Type 2 diabetes mellitus is associated with a combination of resistance to insulin action and impaired insulin secretion [25]. We have unpublished data that both BOF extract and glibenclamide in their oral administration decreased serum glucose level and increased insulin level in serum of alloxan-induced diabetic mice, which show type 2 model of diabetes mellitus [26]. It is also reported that low dose STZ combined with high-energy intake can effectively induce type 2 diabetes through altering the related gene expression [27]. These results demonstrate that BOF improves hyperglycemia through releasing insulin from pancreatic β cell of STZ-diabetic mice.

BOF extract also lowered high levels of serum triglyceride and serum cholesterol of STZ-diabetic mice in a dose-dependent manner. However, glibenclamide decreased only high level of serum cholesterol at a high dose but not affect serum triglyceride even at the high dose (**Figures 3** and **4**). These results indicated that actions of BOF extract on levels of serum triglyceride and cholesterol did not depend on action of released insulin in the STZ-diabetic mice.

GF extract is traditionally classified as antipyretic agent in BOF and decreased high level of serum glucose in STZ-diabetic mice in a dose-dependent manner. However, GF extract did not affect level of insulin in the STZ-diabetic mice (**Figures 5** and **6**). These results demonstrate that anti-hyperglycemic action of GF differed from those of BOF and glibenclamide. The anti-hyperglycemic action of GF is supported by action of geniposide, a main compound of GF on serum glucose level. Yield of geniposide in GF extract is estimated to be 37% and the effect of geniposide is almost 3-time greater than that of GF extract. Geniposide also did not affect serum insulin level of STZ-diabetic mice. These results indicated that anti-hyperglycemic action of GF extract depended on the action of geniposide in the STZ-diabetic mice. The anti-hyperglycemic action of GF extract was independent on release of serum insulin. GF extract also lowered levels of serum triglyceride and serum cholesterol of STZ-diabetic mice in a dose-dependent manner. However, geniposide did not affect level of serum triglyceride and increased level of serum cholesterol (**Figures 7** and **8**), being different from the actions of GF extract for serum levels of triglyceride and cholesterol. The results suggest that some compounds different from geniposide in GF may have a role for the actions of GF extract on serum triglyceride and cholesterol levels.

Mitochondrial uncoupling protein 2 (UCP2) has reported to alter the yield of ATP synthesis from glucose, and is proposed as a negative regulator of glucose-stimulated insulin secretion in pancreatic β cells of type 2 diabetes mellitus model [28]. The absence of mitochondrial UCP2 renders animals more sensitive to the onset of type 1 autoimmune diabetes in mice [29]. UCP2 and UCP3 gene expressions were increased in skeletal muscle of STZ-diabetic rat, while UCP1, UCP2 and UCP3 gene expressions were reduced in brown adipose tissue of these rats [30]. UCP2 also modulates myocardial excitation-contraction coupling [31]. Genipin has been reported to be the bioactive compound of geniposide, one of major effective compounds of GF and a natural cross-linking agent. Genipin is inhibitor of UCP2. UCP2 deficiency improves obesity- and high glucose-induced β cell dysfunction and consequently improves type 2 diabetes [22]. The inhibition of UCP2 with genipin also sensitizes multidrug-resistant cancer cells to cytotoxic agents [32]. The present study demonstrate that administered GF extract and geniposide decreased high serum glucose level but not affect serum insulin level in the

STZ-diabetic mice. Genipin may involve the anti-hyperglycemic and anti-hyperlipidemic actions of GF extract. We need further experiments for high doses of geniposide and genipin, and duration of their treatment.

5. Conclusions

BOF extract improved abnormal levels of serum glucose, insulin, triglyceride and cholesterol in the STZ-diabetic mice. Extract of GF in BOF also improved levels of serum glucose, triglyceride and cholesterol but not level of serum insulin in the diabetic mice. These results indicate that GF extract has an important role in a part of improving actions of BOF in the diabetic mice. Anti-hyperglycemic action of BOF extract may have two, insulin release-dependent and non-insulin release-dependent mechanisms in the STZ-diabetic mice.

6. Acknowledgements

This work was supported in part by a grant (to SK) for the "Academic Frontier" project for Private Universities (2005-2009) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, Japan.

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