Antidiabetic effects of Kampo medicines in a non-insulin-dependent diabetes mellitus model using KK-Ay mice

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Abstract

The effects of Kampo medicine, Dai-saiko-to (DST; 大柴胡湯), Dai-saiko-to-ka-jio (DSJ; 大柴胡湯加地黄), Byakko-ka-ninjin-to (BNT; 白虎加人参湯), Hachimi-jio-to (HJT; 八味地黄湯) on KK-Ay mice, a hereditary animal model of non-insulin-dependent diabetes mellitus, were examined. DST and DSJ at 100 mg/kg inhibited this increase in serum glucose levels after 4 weeks of treatment. DST and DSJ at 500 mg/kg also clearly inhibited hyperglycemia in KK-Ay mice after 4 and 6 weeks of treatment, respectively. BNT and HJT at 100 mg/kg inhibited the increase in serum glucose levels after 4 to 8 weeks of treatment. Anti-hyperglycemic effects of BNT and HJT at 500 mg/kg in KK-Ay mice continued throughout the treatment period. However, none of the Kampo medicines tested inhibited the increases in serum insulin levels in KK-Ay mice. DSJ, BNT and HJT inhibited the increases in body weight. The high levels of serum triglyceride were inhibited by DST, DSJ and BNT. DSJ, BNT and HJT decreased non-esterified fatty acid levels in KK-Ay mice. Moreover, BNT inhibited the increases in G-6-PDH levels in the liver. DSJ significantly decreased levels of the fatty acid synthetic enzyme (ME). BNT and HJT inhibited the decreases in β-oxidation in KK-Ay mice. We demonstrated that the repeated administration of DST, DSJ, BNT and HJT inhibited the increases in serum glucose levels in KK-Ay mice, and the hypoglycemic effects of BNT and HJT were especially long lasting. These results suggested that the hypoglycemic effects of DSJ, BNT and HJT would result in improvement of these diabetes-related symptoms.

Key words Kampo medicines, KK-Ay mice, serum glucose, body weight, TG, NEFA, liver enzymes.

Abbreviations BNT, Byakko-ka-ninjin-to (Bai-Hu-Jia-Ren-Sheng-Tang), 白虎加入参湯 ; DST, Dai-saiko-to (Da-Chai-Hu-Tang), 大柴胡湯 ; DSJ, Dai-saiko-to-ka-jio (Da-Chai-Hu-Tang-Jia-Di-Huang), 大柴胡湯加地黄 ; ELISA, enzyme-linked immunosorbent assay ; G-6-PDH, Glucose 6-phosphate-dyhydrogenase ; HJT, Hachimi-jio-to (Ba-Wei-Di-Huang-Tang), 八味地黄湯 ; ME, Malic Enzyme ; NEFA, non-esterified fatty acid ; NIDDM, non-insulin-dependent-diabetes mellitus ; TC, total cholesterol ; TG, triglyceride.

Introduction

Kampo medicines such as Dai-saiko-to (DST), Dai-saiko-to-ka-jio (DSJ), Byakko-ka-ninjin-to (BNT) and Hachimi-jio-to (HJT) have been widely used for the treatment of Type 2 (NIDDM).[1-5] Prescriptions of Kampo medicines for Type 2 patients are decided according to hyperglycemia and related symptoms such as hydrodipsia, polyuria, hot flushes and hyperlipidemia. Dai-saiko-to (DST) and Dai-saiko-to-ka-jio (DSJ) are prescribed for patients in the early stage

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in Type 2. BNT and HJT are prescribed from the intermediary to late stages of Type 2.[6] Many studies have indicated the antidiabetic effects of Kampo medicines in experimental models of diabetes such as alloxan or streptozotocin-induced diabetic rats and mice and hereditary diabetic KK-Ay mice. However, these previous studies were performed with crude drugs and as a short-term treatment.[7-10] The antidiabetic effects of long-term treatment with clinically useful Kampo medicines for Type 2 and related symptoms in hereditary diabetic KK-Ay mice are not clearly understood. The present study was performed to compare the effects of long-term treatment with DST, DSJ, BNT and HJT in KK-Ay mice.

Materials and Methods

Preparation of Kampo medicines: Four Kampo formulations, Dai-saiko-to (DST, Da-Chai-Hu-Tang; 大柴胡湯), Dai-saiko-to-ka-jio (DSJ, Da-Chai-Hu-Tang-Jia-Di-Huang; 大柴胡湯加地黄), Byakko-ka-ninjin-to (BNT, Bai-Hu-Jia- Ren- Sheng-Tang; 白虎加人参湯) and Hachimi-jio-to (HJT, Ba-Wei-Di-Huang-Tang; 八味地黄湯) were prepared in this study. Formulations of the four Kampo medicines are listed in Table I. The crude drugs were obtained from Uchida Wakan-Yaku Co., Ltd (Tokyo, Japan). The formulations were immersed in a 10-fold (w/w) amount of distilled water, and decocted to half volume. The decoction was centrifuged at 1,500 rpm for

Table I Prescription of Kampo medicines.

Formulation	Name	Herbs	Daily dose (g)
Dai-saiko-to (DST)	saiko	Bupleuri Radix	6
	hange	Pinelliae Tuber	6
	ogon	Scutellariae Radix	3
	shakuyaku	Paeoniae Radix	3
	taiso	Zizyphi Fructus	3
	kijitsu	Auranti Fructus Immaturus	2
	shokyo	Zingiberis Rhizoma	4
	daio	Rhei Rhizoma	1
Dai-saiko-to-ka-jio (DSJ)	saiko	Bupleuri Radix	6
	hange	Pinelliae Tuber	6
	ogon	Scutellariae Radix	3
	shakuyaku	Paeoniae Radix	3
	taiso	Zizyphi Fructus	3
	kijitsu	Auranti Fructus Immaturus	2
	shokyo	Zingiberis Rhizoma	4
	daio	Rhei Rhizoma	1
	jio	Rehmanniae Radix	5
Byakko-ka-ninjin-to (BNT)	chimo	Anemarrhenae Rhizoma	5
	koubei	Oryzae Semen	8
	sekko	Gypsum Fibrosum	15
	kanzo	Glycyrrhizae Radix	2
	ninjin	Ginseng Radix	1.5
Hachimi-jio-to (HJT)	jio	Rehmanniae Radix	5
	sanshuyu	Corni Fructus	3
	sanyaku	Dioscoreae Rhizoma	3
	takusha	Alismatis Rhizoma	3
	bukuryo	Poria	3
	botampi	Moutan Cortex	3
	keihi	Cinnamomi Cortex	1
	bushi	Aconiti Tuber	0.5

20 min, and then the supernatant was immediately passed through filter paper (Advance 2, Toyo Roshi Co., Tokyo Japan). The filtrate was lyophilized and stored at room temparature until use. The yields of lyophilized extracts were 16 %, 19 %, 8 % and 25 % for DST, DSJ, BNT and HJT, respectively.

Animals : Male KK-Ay/Ta Jcl and KK/Ta Jcl, 8 weeks old, were purchased from Clea (Tokyo, Japan). The animals were housed in individual cages under a controlled temperature (23±1°C) and a 12 hour light-dark cycle (06:00-18:00). The mice were allowed free access to breeding food and water.

Experiment procedure : At 10 weeks of age, KK-Ay mice showed blood glucose levels of about 460± 180 mg/dl, which was considered hyperglycemia and oral administration of test formulations was started at this age. Lyophilized extracts of Kampo medicines were dissolved in distilled water, and administered orally once a day at doses of 100 or 500 mg/kg/day for 8 weeks.

Body weight and water intake were measured daily and weekly, respectively. Blood was sampled from the tail vein with glass capillaries tubes at 09:00-12:00 on days of 0,2,4,6 and 8 weeks after Kampo treatment. Urine was sampled every week. After treatment for 8 weeks, mice were anesthetized with sodium pentobarbital (30 mg/kg,i.p.). The studies were started at 09:00-12:00, and biological samples were collected after repeated administration of Kampo medicines at 09:00-12:00.

The abdomen was opened, and blood was collected from the inferior vena cava with a disposable syringe. After perfusion with ice-cold saline, the liver was quickly removed and weighed. All the experiments were performed in non-fasting animals.

Analysis of biochemical factors : Commercial kits (Wako Pure Chemical Industries Ltd.) were used to assay glucose and lipids in serum and urine. Levels of glucose, total cholesterol (TC), triglyceride (TG) and nonesterified fatty acid (NEFA) were determined with the following assay kits : Glucose B, Cholesterol C-II, Triglyceride G and NEFA. Insulin levels in serum were determined with Mause Insulin (Shibayagi) assay kits. Total protein (TP) level in urine was determined by the method of Lowry *et al.*[11]

Analysis of liver enzymes : To assay fatty acid synthetic enzymes (ME: malic enzyme, G-6-PDH: glucose 6-phosphate dehydrogenase), the liver was homogenized in 4 volumes of 0.14 M KCl. The homogenate was centrifuged at 4°C for 60 minutes at 100,000×g, and the resulting supernatant solution was used as the enzyme source.

Two enzyme activities were determined at 25°C by observing changes in absorbance at 340 nm.[12] To determine total β-oxidation enzyme activity, the liver was homogenized in 9 volumes of 0.25 M sucrose-5 mM Tris-HCl (pH7.4). The homogenate was centrifuged at 4°C for 20 minutes at 600×g, and the supernatant thus obtained was further centrifuged at 30,000×g for 30 minutes to obtain the mitochondrial fraction. The mitochondrial pellets were suspended in 0.25 M sucrose - 5mM Tris-HCl. The total β-oxidation activity was also determined at 25°C by observing changes in absorbance at 340 nm.[13]

Statistical analysis : Statistical significance of the results was evaluated using Student's t-test. A p-value of less than 0.05 was considered statistically significant.

Results

Glucose levels in serum and urine

As shown in Fig. 1, serum glucose levels of the KK-Ay mice gradually increased until 10 weeks of age (Fig.1. 0 week), and were higher than those of KK mice. In the KK-Ay controls, maximum glucose levels in serum were observed at 14 to 16 weeks of age, and hyperglycemia continued throughout the experimental period. DST and DSJ at 100 mg/kg significantly inhibited the increases in serum glucose level after 4 weeks of treatment. The level was also significantly inhibited by DST and DSJ at 500 mg/kg after 4 and 6 weeks of treatment, respectively. BNT and HJT at 100 mg/kg significantly inhibited the increases in serum glucose levels after 4 to 8 weeks of treatment. Significant continuous effects of BNT and HJT at 500 mg/kg were observed on the serum glucose levels in KK-Ay mice throughout the treatment period.

Urinary glucose levels in the KK-Ay mice increased with age. DST at 100 and 500 mg/kg and DSJ at 500 mg/kg significantly reduced urinary glucose levels compared to the KK-Ay controls.

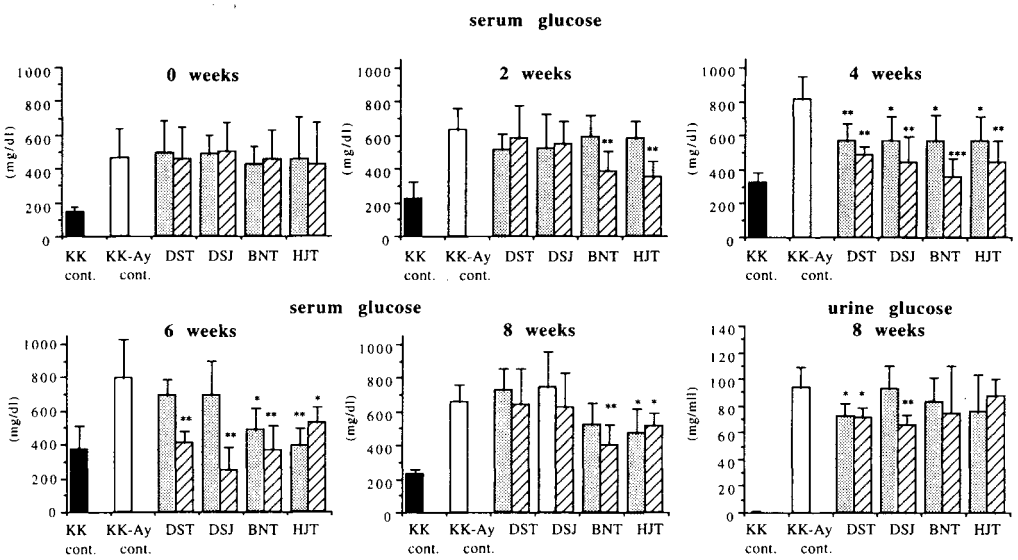

Fig. 1 Effects of Kampo medicines on the serum and urine glucose levels in KK-Ay mice. Each Kampo were Dai-saiko-to (DST), Dai-saiko-to-ka-jio (DSJ), Byakko-ka-ninjin-to (BNT) and Hachimi-jio-to (HJT). ■ KK mice control ; □ KK-Ay mice control ; ▨ Kampo treatment 100 mg/kg and ▨ Kampo treatment 500 mg/kg. Each value represents the mean±S.D. of 5 to 6 male KK-Ay mice.
*$p<0.05$, **$p<0.01$ and ***$p<0.001$: significant difference from KK-Ay control group.

Body weight and water intake

The body weight changes are presented in Table II. During the experimental period, the body weight of KK and KK-Ay mice gradually increased with age, and the body weight gain of KK-Ay mice with age was greater than that of KK-mice. DSJ at 100 mg/kg significantly inhibited the increase in body weight throughout the treatment period. BNT at 100 mg/kg significantly inhibited growth after 4 to 6 weeks of treatment. DSJ at 500 mg/kg, BNT at 500 mg/kg, and HJT at 100 and 500 mg/kg also inhibited these increases after 4 weeks of treatment.

As shown in Table III, the water intake of KK-Ay mice was greater than that of KK-mice. DST at 100 mg/kg reduced the water intake after 4 to 6 weeks of treatment.

Lipid levels in serum

As shown in figure 2, the levels of serum TG in

Table II Effects of Kampo medicines on body weight changes in KK-Ay mice.

Groups	Dose (mg/kg,p.o.)	Body weight (g) 0 (10)	2 (12)	4 (14)	6 (16)	8 weeks of treatment (18) (weeks of age)
KK mice control		34.8±0.8***	37.6±0.6***	39.6±1.8***	41.0±1.0***	42.3±1.2*
KK-Ay mice control		40.3±1.6	43.2±1.6	46.8±1.3	47.4±1.8	46.2±2.6
DST	100	40.5±1.8	42.7±2.5	44.3±2.9	45.7±2.3	44.0±2.6
	500	40.5±1.8	42.5±1.5	45.7±1.6	46.3±2.0	46.0±2.6
DSJ	100	40.1±0.8	40.7±0.8**	43.3±1.5**	43.0±2.6*	42.0±1.4*
	500	40.0±1.3	41.7±2.0	43.7±1.5**	45.2±2.2	44.0±2.1
BNT	100	40.5±1.6	42.7±1.6	43.2±1.6**	43.2±3.6*	45.2±3.6
	500	40.7±1.6	42.3±2.3	43.0±2.7*	44.2±3.0	44.3±2.8
HJT	100	40.7±1.9	42.2±2.4	43.0±2.4*	44.0±3.0	46.2±3.1
	500	41.3±1.4	42.2±2.1	43.5±2.3*	44.8±2.4	46.3±2.4

Each value represents the mean±S.D. of 5 to 6 mice.
*$p<0.05$, **$p<0.01$ and ***$p<0.001$: significant difference from KK-Ay control group.

Table III Effects of Kampo medicines on water intake in KK-Ay mice.

Groups	Dose (mg/kg,p.o.)	Water intake (ml/day) 0 (10)	2 (12)	4 (14)	6 (16)	8 weeks of treatment (18) (weeks of age)
KK mice control		5.7±1.4***	7.1±1.2***	7.6±1.1***	8.8±1.9***	10.4±2.7***
KK-Ay mice control		16.3±4.4	17.7±3.6	19.5±2.4	20.3±3.1	16.7±4.7
DST	100	16.3±3.5	17.2±2.8	16.3±2.4*	15.8±3.0*	15.3±5.5
	500	16.0±3.9	18.1±4.8	19.6±5.3	17.1±4.7	13.2±6.3
DSJ	100	18.8±1.5	20.6±2.4	19.6±3.6	16.8±3.6	17.0±7.4
	500	19.3±2.5	18.4±4.3	15.9±4.0	15.6±3.8	14.3±4.3
BNT	100	16.5±2.5	19.5±3.6	17.0±6.2	17.7±8.7	19.7±2.7
	500	15.2±3.4	18.0±5.8	16.9±6.4	17.6±8.5	16.7±7.6
HJT	100	15.8±2.8	17.9±1.7	14.1±5.7	17.1±8.1	17.7±5.9
	500	16.7±3.4	20.4±4.0	17.8±5.1	22.3±4.0	20.1±2.5

Each value represents the mean±S.D. of 5 to 6 mice.
*$p<0.05$, and ***$p<0.001$: significant difference from KK-Ay control group.

KK-Ay mice were increased compared to KK-mice. DST and BNT at 500 mg/kg and DSJ at 100 and 500 mg/kg significantly inhibited the increases in TG level. However, HJT at 100 mg/kg increased TC levels. DSJ and HJT at 500 mg/kg and BNT at 100 and 500 mg/kg significant decreased NEFA levels.

Liver enzymes

As shown in Table IV, the G-6-PDH activity in the liver of KK-Ay mice was higher than that of KK-mice. BNT at 100 mg/kg prevented the increase in G-6-PDH level. The malic enzyme (ME) activity of KK-Ay mice was almost equivalent to that of KK-mice. DSJ at 100 mg/kg decreased ME levels in KK-Ay mice. The β-oxidation activity in KK-Ay mice was

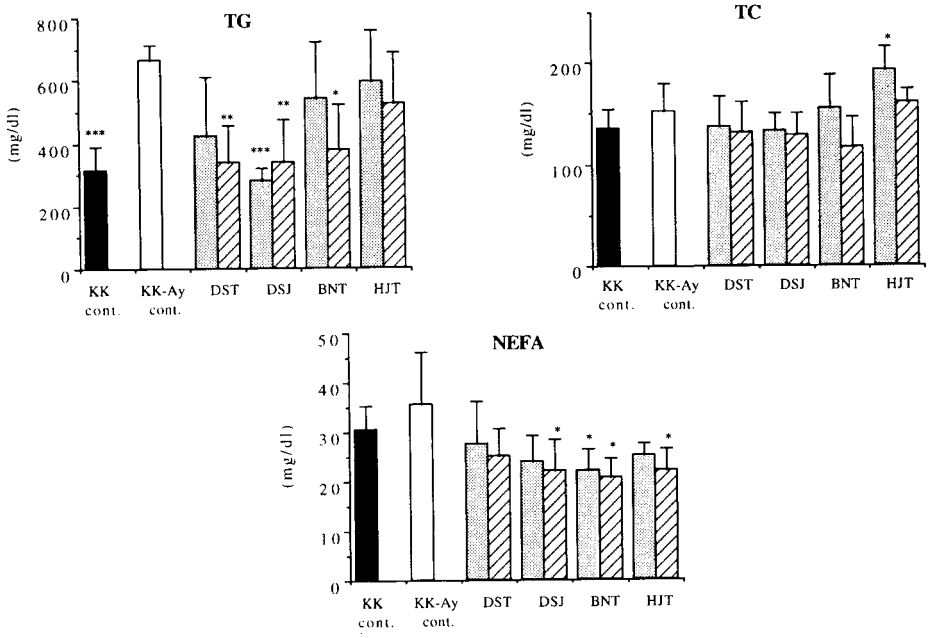

Fig. 2 Effects of Kampo medicines on the TG, TC and NEFA levels in male KK-Ay mice.
■ KK mice control ; □ KK-Ay mice control ; ▨ Kampo treatment 100 mg/kg and ▨ Kampo treatment 500 mg/kg. Each value represents the mean±S.D. of 5 to 6 male KK-Ay mice.
*$p<0.05$, **$p<0.01$ and ***$p<0.001$: significant difference from KK-Ay control group.

Table IV Effects of Kampo medicines on liver enzymes in KK-Ay mice.

Groups	Dose (mg/kg,p.o.)	Liver enzymes		
		G-6-PDH (mU/mg protein)	ME (mU/mg protein)	β-oxidation (m mol AA/mg protein)
KK mice control		37.8±4.9***	15.1±1.8	33.0±4.5**
KK-Ay mice control		51.7±6.3	14.6±1.4	23.1±4.1
DST	100	50.3±10.4	14.4±1.2	20.4±5.2
	500	46.5±9.3	13.7±1.4	21.5±6.3
DSJ	100	47.6±5.9	12.3±1.1*	22.8±2.4
	500	51.7±2.7	13.8±1.2	19.7±4.1
BNT	100	39.2±5.4**	13.5±1.7	32.0±2.9**
	500	51.3±13.0	13.6±0.7	22.3±3.5
HJT	100	44.6±5.0	14.9±2.3	31.4±4.6*
	500	47.0±5.9	13.5±1.2	27.3±1.2*

Each value represents the mean±S.D. of 5 to 6 mice.
*$p<0.05$, **$p<0.01$ and ***$p<0.001$: significant difference from KK-Ay control group.

lower than that of KK-mice. BNT at 100 mg/kg, and HJT at 100 and 500 mg/kg inhibited the decreases in β-oxidation activity.

The serum insulin levels in KK and KK-Ay mice were 3.61±2.14 ng/ml and 14.16±1.82 ng/ml, respectively. The serum insulin levels in the KK-Ay mice were not changed by treatment with DSJ, BNT or HJT (data not shown).

Discussion

KK-Ay mice, an animal model of Type 2 with hyper-insulinemia, show hyperglycemia as a result of insulin resistance.[14]

In the present study, DST and DSJ inhibited the increases in serum glucose levels in the intermediate stage of Type 2 in KK-Ay mice. Further, BNT and HJT clearly showed continuous hypoglycemic effects throughout the experimental period. Kampo medicines have been reported to decrease blood glucose levels in this experimental model. Miura *et al.* reported that the intestinal uptake of glucose increased and hyperglycemia developed in KK-Ay mice. Bakumondo-inshi (BI) treatment significantly inhibited glucose uptake and the expression of SGLUT protein.[15] Also, a single or repeated administration of To-kai-san reduced the glucose and insulin levels of blood in the normal and KK-Ay mice.[16] Byakko-ka-ninjin-to and its component crude drugs inhibited the increases in blood glucose levels in both KK-Ay and alloxan-diabetic mice.[7,9] The intraperitoneal administration of Hachimigan and dietary admixture of Hachimi-jiogan (HMG) also inhibited the increases of blood glucose in alloxan-diabetic and KK-Ay mice.[7,17] The results of the present study indicated that DST, DSJ, BNT and HJT at doses close to those used clinically inhibited the increases in serum glucose level, and the hypoglycemic effects of BNT and HJT were observed over a long-term of 8 weeks as compared with previous reports. The insulin levels in serum of KK-Ay mice were markedly increased relative to KK-mice, but none of the Kampo medicines tested caused any significant changes in insulin levels. However, Kampo medicines significantly improved obesity, hypertriglyceridemia and hyperglycemia. These risk factors have important roles in the development of insulin resistance and type 2. Thus, these Kampo medicines may be able to prevent development of type 2. The differences in the effects of Kampo medicines on the blood insulin levels in diabetic mice between the present study and those reported previously may have been due to differences in route and dose of administration.

The effects of Kampo medicines on type 2 related symptoms in KK-Ay mice were as follows : the inhibition of urinary glucose by DST and DSJ, the inhibition of body weight gain by DSJ, BNT and HJT, and the reduction of water intake by DST. Food intake in KK-

Ay mice increases with the development of diabetes and results in significant obesity. However, Kampo medicines were reported to have no effects on the food intake in KK-Ay mice.[17] In this study, food intake in the Kampo treatment groups did not differ from that of the diabetic controls. Serum TG in KK-Ay mice was significantly reduced by administration of DST, DSJ and BNT. The NEFA levels were also significantly decreased by DSJ, BNT and HJT. Furuya *et al.*[17] recently reported that the high levels of TG and FFA in KK-Ay mice were ameliorated by administration of Hachimi-jio-gan (HMG) included in the powdered diet. In the present study, HJT did not significantly inhibit the increases in TG level. This discrepancy was also considered to be due to the difference in route of administration. The inhibitory effects of Kampo medicines on TG increases in KK-Ay mice were considered to be slightly related to hypoglycemic effects. However, glucose 6-phosphate dehydrogenase, ME, β-oxidative enzymes, fatty acid synthetic enzymes and metabolic enzymes of TG in the liver, were not closely related to changes in TG or NEFA in Kampo medicine-administered KK-Ay mice. These results indicated that the ameliorative effects of Kampo medicines on TG and NEFA levels may be due to direct effects on the excretion of TG and NEFA. On the other hand, it has been shown that free fatty acids cause acceleration of glucose release in the liver due to the inhibition of glucose transporter 4-related glucose uptake.[18] Thus, we regarded the decrease in serum NEFA level as one of the mechanisms of the hypoglycemic effects of DSJ, BNT and HJT. Kuriyama *et al.* demonstrated that hepatic acyl-coenzyme A synthetase (ACS) activity and mRNA levels and microsomal triglyceride transfer protein (MTP) mRNA levels were also increased in obese and hypertriglyceridemic rats.[19] This study suggested that enhanced expression of both ACS and MTP genes associated with visceral fat accumulation before development of insulin resistance may be involved in the pathogenesis of hyperlipidemia in obese animal models of Type 2.[19] Further studies are needed to confirm these suggestions.

In conclusion, we demonstrated that the repeated administration of DST, DSJ, BNT and HJT inhibited the increases of serum glucose in KK-Ay mice, and the hypoglycemic effects of BNT and HJT were especially long lasting. In addition, DSJ, BNT and HJT inhibited body weight gain and decreases in serum NEFA level. The hypoglycemic effects of DSJ, BNT and HJT may improve these diabetes-related symptoms. The results of the present study suggested that DST and DSJ may be effective for the treatment of intermediate to late stages of type 2 related symptoms in KK-Ay mice. Also BNT and HJT were effective throughout the treatment period of type 2 related symptoms in KK-Ay mice. Thus, these Kampo medicines (DST, DSJ, BNT and HJT) may prevent the development of Type 2.

和文抄録

遺伝的NIDDMモデルマウスであるKK-Ayマウスに対する大柴胡湯（DST），大柴胡湯加地黄（DSJ），白虎加人参湯（BNT），八味地黄湯（HJT）の影響について検討した。DST, DSJの100 mg/kgは治療4週で血糖値上昇を抑制した。DST, DSJの500 mg/kgも治療4週，6週でKK-Ayマウスの高血糖状態を抑制した。BNT, HJTの100 mg/kgは血糖値上昇を治療4週から8週まで抑制した。BNT, HJTの500 mg/kgの用量依存的な抗血糖作用は治療期間継続した。しかしながら，これらの漢方薬はKK-Ayマウスのインスリン上昇を抑制しなかった。DSJ, BNT, HJTは体重増加を抑制した。高TGはDST, DSJ, BNTにより抑制された。DSJ, BNT, HJTはKK-AyマウスのNEFAを減少させた。さらに，BNTは肝臓のG-6-PDH増加を抑制した。DSJは脂肪酸合成系酵素MEを減少させた。BNT, HJTはKK-Ayマウスのβ酸化低下を抑制した。我々はDST, DSJ, BNT, HJTの継続的投与がKK-Ayマウスの血糖値上昇を抑制し，特にBNT, HJTの血糖低下作用が持続した事を示した。以上の結果から，DSJ, BNT, HJTの血糖低下作用は上述の糖尿病関連症候改善に起因することが示唆された。

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