Original Article

Anti-hyperglycemic and Anti-hyperlipidemic Activities of *Acanthopanax Senticosus* Herbal Acupuncture in C57BL/6J ob/ob Mice

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Abstract

Objectives : The aim of this study was to investigate the hypoglycemic and hypolipidemic activities and mechanisms of *Acanthopanax senticosus* (AS) herbal acupuncture.

Methods : Anti-diabetic and anti-steatotic activity of the AS herbal acupuncture was investigated on C57BL/6J ob/ob mice. After random grouping at the age of 9 weeks, the herbal acupuncture groups were injected subcutaneously at the left and right Gansu (BL18) corresponding acupuncture points alternately on exactly the same time every day with 0.1ml of either 400 mg/kg or 800 mg/kg of AS (AS400 and AS800) for 8-week period. As a positive control, metformin was administrated at a dose of 300 mg/kg (MT300). Body weights were measured weekly, and on every other week blood was collected for blood glucose analysis. At the end of the study, blood was also collected for determination of plasma insulin and lipid levels, after which they were killed and periepidydimal fat, liver, muscle, and pancreas were immediately removed. The removed tissues were instantly soaked in liquid nitrogen and stored at -70℃ for morphological examination and mRNA analysis.

Results : The AS herbal acupuncture significantly prevented weight gain on C57BL/6J ob/ob mice. The AS herbal acupuncture lowered blood glucose and improved glucose tolerance in C57BL/6J ob/ob mice. The increase of insulin response during the OGTT was inhibited by the AS herbal acupuncture. Insulin sensitivity of skeletal tissue was enhanced. Plasma lipid levels were significantly improved in the AS herbal acupuncture groups. The AS herbal acupuncture decreased hepatic lipogenesis and hepatic triglyceride production, and increased fatty acid (FA) transporter that involves in FA uptake. The AS

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herbal acupuncture inhibited the increase of liver mass by prevention of the accumulation of TG but did not inhibit weight gain of fat tissue on C57BL/6J ob/ob mice.

Conclusion : In summary, we have demonstrated several unique properties of the AS herbal acupuncture in decreasing body weight, and reversing insulin resistance and hepatic steatosis in ob/ob mice. This AS herbal acupuncture acts as an insulin sensitizer and specifically decreases circulating glucose and lipids, and suppresses hepatic lipogenesis.

Key words : *Acanthopanax senticosus*, herbal acupuncture, diabetes mellitus, dyslipidemia, steatosis, lipogenesis

Ⅰ. Introduction

Acanthopanax senticosus (AS), also called the "Siberian ginseng" or "*Eleutherococcus senticosus*", is an oriental herb and has been reported to have effects on immune system[1], hyperglycemia[2], hypertension, ischemia, chronic bronchitis[3], viral disease[4], tumour[5], oxidative stress[6-7], and exercise endurance improvement[8]. The active components of AS have been identified as phenylpropane compounds, lignans, coumarins, polysaccharides and other components[9]. In a previous study on the anti-diabetic and anti-steatotic activities of AS herbal acupuncture, it was revealed that it prevented the development of hypertension, type 2 diabetes mellitus, obesity, dyslipidemia, and disorders of adipose tissue[10].

However, no studies of mechanism have investigated anti-diabetic effect of AS herbal acupuncture. In this study, measurement of the body weight, plasma insulin, lipid levels, morphological examination and mRNA analysis were conducted to evaluate the hypoglycemic and hypolipidemic activities and mechanisms of herbal acupuncture with 50% ethanol extract solution of AS using spontaneous T2DM animal models.

Ⅱ. Materials and Methods

1. Animals

Male, C57BL/6J ob/ob mice were purchased at 8-week-old from Korea Research Institute of Bioscience and Biotechnology (Daejeon, Korea) and were acclimatized for 1 week before being randomly assigned into the groups. The animals were housed in individual cages with free access to water in a room with a 12:12-h light-dark cycle (8:00 am to 8:00 pm), a temperature of 24 ± 1℃, and a humidity of 55 ± 5%, and the animals were weighed periodically. During the acclimatization period, each animal was raised on standard rodent chow (Lab diets, USA) *ad libitum*.

2. Materials

1) Plant material

The *Acanthopanax senticosus* (AS) stem bark was purchased from Gyeongdong Hanyak Market specializing in herbs, Seoul, Korea, and identified and authenticated with the assistance of Prof. Yook Chang-su at the Dept. of Oriental Pharmaceutical Science, Kyung Hee University. A voucher specimen of AS was deposited at the Medicinal Plants Herbarium of the School of Pharmacy, Kyung Hee University, with registration number 221. The AS stem bark was left to dry in the shade at room temperature.

2) Apparatus for herbal acupuncture

1.0 ml syringe (26 gauge needle, Green Cross Medical Equipment, Korea) was used for injection of herbal acupuncture.

3) Preparation of AS extract solution for herbal acupuncture

The stem bark of AS was well dried in the shade to 1,000g at room temperature and was extracted twice at 80℃ in 50% ethanol for 4 h. The whole mass was filtered and concentrated by vacuum evaporation. The extracts were then filtered through filter papers (WHATMAN, England) and the filtrates were concentrated with a vacuum rotary evaporator under low pressure. The residue was freeze-dried in a freezing dryer and was stored in a deep freezer until use. (185g of dried powder was obtained). The AS ethanol extract was dissolved in deionized distilled water to 400 mg/kg or 800 mg/kg before use[11].

3. Grouping and treatment

At the age of 9 weeks, the C57BL/6J ob/ob mice were randomly divided into five groups; two control groups and three experimental groups. The lean (C57BL/6J, lean control, LC) and obese (obese control, OC) mice continued to receive standard rodent chow, and the herbal acupuncture groups were injected subcutaneously at the left and right Gansu (BL18) corresponding acupuncture points[12] alternately on exactly the same time every day with 0.1 ml of either 400 mg/kg or 800 mg/kg of the AS (AS400 and AS800) for 8-week period[10]. As a positive control, metformin (MT) was administrated everyday for 8-week period at a dose of 300 mg/kg (MT300). Body weights were measured weekly, and every other week blood was collected for blood glucose analysis. At the end of the study, blood was also collected for determination of plasma insulin and lipid levels, after which they were killed and periepidydimal fat, liver, skeletal muscle, and pancreas were immediately removed. The removed tissues were instantly soaked in liquid nitrogen and stored at -70℃ for morphological examination and mRNA analysis. The experiments were conducted in accordance with internationally-accepted experimental protocols for laboratory animal use and care as found in the U.S. guidelines.

4. Measurements of outcome

1) Blood sampling and plasma assay

Blood was withdrawn from the orbital venous plexus every other week, using a heparinized capillary tube without anesthesia. The blood samples were placed on ice, centrifuged, and the plasma was stored at -70℃ until assay. The plasma glucose concentration was determined using the glucose oxidase method (Youngdong Pharmaceutical Co., Korea)[13]. The plasma insulin concentration was measured according to the protocol described by the manufacturer of the mouse insulin ELISA (enzyme linked immunosorbent asssay) kit (Shibayagi Co., Japan). Plasma triglyceride, total cholesterol, and HDL cholesterol were determined using commercially available kits (Asan Pharmaceutical Co., Korea). Plasma nonesterified fatty acid (NEFA) concentrations were assayed by an enzymatic colorimetric method (NEFAZYMES-S, Eiken, Japan).

2) Oral glucose tolerance test

An oral glucose tolerance test (OGTT) was performed at the end of the treatment. On the test day, animals were fasted for 10 h, and glucose (1.5 g/kg) was then orally administrated to them. Blood glucose levels were determined from the orbital venous plexus at 0 (before glucose challenge), 30, 60, and 120 min after glucose administration.

3) Determination of hepatic glycogen

Hepatic glycogen was purified from whole liver

according to the method of extraction with chloroform[14]. Frozen liver (50~70 mg) was digested in 1 ml of 30% KOH for 20 min at 10 0℃. Digestion was completed by addition of one-fifth volume of 20% $NaSO_4$. The pellet was precipitated by addition of two volumes of absolute ethanol and incubated at -20℃ for 16 h. The pellet were washed twice with 70% ethanol and dried, and then glycogen was hydrolyzed by digestion in 200ℓ of 4N H_2SO_4 for 10 min at 10 0℃. The solution was neutralized with the addition of 200ℓ of 4N NaOH and released glucose was determined using a glucose oxidase method.

4) Determination of triglycerides in non-adipose tissues

For determination of triglycerides in non-adipose tissues, 50~70 mg of liver, skeletal muscle or pancreas from each mouse was homogenized in 4 ml of chloroform-methanol (2:1). 0.8 ml of 50 mmol NaCl was added to each homogenate, and the solution was incubated at 4℃ for 12 h. The sample was centrifuged for 5 min at 1,300 g. The organic layer was removed and dried using Speed Vac (Savant, USA). The resulting pellet was dissolved in phosphate buffer saline containing 1% Triton X-100 and then assayed for triglyceride as described above[15].

5) RNA extraction

The total RNA from liver or white adipose tissue was prepared using a guanidine thiocyanate-water saturated phenol/chloroform extraction method and subsequent precipitation with acidic sodium acetate[16]. The total RNA in the aqueous phase was precipitated with ice-cold isopropyl alcohol, and isolated RNA was determined by spectrophotometric analysis at 260 nm and 280 nm.

6) RT-PCR

One μg of total RNA was reverse transcribed into cDNA using the Moloney murine leukemia virus transcriptase and random hexamers (Promega, USA) as primers. The list of specific primers were: sterol regulatory element binding protein 1 (SREBP1a and c) sense GCG CTA CCG GTC TTC TAT CA, anti-sense TGC TGC CAA AAG ACA AGG G, accession number AF374266; fatty acid synthase (FAS) sense GAT CCT GGA ACG AGA ACA C, anti-sense AGA CTG TGG AAC ACG GTG GT, accession number X13135; glycerol-3-phosphate acyltransferase (GPAT) sense GGT AGT GGA TAC TCT GTC GTC CA, anti-sense CAT CAG CAA CAT CAT TCG GT, accession number NM_008149; steroyl-CoA desaturase (SCD)-1 sense CGA GGG TTG GTT GTT GAT CTG T, anti-sense ATA GCA CTG TTG GCC CTG GA, accession number NM_009127; peroxisome proliferlator activated receptor alpha (PPARa) sense CCC TGA ACA TCG AGT GTC GA, anti-sense CTT GCC CAG AGA TTT GAG GTC T, accession number NM_011144; CD36 sense TCC TCT GAC ATT TGC AGG TCT ATC, anti-sense GTG AAT CCA GTT ATG GGT TCC AC, accession number L23108; carnitine palmitoyl transferase (CPT-1a) sense CCT GGG CAT GAT TGC AAA G, anti-sense ACA GAC TCC AGG TAC CTG CTC AC, accession number AF017175; acylCoA oxidase (ACOx) sense CAC AGC AGT GGG ATT CCA AAT, anti-sense CTT CCT TGC TCT TCC TGT GAC TC, accession number AF006688; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) sense CAA CTT TGG CAT TGT GGA AGG, anti-sense ATG GAA ATT GTG AGG GAG ATG C accession number BC083149. The primers were added at final concentration of 0.5 μM to a 25 μℓ reaction mixture containing 20 mmol Tris-HCl (pH 8.4), 50 mmol KCl, 1.5 mmol MgCl2, 0.5 mmol each dNTP, 5 μℓ of cDNA, and 2.5 units of Taq DNA polymerase (TaKaRa medicals, Japan).

The PCR conditions were denaturation at 94℃ for 1 min, annealing at 57.5℃ for 1 min, and extension at 72℃ for 1 min. The RT-PCR

products were electrophorosed in 1% agarose gels under 100 V and was stained with 0.5 ㎍/㎖ ethidium bromide. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was amplified as a control gene. The density of the PCR product was measured using a GS-700 imaging densitometer. The level of mRNA was expressed as the ratio of signal intensity for each gene relative to that of GAPDH.

7) Preparation of DIG-labeled cRNA probes

The cRNA probes for GK, phosphoenol pyruvate carboxykinase (PEPCK), G6Pase, GLUT4, PPAR-g and cyclophilin were synthesized in vitro from linearized expression vectors which contained SP6 or T7 viral promoter. One ㎍ of linearized plasmid was mixed with RNA labelling mixture containing ATP, CTP, GTP, Dig(digoxigenin)-labeled-UTP, transcription buffer, and SP6 or T7 RNA polymerase. After incubation at 37℃ for 2 h, the mixture was co-incubated with DNase I (RNase free) at 37℃ for 15 min, precipitated in ethanol containing lithium chloride at -70℃ for 30 min, and washed with 70% chilled ethanol.

8) Northern blot analysis

Extracted RNA samples were dissolved in 30~50 ℓ water and 500 X diluted RNA solution was measured for spectrophotometric analysis at 260 and 280 ㎚. 10 ㎍ of the total RNA and equal volume of RNA loading buffer, containing 50% glycerol and 1 mmol EDTA, were denatured in 6 5℃ for 30 min and subsequently cooled on ice. The denaturated RNA samples were electrophorosed in 1% agarose-formaldehyde gels under 60-80 V and transferred to nylon hybond-N hybridization membrane (Amersham, England) and cross-linked with UV. After the membranes were pre-hybridized at 68℃ for 1 h in prehybridization buffer (5 X SSC, 50% formamide, 0.02% SDS, 0.1% sodium N-lauroyl sarcosine, and 2% blocking reagent), the membranes were incubated overnight at 68℃ in prehybridization buffer with the digoxigenin (DIG)-labeled probe, using the hybridization incubator. The membrane was washed twice for 30 min in 2 X washing solution (2 X SSC and 0.1% SDS) at room temperature and washed for 1 h in 0.1 X washing solution. After equilibrating the membrane in Buffer I (100 mmol maleic acid, 150 mmol NaCl, and pH 7.5) for 5 min, the membrane was gently shaken in Buffer II (1% blocking reagent in Buffer I) for 60 min. The membrane was hybridized with the diluted anti-DIG-alkaline phosphatase (1:10,000, 75 mU/ml) in Buffer II for 30 min. After washing the membrane twice for 20 min per wash in 0.3% Tween 20 (in Buffer I), the membrane was equilibrated in Buffer III (100 mmol Tris-HCl, pH 9.5, 100 mmol NaCl, and 50 mmol $MgCl_2$) for 2 min. CSPD solution (Roche, Germany) was spread over the surface of the membrane. After incubation of the membrane at 37℃ for 15-30 min, the membrane was exposed to Hyperfilm-ECL (Amersham) for detection of the chemiluminescent signal. For rehybridization, the membrane was washed for 20 min at room temperature in sterilized water, and shaken for overnight at 65℃ in 50 mmol Tris-HCl (pH 8.0), 50% dimethylformamide and 1% SDS to remove the hybridized probe. After removal of the former probe, the membrane was rehybridized to the DIG(digoxigenin)-labeled rat cyclophilin cRNA probe to normalize.

9) Histology and microscopy

After fasting overnight, the mice were deeply anesthetized with urethane (0.9 ㎖/100 g body weight of 20% solution) and were perfused transcardially with 10% buffered formalin. The mice were killed by decapitation, and WAT was removed from periepidydimal fat pad, and subsequently embedded in paraffin. Paraffin sections with thickness of 6 ㎛ were prepared using a microtome (RM2125RT, Leica, Germany),

and these sections were mounted on slides. The tissue section was deparaffinized and then stained with hematoxylin and eosin.

10) Statistical analysis

All data were expressed as a mean ± S.E. For multiple comparisons, an analysis of variance (ANOVA) was carried out, followed by significant difference test with Bonferroni test. A value of $P<0.05$ was considered significant.

Ⅲ. Results

1. Effect on body weight and weight gain

The body weight of the OC group was increased after 8 weeks, compared to the LC group (Table 1). Weight gains in the LC and OC groups during the 8-week period were 4.2 g (20% increase over the initial body weight) and 14.2 g (37% increase), respectively. On the other hand, the body weight and weight gain of the AS herbal acupuncture groups were significantly less than those of the OC group. The AS400 and AS800 herbal acupuncture groups significantly prevented the weight gain by 54% and 32% respectively, compared to the OC group. However, the MT300 group did not show significant prevention of weight gain.

2. Effect on plasma glucose, insulin, and insulin resistance index

The hyperglycemia and hyperinsulinemia were developed in the OC group when compared to the LC group after 8 weeks (Table 2). However, the AS herbal acupuncture groups showed a significant decrease in plasma glucose and insulin levels when compared to the OC group. Plasma insulin levels at 8 weeks were increased 10-fold in the OC group compared to the LC group. As a result of increased plasma glucose and insulin levels, the insulin resistance index (IRI), calculated by insulin (μU/ml) × glucose (mmol) / 22.5, of the OC group was 14.7 times higher than that of the LC group. However, the insulin resistance indices in the AS400 and AS800 herbal acupuncture groups were significantly reduced by 58% and 28% respectively, when compared to the OC group. Amelioration of insulin resistance in the AS400 herbal acupuncture group was significant

Table 1. Effect of AS Herbal Acupuncture on Body Weight and Weight Gain

Group	Body weight (g)		Weight gain (g)
	Initial	Final	
LC	21.0 ± 0.6	25.2 ± 0.3	4.2 ± 0.7
OC	38.5 ± 2.2	52.7 ± 2.7† † †	14.2 ± 2.1† † †
AS400	38.9 ± 2.4	45.4 ± 4.7**	6.5 ± 2.9***
AS800	38.4 ± 2.7	48.0 ± 2.3**	9.6 ± 1.1**
MT300	39.1 ± 2.7	52.2 ± 2.9	13.0 ± 0.5

AS, *Acanthopanax senticosus*; LC, lean control (lean type, C57BL/6J mice); OC, obese control (obese type, C57BL/6J ob/ob mice); AS400, 400 mg/kg of AS herbal acupuncture at Gansu(BL18) to C57BL/6J ob/ob mice; AS800, 800 mg/kg of AS herbal acupuncture at Gansu(BL18) to C57BL/6J ob/ob mice; MT300, administrated 300 mg/kg of metformin to C57BL/6J ob/ob mice. Values represent the mean ± SD (n=6) † † † $P<0.001$ vs. LC; *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs. OC

Table 2. Effect of AS Herbal Acupuncture on Plasma Glucose, Plasma Insulin, and Homeostasis Model Assessment Values for Insulin Resistance (HOMA-IR)

Group	Plasma glucose (mmol/l)	Plasma insulin (μU/ml)	HOMA-IR
LC	6.6 ± 0.4	21.2 ± 7.6	6.4 ± 2.2
OC	10.0 ± 0.4† † †	212.5 ± 7.1† † †	93.8 ± 3.0† † †
AS400	5.9 ± 0.8**	153.6 ± 23.8*	39.7 ± 7.9***
AS800	7.2 ± 0.2***	212.4 ± 13.4	68.0 ± 4.7***
MT300	7.3 ± 0.5**	181.1 ± 8.5*	60.3 ± 3.6***

Plasma glucose and insulin were analyzed in plasma samples obtained from blood of 4h-fasted (9:00 am ~ 1:00 pm) mice. Homeostasis model assessment was used to calculate an index of insulin resistance as glucose (mmol/l) × insulin (μU/ml) / 22.5. Values represent means ± SE (n=6). † † † P<0.001 vs. LC; *P<0.05, **P<0.01, ***P<0.001 vs. OC

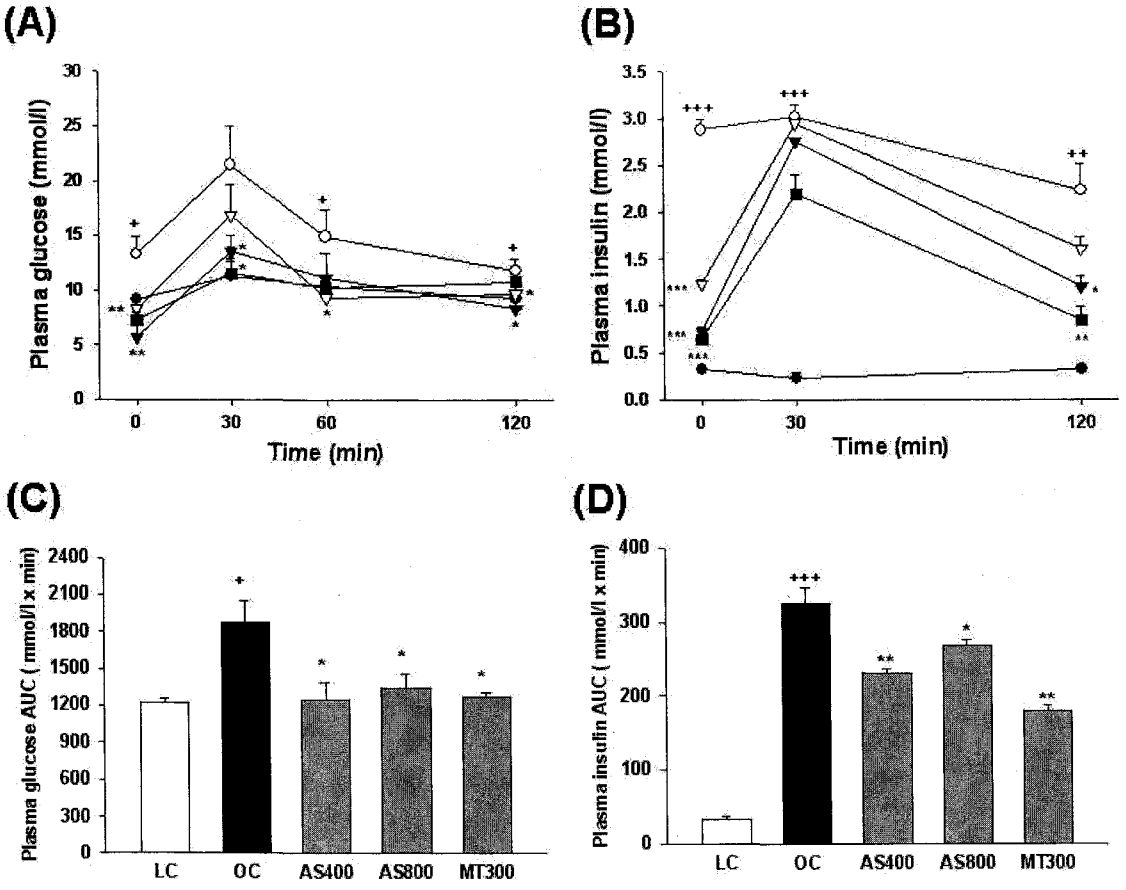

Fig. 1. Plasma glucose (A) and insulin (B) responses to an oral glucose challenge (1.5 g/kg) after 10 h of food deprivation at 8 weeks. Area under the curve (AUC) of plasma glucose (C) and insulin (D) during oral glucose tolerance test

LC (●), lean control; OC (○), obese control; AS400 (▼), mice treated with 400 mg/kg of AS herbal acupuncture at Gansu(BL18); AS800 (▽), mice treated with 800 mg/kg of AS herbal acupuncture at Gansu(BL18); MT300 (■), mice administrated with 300 mg/kg of metformin. Values represent the means ± SE (n=6). P<0.05, † † † P<0.001 vs. LC; *P<0.05, **P<0.01, ***P<0.001 vs. OC

Table 3. Effect of AS Herbal Acupuncture on Plasma Lipid Levels

Group	NEFA (mg/dl)	Triglyceride (mg/dl)	Total cholesterol (mg/dl)	LDL cholesterol (mg/dl)
LC	1193.8 ± 50.3	79.0 ± 4.1	85.5 ± 4.7	11.2 ± 3.2
OC	1254.3 ± 45.8	100.8 ± 3.6† †	196.3 ± 10.1† † †	81.4 ± 4.5† † †
AS400	1100.3 ± 48.5*	71.0 ± 2.5***	171.5 ± 4.6*	68.3 ± 4.9*
AS800	1061.8 ± 68.9*	82.5 ± 7.0*	177.8 ± 20.6	71.8 ± 14.2
MT300	1201.3 ± 96.9	61.3 ± 2.8***	136.0 ± 3.0**	27.0 ± 5.3***

Non-esterified fatty acid (NEFA), triglyceride, total cholesterol, and HDL-cholesterol were determined in plasma after they were killed. LDL-cholesterol (mg/dl) = Total cholesterol - HDL cholesterol - triglyceride/5. Values represent mean ± SE (n=6).
† † P<0.01, † † † P<0.001 vs. LC; *P<0.05, **P<0.01, ***P<0.001 vs. OC

(54% reduction) and even better than that in the MT300 group. This result suggests that the AS herbal acupuncture was able to lower the blood glucose level primarily due to the improvement of insulin resistance.

3. Effect on oral glucose tolerance test

To examine whether the insulin resistance of the AS herbal acupuncture groups was actually improved as calculated IRI, an oral glucose tolerance test (OGTT) was carried out at the end of the experiment (Fig. 1). Glucose challenge dramatically increased the blood glucose levels in the OC group compared to those in the LC group, while the AS herbal acupuncture groups significantly prevented the blood glucose levels from rising, especially at the 30 and 60 min time point (Fig. 1A). When the area under the curve (AUC) was compared between groups, the AS400 and AS800 herbal acupuncture groups showed 34% and 28% reduction respectively, in the AUC compared to that in the OC group (Fig. 1C). Improving the glucose intolerance of the AS herbal acupuncture groups was similar to that of the MT300 group (32% reduction compared to the OC group). The insulin response during the OGTT was considerably increased in the OC group compared to the LC group, which indicates insulin resistance (Fig. 1B). Plasma insulin AUC levels in the AS400 and AS800 herbal acupuncture groups were also markedly decreased by 29% and 18%, respectively (Fig. 1D).

4. Effect on plasma lipid levels

The effect of the AS herbal acupuncture on plasma lipid levels were examined at the end of the treatment (Table 3). The plasma lipid levels in the OC group were dramatically elevated compared to the levels in the LC group except for non-esterified fatty acid (NEFA) level. In the OC group, plasma triglyceride (TG) was increased by 1.3-fold (79.0 to 100.8 mg/dl), LDL cholesterol (LDL-C) increased by 7.3-fold (11.2 to 81.4 mg/dl) and total cholesterol increased by 2.3-fold (85.5 to 196.3 mg/dl) compared to those in the LC group. However, plasma lipid levels of the AS400 herbal acupuncture group were significantly improved by 12% diminution of NEFA, 30% reduction of TG, and 16% decrease of LDL-C, compared to the OC group.

5. Effect on liver mass

As the increase of circulating fatty acids causes the accumulation of triglycerides in liver, liver mass is increased. The % liver / body weight of the OC group (7.1%) was increased 1.7-fold compared to the LC group (4.2%, Fig. 2).

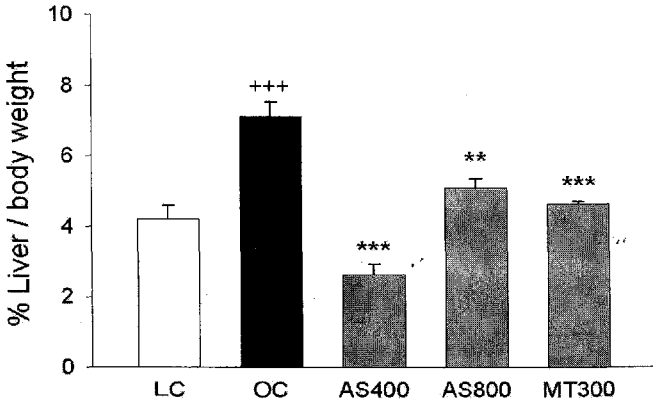

Fig. 2. Liver mass of the Control and the AS herbal acupuncture groups
Values represent mean ± SE (n=6). † † † P<0.001 vs. LC; *P<0.05, ***P<0.001 vs. OC

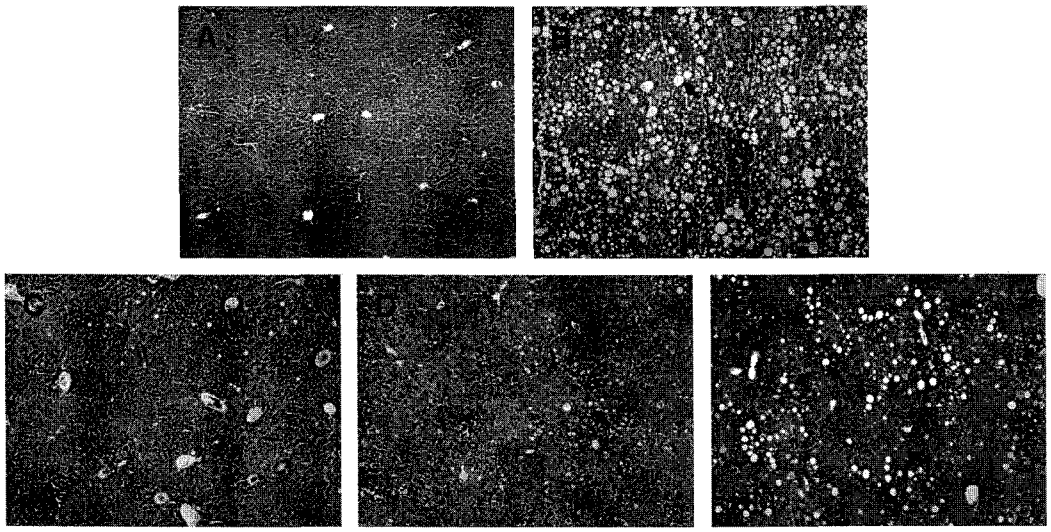

Fig. 3. Morphology of liver from the Control and the AS herbal acupuncture groups in mice. Hematoxylin and eosin-stained paraffin sections of liver from lean control (LC, A), obese control (OC, B), AS400 herbal acupuncture (AS400, C), AS800 herbal acupuncture (AS800, D), and metformin 300 mg/kg (MT300, E) treated mice from the 8-week treatment. Magnification of histological sections × 100

Although effect of the AS herbal acupuncture on liver mass was not in a dose-dependent manner, the % liver / body weight of the AS400 and AS800 herbal acupuncture groups were significantly reduced 63% and 27% respectively compared to the OC group. The liver mass of the AS400 herbal acupuncture group especially was lower than that of the MT300 group (35%, compared to the OC group).

6. Effect on morphology of liver

The liver of the OC group showed intracytoplasmic large and small lipid droplet (arrows) in the pericentral region and relative sparing of portal areas (Fig. 3B), but the LC group had no identifiable fat accumulation (Fig. 3A) and no histological evidence of liver diseases, such as inflammation or hepatocytes injury. The

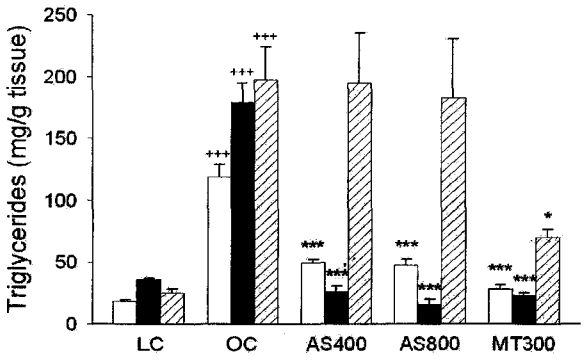

Fig. 4. Triglycerides in non-adipose tissues; liver(□), skeletal muscle (■), and pancreas (▨)
Values represent mean ± SE (n=6). † † † P<0.001 vs. LC; *P<0.05, ***P<0.001 vs. OC

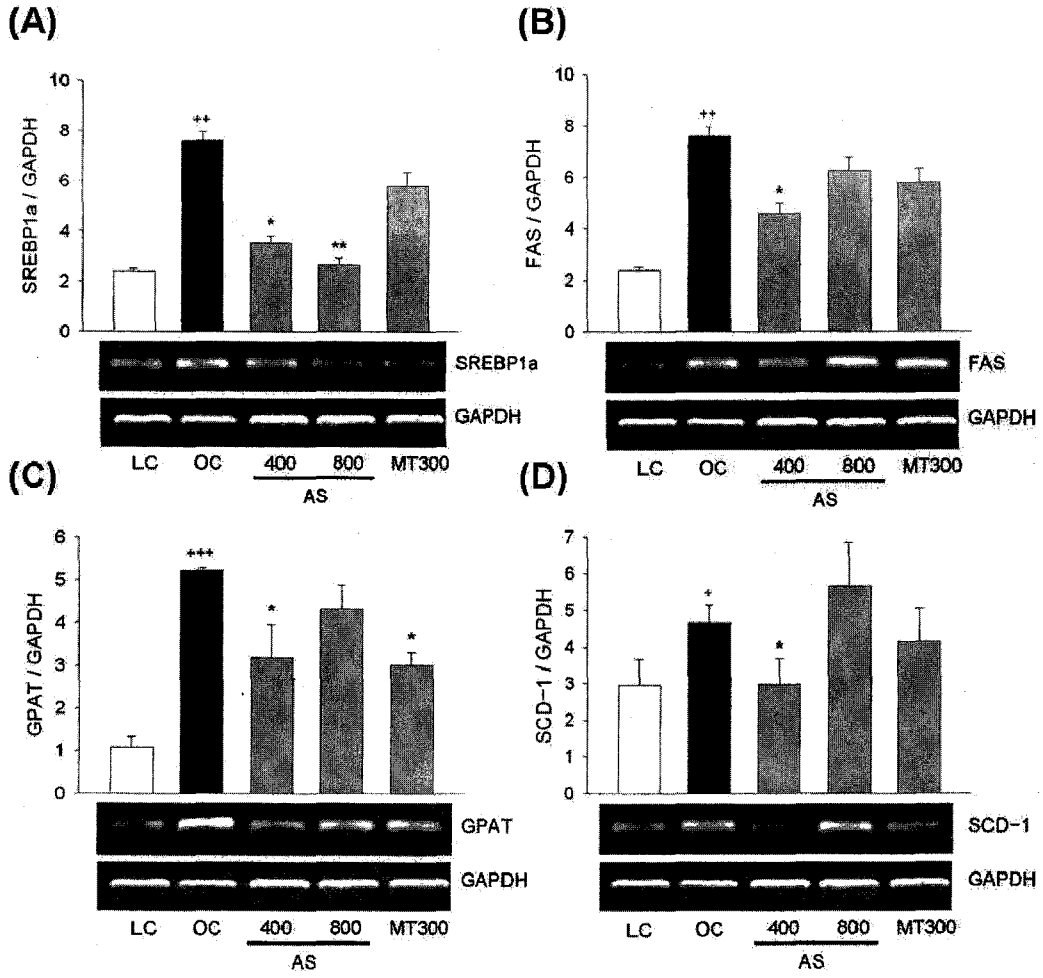

Fig. 5. mRNA expression of genes related to lipogenesis. Regulation of SREBP1a (A), FAS (B), GPAT (C) and SCD-1 (D) mRNA expression normalized by GAPDH in liver
Values represent mean ± SE (n=6). † P<0.05, † † P<0.01, † † † P<0.001 vs. LC; *P<0.05, **P<0.01, ***P<0.001 vs. OC

AS400 and AS800 herbal acupuncture groups, and the MT300 group showed dramatic decrease in lipid droplets and mitigated hepatic steatosis of C57BL/6J ob/ob mice (Fig. 3C-E).

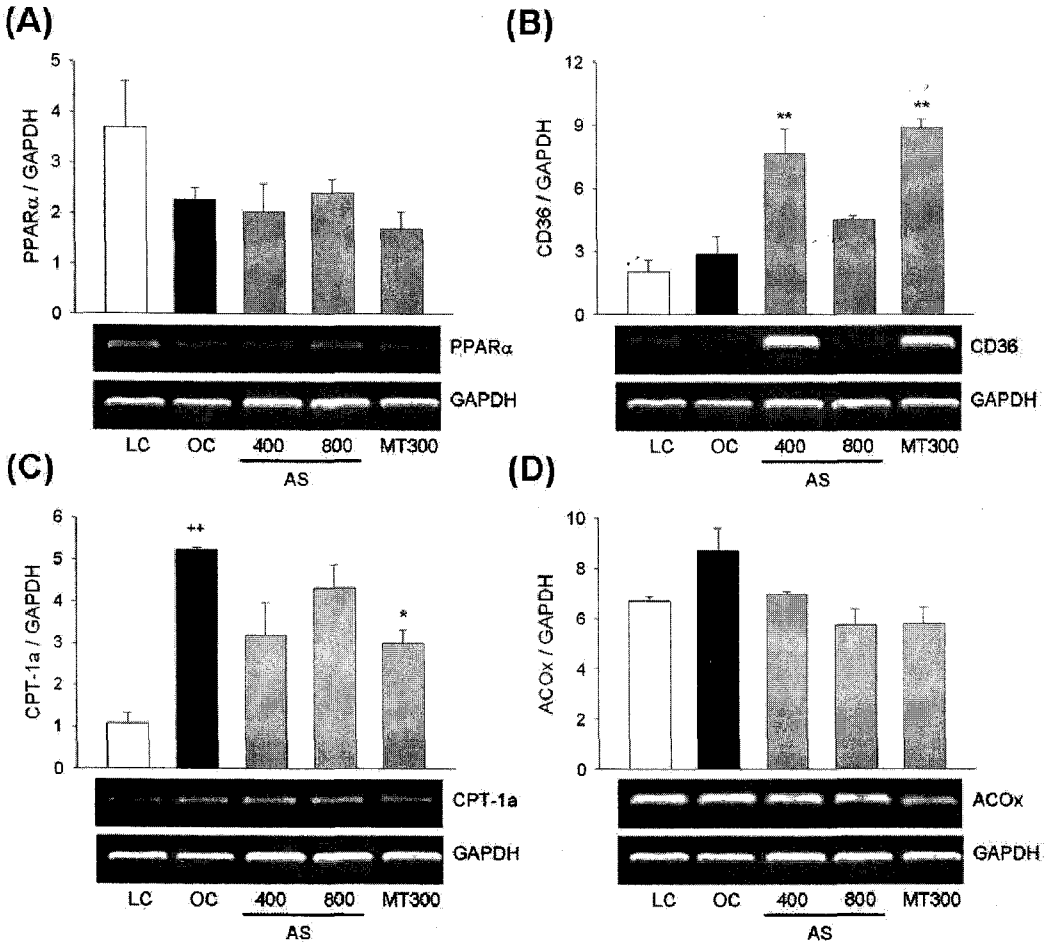

Fig. 6. mRNA expression of genes related to lipolysis. Regulation of PPARα (A), CD36 (B), CPT-1a (C) and ACOx (D) mRNA expression normalized by GAPDH in liver
Values represent mean ± SE (n=6). † † P<0.05 vs. LC; *P<0.05, **P<0.01, ***P<0.001 vs. OC

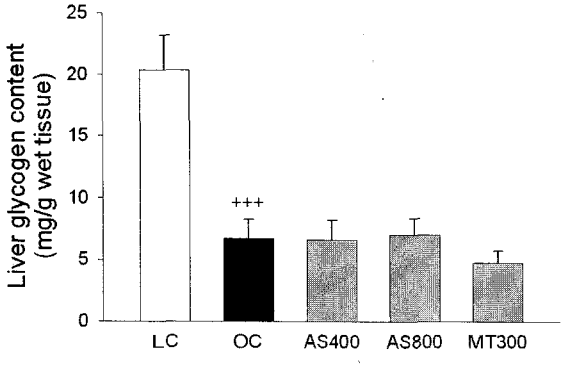

Fig. 7. Hepatic glycogen
Values represent mean ± SE (n=6). † † † P<0.001 vs. LC

7. Effect on triglycerides in non-adipose tissues

The accumulation of triglycerides (TG) was evaluated in non-adipose tissues, as it could be associated with lipotoxicity and insulin resistance. The OC group resulted in a five- to eight-fold increase in TG levels of non-adipose tissues, when compared to those levels in the LC group (Fig. 4). The TG levels in liver of the AS400 and AS800 herbal acupuncture groups were significantly decreased by 58% and 60%, and those in skeletal muscle of the AS400 and AS800 herbal acupuncture groups were reduced by 85% and 91% respectively compared to the OC group. However, the AS herbal acupuncture did not prevent the accumulation of TG in pancreas. The AS800 herbal acupuncture group especially showed similar effects shown in the MT300 group.

8. Effect on lipogenesis and lipolysis in liver

From examination on the expression of genes involved in hepatic lipogenesis, the OC group significantly increased the mRNA expression of sterol regulatory element binding protein 1a (SREBP1a) by 3.2-fold, fatty acid synthase (FAS) by 3.2-fold, glyceraldehyde 3-phosphate dehydrogenase (GPAT) by 4.8-fold and steroyl-CoA desaturase (SCD-1) by 1.6-fold, compared to the LC group (Fig. 5). However, AS herbal acupuncture groups suppressed the mRNA levels of genes involved in lipogenesis compared to the OC group (SREBP1a by 54%, FAS by 40%, GPAT by 39% and SCD-1 by 36%), but did not show a dose-dependent pattern except for SREBP1a. In contrast, the mRNA levels of the fatty acid transporter CD36, related to hepatic lipolysis was significantly increased 2.7-fold in the AS800 herbal acupuncture group compared to the OC group (Fig. 6).

9. Effect on hepatic glycogen

The hepatic glycogen of the OC group (6.7 ± 1.6 mg/g wet tissue; Fig. 7) was decreased by 67%, compared to the LC group (20.4 ± 2.8 mg/g wet tissue). However, there were no difference between the AS herbal acupuncture groups and the OC group.

10. Effect on mRNA expression of PEPCK and G6Pase on hepatic gluconeogenesis

The quantitative analysis of phosphoenol-pyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase), which mediate gluconeogenesis in the liver, was performed using Northern blotting normalized by cyclophilin (CPN). mRNA level of G6Pase in the OC group was increased by 2.2-fold compared to that of the LC group, but that of PEPCK was not a significant difference between the LC and OC group (Fig. 8). On the other hand, the mRNA expression of G6Pase in the AS400 and AS800 herbal acupuncture groups were significantly decreased by 57% and 52% respectively, compared to the OC group. PEPCK and G6Pase in hepatocytes is suppressed by metformin treatment. mRNA level of G6Pase in the MT300 group was reduced by 75% compared to the OC group, but we could not confirm inhibition of PEPCK in the MT300 group.

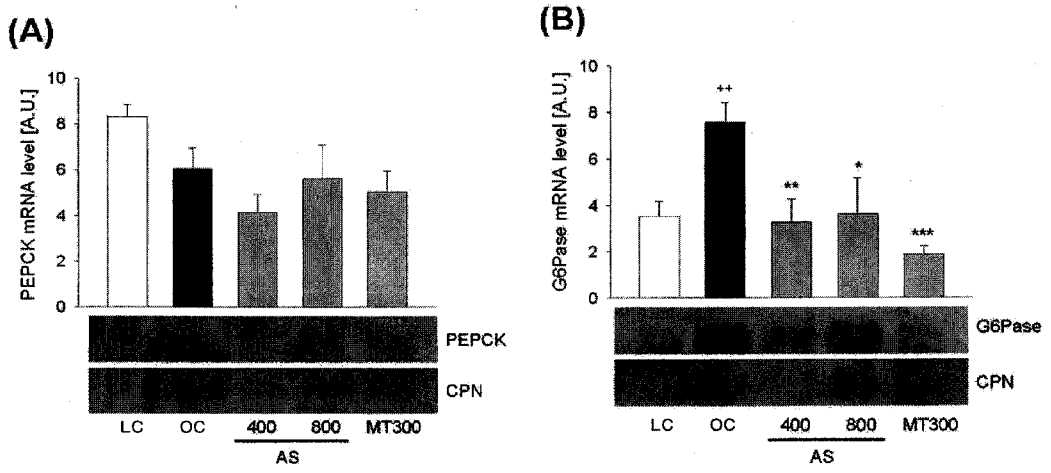

Fig. 8. mRNA expression of PEPCK (A) and G6Pase (B) in the liver. Representative Northern blot analysis and densitometric quantitation of PEPCK and G6Pase mRNA expression normalized by CPN
Values represent mean ± SE (n=6). † † P<0.01 vs. LC; *P<0.05, **P<0.01, ***P<0.001 vs. OC

Table 4. Effect of AS Herbal Acupuncture on Area and Diameter of Periepididymal Fat Pad

Group	Mass (g)	Area (μm2)	Diameter (μm)
LC	0.53 ± 0.21	2952.9 ± 105.4	53.8 ± 1.2
OC	3.20 ± 0.26	12501.9 ± 385.9† † †	129.6 ± 2.9† † †
AS400	3.13 ± 0.20	5894.3 ± 124.6***	75.0 ± 2.0***
AS800	3.47 ± 0.17	8115.5 ± 170.5***	90.3 ± 1.1***
MT300	3.83 ± 0.12	8076.1 ± 113.4***	90.8 ± 1.4***

Values represent mean ± SD (n=4). † † † P<0.001 vs. LC; ***P<0.001 vs. OC

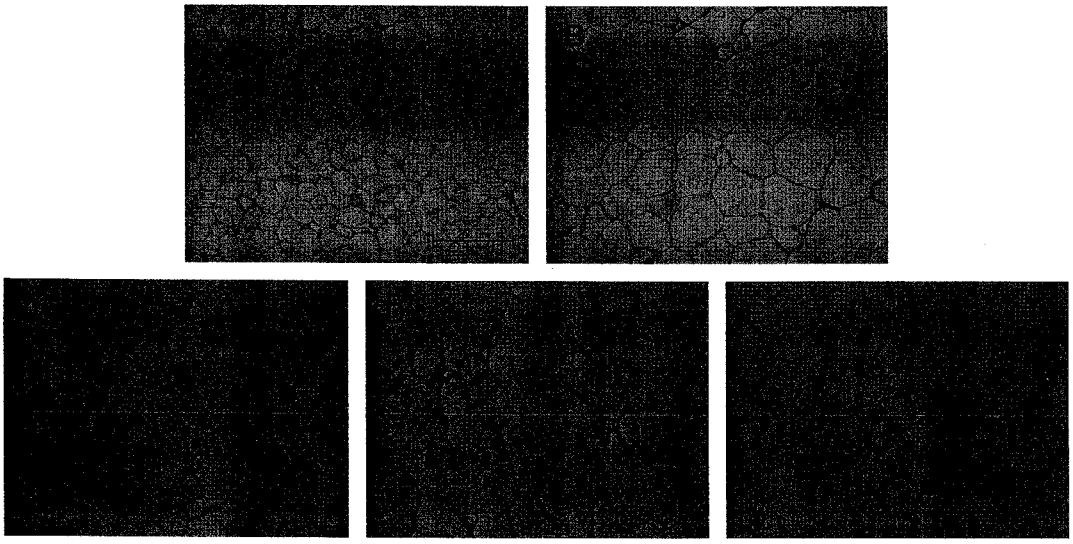

Fig. 9. Morphology of periepididymal fat pad from the Control and the AS herbal acupuncture in mice. Hematoxylin and eosin-stained paraffin sections of liver from LC (A), OC (B), AS400 (C), AS800 (D), and MT300 (E) from the 8-week treatment. Magnification of histological sections × 200

11. Effect on mass and morphology of periepididymal fat pad

The weight gain of the AS400 herbal acupuncture group was decreased by 54% compared to the OC group (Table 1). However, there was no significant difference in the masses of periepididymal fat pad of the AS400 herbal acupuncture group, respectively, compared to the OC group (Table 4). The loss of body weight in the AS herbal acupuncture group was caused not by the inhibition of hypertrophy in periepididymal fat pad but by the suppression of the accumulation of TG in liver. In spite of no difference of periepididymal fat pad mass in the AS herbal acupuncture groups and the OC group, the histological analysis of periepididymal fat pad in the AS herbal acupuncture groups showed reduction of area and diameter compared to the OC group. The area and diameters of periepididymal fat pad were decreased by 53% and 42% respectively, in the AS400 herbal acupuncture group compared to the OC group.

12. Effect on GLUT4 mRNA expression in skeletal muscle

mRNA expression of glucose transporter (GLUT4),

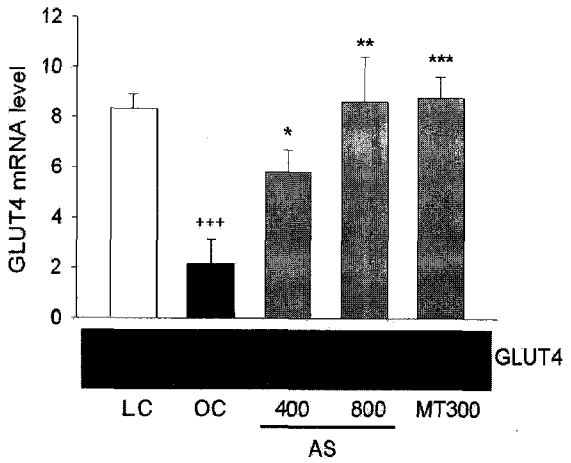

Fig. 10. mRNA expression of GLUT4 mRNA in the skeletal muscle. Representative Northern blot analysis and densitometric quantitation of GLUT4 mRNA expression

Values represent mean ± SE. † † † P<0.001 vs. LC; *P<0.05, ***P<0.001 vs. OC

from the skeletal muscle of each group, were determined by using northern blotting (Fig. 10). mRNA level of GLUT4 in the OC group was decreased by 74% compared to that of the LC group. However, the AS400 and AS800 herbal acupuncture groups had an increased GLUT4 mRNA level up to 2.7- and 4.0-fold respectively, compared to the OC group. mRNA expression of GLUT4 in the MT300 group was also significantly increased by 4.1-fold compared to the OC group.

IV. Discussion

Acanthopanax senticosus is one of the Araliaceae family. It is a deciduous and suckering shrub. Fourteen kinds of *Acanthopanax senticosus* are either farmed or found in the wilderness of the Korean peninsula. It has a spiny stem abundant with thin thorns[17]. No detailed records of *Acanthopanax senticosus* can be found in ancient literatures other than the records of *Senticosus*. *Acanthopanax senticosus* and *Senticosus* is divided into two different herbs in the Pharmacopoeia of People's Republic of China, published in 1977[18].

Acanthopanax senticosus (AS) is pungent and bitter in taste and warm in nature. It enters the kidney and spleen meridians. It nourishes the kidney and strengthens the lower back. It also nourishes the qi and calms the mind. It circulates the blood and collateral channels. It is effective for stroke, diabetes, weak body due to deficiency of the kidney, low back pain and knee ache, fatigue due to deficiency of the spleen, edema due to deficiency of qi, chest pain, and tingling due to pathogenic wind, cold and damp[18].

It was reported that oral administration of AS extract decreases LDL and VLDL blood concentration and increases the HDL blood concentration[19], that it decreases the blood concentration of glucose, cholesterol and triglyceride[20], and that lipid peroxidation is suppressed[21]. And it was also revealed that the increase of urinary albumin was suppressed and average blood pressure was decreased[22] by AS herbal acupuncture, that it prevents diabetes and supresses the increase of glucose[23], and that AS herbal acupuncture inhibits the development of risk factor of metabolic syndrome (obesity, hypertension, type 2 diabetes mellitus, and disorders of adipose tissue)[10]. But the study on the mechanism of anti-diabetic and anti-steatotic activities of the AS herbal acupuncture had not been accomplished.

Herbal acupuncture is a treatment concerning injection of various herbal extract solution into acupuncture points, positive reaction points, and pain response points. It is reported that herbal acupuncture has strong analgesic, detoxicant, anticonvulsant, antihypertensive, anti-tumor and anti-inflammatory effects. It is also effective for blood enhancement, sugar control and immunological enhancement. Active researches concerning the immunological enhancement effect are in progress[24].

Gansu (BL18) is located at 1.5 cun lateral to the lower border of the spinous process of the

ninth thoracic vertebra (T9)[12]. This acupuncture point is selected for treatment of various diseases such as liver deficiency syndrome, asthenic disease, distention and pain of the lateral costal region, epigastric pain, abdominal mass, rigidity of the neck and spine, cramps, tetany, blurred vision, redness of the eyes, and lacrimation dysfunction from its action as spreading liver qi, regulating and nourishing liver blood, pacifying wind, cooling fire and clearing damp-heat, benefiting the eyes, and benefiting the sinews[25].

In this study, we investigated anti-diabetic and anti-steatosis activity of the AS herbal acupuncture using C57BL/6J ob/ob mice. Obesity of ob/ob mice is due to a mutation in the obese gene that codes for leptin[26]. Leptin is a 167 amino acid protein hormone produced by adipose tissue and acts on specific receptor located in the hypothalamus to decrease appetite and increase energy expenditure[27]. Animals with defect in leptin show a hyperphagia and develop an extreme obesity[28]. Obesity may play a primary role for the progress of insulin resistance, whereas insulin resistance itself may lead to hyperglycemia and dyslipidemia[29]. Even if ob/ob mice consequently do not reflect common human obesity, they indicate morbid obesity and metabolic abnormalities such as hyperglycemia, glucose intolerance and hyperinsulinemia that phenotypically present similar to human with T2DM[28, 30].

The AS herbal acupuncture lowered blood glucose and improved glucose tolerance in obese ob/ob mice. The AS herbal acupuncture groups also needed low plasma insulin for glucose homeostasis and thus enhanced insulin sensitivity. The relationship between body weight and the risk of diabetes was successive and graded[31]. Insulin sensitivity in T2DM improves with weight loss[32]. The hypoglycemic effect of the AS herbal acupuncture was caused by inhibiting hepatic glucose production, losing body weight and directly stimulating a glucose transporter (insulin-dependent) in skeletal muscle.

Disturbed glucose metabolism has been suggested as a factor of insulin resistance in patients with T2DM which could be a primary or acquired defect in the pathogenesis of diabetes[33]. The PEPCK is the rate-limiting enzyme of gluconeogenesis and enhanced expression of the PEPCK gene in liver is presented in most models of diabetes, and is thought to be conductive to the increased hepatic glucose output in T2DM[34]. The microsomal G6Pase catalyzes hepatic glucose production because this enzyme catalyzes the hydrolysis of glucose-6-phosphate (coming from gluconeogenesis and glycogenolysis) in glucose[35]. Gluconeogenesis of ob/ob mice in liver was promoted by overexpression of G6Pase in spite of hyperinsulinemia, but the AS herbal acupuncture suppressed mRNA levels of G6Pase. However, the AS herbal acupuncture has no significant effect on inhibiting PEPCK. Although plasma insulin levels in the OC group were high, the OC group could not repress hepatic gluconeogenesis and show insulin resistance in liver. It can be suggested that the AS herbal acupuncture has effects of inhibiting hepatic glucose output.

The liver plays an important role in the progress of T2DM, contributing to dyslipidemia as well as hyperglycemia[29,36]. Over-intake of carbohydrate and fat may challenge the liver, thereby influencing the hepatic glucose homeostasis and the degree of hepatic lipoprotein accumulation, which leads to steatosis (non-alcoholic fatty liver disease, NAFLD), a phenomenon linked to insulin resistance[36]. NAFLD in ob/ob mice was prevented by improving insulin resistance and inhibiting fat accumulation through treatment of the AS herbal acupuncture and metformin. NAFLD in ob/ob mice is characterized by over-expression of lipogenic genes, and suppression genes which participate in hepatic lipolysis[15,37]. The AS herbal acupuncture decreased hepatic lipogenesis and hepatic TG production, and increased FA transporter that involves in FA uptake. SREBP1a, FAS, GPAT, and SCD-1 were repressed in ob/ob mice by the AS herbal

acupuncture. Fatty acid transporter CD36 was increased, but other lipolytic genes were not influenced by the AS herbal acupuncture. These results suggest that the anti-steatotic activity of the AS herbal acupuncture is mediated mainly through inhibition of lipogenesis and regulation of lipid metabolism.

The increased TG content in the non-adipose tissues, such as liver, muscle and pancreas, has been proposed as an important mechanism causing insulin resistance (the lipotoxicity hypothesis)[38-40]. The lowered plasma lipids in the AS herbal acupuncture group reduced the accumulation of TG in non-adipose tissues and insulin sensitivity of each tissue was enhanced. Insulin resistance of ob/ob mice in skeletal muscle is associated with a down-regulation of GLUT4. The role of GLUT4 down-regulation in phathogenesis of insulin resistance and glucose tolerance has been confirmed in mice with muscle selective ablation of GLUT4[41]. Therefore, the higher mRNA expression of GLUT4 in the AS herbal acupuncture group reflects increase of glucose flux into skeletal muscle in response to the ameliorated insulin resistance. Dysfunction of β-cell in rats with T2DM is related to the accumulation of TG in islet[42], which leads to increased production of nitric oxide[43] and β-cell apoptosis according to lipotoxicity[44-45]. AS herbal acupuncture prevented the accumulation of TG in pancreas and reduced lipotoxicity by fatty acids

The AS herbal acupuncture also had an effect on weight loss that did not reduce weight of fat tissue but inhibited increase on liver mass by prevention of the accumulation of TG. Although the mass of fat in each group was not different, the area and diameter of adipocytes in the AS herbal acupuncture groups were decreased. Increased TG store and lipolysis of adipocytes were caused by hyperinsulinemia[40]. Because of the repressed plasma insulin levels via improvement of insulin resistance by the AS herbal acupuncture, decreased TG store in adipose tissue and the area and diameter of adipocytes in periepidydimal fat were reduced.

The AS herbal acupuncture did not show an exactly dose-dependent manner in all parameters. The reason of this phenomenon may be that the AS contains several different ingredients with synergistic or antagonistic effects, or high dose of the AS can affect bioavailability of active ingredients.

In summary, The AS herbal acupuncture significantly reduced hyperglycemia, hyperinsulinemia and hyperlipidemia, and improved insulin resistance in liver by repressing G6Pase in ob/ob mice. The AS herbal acupuncture also suppressed obesity, hepatic lipogenesis and hypertrophy of adipocytes. Improved insulin resistance in non-adipose tissues was caused by reduced lipotoxicity, and whole glucose homeostasis was ameliorated compared to the OC group. Future studies will determine which active ingredient(s) of the AS is responsible for this action, and whether the AS herbal acupuncture is able to reverse other abnormalities in non-alcoholic fatty liver disease (NAFLD), including inflammation, necrosis and fibrosis. Consequently, the AS herbal acupuncture showed potential as a treatment agent for type 2 diabetic patients who has genetic predisposition.

V. Conclusions

In conclusion, we have demonstrated several unique properties of the AS herbal acupuncture in decreasing body weight, reversing insulin resistance and hepatic steatosis, inhibition of liver mass, decreasing hepatic lipogenesis and hepatic triglyceride production, and increasing fatty acid transporter and glucose transporter in C57BL/6J ob/ob mice. The AS herbal acupuncture acts as an insulin sensitizer and shows potential as a treatment agent for type 2 diabetes mellitus for who has genetic predisposition.

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