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## Increase of insulin sensitivity in diabetic rats received Die-Huang-Wan, a herbal mixture used in Chinese traditional medicine

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**KEY WORDS** insulin sensitivity; Die-Huang-Wan; obese Zucker rats; insulin resistance

### ABSTRACT

**AIM:** Effects on insulin sensitivity of Die-Huang-Wan, the herbal mixture widely used to treat diabetic disorder in Chinese traditional medicine, were investigated *in vivo*. **METHODS:** The obese Zucker rats were employed as insulin-resistant animal model. Also, insulin-resistance was induced by the repeated intraperitoneal injections of long-acting human insulin at 0.5 U/kg three times daily into adult male Wistar rats. Insulin resistance was identified using the loss of tolbutamide (10 mg/kg) or electroacupuncture (EA)-induced plasma glucose lowering action. The plasma glucose concentration was examined by glucose oxidase assay. **RESULTS:** The plasma glucose-lowering action induced by tolbutamide was significantly enhanced in obese Zucker rats receiving the repeated administration of Die-Huang-Wan at dosage of 26 mg/kg for 3 d. Furthermore, administration of Die-Huang-Wan delayed the formation of insulin resistance in rats that were induced by the daily repeated injection of human long-acting insulin at 0.5 U/kg three times daily and identified by the loss of tolbutamide- or EA-induced hypoglycemia. In streptozotocin-induced diabetic rats, oral administration of metformin at 320 mg/kg once daily made an increase of the response to exogenous short-acting human insulin 15 d later. This is consistent with the view that metformin can increase insulin sensitivity. Similar treatment with Die-Huang-Wan at an effective dose (26.0 mg/kg) also increased the plasma glucose lowering action of exogenous insulin at 10 d later. The effect of Die-Huang-Wan on insulin sensitivity seems to produce more rapidly than that of metformin. **CONCLUSION:** The present study found that oral administration of Die-Huang-Wan increased insulin sensitivity and delayed the development of insulin resistance in rats.

### INTRODUCTION

Insulin is one of the important hormones in human metabolism. Before the advent of insulin therapy, diet abstinence and herbal medication were the mainstay in anti-diabetic therapies<sup>[1]</sup>. Due to the remarkable therapeutic effect of insulin, herbal medication was gradually abandoned in European area. However, com-

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plications in macrovascular, retinal and neuropathic functions are still associated in the patients receiving insulin injection<sup>[2]</sup>. Also, insulin resistance is another serious problem in clinic. Insulin resistance, defined as impaired insulin-mediated glucose disposal, is a common consequence of excess body weight and a cause of impaired glucose tolerance in type II diabetes<sup>[3]</sup>. In clinics, insulin resistance and hyperinsulinemia are usually observed in association with hypertension<sup>[4]</sup>. Recent decades have seen a resurgent interest in the development of insulin sensitizing agent for diabetes.

In Chinese traditional medicine (CTM), Die-Huang-Wan is one of the prescriptions for handling of diabetic disorders and this herbal mixture has also been used for a long time in Japan and other Asian area<sup>[5]</sup>. As shown in Tab 1, Die-Huang-Wan was prepared by a mixture of 6 herbs and this product is commercial available in China. An antihyperglycaemic activity of this herbal mixture has ever been observed in diabetic mice<sup>[5]</sup>. In an attempt to develop agents without side effect for good handling of DM, Die-Huang-Wan was investigated in the present study. From the *in vivo* data, we demonstrated that Die-Huang-Wan is helpful to reduce insulin resistance associated with an increase of insulin sensitivity in diabetic rats.

**Tab 1. The herbs contained in Die-Huang-Wan.**

Die-Huang-Wan	
Ingredients	Compositions
Rehmanniae Radix et Rhizoma	8 g, wet weight
Corni Fructus	4 g, wet weight
Dioscoreae Rhizoma	4 g, wet weight
Moutan Radics Cortex	3 g, wet weight
Alismatis Rhizoma	3 g, wet weight
Hoelen	3 g, wet weight

Extract of total ingredients was concentrated to 4 g of dry weight to meet the ratio of 6.25:1 in the granule of Die-Huang-Wan (6 g).

## MATERIALS AND METHODS

**Animals** Male Wistar rats, aged 8-10 weeks, were obtained from the Animal Center of National Cheng Kung University. Male obese Zucker rats, aged 8-10 weeks, were obtained from Prof HH LOH (Department of Pharmacology, University of Minnesota Medical Center, Minneapolis, USA). Streptozotocin (STZ)-induced dia-

betic rats, used as the type I-diabetes like model or the insulin-dependent diabetes mellitus (IDDM) model, were prepared by an intravenous injection of STZ (Sigma Chemical Co, St Louis, USA) (60 mg/kg) into rats after fasting for three days. Rats with plasma glucose concentration of 20 mmol/L or greater in addition to polyuria and other diabetic disorders were considered as IDDM. All studies were carried out 2 weeks after the induction of diabetes. Also, insulin-resistance was induced in male Wistar rats aged 8 weeks by the intraperitoneal (ip) injections of long-acting human insulin (Monotard<sup>®</sup> HM, Novo Nordisk A/S, Bagsvaerd, Denmark) at 0.5 U/kg three times daily (tid) for 15 d<sup>[6]</sup>. Rats with insulin-resistance were employed as the model of non-insulin-dependent diabetes mellitus (NIDDM).

**Effect of Die-Huang-Wan on the formation of insulin resistance in obese Zucker rats** Oral treatment with Die-Huang-Wan at dose of 26.0 mg/kg decreased the plasma glucose concentration at 1 h later in normal Wistar rats in the previous study<sup>[7]</sup>. Thus, granules of Die-Huang-Wan (Cheng-Hoo Pharmaceutical Co, Tainan, Taiwan) were dissolved in distilled water containing 0.9 % NaCl for oral administration at the effective dose of 26.0 mg/kg into obese Zucker rats. The obese Zucker rats were separated into two groups. One group received oral administration of Die-Huang-Wan at 26.0 mg/kg once a day and the other group received the same volume of vehicle that was used to dissolve Die-Huang-Wan. After treatment for 3 d, insulin-resistance was identified using the loss of tolbutamide-induced plasma glucose lowering action<sup>[6,8,9]</sup>. In brief, obese Zucker rats under anesthesia of sodium pentobarbital (30 mg/kg, ip) were used to receive an ip injection of 10 mg/kg tolbutamide (Sigma Chemical Co, St Louis, USA) at 5 h later of the treatment with Die-Huang-Wan or vehicle. Effects on plasma glucose were determined using the blood samples collected from femoral vein of rats at 1 h after tolbutamide injection.

**Effect of Die-Huang-Wan on formation of insulin resistance in Wistar rats induced by repeated insulin injection** Two groups of Wistar rats were used to receive the ip injection of long-acting human insulin to induce insulin-resistance. Injection of long-acting human insulin was performed at 0.5 U/kg, every 8 h, three times daily. One group received oral administration of Die-Huang-Wan at 26.0 mg/kg once a day and the other group received the same volume of vehicle. Insulin-resistance was identified using the loss of tolbutamide-induced plasma glucose lowering action as

described above. Otherwise, the loss of plasma glucose lowering action by electroacupuncture (EA) stimulation, as described previously<sup>[9]</sup>, was also used to characterize the formation of insulin resistance. This identification was carried out in the same manner as that of tolbutamide on indicated date in the separate experiment.

**Effect of Die-Huang-Wan on insulin sensitivity in STZ-diabetic rats** STZ-diabetic rats were used to investigate the response to exogenous insulin. These rats received an ip injection of long-acting human insulin at 1 U/kg once daily to normalize the insulin sensitivity<sup>[10]</sup>. Three days later, the STZ-diabetic rats were divided into two groups for experiment. One group of STZ-diabetic rats received an oral administration of Die-Huang-Wan at 26.0 mg/kg every 8 h, three times daily; and the other group received similar treatment with same volume of vehicle. The injection of long-acting human insulin at 1 U/kg was also continued once a day in each group of STZ-diabetic rats. After ten days of treatment, all rats were used to challenge with exogenous insulin. According to the method as described previously<sup>[11]</sup>, an intravenous insulin challenge test was performed by giving 0.05 to 2.5 U/kg of short-acting human insulin (Actrapid® HM, Novo Nordisk A/S, Bagsvaerd, Denmark) into these STZ-diabetic rats. Blood samples (0.2 mL) from the femoral vein were drawn at 30 min following the intravenous insulin challenge test for the measurement of the plasma glucose concentrations. The difference in the response to exogenous insulin was compared in two groups. Otherwise, as the positive control, STZ-diabetic rats receiving an oral administration of metformin (Glucophage®, Lipha, UK) at 320 mg/kg, the effective dose used in Zucker rat<sup>[12]</sup>, were used to compare the vehicle-treated group. However, different to that performed in Die-Huang-Wan-treated group, administration of metformin at the dose of 320 mg/kg every 8 h, three times daily was performed for 15 d; the time to produce effect. STZ-diabetic rats received oral administration of metformin (320 mg/kg) or the same volume of vehicle was used to carry out the intravenous insulin challenge test in a same way. The difference between two groups in the response to exogenous insulin was also compared.

**Determination of plasma glucose** Blood sample (0.2 mL) was collected by a chilled syringe containing 10 U heparin from the femoral vein of rats under anesthesia with sodium pentobarbital (30 mg/kg, ip). Blood samples were then centrifuged at 13 000 rpm for 3 min

and an aliquot (15 mL) of plasma was added to 1.5 mL of glucose kit reagent (Biosystems SA, Barcelona, Spain) and incubated at 37 °C in a water bath (Yamato-BT-25, Tokyo, Japan) for 10 min. The concentrations of plasma glucose were then estimated via an analyzer (Quik-Lab, Ames, Miles Inc, Elkhart, Indiana 46515, USA) run in duplicate. Die-Huang-Wan or metformin did not influence this determination of glucose in our preliminary experiments ( $n=6$ ).

**Statistical analysis** Data are expressed as the Mean±SD for the number ( $n$ ) of animals in the group indicated in figures. Repeated measures analysis of variance (ANOVA) was used to analyze the changes in plasma glucose and other parameters. The Dunnett range *post-hoc* comparisons were used to determine the source of significant differences where appropriate. The obtained  $P$  value of 0.05 or less was considered statistically significant.

## RESULTS

**Effect of Die-Huang-Wan on the formation of insulin resistance in obese Zucker rats** After 3-d treatment with Die-Huang-Wan (26.0 mg/kg), the plasma glucose level declined to (5.7±0.2) mmol/L in obese Zucker rats, but the value was not markedly ( $P>0.05$ ) different from that (6.2±0.3) mmol/L of vehicle-treated control (Tab 2). According to the previous study<sup>[6]</sup>, the plasma glucose-lowering activity induced by tolbutamide at 10 mg/kg in Wistar rats was approximately (28.3±3.4)%. Though the action of tolbutamide at 10 mg/kg in obese Zucker rats treated with Die-Huang-Wan was not as effective as that produced in normal rats, the value was indeed higher ( $P<0.05$ )

**Tab 2. Changes of the plasma glucose concentration and the plasma glucose lowering activity of tolbutamide (10 mg/kg, ip) in obese Zucker rats that received Die-Huang-Wan treatment for 3 d.  $n=10$ . Mean±SD. <sup>b</sup> $P<0.05$  vs data obtained from animals treated with vehicle.**

	Plasma glucose/ mmol·L <sup>-1</sup>	Plasma glucose lowering activity of tolbutamide/%
Vehicle-treated	6.2±0.3	13.2 ± 2.7
Die-Huang-Wan-treated	5.7±0.2	20.9 ± 2.9 <sup>b</sup>

The distilled water containing 0.9 % NaCl was used as vehicle to dissolve Die-Huang-Wan.

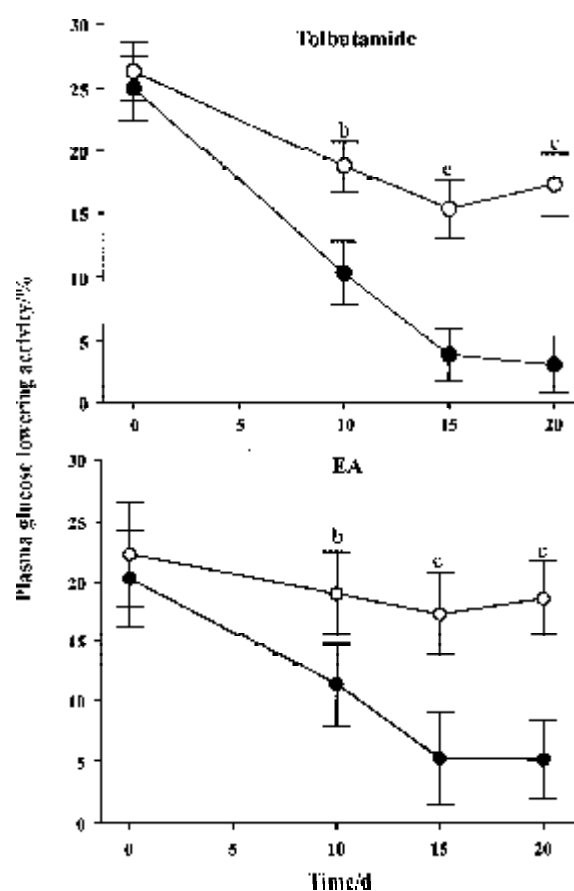
than that from vehicle-treated group (Tab 2).

**Effect of Die-Huang-Wan on the formation of insulin resistance in Wistar rats induced by repeated insulin injection** The basal glucose concentration of Wistar rats was  $(5.4 \pm 0.3)$  mmol/L ( $n=10$ ). An injection of long-acting human insulin at 0.5 U/kg into peritoneal cavity of rats was repeated every 8 h daily. The marked increase ( $P < 0.01$ ) of plasma glucose to  $(6.9 \pm 0.4)$  mmol/L ( $n=10$ ) was then observed in these rats at d 15 and remained at  $(8.2 \pm 0.3)$  mmol/L on d 20 of injections. An induction of insulin-resistance was obtained as previously reported<sup>[6]</sup>. However, the plasma glucose level was not increased but decreased from  $(5.5 \pm 0.3)$  mmol/L to  $(4.2 \pm 0.5)$  mmol/L ( $P < 0.05$ ) in rats receiving Die-Huang-Wan in combination during the induction of insulin-resistance at d 15 ( $n=10$ ). Die-Huang-Wan treatment made the plasma glucose level obtained at d 20 of rats receiving the induction of insulin-resistance to  $(5.2 \pm 0.4)$  mmol/L, which was not different ( $P > 0.05$ ) from the basal level  $(5.4 \text{ mmol/L} \pm 0.4 \text{ mmol/L})$ .

As shown in Fig 1, the plasma glucose lowering-activity of tolbutamide (10 mg/kg, ip) decreased gradually in rats that received daily injections of long-acting insulin. The activity of tolbutamide was decreased to  $(3.9 \pm 2.0)$  % at d 15 and  $(3.1 \pm 2.3)$  % at d 20 after the induction of insulin-resistance. However, the plasma glucose-lowering activity of tolbutamide was still observed in rats that received daily oral administration of Die-Huang-Wan in combination during the induction of insulin-resistance at d 15 or d 20 (Fig 1).

Otherwise, a decrease of EA-induced hypoglycemia was also observed in rats that received the induction of insulin-resistance. The plasma glucose-lowering activity of EA-stimulation at the Zhongwan acupoint in Wistar rats was  $(20.3 \pm 5.0)$  % ( $n=10$ ). The activity of this EA-stimulation became  $(5.2 \pm 3.7)$  % only at d 15 and  $(5.1 \pm 7.2)$  % at d 20 during the induction of insulin-resistance. However, the plasma glucose-lowering activity of EA-stimulation remained  $(18.3 \pm 4.0)$  % at d 15 and  $(19.6 \pm 6.1)$  % at d 20 in rats that received daily oral administration of Die-Huang-Wan in combination during the induction of insulin-resistance (Fig 1).

**Effect of Die-Huang-Wan on the insulin sensitivity in STZ-diabetic rats** In agreement with the previous study<sup>[7]</sup>, after Die-Huang-Wan treatment (26.0 mg/kg, tid) for 10 d, the plasma glucose level obtained from STZ-diabetic rats was  $(24.3 \pm 2.5)$  mmol/L, being similar to that  $(25.9 \pm 3.4)$  mmol/L before



**Fig 1.** Plasma glucose-lowering activity of tolbutamide or electroacupuncture (EA) at Zhongwan acupoint in rats with insulin-resistance. Wistar rats aged 8 weeks received the ip injection of long-acting human insulin at 0.5 U/kg combined with an oral administration of Die-Huang-Wan at 26.0 mg/kg once a day were performed to induce insulin resistance (opened circle). The distilled water containing 0.9 % NaCl was used as vehicle to dissolve Die-Huang-Wan. Animals receiving the same volume of vehicle during the induction of insulin resistance were expressed in closed circle. Changes of the plasma glucose lowering activity (%) of tolbutamide (10 mg/kg, ip) were indicated in upper while the lower shows changes of the plasma glucose lowering activity of EA stimulation.  $n=8$ . Mean  $\pm$  SD. <sup>b</sup> $P < 0.05$ , <sup>c</sup> $P < 0.01$  vs vehicle, respectively.

treatment. However, the plasma glucose-lowering activity of short-action human insulin at the dose of 0.05 to 2.5 U/kg in these STZ-diabetic rats received Die-Huang-Wan was higher than that in the control group receiving the same volume of vehicle. The plasma glucose-lowering activity of exogenous insulin was more prominent at the dose  $> 1$  U/kg (Fig 2).

Metformin was administrated at the dose of 320 mg/kg three times daily as described previously<sup>[12]</sup>. Similar intravenous insulin challenge was performed in STZ-

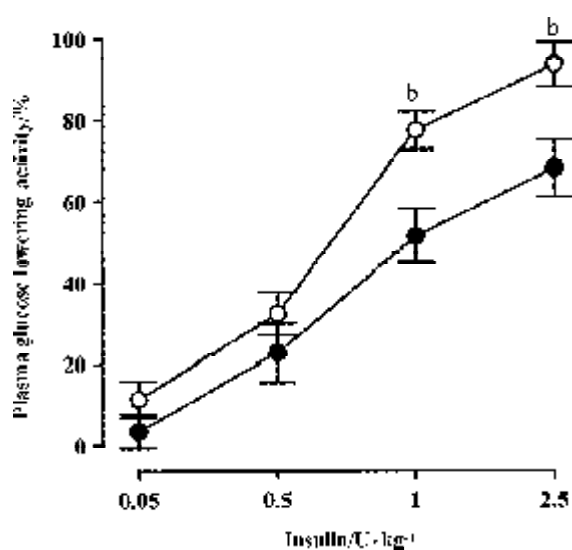


Fig 2. Change of intravenous insulin challenge test in STZ-diabetic rats treated with an oral administration of Die-Huang-Wan at 26.0 mg/kg, three times daily, every 8 h for 10 d (opened circle). Vehicle of 0.9 % NaCl in distilled water was used to dissolve Die-Huang-Wan. Animals receiving the same volume of vehicle were taken as the control (closed circle).  $n=8$ . Mean $\pm$ SD. <sup>b</sup> $P<0.05$  vs vehicle.

diabetic rats received metformin or vehicle. Different from that in Die-Huang-Wan, after 10 d, the plasma glucose-lowering action of short-action insulin in STZ-diabetic rats was similar between metformin-treated group and vehicle-treated group. Fifteen days later, intravenous challenge with short-action insulin at the dose of 1 U/kg caused plasma glucose-lowering activity about (68.1 $\pm$ 5.2) % in STZ-diabetic rats that received metformin treatment. This value was significantly ( $P<0.05$ ) higher than that produced in vehicle-treated STZ-diabetic rats (50.3 % $\pm$ 4.2 %).

## DISCUSSION

In the present study, we assessed the role of Die-Huang-Wan in the modulation of insulin sensitivity using an animal model of obesity-associated insulin resistance, the obese Zucker rats, which accompanied by mild hyperglycemia, glucose intolerance and hyperinsulinemia<sup>[13]</sup>. It has been documented that tolbutamide-induced hypoglycemia occurs through the stimulation of endogenous insulin release<sup>[6,8,9]</sup>. Whole-body insulin sensitivity, as reflected in the plasma glucose lowering action of tolbutamide, was significantly enhanced in obese Zucker rats receiving the repeated administration of Die-Huang-Wan for 3 d. However, the plasma glucose concentrations in these insulin-re-

sistant animals with same treatment did not change significantly. These results suggest that Die-Huang-Wan might enhance insulin sensitivity in the insulin-resistant animal.

Then, another insulin-resistant animal model induced by repeatedly injection of long action human insulin was used to confirm this hypothesis. A marked response to tolbutamide-induced hypoglycemia persisted in rats that received Die-Huang-Wan in 15 d after the induction of insulin-resistance. In contrast, tolbutamide was not effective in rats that received chronic injections of long-acting insulin during the same period without Die-Huang-Wan treatment. The effect due to combined treatments can be ruled out because tolbutamide was challenged at 5 h after the oral administration of Die-Huang-Wan. In addition, no increase of plasma glucose level was obtained during the induction of insulin-resistance in rats receiving combined treatment. Thus, delaying the formation of insulin-resistance in rats by repeatedly injection of long action insulin might be associated with the action of Die-Huang-Wan to enhance whole-body insulin sensitivity. Results of the present investigation are consistent with those from obese Zucker rats.

Otherwise, EA-stimulation at the Zhongwan acupoint to produce a marked reduction of plasma glucose in rats was also through the secretion of endogenous insulin<sup>[9]</sup>. Loss of the plasma glucose lowering response to EA-stimulation can thus be interpreted as the development of insulin-resistance. Therefore, EA-stimulation was then used to identify the influence of Die-Huang-Wan on the formation of insulin-resistance. Challenge of EA-stimulation was also performed at 5 h later of the oral administration of Die-Huang-Wan to exclude the interaction between these treatments. The plasma glucose-lowering action of EA stimulation was decreased and/or disappeared in rats that received chronic injections of long-acting insulin over the period of 15 d to 20 d in vehicle-treated group. Furthermore, the plasma glucose was elevated in these rats with insulin-resistance. Similar to the results obtained from tolbutamide, the plasma glucose-lowering action response to EA-stimulation was still observed in insulin-resistance induction rats that received Die-Huang-Wan in combination 15 d after the induction period. However, insulin resistance was not produced even at 20 d after Die-Huang-Wan treatment in rats. Thus, delay of insulin resistance in rats by Die-Huang-Wan can be considered.

Clinically, the patient with NIDDM was characterized by the change of insulin secretion and/or insulin action<sup>[14]</sup>. In the insulin-resistant animal model induced by insulin repeated injection, as that in clinic<sup>[14]</sup>, change of insulin action seems more important than the alteration of insulin secretion. Actually, Die-Huang-Wan has an ability to increase insulin secretion<sup>[7]</sup>. Higher insulin may enhance the down-regulation of insulin receptor stimulated by long action exogenous insulin repeatedly. However, induction of insulin resistance was reduced by Die-Huang-Wan in the present study. The possible mechanism is related to the change of insulin sensitivity in our findings. Thus, we investigated the effect of Die-Huang-Wan on insulin sensitivity.

The first method developed to evaluate insulin sensitivity *in vivo* was using the intravenous insulin challenge test, which is based on the change of plasma glucose level after a bolus injection of regular insulin<sup>[10]</sup>. Then, the intravenous insulin challenge test was performed in STZ-diabetic rat. The advantage of using STZ-diabetic rats in this study is the negligible endogenous insulin. Plasma glucose lowering action is directly due to the activity of exogenous insulin in STZ-diabetic rat. The obtained result can be used to indicate insulin sensitivity. We found that daily treatment with Die-Huang-Wan at the effective dose (26.0 mg/kg) for 10 d enhanced the plasma glucose lowering action of exogenous insulin in STZ-diabetic rats. Because Die-Huang-Wan failed to modify the plasma glucose in STZ-diabetic rats<sup>[10]</sup>, increase of exogenous insulin action by this herbal preparation was associated with an improvement of insulin sensitivity. Thus, enhancement of insulin action brought about by Die-Huang-Wan might produce a synergistic effect on the plasma glucose lowering action of insulin in STZ-diabetic rats.

Also, we used the effect of metformin as positive control in the present study. Metformin is believed to alleviate insulin resistance in the presence of insulin<sup>[15]</sup>. Actually, an increase of response to exogenous insulin was observed in STZ-diabetic rats that received metformin at an effective dose as described previously<sup>[16]</sup>. The action of Die-Huang-Wan to improve insulin sensitivity was more effective than that of metformin, because the influence of metformin was achieved over 15 d of treatment in STZ-diabetic rat that was 5 d later than Die-Huang-Wan. Metformin has been shown to improve the insulin sensitivity in NIDDM subjects by activating post-receptor insulin signaling pathways<sup>[15]</sup>. However, the detailed mechanisms of action by which

Die-Huang-Wan improves the insulin sensitivity need further investigation.

Different from purified synthetic drugs used in clinic, Die-Huang-Wan is a mixture of 6 herbs (Tab 1). This product has widely been used in China for a long time and there has been no serious reported toxicity. Currently, an agent that can increase insulin sensitivity is needed even the precise mechanisms are largely unknown<sup>[17]</sup>. An agent that improves insulin sensitivity, named as insulin sensitizer, has recently been developed<sup>[18]</sup>. However, the clinical application remains limited due to its toxic effect<sup>[19]</sup>. Therefore, the herbal preparation of Die-Huang-Wan seems helpful to the diabetic patients as an alternative medication.

In conclusions, we provided the scientific evidence that Die-Huang-Wan could delay the formation of insulin-resistance in association with an increase of insulin sensitivity. Thus, Die-Huang-Wan may be used as an adjuvant for the handling of the diabetic patients with clinically manifested insulin resistance.

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