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Corni fructus as the major herb of Die-Huang-Wan for lowering plasma glucose in Wistar rats

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Abstract

Die-Huang-Wan is a mixture of six herbs used to lower plasma glucose by increasing insulin secretion in normal rats. Die-Huang-Wan contains the herbs dioscorea (*Dioscoreae rhizoma*), cornus (*Corni fructus*), alisma (*Rhizoma alismatis*), holelen (*Poria*), rehmannia (*Rehmanniae radix*) and tree peony bark (*Moutan radicis cortex*). The present study was designed to clarify the major herb contributing to the plasma glucose-lowering action of Die-Huang-Wan in rats. A decrease in plasma glucose was not observed in Wistar rats treated with the cornus-deleted formula of Die-Huang-Wan; however, the action was retained in the other herb-deleted formulas containing cornus. In normal rats, the decrease in plasma glucose and increase in plasma insulin concentrations were dependent on the dose of cornus and were similar to those produced by Die-Huang-Wan. Treatment of Wistar rats with each of the other five herbs separately did not result in a decrease in plasma glucose. Moreover, the increase in plasma insulin or reduction in plasma glucose resulting from cornus treatment was blocked by atropine or 4-diphenylacetoxy-N-methylpiperidine methiodide mustard, indicating mediation of muscarinic M_3 receptors similar to that caused by Die-Huang-Wan. These results suggest that cornus is the major contributor to the plasma glucose-lowering action in Die-Huang-Wan in normal rats.

Introduction

Diabetes mellitus, which ranks among the top 10 causes of mortality throughout the world, often leads to disability from vascular complications and limb amputation, neurological complications, and premature death (Lopez-Candales 2001). Novel treatments with fewer side effects are needed for the long-term control of this disorder.

In Chinese traditional medicine, Die-Huang-Wan is used to treat diabetic disorders (Suzuki & Kimura 1984; Kamei et al 1987). Previous studies have demonstrated that the plasma glucose-lowering action of Die-Huang-Wan is associated with an increase in insulin secretion via release of acetylcholine (ACh) from nerve terminals, which stimulates muscarinic cholinoceptors in the pancreatic islets of normal Wistar rats (Liou et al 2002). However, Die-Huang-Wan does not lower plasma glucose levels in streptozotocin-induced diabetic rats, a model for type 1 diabetes (Cheng et al 2001). Die-Huang-Wan therefore appears to be a suitable treatment for type 2 diabetes.

Die-Huang-Wan is a mixture of six herbs: dioscorea (*Dioscoreae rhizoma*), cornus (*Corni fructus*), alisma (*Rhizoma alismatis*), holelen (*Poria*), rehmannia (*Rehmanniae radix*) and tree peony bark (*Moutan radicis cortex*). However, the herb that offers the greatest contribution to the activity of Die-Huang-Wan has not been identified. The present study was designed to determine the major active constituent of Die-Huang-Wan.

Materials and Methods

Materials

Granules of Die-Huang-Wan were donated by the Cheng-Hoo Pharmaceutical Co. (Hsin-Yin City, Tainan Shian, Taiwan). The composition of Die-Huang-Wan included dioscorea (4.2 mg kg^{-1}), cornus (4.2 mg kg^{-1}), alisma (3.1 mg kg^{-1}), holelen (3.1 mg kg^{-1}), rehmannia (8.3 mg kg^{-1}) and tree peony bark (3.1 mg kg^{-1}). The extract of the total

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ingredients was concentrated to 4 g of dry weight to meet the ratio of 6.25:1 in the granules of Die-Huang-Wan (6 g). Granules of dioscorea, cornus, alisma, holelen, rehmannia and tree peony bark were also received from the Cheng-Hoo Pharmaceutical Co (Hsin-Yin City, Tainan Shian, Taiwan). Atropine sulfate was purchased from Sigma-Aldrich, Inc. (St Louis, MO). 4-Diphenyl-acetoxy-N-methylpiperidine methiodide (4-DAMP) was purchased from Tocris Cookson Inc. (Missouri). A commercial kit for plasma insulin-like immunoreactivity (IRI) was purchased from Peninsula Lab. Inc. (California).

Animals

Male Wistar rats weighing 200–250 g were obtained from the Animal Center of the National Cheng Kung University Medical College. Rats were housed in a temperature-controlled room (25 ± 1 °C) and kept on a 12:12 light–dark cycle (light on at 0600 h). Water and standard laboratory diet were freely available. All animal procedures were conducted according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, as well as the guidelines of the Animal Welfare Act.

Experimental protocols

Three experiments were performed:

Experiment 1: Granules of Die-Huang-Wan were dissolved in saline for oral administration at an effective dose of 26.0 mg kg^{-1} to fasting Wistar rats as described previously (Cheng et al 2001).

Experiment 2: Each herb contained in Die-Huang-Wan was deleted separately, and plain flour was substituted for the deleted ingredient. The deletion preparation was dissolved in saline for oral administration at 26.0 mg kg^{-1} to fasting Wistar rats as described above.

Experiment 3: Granules of each individual herb contained in Die-Huang-Wan, dioscorea (4.2 mg kg^{-1}), cornus (4.2 mg kg^{-1}), alisma (3.1 mg kg^{-1}), holelen (3.1 mg kg^{-1}), rehmannia (8.3 mg kg^{-1}) and tree peony bark (3.1 mg kg^{-1}), were dissolved in saline for oral administration to fasting Wistar rats at specified dosages that matched the concentrations of each ingredient in Die-Huang-Wan.

Changes in plasma concentrations of glucose and insulin were determined in blood samples collected 1 h after oral administration, which is the time required for Die-Huang-Wan maximal action (Cheng et al 2001). Wistar rats receiving vehicle (saline) only at the same volume were used as controls. Pharmacological inhibitors (atropine sulfate and 4-DAMP) were injected intravenously into fasting rats 30 min before oral administration of testing agents.

Laboratory determination

Blood samples (0.2 mL) were collected from the tail vein of rats under anesthesia with sodium pentobarbital (30 mg kg^{-1}, i.p.) using a chilled syringe containing heparin (10 IU). Blood samples were then centrifuged at 13 000 rpm for 3 min, and an aliquot (15 μL) of plasma was added to 1.5 mL of the glucose kit reagent and incubated at 37°C in a water bath (Yamato-BT-25, Tokyo, Japan) for 10 min. Plasma glucose concentrations were determined in duplicate using an analyser (Quik-Lab, Ames, Miles Inc., Elkhart, IN). Plasma insulin was determined by enzyme-linked immunosorbent assay (ELISA) using a commercial kit. Samples were analysed in triplicate and results were expressed as pmol of IRI per litre of plasma.

Statistical analysis

Data are expressed as mean ± s.e.m. for the number (n) of animals in the group. A one-way ANOVA was used to analyse the changes in plasma glucose and other parameters. Dunnett range post-hoc comparisons were used to determine the source of significant differences where appropriate. A P value of 0.05 or lower was considered statistically significant.

Results

Similar to previous studies (Cheng et al 2001; Liou et al 2002), the maximum plasma glucose-lowering activity of Die-Huang-Wan at 26.0 mg kg^{-1} in Wistar rats was approximately 25.27 ± 2.69% (Figure 1). In the absence of rehmannia, the plasma glucose concentration in Wistar rats treated with a combination of the other five herbs at a dosage of 26 mg kg^{-1} was reduced from 5.42 ± 0.19 mmol L^{-1} to 4.40 ± 0.14 mmol L^{-1}; the plasma glucose-lowering activity remained approximately 18.82 ± 1.34% (Figure 1). Moreover, the plasma glucose-lowering activity of the

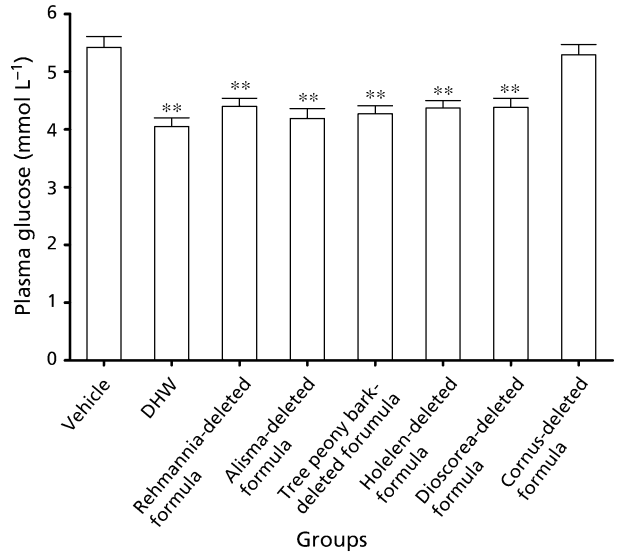

Figure 1 Effect of formulations lacking one ingredient on plasma glucose level in Wistar rats. Each herb contained in Die-Huang-Wan (DHW) was deleted separately and substituted with plain flour of equal weight. Values (mean ± s.e.m.) were obtained from each group of eight animals. The basal level was obtained from animals that received oral administration of the same volume of vehicle. **$P < 0.01$ vs data from the basal value.

alisma-deleted preparation (26.0 mg kg^{-1}) remained at 22.69 ± 1.43%, not significantly different from that obtained with Die-Huang-Wan (26 mg kg^{-1}) (Figure 1). Similarly to the value obtained from the tree peony bark-deleted preparation, the plasma glucose concentration of Wistar rats treated with the holelen- or dioscorea-deleted formula at 26.0 mg kg^{-1} was also reduced significantly and was similar to that produced by Die-Huang-Wan at the same dosage (Figure 1). However, plasma glucose was not reduced in Wistar rats receiving the cornus-deleted formula at 26.0 mg kg^{-1} (2.3 ± 0.96%) (Figure 1).

As shown in Figure 2, the plasma glucose concentration of Wistar rats was not influenced by oral administration of rehmannia (8.3 mg kg^{-1}); the value (5.05 ± 0.17 mmol L^{-1}) was not markedly different to that produced by the vehicle-treated controls (5.37 ± 0.18 mmol L^{-1}). The plasma glucose concentration in the alisma group (3.1 mg kg^{-1}) was 5.11 ± 0.18 mmol L^{-1}, a level similar to that observed in the vehicle-treated group. Similarly, plasma glucose-lowering activity in Wistar rats was not changed significantly by oral treatment with tree peony bark (3.1 mg kg^{-1}), holelen (3.1 mg kg^{-1}) or dioscorea (4.2 mg kg^{-1}), which lowered the glucose level by 4.66 ± 0.18%, 5.40 ± 0.21% and 4.47 ± 0.24%, respectively (Figure 2).

A dose-dependent decrease in plasma glucose concentration was observed in Wistar rats receiving oral administration of cornus; the maximal effect (21.04 ± 1.43%) was achieved by 4.2 mg kg^{-1} of cornus (Figure 3A). Increasing the oral dose of cornus to 8.4 mg kg^{-1} showed no further decrease in the plasma glucose level (21.12 ± 1.41%). The plasma glucose lowering activity of cornus (4.2 mg kg^{-1}) was similar to that produced by Die-Huang-Wan (26.0 mg kg^{-1})

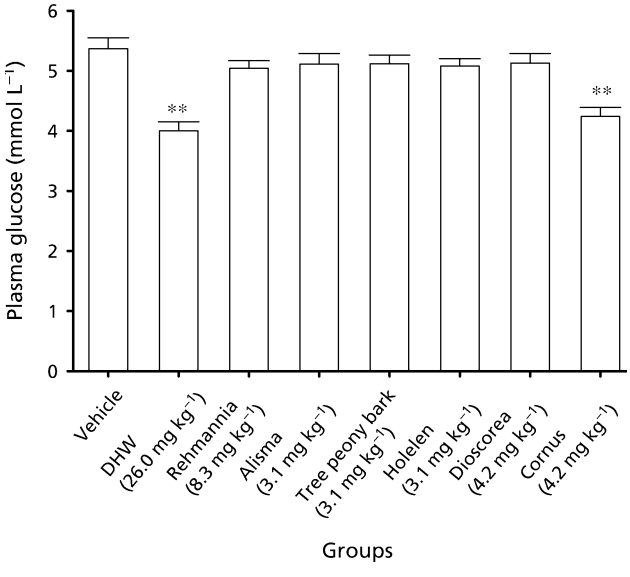

Figure 2 Effects of each herb in Die-Huang-Wan (DHW) on plasma glucose level in Wistar rats. Values (mean ± s.e.m.) were obtained from each group of eight animals. The basal level was obtained from animals that received oral administration of the same volume of vehicle. **P < 0.01 vs data from the basal value.

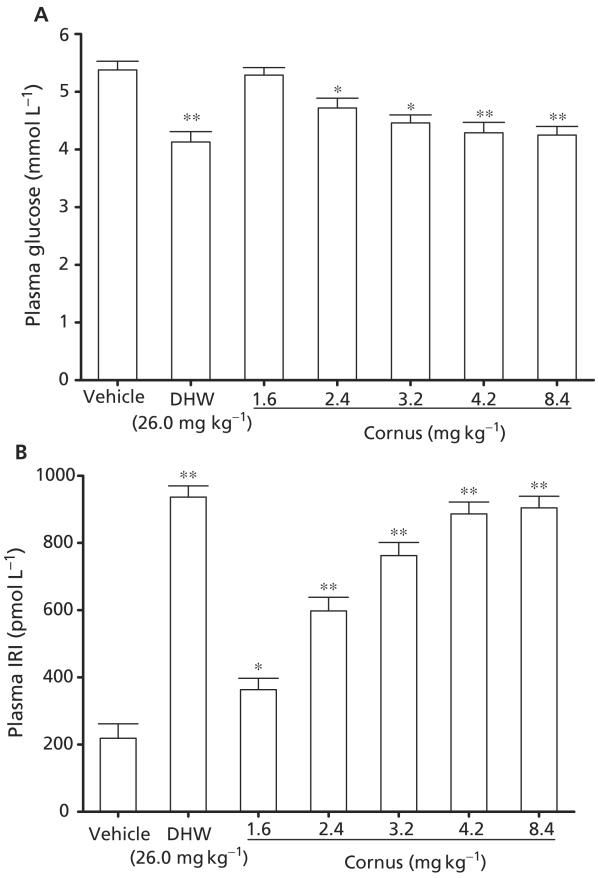

Figure 3 Effects of cornus on (A) plasma glucose level and (B) plasma IRI in Wistar rats. Values (mean ± s.e.m.) were obtained from each group of eight animals. The basal level was obtained from animals that received oral administration of the same volume of vehicle. *P < 0.05 and **P < 0.01 vs data from the basal value.

in Wistar rats (Figure 3A). The decrease in plasma glucose concentration by cornus paralleled the increase in plasma insulin. Oral administration of cornus into Wistar rats at a dose (4.2 mg kg^{-1}) that effectively lowers plasma glucose increased the IRI from 218.60 ± 43.20 pmol L^{-1} to 886.35 ± 35.29 pmol L^{-1}, close to the effect produced by Die-Huang-Wan (26.0 mg kg^{-1}) in Wistar rats (Figure 3B). However, the stimulatory action of cornus at 8.4 mg kg^{-1} on the plasma IRI level was similar to that obtained at the 4.2 mg kg^{-1} dose (P > 0.05), as shown in Figure 3B.

In the presence of atropine, the plasma glucose-lowering action of cornus at 4.2 mg kg^{-1} was reduced in Wistar rats; atropine inhibited cornus activity in a dose-dependent manner. At the effective dose (0.15 mg kg^{-1}), atropine suppressed the plasma glucose-lowering action of cornus (4.2 mg kg^{-1}) in Wistar rats (Table 1). In addition, atropine (0.15 mg kg^{-1}) inhibited the increase in IRI caused by cornus (4.2 mg kg^{-1}) to near basal level (Table 1). Although plasma IRI in Wistar rats was lowered slightly by intravenous injection of atropine alone, the value was not statistically different from the effect produced in vehicle-treated control rats (P > 0.05) (Table 1). The plasma

Table 1 Effects of muscarinic receptor antagonists on plasma glucose level and IRI in normal Wistar rats treated with oral cornus

	Plasma glucose ($mmol\,L^{-1}$)	Plasma IRI ($pmol\,L^{-1}$)
Basal	5.41 ± 0.21	217.52 ± 34.73
Die-Huang-Wan ($26.0\,mg\,kg^{-1}$)	4.03 ± 0.15**	930.41 ± 33.46**
Cornus ($4.2\,mg\,kg^{-1}$)		
+ vehicle	4.21 ± 0.16**	892.43 ± 31.28**
+ atropine ($mg\,kg^{-1}$)		
0.05	4.83 ± 0.18*	787.54 ± 35.63**
0.10	5.08 ± 0.14	412.38 ± 32.96*
0.15	5.32 ± 0.24	257.33 ± 20.76
+ 4-DAMP ($\mu g\,kg^{-1}$)		
1	4.73 ± 0.19**	745.29 ± 41.36**
5	5.04 ± 0.21	456.37 ± 39.42*
10	5.27 ± 0.16	276.48 ± 30.73
Atropine ($0.15\,mg\,kg^{-1}$)	5.52 ± 0.27	204.43 ± 37.24
4-DAMP ($10\,\mu g\,kg^{-1}$)	5.48 ± 0.27	210.86 ± 40.38

Antagonists were administered by intravenous injection 30 min before oral administration of cornus. Saline was used to dissolve the antagonists. Values (mean ± s.e.m.) were obtained from groups of eight animals in each experiment. The basal level was obtained from animals receiving an injection of an equivalent volume of vehicle. *$P < 0.05$ and **$P < 0.01$ vs basal value.

glucose level increased slightly in Wistar rats receiving atropine alone, but the value was not significantly different from the basal level ($P > 0.05$) (Table 1).

4-DAMP attenuated the plasma glucose-lowering activity of cornus ($4.2\,mg\,kg^{-1}$) in a dose-dependent manner in Wistar rats (Table 1). However, the plasma glucose level remained unchanged after intravenous injection with 4-DAMP alone at a concentration ($10\,\mu g\,kg^{-1}$) sufficient to block muscarinic M_3 receptors. The plasma glucose concentration was $5.48 \pm 0.27\,mmol\,L^{-1}$ in rats treated with 4-DAMP alone, a value equivalent to that obtained in vehicle-treated controls. The increase in plasma IRI by cornus ($4.2\,mg\,kg^{-1}$) in Wistar rats was reduced in a dose-dependent manner by 4-DAMP (Table 1). However, 4-DAMP at the maximal dose of $10\,\mu g\,kg^{-1}$ did not modify basal plasma IRI. Plasma IRI in Wistar rats treated with 4-DAMP alone was $210.86 \pm 40.38\,pmol\,L^{-1}$, not significantly different from the value obtained in vehicle-treated controls ($P > 0.05$).

Discussion

The results of this study confirmed the plasma glucose-lowering activity of Die-Huang-Wan in Wistar rats (Cheng et al 2001; Liou et al 2002). Because Die-Huang-Wan is a mixture of six herbs, we also determined if one herb provided the major biological effects. Each herb in Die-Huang-Wan was deleted one at a time to compare the activity of the modified preparations to the activity of Die-Huang-Wan. Deletion of cornus eliminated the plasma glucose-lowering response in Wistar rats. However, plasma glucose-lowering activity was retained in Wistar rats receiving modified preparations containing cornus but lacking each of the other herbs. Thus, cornus appears to be the major ingredient of Die-Huang-Wan for the plasma glucose-lowering action in animals with normal functioning pancreatic β-cells. The other five herbs constituting Die-Huang-Wan are minor ingredients in the activity of the preparation.

ACh, the major parasympathetic neurotransmitter, is released by intrapancreatic nerve endings during the preabsorptive and absorptive phases of feeding (Gilon & Henquin 2001). Following release from the nerve terminals, ACh binds with cholinoceptors for activation or is separated into choline and acetate by acetylcholinesterase. Muscarinic receptors exist in multiple subtypes, each of which has been distinguished pharmacologically and structurally (Nathanson 1987; Caulfield 1993). The regulation of insulin secretion by the cholinergic nervous system is mediated by muscarinic M_3 receptors (Iversen 1973; Henquin & Nenquin 1988). In fact, binding of ACh to muscarinic M_3 receptors on β-cells is important for the early phase of insulin secretion, which may decrease the exaggerated second phase and thus total insulin response, potentially providing protection from macroangiopathy development (Gilon & Henquin 2001). The insulinotropic effect of ACh might result from two mechanisms: one involves a rise in free cytosolic Ca^{2+} concentration and the other involves a marked PKC-mediated increase in the efficiency of Ca^{2+} on exocytosis (Gilon & Henquin 2001). In a previous study, we demonstrated that Die-Huang-Wan accelerated ACh release from the cholinergic neuronal terminals in Wistar rats to activate muscarinic M_3 receptors, thereby increasing the insulin secretion that lowered plasma glucose in normal rats; however, the nicotinic cholinoceptor was not involved in the action of Die-Huang-Wan (Liou et al 2002). Instead, a dose-dependent increase of plasma IRI was observed in Wistar rats on oral administration of cornus. The increase in plasma IRI was associated with the plasma glucose-lowering action of cornus in Wistar rats in a manner similar to that of Die-Huang-Wan (Cheng et al 2001; Liou et al 2002). Muscarinic antagonists were therefore employed to confirm the effect of cornus on plasma glucose in Wistar rats. Similar to the results obtained after administration of Die-Huang-Wan, the stimulatory action of cornus was antagonized by the muscarinic antagonist atropine (Ali-Melkkila et al 1993), indicating that the muscarinic receptor is involved in the plasma glucose-lowering action of cornus. These results reinforce the view that endogenous ACh is involved in the glucose-lowering action of Die-Huang-Wan in Wistar rats (Liou et al 2002).

Classic antagonists of muscarinic receptors, such as atropine, cannot distinguish among the subtypes of muscarinic receptors. 4-DAMP selectively inhibits muscarinic M_3 receptors without influencing other muscarinic receptor subtypes (Barlow et al 1991; Eglen & Watson 1996). 4-DAMP was therefore chosen to examine the role of muscarinic M_3 receptors in the activity of cornus. We found that prior blockade of muscarinic M_3 receptors by the antagonist 4-DAMP impeded the elevation of plasma IRI induced by cornus in a manner similar to that produced by Die-Huang-Wan. Thus, the effect

of cornus on glucose homeostasis in Wistar rats is related to the activation of muscarinic M_3 receptors. The influence of cornus on pancreatic ACh levels was difficult to determine, and the excitation of synaptic potential by cornus was not recorded. In a previous study (Liou et al 2002), pharmacological inhibitors that blocked the action of Die-Huang-Wan at concentrations sufficient to inhibit cholinergic neurotransmission demonstrated the involvement of ACh in the regulation of plasma glucose in normal Wistar rats. The plasma glucose-lowering action of cornus is therefore likely to occur via an increase in insulin secretion through the release of ACh, which stimulates muscarinic M_3 receptors. However, a detailed mechanism requires further investigation.

Cornus has been used in traditional Chinese medicine to treat disorders of the liver and kidney (Hsu & Peacher 1982). In addition to reducing tinnitus, this herb also has beneficial effects on the urogenital system, such as improving impotence and decreasing excessive urination (Hsu & Peacher 1982). Although the application of cornus in diabetic disorders has not been reported, these data strengthen the basis of its use to improve insulin action and benefit patients with type 2 diabetes.

Conclusions

We have demonstrated that cornus is the major ingredient in Die-Huang-Wan and plays an essential role in the plasma glucose-lowering action of Die-Huang-Wan within an insulin-sufficient state.

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