Effect of *Danhong Huayu Koufuye* combined with insulin on prevention and progression of early diabetic nephropathy in rats

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Abstract *Danhong Huayu Koufuye* (DHK), a traditional Chinese prescription, has activities of promoting blood circulation to remove blood stasis and promoting *qi* circulation to remove meridian obstruction. The aim of the study was to investigate effects of DHK, insulin and their combination on diabetic nephropathy (DN) in streptozotocin (STZ, 50 mg/kg, ip)-induced diabetic Sprague-Dawley rats. Rats were divided into five groups: normal control, model control, insulin, DHK, and DHK plus insulin. The animals were treated once daily for 15 weeks starting one week after STZ injection. The combination of DHK with insulin resulted in more significant effects than insulin or DHK alone on decreasing fasting blood glucose, 24 h water intake, diet intake and urine volume, reducing serum urea level and urea-to-Cr ratio, promoting kidney hypertrophy and renal damage, and increasing serum Na^+ and Cl^- level as well. These results suggest that DHK may be a valuable adjuvant therapy for DN.

Key words *Danhong Huayu Koufuye*; diabetic nephropathy; streptozotocin; diabetic mellitus; insulin resistance

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丹红化瘀口服液联合胰岛素对早期糖尿病肾病的防治作用

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摘 要 丹红化瘀口服液是传统中药复方,具活血化瘀、行气通络的功效。观察丹红化瘀口服液、胰岛素及两者联合用药对糖尿病肾病大鼠的治疗效果。采用链脲佐菌素(STZ)诱导糖尿病肾病大鼠模型,STZ 注射后 1 周后将造模成功的大鼠随机分为 4 组:糖尿病模型组、胰岛素组、丹红化瘀口服液组,丹红化瘀口服液联合胰岛素组,另设一正常组,每天给药 1 次,连续 15 周。丹红化瘀口服液联合胰岛素用药比两者单独用药能更显著降低空腹血糖、24 h 饮水量、摄食量和尿量,降低血清尿素与尿素/肌酐比,改善肾脏肥大和肾组织病理损伤,同时提高血清 Na^+ 与 Cl^- 水平。结果提示:丹红化瘀口服液可辅助胰岛素治疗糖尿病肾病。

关键词 丹红化瘀口服液;糖尿病肾病;链脲佐菌素;糖尿病;胰岛素抵抗

1 Introduction

Diabetic nephropathy (DN), one of the complications of diabetic mellitus, is a predominant cause of morbidity and mortality in diabetic patients. Approximately 30% of diabetic patients with either type 1 or type 2 diabetes will develop DN 10 to 20 years from the onset of diabetic mellitus[1]. DN, which is characterized by renal morphological and functional alterations, affects about 15%-25% of type 1 diabetes patients and 30%-40% of patients with type 2 diabetes[2]. Poor blood glucose control and elevated systolic

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blood pressure further accelerate the disease to renal failure[3].

Since DN is a major cause of end-stage renal disease, the prevention and early treatment to delay its progress are desperately needed with great social and economic impacts. Current treatments for DN have been geared toward the regulation of blood glucose by insulin injection or oral therapeutic agents and control of blood pressure[4].

Danhong Huayu Koufuye (DHK), manufactured by Baiyunshan Hutchison Whampoa Chinese Medicine Co., is a traditional Chinese prescription. It contains SALVIAE MILTIORRHIZAE RADIX ET RHIZOMA (Roots of *Salvia miltiorrhiza*), ANGELICAE SINENSIS RADIX (Root of *Angelicae sinensis*), CHUANXIONG RHIZOMA (Roots of *Ligusticum*), PERSICAE SEMEN (Seed of *Prunus persica*), CARTHAMI FLOS (Flower of *Carthamus tinctorius*), BUPLEURI RADIX (Root of *Bupleurum folium*), as well as AURANTII FRUCTUS (Fructus of *Citrus auantium*). The quantities of the ingredients are 29%, 11.5%, 15%, 11.5%, 11.5%, 11.5% and 10% of the total weight, respectively. DHK has shown activities of promoting blood circulation to remove blood stasis and promoting *qi* circulation to remove meridian obstruction, so it has been applied for the treatment of blurred vision induced by stagnation of *qi* and blood stasis, and central retinal vein occlusion[5]. Our previous research found that DHK had anti-hyperglycemic activity, restored retinal function as well as improved microvascular perfusion in STZ-induced diabetic rats[6], thus DHK was hypothesized to prevent and/or treat DN.

There have been no reports about effects of DHK and the combination of DHK with insulin on the attenuation of DN so far. Therefore, the pharmacological activities of DHK, insulin and their combination on kidney weight, kidney index (KI), serum levels of urea, creatinine (Cr), Na^+, K^+ and Cl^-, proteinuria, as well as renal morphological changes were evaluated in streptozotocin (STZ)-induced diabetic rats in this study.

2 Materials and methods

2.1 *Animals*

Sixty male Sprague-Dawley (SD) rats weighing 220-260 g were purchased from Experimental Animal Center, Guangzhou University of Chinese Medicine (Animal Certificate No: 0110336). They were housed in a group of 2-3 rats per cage and provided with free access to balanced pellet food and water. All rats were kept at (24 ± 2) °C and 40%-70% humidity using a 12/12 h light/dark cycle. The experiment was performed in accordance with the Animal Ethics Committee of Guangzhou University of Chinese Medicine.

2.2 *Materials*

STZ, isophane protamine recombinant human insulin injection, and pentobarbital sodium were obtained from Sigma-Aldrich Chemical Co., Novo Nordisk A/S, and Merck KG Co., respectively. Urinalysis reagent strips were purchased from ACON Biological Technology (Hangzhou) Co.. DHK (Lot number: D0A003) was kindly donated by Guangzhou Baiyunshan Hutchison Whampoa Chinese Medicine Co..

2.3 *Induction of diabetic mellitus*

Diabetic rats were induced as our previous method[6]. Seven days after administration of STZ, forty-five rats with fasting blood glucose (FBG) between 14.0 and 33.0 mmoL/L were selected as diabetic rats. FBG was measured using FreeView Blood Glucose Monitoring Meter and Test Strips (Guangzhou Wondfo Biotech, Co., Ltd.).

2.4 *Method of drug administration*

Animals were randomly divided into five groups. Distilled water, insulin, and DHK administered once daily began on the 7th day after STZ injection. All rats were treated for 15 weeks as follows: Group A (n = 10), normal control (distilled water, *po*, 3.2 mL/kg); Group B (n = 11), model control (distilled water, *po*, 3.2 mL/kg); Group C (n = 11), diabetic + insulin (sc, 1.5 and 3.0 U/kg for rats with FBG of 14.0-25.0 and 25.1-33.0 mmoL/L, respectively); Group D (n = 11), diabetic DHK (*po*, 3.2 mL/kg); Group E (n = 12), diabetic + insulin (sc, 1.5 and 3.0 U/kg for rats with FBG of 14.0-25.0 and 25.1-33.0 mmoL/L, respectively) + DHK (*po*, 3.2 mL/kg).

2.5 *Measurement of body weight, FBG, diet intake, water intake, and urine volume*

Non-fasting body weight was measured weekly. FBG was monitored once every two weeks after rats were fasted for 8 h. Diet and water intake over 24 h was also carried out once every two weeks. All rats were placed into metabolic cages to record 24 h urine volume at 7 and 15 weeks after STZ injection.

2.6 *Determination of renal parameters*

Proteinuria was measured weekly with urinalysis reagent strips. At the end of treatment, rats were anesthetized with pentobarbital sodium at 30 mg/kg by intraperitoneal injection. Abdominal aorta blood was then collected into tubes without anticoagulant and centrifuged at 3 820 r/min at 4 °C for 15 min. Serum was separated and levels of Cr, urea, Na^+, K^+, Cl^- were measured using Automatic Biochemical Analyzer (Hitachi 7020, Japan).

2.7 *Analysis of renal histology and morphology*

Kidneys were immediately removed and weighed after the animals were sacrificed, and then fixed in 4% poly-formaldehyde. Eight samples of each group were cut and stained with hematoxylin and eosin (HE) and examined under light microscope. Histopathological alterations were analyzed for glomerular hypertrophy, glomeruloslerosis, tubular necrosis and glycogen formation. KI was calculated using the equation of KI = KW/FBW, where in KW was kidney weight and FBW the fasting body weight of rats.

2.8 *Statistical analysis*

All data were presented as $\bar{x} \pm s$ and analyzed by the Statistical Package for the Social Sciences version 17.0 (SPSS 17.0). One-way analysis of variance (ANOVA) test was performed and post hoc multiple comparisons were conducted with LSD. $P < 0.05$ was assumed to be significant.

3 Results

3.1 *Effects on body weight, FBG, diet intake, water intake and urine volume*

The body weights of diabetic rats was markedly decreased by 40.6% after 16 weeks of diabetes induction ($P < 0.01$ *vs* normal control). Treatment with insulin alone prevented the loss of body weight ($P < 0.05$ *vs* model control group). Treatment with DHK combined with insulin (448.9 ± 71.3 g) displayed more significant effect on increasing body weight than insulin group (382.4 ± 45.2 g, $P < 0.05$) after 15 weeks of treatment (Table 1).

STZ-induced hyperglucose had been maintained over 16 weeks ($P < 0.01$ *vs* normal control). Insulin, DHK, and the combination of insulin and DHK significantly reduced FBG of diabetic rats by 68.0% ($P < 0.01$), 14.5% ($P < 0.01$) and 78.5% ($P < 0.01$) as compared with model control rats after 15 weeks of treatment, respectively. The combination usage significantly reduced 24 h diet intake, water intake, as well as urine volume as compared with diabetic control rats after 15 weeks of treatment (Table 1).

Table 1 Effects on body weight, FBG, diet intake, water intake and urine volume of rats after 15 weeks of treatment ($\bar{x} \pm SD$, $n = 10$-12)

	Group	*n*	Dose	Body weight/g	FBG/(mmoL/L)	24 h Diet intake/g	24 h Water intake /mL	24 h Urine volume/mL
A	Normal	10	–	548.5 ± 57.9	6.0 ± 0.5	30.4 ± 3.5	41.2 ± 6.0	12.4 ± 4.4
B	Model	11	–	325.6 ± 36.9**	27.6 ± 2.9**	66.7 ± 6.0**	282.3 ± 40.4**	230.3 ± 27.0**
C	Insulin	11	1.5 U/kg 3.0 U/kg	382.4 ± 45.2[a]	8.5 ± 2.3[a]	60.3 ± 5.7	293.3 ± 68.7	227.0 ± 55.5
D	DHK	11	3.2 mL/kg	336.5 ± 14.1	21.9 ± 4.0[a]	63.6 ± 6.7	289.5 ± 66.4	213.5 ± 62.8
E	Insulin + DHK	12	1.5 U/kg + 3.2 mL/kg 3.0 U/kg + 3.2 mL/kg	448.9 ± 71.3[a, b, c]	5.5 ± 1.2[a, b, c]	50.5 ± 8.9[a, c]	210.1 ± 104.0[a, b, c]	157.3 ± 93.2[a, b, c]

** $P < 0.01$ *vs* normal control; [a] $P < 0.05$ *vs* model control; [b] $P < 0.05$ *vs* insulin group; [c] $P < 0.05$ *vs* DHK group

3.2 *Changes in the kidney parameters*

Significant increases of kidney weight ($P < 0.01$) and KI ($P < 0.01$) were detected in diabetic rats as compared with normal rats. DHK other than insulin showed the tendency of down-regulation of kidney weight in diabetic rats. Although either insulin or DHK decreased KI of diabetic rats, combination treatment produced more significant effect (both $P < 0.05$ *vs* diabetic treated with insulin and DHK group, Table 2).

There was no proteinuria in diabetic rats after 16 weeks of diabetic induction (data not shown). As shown in Table 2, serum urea ($P < 0.01$) and urea-to-Cr ratio ($P < 0.01$) in diabetic rats were markedly higher than in those age-matched normal rats. Treatment with DHK alone had no effect on serum urea or urea-to-Cr ratio, whereas treatment with insulin alone or combination of DHK with insulin significantly decreased urea level by 23.8% and 42.6% and urea-to-Cr ratio by 24.9% and 41.0% in diabetic rats,

respectively. Addition of DHK to insulin showed a significant reduction of urea level ($P < 0.05$) and urea-to-Cr ratio ($P < 0.05$) as compared with treatment of insulin alone. Serum levels of Na^+ and Cl^- were both significantly lower in diabetic rats than in normal ones. Treatment with DHK combined with insulin significantly up-regulated serum Na^+ and Cl^- levels (both $P < 0.01$ *vs* diabetic model group).

Table 2 Effects on kidney parameters ($\bar{x} \pm s$, $n = 10$-12)

	Normal	Model	Insulin	DHK	Insulin + DHK
Left kidney weight/g	1.43 ± 0.11	1.70 ± 0.19 * *	1.74 ± 0.18	1.49 ± 0.14[a]	1.67 ± 0.17
Fasting body weight/g	518.46 ± 57.92	279.88 ± 39.92 * *	342.43 ± 45.15[a]	284.04 ± 14.11	401.23 ± 81.31[a, b, c]
KI of left kidney/(mg/g)	2.76 ± 0.22	6.07 ± 0.69 * *	5.08 ± 0.52[a]	5.24 ± 0.49[a]	4.17 ± 0.43[a, b, c]
Urea/(mmoL/L)	6.31 ± 2.21	12.46 ± 2.29 * *	9.50 ± 2.29[a]	13.86 ± 3.52	7.15 ± 1.82[a, b, c]
Cr/(μmol/L)	93.49 ± 15.03	89.46 ± 8.74	91.30 ± 8.57	87.00 ± 5.7	88.48 ± 9.92
Urea/Cr	66.55 ± 12.76	139.11 ± 19.42 * *	104.47 ± 24.16[a]	153.00 ± 37.32	82.12 ± 16.01[a, b, c]
Na^+/(mmoL/L)	144.60 ± 1.34	136.14 ± 3.56 * *	138.45 ± 3.14	139.14 ± 4.04	142.26 ± 5.08[a, b]
K^+/(mmoL/L)	4.78 ± 1.55	4.50 ± 0.66	3.95 ± 0.56	4.10 ± 0.54	4.00 ± 0.51
Cl^-/(mmoL/L)	108.32 ± 2.19	97.36 ± 5.04 * *	99.95 ± 3.00	101.69 ± 4.82	104.55 ± 4.23[a, b]

* * $P < 0.01$ *vs* normal control; [a] $P < 0.05$ *vs* model control; [b] $P < 0.05$ *vs* insulin group; [c] $P < 0.05$ *vs* DHK group

3.3 *Effects on glomerular damage*

Normal control kidneys showed normal glomerular structure, no tubular lesions and glycogen formation (Figure 1, A). Diabetic rats developed severe glomerular hypertrophy, mesangium enlargement, matrix expansion, glycogen formation, as well as glomerulosclerosis (Figure 1, B). These alterations were ameliorated by insulin and DHK treatment alone (Figure 1, C and D). Treatment with DHK combined with insulin showed more remarkable reversion effect on these damages than those achieved with insulin or DHK alone (Figure 1, E).

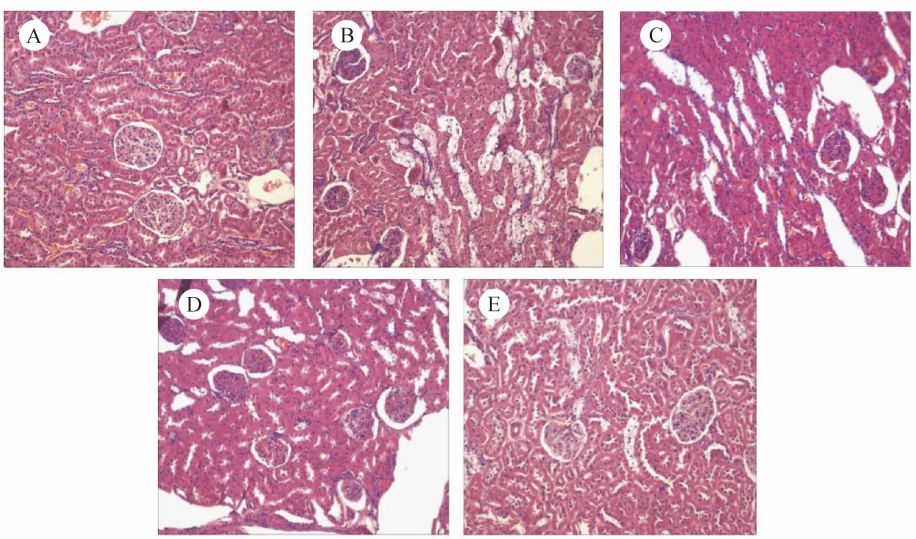

Figure 1 Light microscopy of renal tissue with HE staining (×100)
A: Normal; B: Model; C: Insulin group; D: DHK (3.2 mL/kg); E: DHK combined with insulin group

4 Discussion

The Diabetes Control and Complications Trial and the United Kingdom Prospective Diabetic Study have demonstrated in type 1 and type 2 diabetes that intensive glycemic control significantly slows the progression of DN[7]. Achieving optimal glycemic control for type 1 and type 2 diabetes usually necessitates the use of exogenous insulin. However, complications in retinal, renal and neuropathic functions are still associated with the patients receiving insulin injection because of insulin resistance[8]. In the present study, the results showed that DHK combined with insulin significantly and steadily reversed the increase of FBG, 24 h water and diet intake and urine volume, as well as the decrease of body weight throughout the

experiment. The results indicate that the combination of insulin with DHK contributes to maintain glycemic control, and thus improves insulin resistance[9] and slows the progress of DN.

No proteinuria was detected in diabetic rats during our study, possibly because it was at early stage of DN[10]. Increase in serum urea and urea-to-Cr ratio, markers of renal dysfunction, have been observed in DN[11]. Our data showed that diabetic rats had increased serum urea and urea-to-Cr ratio (both $P<0.01$ *vs* normal rats), which suggests that kidneys of diabetic rats were abnormal. There was no statistical difference in serum Cr level in diabetic SD rats (Table 2), which indicates that it is at the early stage of DN and is consistent with Hartner's report[12]. Furthermore, reduced serum concentrations of Na^+ and Cl^- in diabetic rats might indicate a greater reduction in absolute and fractional reabsorption in kidney[13]. The combination usage of DHK and insulin had significant effect on reversing the increase of urea level ($P<0.05$) and urea-to-Cr ratio ($P<0.05$), and decrease of serum Na^+ ($P<0.05$) and Cl^- ($P<0.05$) compared with insulin alone (Table 2), which suggests that combination therapy has more beneficial effects on renoprotective action than insulin alone.

Histologically the major pathology of DN is confined to the glomerulus which includes nodular changes in the glomerulus[14]. At present, treatment with insulin plus DHK showed more obvious repair in renal damages than treatment of insulin or DHK alone (Figure 1), whichis consistent well with changes in renal hypertrophy with increasing of kidney weight and KI (Table 2). The renoprotective effect of the combination may be due to better glycemic control and reduced glycogen formation in kidney. Moreover, DHK's beneficial effect on improving microvascular perfusion cannot be neglected[6].

In conclusion, DHK in combination with insulin achieved and maintained glycemic control, then reduced and delayed the onset of DN. This study suggests that DHK may be a valuable adjuvant therapy for DN.

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Disclosure

The authors declare no potential confilict of interests.

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