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Oriental Pharmacy and Experimental Medicine 2009 **9(1)**, 20-38
DOI 10.3742/OPEM.2009.9.1.020

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HDDM, a formula consisting of seven herbs, had anti-diabetic but no immunomodulatory activities in multiple low doses of streptozotocin-treated female of B6C3F1 mice

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Received for publication July 28, 2008; accepted February 09, 2009

SUMMARY

The objectives of this study were to determine the effect of herb formula HDDM, a modification of Huangdan decoction that has been shown to be effective in the treatment of glomerulonephritis and chronic renal failure, on the blood glucose levels in multiple low doses (MLD; 50 mg/kg for five consecutive days) of streptozotocin (STZ)-treated female B6C3F1 mice. Initial studies were performed to compare diabetes induction in five strains (e.g., B6C3F1, NOD, CD-1, C3H/HeN and C57BL/6) of mice by MLD-STZ, and immune changes following the treatment. The results suggested that the order of susceptibility to diabetes induction was NOD ≈ CD-1 > B6C3F1 ≈ C3H > C57BL/6. Furthermore, STZ modulation of T cell development, differentiation and activation might play a role in diabetes induction by MLD-STZ treatment. MLD-STZ-induced diabetes in female B6C3F1 mice was moderate, which allowed the evaluation of drug-induced protection or exacerbation of diabetes to be performed. As such, modulation of blood glucose by HDDM, which consisted of Da Huang (Radix Et Rhizoma Rhei), Huang Qi (Radix Astragali Seu Hedysari), Dan Shen (Radix Salviae Miltiorrhizae), Yin Yang Huo (Herba Epimedii), Yi Yi Ren (Semen Coicis or Coix lacryma-jobi), Mai Dong (Radix Ophiopogonis) and Shan Zhu Yu (Fructus Corni), was evaluated in MLD-STZ-treated female B6C3F1 mice. The results suggested that HDDM could lower the blood glucose levels, but it had no immunomodulatory activities. Additionally, HDDM-treated mice exhibited improved glucose tolerance. In conclusion, these studies have suggested that MLD-STZ-induced diabetes in female B6C3F1 mice is a useful model to evaluate drug modulation of diabetes, and that the herb formula HDDM possesses anti-diabetic effects.

Key words: diabetes; herbs; streptozotocin; B6C3F1 mice

INTRODUCTION

Diabetic nephropathy (DN) is the single most common cause of end stage renal disease (ESRD) in the United States, and it is the major predictor of mortality (Striker *et al*., 1992; Striker and Striker, 1996; Jones *et al*., 2005). Approximately 40 - 60% patients who progressed to ESRD have diabetes (Snyder and Pendergraph, 2005). Both patients with type 1 and type 2 diabetes are prone to the development of DN (Ziyadeh *et al*., 2000; Hovind *et al*., 2004; Finne *et al*., 2005; Rossing *et al*., 2005; Jorsal *et al*., 2008; Ziyadeh and Wolf, 2008). Currently, major therapeutic interventions include blood

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glucose control, antihypertensive treatment and dietary protein restriction (Rossing *et al*., 2005). Despite the success in implementing antihypertensive treatment in patients with type 1 or type 2 diabetes and widespread use of angiotensin-converting enzyme inhibition, no tempering in the rising risk of diabetes-related ESRD has yet appeared in any age categories in the United States population (Chow *et al*., 2004; Jones *et al*., 2005). Furthermore, the development of newly onset diabetes mellitus that seems to be initiated by insulin resistance caused by certain antihypertensive regimens has emerged as a strong, independent predictor of all-cause mortality (Copley and Rosario, 2005). Therefore, it is important to identify new strategies and additional therapeutic targets for treating DN.

For more than 5,000 years, Chinese herbal medicine (CHM) has helped people live a longer and healthier life. It has been used clinically for over 2,500 years, playing a crucial role in traditional Chinese medicine (TCM). A substantial body of evidence suggests that some Chinese herbs possess a wide range of important pharmacological properties in retarding progressive chronic kidney diseases (CKD; Peng *et al*., 2005). Development of new strategies using CHM in the treatment of chronic glomerulonephritis (CG) and chronic renal failure (CRF) can be complementary for, and sometimes even have more advantages than the current therapies because of their less side effects and appropriateness for long-term usage. CHM can be used in conjunction with current therapies to enhance their therapeutic outcomes and reduce their side effects (Yang *et al*., 1998; Sun *et al*., 2000; Song *et al*., 2007; He *et al*., 2007). However, ancient TCM practitioners could not measure renal function using parameters such as urinary protein, serum creatinine (Scr) and blood urea nitrogen (BUN), and thus, the attempt to employ TCM to treat CG and CRF according to changes in renal function was not started until 1950s. In 1960s, there was a vigorous debate in Chinese medical fields on treatment of CRF using the concept of promoting circulation and removing blood stasis. After several decades of research, general agreements on the pathogenesis and principles to treat CG and CRF using TCM have been reached, which were to enhance immune defense to eliminate the invading pathogens; to strengthen the function of the Spleen and tonicize the function of the Kidney; and to promote circulation and remove blood stasis and dampness. Our previous studies (both animal and clinical) have demonstrated the efficacy of Huangdan decoction (HDD) or Huangdan capsule (HDC) in the treatment of CG and CRF. In determining the effect of HDD on membranous glomerulonephritis (MGN), the rat model of MGN was established using self-made cationic bovine serum albumin according to Furness' methods (Furness and Turner, 1987). At the 16th week of HDD treatment, decreases in proteinuria, Scr and BUN were apparent when compared to the controls (Zheng and Chen, 1993). HDD-treated groups also displayed less kidney damage as compared to the control group. In determining the effect of HDC on the progression of CRF, the 5/6 nephrectomized CRF rat model was employed (Zheng *et al*., 1997). Fourteen weeks after operation, the levels of BUN and Scr in the 5/6 nephrectomized rats were significantly higher than those in the sham controls. Treatment with HDC significantly attenuated these increases. Additionally, HDC treatment slowed the progression of CRF in the 5/6 nephrectomized rats possibly by increasing the antioxidant activities of superoxide dismutase in renal cortex and erythrocytes, and reducing the levels of lipid peroxidation, plasma cGMP and atrial natriuretic peptide. Furthermore, HDC delayed the damage of remnant kidneys in these rats. In clinical studies (Zheng *et al*., 1998), patients with CKD were randomly divided into two groups: HDD group (47 cases) and CAO (Coated aldehyde oxystarch) group (38 cases). CAO is commonly used in clinical practices to decrease the level of BUN (Feng *et al*., 1988). HDD treatment showed efficacy in 28 cases and improvement in 12 cases, and the overall rate

was 85.1%. CAO treatment showed efficacy in 14 cases and improvement in 8 cases, and the overall rate was 57.6%, which was significantly lower than HDD group.

HDD, which mainly consisted of five herbs, was prepared in such a way that it could strengthen the function of Spleen and tonicize the function of Kidney, promote circulation and eliminate stasis and dampness (Zheng and Chen, 1993). These five herbs were: Da Huang (Radix Et Rhizoma Rhei), Huang Qi (Radix Astragali Seu Hedysari), Dan Shen (Radix Salviae Miltiorrhizae), Yin Yang Huo (Herba Epimedii), Yi Yi Ren [Semen Coicis or Coix lacryma-jobi (Poaceae)]. Da Huang, which was bitter and cold, could activate viscera to eliminate wastes, remove dampness, dispel toxic substances, and remove blood stasis; it worked as the Chief or Principal (Jun) in the formula. Huang Qi, sweet and slightly warm, could invigorate Qi (or energy) to elevate the Spleen Yang, and induce diuresis to alleviate edema; it worked as the Adjuvant (Chen). Yin Yang Huo, which was pungent, sweet and warm, could invigorate Kidney and strengthen Yang, dispel the wind and resolve dampness; it invigorated Yang without causing damage to Yin, and worked as the Adjuvant (Chen). Dan Shen, bitter and slightly cold, removed blood stasis and promoted blood circulation; it worked as the Assistant (Zuo). Yi Yi Ren, sweet and slightly cold, could remove dampness, induce diuresis, and invigorate the Spleen; it worked as the Guide (Shi). Taken together, we have adopted the therapeutic principles by strengthening the host defense and eliminating the invading pathogens for treatment of CG and CRF.

The selection of these herbs could be also justified by their cellular and molecular mechanisms of action. Da Huang inhibited the synthesis of urea in liver and kidneys and decreased the levels of BUN and Scr by reestablishing the balance of amino acids (Wu, 1988). It could also increase the excretion of waste nitrogen through intestines (Chen *et al*., 1987), prevent the hyperactive metabolism of the remnant kidney in CRF patients and inhibit the proliferation of glomerular mesenchymal cells and renal tubular epithelial cells (Yang *et al*., 1993, 1994; Yu *et al*., 1995; Li, 1996). The effectiveness of Da Huang in CRF patients might also be due to its inhibition on proteinuria (Zhang and el Nahas, 1996), IL-6 production (Song *et al*., 2000) and fibronectin secretion (Wei *et al*., 1997), and its antimicrobial activity (Tegos *et al*., 2002). In addition, Da Huang could antagonize kidney-stimulating factor to restore lipid metabolism (Li, 1991), and it was effective in treating hypertension (Zhang *et al*., 1994; Wang and Song, 1999). The cholesterol-lowering effect of Da Huang might be attributed to its potent inhibitory activity on squalene epoxidase, a rate-limiting enzyme of cholesterol biogenesis (Abe *et al*., 2000), and its enhancing effects on the excretion of bile acids and the expression of cholesterol 7α-hydroxylase (Goel *et al*., 1997, 1999). The purified compounds from Da Huang, (-)-epicatechin 3-O-gallate and procyanidin B-2 3,3'-di-O-gallate, could decrease BUN and Scr in rats with adenine-induced renal failure (Yokozawa *et al*., 1991), and increase glomerular filtration rate, renal plasma flow and renal blood flow (Yokozawa *et al*., 1993). Interestingly, these gallate compounds could inhibit cholera toxin-induced activities including ADP-ribosylation, elongation of Chinese hamster ovary cells, and fluid accumulation in ileal loops (Oi *et al*., 2002). However, single use of Da Huang in CRF patients was discouraged because it required larger doses and induced side effects (Shi *et al*., 2001; Mantani *et al*., 2002a,b).

Huang Qi, traditionally used in combination with other Chinese herbs (Peng *et al*., 2005), contained numerous active components, including flavonoids, polysaccharides, triterpene glycosides (e.g., astragalosides I-VII), amino acids, and trace minerals. Huang Qi could improve renal function by decreasing BUN levels (Zhang *et al*., 1984; Yang *et al*., 1997; Zhao *et al*., 2000), proteinurea, renal fibrosis (Ding *et al*., 1998) and renal damage (Zhao and Li, 1983; Shi *et al*., 2002; Peng *et al*., 2005).

Huang Qi could retard the progression of CKD using mechanisms such as improving water and sodium retention (Ma *et al*., 1996; Wang *et al*., 2002) and modulating the immune responses (Chen *et al*., 2000; Cai *et al*., 2001; Jang *et al*., 2003; Peng *et al*., 2005), which were independent of the local renal angiotensin system (Yu *et al*., 2000; Wang *et al*., 2002; Min *et al*., 2003).

Yin Yang Huo had vasodilatory effects, and it was especially effective in treating hypertension in patients with kidney diseases (Wu, 1982; Zhao *et al*., 2007; Xu and Huang, 2007a,b). It warmed Yang and nourished Yin by increasing the expression glucocorticoid receptor (GR) on lymphocytes and GR's affinity for its ligands (Du *et al*., 2008). Yin Yang Huo treatment also reduced TNF-α production and inhibited NF-κB activity (Xie *et al*., 2006). Icariin ($C_{33}H_{40}O_{15}$), the major flavonoid glycoside in Yin Yang Huo, possessed antioxidative property (Zhao *et al*., 2007), and could enhance the immune responses (He *et al*., 1995; Kim *et al*., 2001). The breakdown of Ca^{2+} homeostasis in cells was an early and important indicator of cell injury, and the Ca^{2+} antagonists were being used to treat diseases of hypertension, renal failure, renal colic (Yang *et al*., 2006). There was evidence that Yin Yang Huo could prevent Ca^{2+} from entering cells, and facilitate efflux of intracellular Ca^{2+} by modulating the expression of calmodulin (Jiang *et al*., 2004; Yang *et al*., 2006).

Dan Shen could improve microcirculation and increase the glomerular filtration rate. Endothelial nitric oxide synthase in the vascular system has been shown to be a molecular target for Dan Shen in hypertension (Kim *et al*., 2007). There was evidence that Dan Shen could decrease cyclosporin A-induced expression of TGF-β1 and rennin, and the accumulation of matrix proteins in rat kidneys (Qiao *et al*., 2001). Dan Shen has been shown to decrease renal damage in glycerol-induced acute renal failure in rats (Jin *et al*., 1997). Magnesium lithospermate B isolated from Dan Shen could inhibit the accumulation of fibronectin in mesangial cell line (Jung *et al*., 2002) and decrease the blood pressure in rats with sodium-induced hypertension and renal failure, with the latter process being due in part to enhancement of the kallikrein-prostaglandin system (Yokozawa *et al*., 1992; Chung *et al*., 1995). An aqueous extract of Dan Shen could inhibit LPS-induced proliferation and endothelin production by mesangial cells (Xu *et al*., 2001). Dan Shen also exerted a protective effect against renal cell injury, and its effect might be due to its antioxidant action (Yokozawa and Chen, 2000; Jeong *et al*., 2001). In post-operative patients with obstructive jaundice, Dan Shen protected renal function by inhibiting the release of inflammatory mediators and improving blood dynamics (Peng *et al*., 2001).

Yi Yi Ren could induce diuresis and modulate immunity (Chen *et al*., 1987; Kim *et al*., 2004). The active ingredients from Yi Yi Ren (Kang-Lai-Te) could inhibit mesangial cell proliferation (Hu *et al*., 2005). There were evidences that Yi Yi Ren could suppress the production of IgE against injected antigen ovalbumin in a mouse model (Shyu *et al*. 1998) by shifting the immune balance from Th2 to Th1 (Hsu *et al*. 2003). On the other hand, ingestion of Yi Yi Ren could increase the number of cytotoxic T-lymphocytes (CD3+, CD56+) and natural killer (NK) cells (CD16+, CD57-) in humans (Hidaka et al., 1992). Using a NF-κB-dependent reporter assay, Woo *et al*. (2007) demonstrated a dose-dependant inhibition of NFκB signaling by Yi Yi Ren extract, which was associated with a reduced translocation of the Rel-A/p65 subunit of NF-κB to the nucleus. Additionally, Yi Yi Ren extract also inhibited the activity of protein kinase C, a major mediator of signal transduction and activator of NFκB (Woo *et al*., 2007). Yi Yi Ren also possessed anti-oxidant activity (Kuo *et al*., 2002; Huang *et al*., 2005) and inhibited nitric oxide and superoxide production (Seo *et al*., 2000). Yi Yi Ren could lower fibrinogen levels and reduce fibrinolytic activity in Wistar rats (Check and K'Ombut, 1995), which was important since increased fibrinogen levels have been associated with reduced kidney functions in

individuals with stage 3 to 4 CKD (Weiner *et al*., 2008).

It is also possible that HDD possesses the properties to retard the progression of DN. Glucose transporter-1 (GLUT1) is a membrane-embedded protein that mediates the uptake of glucose into the cells. TGF-β1 stimulates glucose uptake by enhancing the expression and function of GLUT1 in cultured mesangial cells, resulting in excessive glucose consumption and the production of extracellular matrix in DN (Zhang *et al*., 2000). Huang Qi has been shown to significantly reduce proteinuria in DN patients with massive macroalbuminuria (Chen *et al*., 1998; XY *et al*., 1999) and diabetic renal hypertrophy in experimental animals (Xu *et al*., 1997). Huang Qi can upregulate the expression of hepatocyte growth factor (HGF) that further inhibits glucose-induced overexpression of TGF-β1 in human kidney fibroblast cells (Peng *et al*., 2005). There are also substantial amount of evidences to demonstrate that Da Huang can retard the progression of DN (Dai *et al*., 1999; Guo *et al*., 2002a,b; Zhu *et al*., 2002). Da Huang can antagonize the effect of TGF-β1 in mesangial cells to reduce the expression of GLUT1 (Zhang *et al*., 1999). The purified compound emodin (3-methyl-1,6,8 trihydroxyanthraquinone) from Da Huang can decrease the gluconeogenesis of tubular cells (Li, 1996a) and inhibit postprandial hyperglycemia (Choi *et al*., 2005), and it can decrease glucose-induced matrix synthesis in human peritoneal mesothelial cells and cultured mesangial cells (Chan *et al*., 2003). Hexosamine biosynthesis pathway plays an important role in the development of insulin resistance. Da Huang inhibits the activity of fructose 6-phosphate aminotransferase, a rate-limiting enzyme in the hexosamine pathway, in mesangial cells. Yi Yi Ren can decrease the concentration of leptin (Huang *et al*., 2005) and the lipid components in plasma and feces in rats fed high fat and cholesterol diet (Kim *et al*., 2004), which are responsible for its anti-obesity effects (Kim *et al*., 2007a). Yi Yi Ren-containing formula has insulin-like action and insulin sensitizer property in type 2 diabetic models (Chang *et al*., 2006). Coixans A, B, and C isolated from Yi Yi Ren have hypoglycemic activity in rats (Takahashi *et al*., 1986). Supplementation with Yi Yi Ren has also decreased plasma lipids and inhibited LDL oxidation in hyperlipidemic individuals (Yu *et al*., 2004). It is likely that the combination of Huang Qi, Da Huang and other herbs in HDD may exert a greater protective effect on diabetic renal damage. It would be a great advantage if an herb formula that was designed to treat DN could also reduce the blood glucose level. This study reported here represented a first series of our attempts to address this issue. Mai Dong (Ophiopogon; Radix Ophio-pogonis) and its components have been used to reduce blood sugar (Chen *et al*., 1998; Wu *et al*., 2006); Shan Zhu Yu (Fructus Corni) could suppress hepatic gluconeogenesis-related gene transcription, enhance pancreatic β-cells glucose responsiveness, and prevent toxin-induced β-cell death (Chen *et al*., 2008). Therefore, in addition to HDD, a modified HDD (HDDM) that included additional herbs such as Mai Dong and Shan Zhu Yu has been tested in a multiple low doses (MLD) streptozotocin (STZ)-induced diabetic model. We used B6C3F1 mouse, a hybred of male C3H/HeN and female C57BL/6, in our studies because it was preferred over randomly bred mice in order to decrease the variation between individual animal's responses and reduce the number of animals for each experiment, and yet have the vigor associated with the heterozygosity. Furthermore, our data on several strains of mice (NOD, CD-1, C3H/HeN, C57BL/6 and B6C3F1) have suggested that MLD-STZ-induced diabetes in B6C3F1 mice was moderate, and this model had the ability to detect either protection or exacerbation of diabetes by the treatments.

MATERIALS AND METHODS

Animals

Female NOD and B6C3F1 mice (6 - 8 week old)

were purchased from Taconic Farms (Germantown, NY), and female CD-1, C3H/HeN and C57BL/6 mice (6 - 8 week old) from Charles River Laboratories. Animals were quarantined for at least a week prior to use. Mice were randomized by weight, ear-punched for identification, and transferred to plastic shoebox cages (3 - 4 animals per cage) for testing. The cages were bedded with hardwood sawdust and covered with a filter bonnet; mice were provided with tap water and fed certified Zeigler rodent chow ad libitum for the duration of the study. Room temperature was maintained at 22 - 24 °C, and the relative humidity between 40 and 70%. The light/dark cycle was maintained on 12 h intervals. Mice were determined to be free of hepatitis and Sendai virus by serology testing. All animal procedures were conducted under an animal protocol approved by the VCU Institutional Animal Care and Use Committee (IACUC).

Treatment with STZ

For STZ treatment, female mice (NOD, CD-1, B6C3F1, C3H/HeN and C57BL/6) were injected (i.p.) with STZ (Sigma-Aldrich) solutions, prepared immediately before use, in citrate buffer (pH 4.5). The dosing regimen for STZ consisted of five consecutive daily 50 mg/kg doses. The vehicle (VH) group received the same amount of 0.1 M, pH 4.5 citrate buffer (i.p.) for five consecutive days as detailed by the NIDDK Consortium for Animal Models of Diabetic Complications' (AMDCC) protocol (available from http://www.amdcc.org).

Herb preparation and dosing

The herb extracts in powder form were purchased from Crane Herb Company (Mashpee, MA). These herbs were mixed well and resuspended in distilled water and heated to dissolve, and administered at dose of 1000 mg/kg by gavage daily. The formulation of HDD has been reported previously (Zheng and Chen, 1993). For HDDM, two additional herbs Mai Dong and Shan Zhu Yu were included, and the percentages of both herbs were 22%.

Measurement of blood glucose levels

Monitoring of glycemic status was performed prior to the beginning of treatment to ensure that there were no significant differences between groups in blood glucose levels and no outlier animals. Thereafter, the animals were monitored for blood glucose changes every week for STZ-treated mice. Non-fasting blood glucose was measured directly in small samples of venous blood using Accu-Chek Diabetes monitoring kit (Roche Diagnostics, Indianapolis, IN). Mice with a serum blood glucose concentration above 250 mg/dl were considered diabetic and above 400 mg/dl were considered as severely diabetic as described by Li et al. (2001).

Glucose Tolerance Test (GTT)

For GTT, a fasting blood sample was taken from all groups of mice. Four more blood samples were collected at 30, 60, 90, and 120 min intervals after administration (i.p.) of a sterile glucose solution at the dose of 2 g/kg of body weight.

Cell isolation and determination of cell number

The quantification of splenocyte subsets was performed as previously described (Auttachoat *et al.*, 2007). Single cell suspensions of individual spleens were prepared by mashing the excised organs between the frosted ends of two microscope slides. After washing, cells were resuspended in RPMI complete medium and counted using a Coulter Counter ZII with the red blood cells lysed by a ZAP-O-GLOBIN II lytic reagent (Coulter Corporation, Miami, FL).

Flow cytometric analysis

Splenocytes and thymocytes were labeled with the appropriate monoclonal antibody (mAb), conjugated directly with a fluorescent molecule for visualization. Cells were dual stained with phycoerythrin (PE)-conjugated antibody and fluorescein isothiocyanate (FITC)-conjugated antibody or three-way stained using antibodies conjugated with FITC, PE and peridinin chlorophyll protein (PerCP). The antibodies

were purchased from BD PharMingen (San Jose, CA). The mAb used were anti-mouse Ly-6G (Gr-1) conjugated with FITC, anti-mouse IgM with FITC, anti-mouse CD8 with PE, anti-mouse CD4 with FITC or PerCP, anti-mouse CD3e with PerCP, anti-mouse NK1.1 with PE and anti-mouse Mac-3 with PE. The antibodies were diluted (1:80) in 50% fetal bovine serum-phosphate buffered saline (FBS-PBS). Isotype-matched irrelevant antibodies were used as controls. Following the addition of the reagents, the cells were incubated at 4 °C in the dark for at least 30 min. Thereafter, the cells were washed 2X, and enumeration performed on a Becton Dickinson FACScan Flow Cytometer in which log fluorescence intensity was read with a forward scatter threshold high enough to eliminate red blood cells. The data were analyzed using CELLQuest software. A minimum of 5,000 events was acquired.

Natural killer cell activity

The activity of NK cells was assayed as described (Reynolds and Herberman, 1981) with modification. Single cell suspensions were adjusted to four concentrations: 1×10^7, 5×10^6, 2.5×10^6 and 1.25×10^6 cells/ml. The target cells, YAC-1 cells, were labeled with ^{51}Cr and added to each well of a 96-well plate in a volume of 0.1 ml. The effector cells (0.1 ml) were added to each of two replicate wells of target cells at each effector concentration to obtain effector:target (E:T) ratios of 100:1, 50:1, 25:1, and 12.5:1. The spontaneous release and the maximum release were determined by adding 0.1 ml of medium and Triton X-100 (0.1%) to each of 12 replicate wells containing the target cells, respectively. Following 4 h incubation, the plates were centrifuged, and 0.1 ml of the supernatant was removed from each well and the radioactivity counted. The mean percentage of cytotoxicity at each E:T ratio was determined.

Anti-CD3 antibody-mediated spleen T cell proliferation

The proliferation of splenocytes in the presence of anti-CD3 antibody was performed as described (Guo *et al.*, 2001). Briefly, a single spleen cell suspension was prepared and resuspended in RPMI medium (Roswell Park Memorial Institute) supplemented with fetal bovine serum (FBS; 10%), sodium bicarbonate, HEPES, L-glutamine, gentamicin and 2-mercaptoethanol (0.00035%). The splenocytes (2×105/well) were cultured in the microtiter wells coated with anti-CD3 antibody (1 mg/ml; Phar Mingen) or in wells without antibody coating at 37 °C at 5% CO_2 and 95% humidity. Prior to harvest on day 3, the cells were pulsed with ^3H-thymidine for 18 - 24 h. The incorporation of ^3H-thymidine into the proliferating cells was used as the endpoint of the assay, and the data were expressed as CPM/2×10^5.

Statistics

The data were expressed as mean ± S.E. The results were tested for variance homogeneity using Bartlett's Test, and all the data reported here were homogeneous. Homogeneous data were analyzed using a one-way analysis of variance; when significant, Dunnett's *t* Test was used to determine differences between the experimental and vehicle control group. For diabetes incidence, the Fisher's Exact Test was used to determine if a group was statistically significant from the control group. A group was considered statistically significant from the control group if $P < 0.05$.

RESULTS

Induction of diabetes in female B6C3F1, C3H/HeN, C57BL/6, CD-1 and NOD mice by MLD-STZ

To determine the strain difference in MLD-STZ-induced diabetes, female mice of five strains were included: B6C3F1, C3H/HeN, C57BL/6, CD-1 and NOD. Among them, NOD and CD-1 were the most susceptible strains (Fig. 1). Treatment with MLD-STZ produced a marked increase in the incidence of diabetes in female NOD mice (Fig. 1A). Of eight NOD mice in the group, five mice

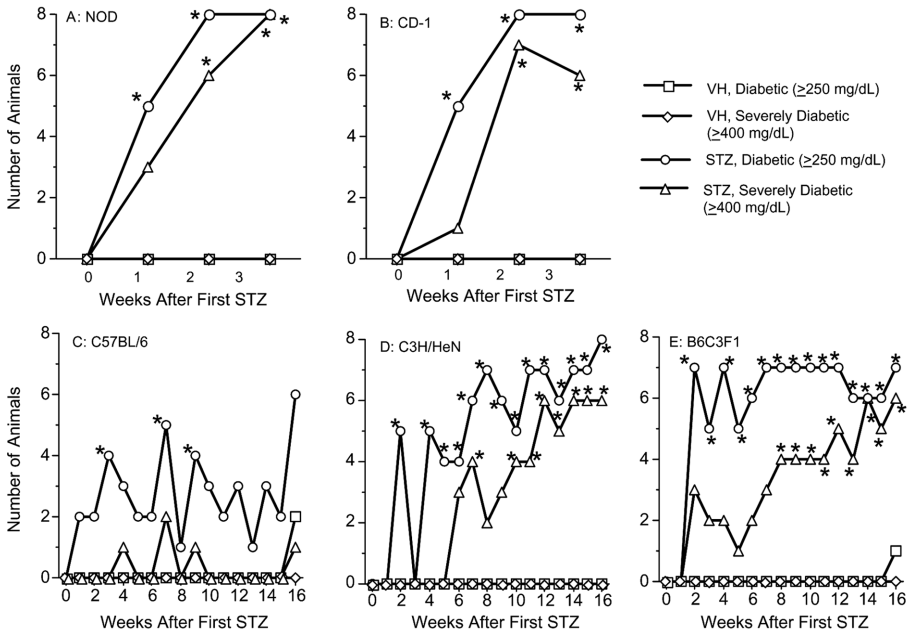

Fig. 1. Strain differences in MLD-STZ-induced diabetes. (A) Female NOD mice, (B) female CD-1 mice, (C) female C57BL/6 mice, (D) female C3H/HeN mice, and (E) female B6C3F1 mice. Animals were treated with MLD-STZ (50 mg/kg for five consecutive days); the development of diabetes monitored for 3 weeks for NOD and CD-1 mice, and 16 weeks for C57BL/6, C3H/HeN and B6C3F1 mice as described. *$P \leq 0.05$ when compared to vehicle (VH). N = 8.

developed diabetes at week 1 after the first STZ injection, and all mice developed diabetes at week 2 after the first STZ injection. Six of the eight NOD mice developed severe diabetes at week 2 after the first STZ injection, and all mice developed severe diabetes at week 3 after the first STZ injection. For CD-1 strain (Fig. 1B), five of the eight CD-1 mice developed diabetes at week 1 after the first STZ injection, and all mice developed diabetes at week 2 after the first STZ injection. Seven of the eight CD-1 mice developed severe diabetes at week 2 after the first STZ injection, and six of the eight mice developed severe diabetes at week 3 after the first STZ injection. On the other hand, C57BL/6 was the least susceptible strain (Fig. 1C): In the period of 16 weeks, the incidence of diabetes only reached the levels of statistical significance at three time points, e.g., week 3, 7 and 9 following the first STZ injection, when compared to the vehicle control; the incidence of severe diabetes was not significantly changed at any time points. The susceptibilities of C3H/HeN and B6C3F1 mice were identical (Fig. 1D and 1E): when compared to the control, the incidence of diabetes reached the levels of statistical significance for 14 and 15 times in the assessed 16 time points for C3H/HeN and B6C3F1 mice, respectively; the incidence of severe diabetes reached the levels of statistical significance for 8 and 9 times in the assessed 16 time points for, C3H/HeN and B6C3F1 mice, respectively.

MLD-STZ treatment produced decreases in the terminal body weights in mice of all these strains (Table 1). Increases in the weights of liver were observed in mice of both the C3H/HeN and B6C3F1 strains following the MLD-STZ treatment. MLD-STZ treatment also produced a decrease in spleen weight in NOD mice. No significant changes were observed for the weights of spleen, and kidneys in mice of any other strains (Table 1).

Table 1. MLD-STZ treatment on body weight and organ weights in female mice of various strains

Mouse Strains	Treatment	Body Weight (g)	Spleen (mg)	Kidneys (mg)	Livers (mg)
NOD	VH	24.1 ± 0.6	72.0 ± 2.3	ND	ND
	MLD-STZ	17.8 ± 0.6*	54.3 ± 2.6*	ND	ND
CD-1	VH	28.2 ± 0.5	107.0 ± 9.3	ND	ND
	MLD-STZ	24.1 ± 0.3*	113.1 ± 7.2	ND	ND
C57BL/6	VH	29.6 ± 1.3	97.3 ± 4.8	343.8 ± 18.6	1156.3 ± 75.3
	MLD-STZ	25.7 ± 0.8*	104.1 ± 4.1	301.1 ± 12.9	1282.9 ± 64.7
C3H/HeN	VH	35.2 ± 1.5	135.8 ± 14.2	437.8 ± 20.8	1477.1 ± 102.8
	MLD-STZ	27.2 ± 0.7*	129.9 ± 9.0	460.5 ± 20.6	1813.9 ± 63.6*
B6C3F1	VH	32.7 ± 2.1	80.0 ± 3.3	378.8 ± 13.2	1150.1 ± 29.2
	MLD-STZ	25.2 ± 0.5*	88.6 ± 3.4	394.9 ± 18.9	1370.5 ± 85.2*

Animals were sacrificed three weeks after the first STZ injection for NOD and CD-1 mice, and after 16 weeks for mice of other strains. ND = not determined. *$P \leq 0.05$ when compared to vehicle (VH). N = 8.

Immunomodulation in female B6C3F1, C3H/HeN, C57BL/6, CD-1 and NOD mice by MLD-STZ

MLD-STZ-induced diabetes is considered a disease of immune origin since it is associated with a secondary autoimmune insulitis following apoptotic injury of the pancreatic β-cells by STZ in contrast to diabetes induced by a single high dose of STZ (- 200 mg/kg) that causes a direct death of islet β-cells (Like and Rossini, 1976; Ablamunits *et al*., 1999). Three assays were conducted to determine if MLD-STZ treatment would affect the immune response in mice of these strains: anti-CD3 antibody-mediated splenocyte proliferation, NK cell activity assay and flow cytometric analysis of differential splenocytes. Increases in both the basal and anti-CD3 antibody-mediated splenocyte proliferation

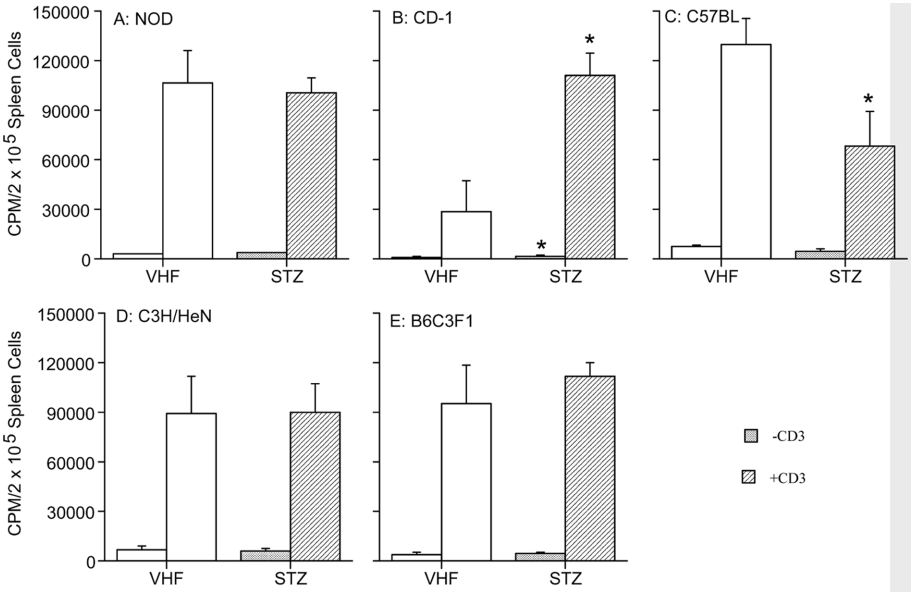

Fig. 2. Modulation of anti-CD3 antibody-mediated splenocyte proliferation in female NOD (A), CD-1 (B), C57BL/6 (C), C3H/HeN (D) and B6C3F1 (E) mice following MLD-STZ treatment. Animals were sacrificed three weeks after the first STZ injection for NOD and CD-1 mice, and 16 weeks for mice of other strains; splenocyte proliferation was determined in the presence or absence of anti-CD3 antibodies as described. *$P \leq 0.05$ when compared to vehicle (VH). N = 8.

were observed in CD-1 mice following MLD-STZ treatment (Fig. 2B). On the other hand, a decrease in anti-CD3 antibody-mediated splenocyte proliferation, but not the basal splenocyte proliferation, was observed in the C57BL/6 mice following MLD-STZ treatment (Fig. 2C). No significant changes were produced in either the basal or anti-CD3 antibody-mediated splenocyte proliferations in mice of the other three strains: NOD, C3H/HeN and B6C3F1 (Figs. 2A, D and E).

Treatment with MLD-STZ did not affect the NK cell activity in mice of these strains except that there were significant decreases in the C57BL/6 mice (Fig. 3) with statistically significant changes observed at the E:T ratios of 50:1, 25:1 and 12.5:1. MLD-STZ treatment did not affect the percentages of splenic NK ($NK1.1^+CD3^-$) cells in any of the strains (data not shown).

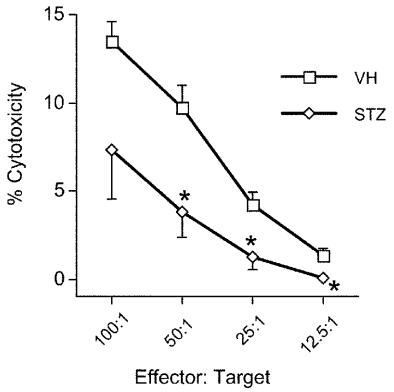

Fig. 3. MLD-STZ treatment decreased splenic NK cell activity in female C57BL/6 mice. Animals were treated with MLD-STZ, and the NK cell activity determined using YAC-1 cells as the target as described. *$P \leq 0.05$ when compared to vehicle (VH). N = 8.

Flow cytometric analysis of spenocytes did not reveal any changes in the percentages of IgM^+ $CD3^-$ B cells, $CD4^-CD8^+$ T cells, $CD4^+CD8^-$ T cells, and neutrophils ($Gr\text{-}1^+MAC3^-$) in mice of CD-1, C3H/HeN, C57BL/6 and B6C3F1 strains following MLD-STZ treatment except that an increase in the neutrophils was observed in the B6C3F1 mice (Table 2). In NOD mice, MLD-STZ treatment induced decreases in the percentages of both IgM^+CD3^- B cells and $Gr\text{-}1^+Mac\text{-}3^-$ neutrophils, and increases in the percentages of both $CD4^+$ $CD8^-$ and $CD4^-CD8^+$ T cells (Table 2). MLD-STZ treatment did not affect the total number of white blood cells in the spleen (data not shown). When the thymocytes were evaluated in NOD mice, a decrease in the percentages of $CD4^+CD8^+$ thymocytes while an increase in the percentages of $CD4^-CD8^-$ thymocytes was observed following MLD-STZ treatment (Table 3), suggesting that MLD-STZ treatment might affect T-cell development. A decrease

Table 2. MLD-STZ treatment on the percentages of splenocyte differentials in female mice of various strains

Mouse strains	Treatment	IgM^+CD3^-	$CD4^-CD8^+$	$CD4^+CD8^-$	$Gr\text{-}1^+MAC3^-$
		(%)			
NOD	VH	53.83 ± 2.90	4.91 ± 0.78	16.6 ± 2.1	4.09 ± 1.00
	MLD-STZ	39.27 ± 1.31*	8.76 ± 0.41*	24.13 ± 0.61*	1.30 ± 0.24*
CD-1	VH	50.26 ± 1.56	5.12 ± 0.61	15.43 ± 1.71	4.92 ± 1.53
	MLD-STZ	52.26 ± 1.46	4.04 ± 0.45	14.31 ± 1.59	3.15 ± 0.71
C3H/HeN	VH	41.04 ± 4.38	2.45 ± 0.42	8.66 ± 1.16	7.55 ± 0.66
	MLD-STZ	41.39 ± 1.91	3.41 ± 0.49	9.32 ± 0.95	7.05 ± 1.08
C57BL/6	VH	43.89 ± 1.33	4.30 ± 0.18	14.72 ± 0.70	1.40 ± 0.11
	MLD-STZ	39.29 ± 3.67	4.07 ± 0.40	14.12 ± 0.44	1.55 ± 0.23
B6C3F1	VH	45.67 ± 4.40	5.33 ± 0.54	14.44 ± 0.87	1.62 ± 0.11
	MLD-STZ	49.44 ± 1.18	5.33 ± 0.24	15.60 ± 0.67	2.20 ± 0.14*

Animals were sacrificed three weeks after the first STZ injection for NOD and CD-1 mice, and after 16 weeks for mice of other strains; flow cytometric analysis was performed as described. *$P \leq 0.05$ when compared to vehicle (VH). N = 8.

Table 3. MLD-STZ treatment on thymus and thymocytes in female NOD and CD-1 mice

Mouse strains	Treatment	Thymus Weight (mg)	$CD4^+CD8^-$	$CD4^-CD8^+$	$CD4^+CD8^+$	$CD4^-CD8^-$
			(%)			
NOD	VH	54.4 ± 2.6	16.29 ± 0.52	5.21 ± 0.57	63.24 ± 5.12	15.27 ± 5.44
	MLD-STZ	36.4 ± 3.4*	15.79 ± 1.67	5.57 ± 0.45	37.75 ± 2.11*	40.90 ± 2.29*
CD-1	VH	46.3 ± 2.9	16.59 ± 0.92	5.11 ± 0.55	71.55 ± 1.19	6.75 ± 0.55
	MLD-STZ	41.1 ± 2.2	18.25 ± 1.48	4.63 ± 0.53	69.63 ± 2.84	7.50 ± 0.98

Animals were sacrificed three weeks after the first STZ injection, and flow cytometric analysis of thymocytes performed as described. *$P \leq 0.05$ when compared to vehicle (VH). N = 8.

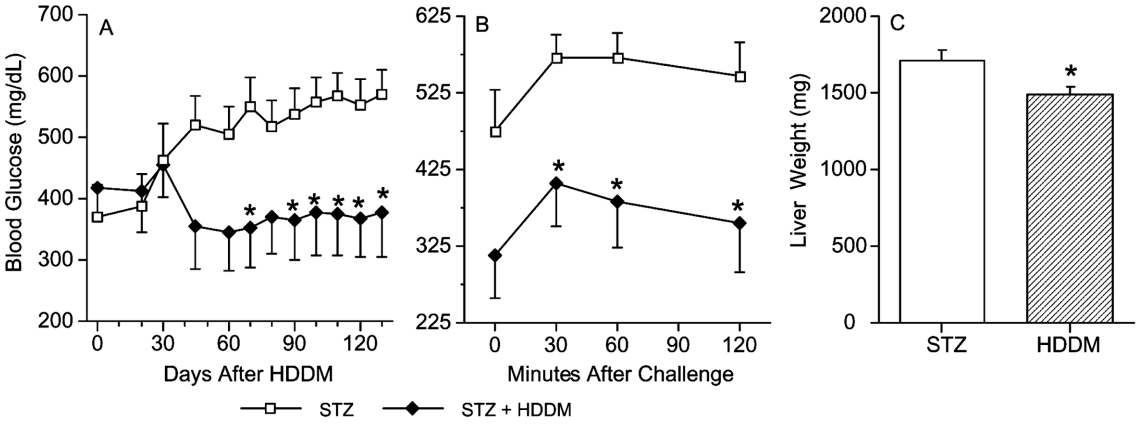

Fig. 4. HDDM reduced blood glucose levels (A), improved glucose tolerance (B), and decreased liver weight (C) in MLD-STZ-treated female B6C3F1 mice. One month after diabetes induction by MLD-STZ, female B6C3F1 mice were dosed with HDDM daily by gavage, and various parameters determined as described. *$P \leq 0.05$ when compared to the control. N = 8-11.

in the thymus weight was also observed in MLD-STZ-treated female NOD mice (Table 3). However, MLD-STZ treatment did not affect the percentages of thymocytes in CD-1 mice.

Anti-diabetic effect of HDD and HDDM in female B6C3F1 treated with MLD-STZ

Above all, the B6C3F1 mice treated with MLD-STZ had moderate diabetes, which was the ideal model to determine the modulatory effect of herb formula on the development of diabetes. One month after diabetes induction by MLD-STZ in female B6C3F1 mice, animals were dosed with HDD or HDDM by daily gavage as described. Treatment with HDD had no effect on the blood glucose levels (data not shown); however, HDDM treatment decreased the blood glucose levels starting at 45 days after the dosing with statistically significant changes observed after 70 days of treatment when compared to the MLD-STZ treatment (Fig. 4A). GTT also suggested that HDDM treatment improved glucose tolerance in these mice (Fig. 4B). In addition, HDDM treatment decreased the liver weight when compared to the MLD-STZ treatment control (Fig. 4C). No significant changes in the body weight (either during the study or at the termination) and kidney weight were produced by HDDM treatment (data not shown).

HDDM had no effect on the immune responses in female B6C3F1 treated with MLD-STZ

As demonstrated above, MLD-STZ treatment had no substantial effects on the immune response in female B6C3F1 mice. Therefore, we determined the immunomodulatory effect of HDDM in MLD-STZ-

treated animals, which was important because of the reported effects of these herbs on the immune system. Following immune parameters were measured: spleen weight, total number of splenocytes, NK activity, anti-CD3 antibody-mediated spenocyte proliferation, the percentages of splenic neutrophils, NK cells, $CD3^+$ T cells and B cells (IgM^+). Treatment with MLD-STZ had no effects on these parameters (data not shown).

DISCUSSION

Among various strains of mice, different susceptibility to STZ-induced diabetes has been reported, indicating that genetic background is playing an important role in this model (Babaya *et al*., 2005). In this study, we compared MLD-STZ-induced diabetes in female mice of various strains, including NOD, CD-1, C3H/HeN, C57BL/6 and B6C3F1. The order of susceptibility to diabetes induction by MLD-STZ treatment was NOD CD-1 > B6C3F1 C3H > C57BL/6. This was surprising since it had been shown that C3H/HeN mice were insensitive, while C57BL/6 mice were sensitive, to diabetes induction by STZ (Kaku *et al*., 1989; Gonzalez *et al*., 2003). One possible reason was that the mice used in our studies were female since there were sex differences in STZ-induced diabetes; for example, female mice were less susceptible than male mice to STZ-induced hyperglycemia in general (Leiter, 1982; Gurley *et al*., 2006). B6C3F1 mouse is a hybred of male C3H/HeN and female C57BL/6 mice, and further study is needed to determine if the susceptibility to MLD-STZ-induced diabetes in female B6C3F1 mice is inherited from male C3H/HeN mice.

Paik *et al*. (1980) have shown that thymus-dependent functions played an important role in the MLD-STZ-induced diabetes. Nude mice developed milder diabetes than the thymus-intact mice following MLD-STZ treatment (Buschard, 1985). In our studies, MLD-STZ treatment induced an increase in the percentage of $CD4^-CD8^-$ thymocytes and a decrease of the $CD4^+CD8^+$ thymocytes in female NOD mice. On the other hand, increased percentages of splenic $CD4^+$ and $CD8^+$ T cells in these mice were produced following the MLD-STZ treatment. These observations suggested that some immature $CD4^-CD8^-$ T cells exited the thymus and matured without further positive and negative selection. MLD-STZ treatment also enhanced T cell response in female CD-1 mice, which exhibited high STZ susceptibility. In contrast, MLD-STZ treatment decreased T cell response in female C57BL/6 mice, which exhibited the least STZ susceptibility. Thus, changes in T cell function might be partially responsible for diabetes induction following MLD-STZ treatment, which was consistent with the reports that cyclophosphamide could suppress streptozotocin-induced diabetes in mice (Yanagawa *et al*., 1989) and anti-β cell cytotoxic autoimmune response was involved in the diabetes induction (McEvoy *et al*., 1987). The activity of myeloperoxidase was found to be 3-fold higher in the pancreas following MLD-STZ treatment indicative of an invasion of the pancreatic tissue by polymorphonuclear leukocytes in BALB/c strain (Mabley *et al*., 2003). However, the contributions of neutrophils to the induction of diabetes by MLD-STZ treatment are currently unclear because MLD-STZ treatment induced an increase of splenic neutrophils in B6C3F1 mice while a decrease in female NOD.

MLD-STZ-induced diabetes in the B6C3F1 mice has been shown be a useful model for the evaluating diabetes exacerbation or attenuation in our studies. Hepatomegaly has been observed in STZ-induced experimental diabetes, which may be due to early hyperplasia, and later decreased apoptosis in liver (Herrman *et al*., 1999). In our studies, MLD-STZ treatment also induced an increase in the liver weight in both female B6C3F1 and C3H/HeN mice. Interestingly, the increase of liver weight in MLD-STZ-treated B6C3F1 mice was attenuated following HDDM treatment, which was consistent with reduced blood glucose levels in these mice. The anti-diabetic effect of HDDM was further supported by the findings that HDDM treatment

could also decrease blood glucose levels in the db/db diabetic mice and improve their glucose tolerance (data not shown). In STZ-induced diabetic rats, Yeh *et al*. (2006) has demonstrated that Yi Yi Ren can regulate lipid and glucose metabolisms. However, Yi Yi Ren-containing HDD had no effect on blood glucose level in MLD-STZ-induced diabetes in B6C3F1 mice. Thus, it was likely that Mai Dong and Shan Zhu Yu were responsible for the anti-diabetic effect of HDDM. Mai Dong and its components have blood glucose lowering activity (Chen *et al*., 1998; Wu *et al*., 2006). Oleanolic acid, one of the active principles of cornus fruit can increase the release of ACh from nerve terminals, which in turn to stimulate muscarinic M3 receptors in the pancreatic cells and augment the insulin release (Hsu *et al*., 2006). In addition, Shan Zhu Yu could also increase renal function, and consequently ameliorate glycation-associated renal damage in DN (Xu and Hao, 2004; Yamabe *et al*., 2007).

In addition to type 1 diabetes, type 2 diabetes was also suggested to be a manifestation of the inflammatory host response (Trabattoni *et al*., 2006). Furthermore, some herbs (e.g., Panax ginseng) possess both anti-diabetic effect and immune modulatory effects (Cho *et al*., 2006). Although several herbs (e.g., Huang Qi, Yi Yi Ren, Yin Yang Huo, Dan Shen) in HDDM have immune modulatory effects, we did not observe significant changes in all the immune parameters measured. In future studies, it will be interesting to examine tissue-specific immune responses (e.g., pancreas, kidneys) to determine if immunomodulation is contributing to the anti-diabetic effect of HDDM.

In summary, the studies reported here have suggested that MLD-STZ-induced diabetes in female B6C3F1 mice is a useful model to evaluate drug modulation of diabetes, and that the herb formula HDDM possesses anti-diabetic effects. Future studies in DN patients will lend further support that HDDM can be used to treat renal damage in DN as well as reducing blood glucose levels.

ACKNOWLEDGEMENT

This work was supported in part by the NIEHS Contract ES 55387.

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