DOI 10.1002/mnfr.200500252 Mol. Nutr. Food Res. 2006, *50*, 732–738

Effect of a *Siraitia grosvenori* extract containing mogrosides on the cellular immune system of type 1 diabetes mellitus mice

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The purpose of this study was to observe the islet changes of pancreas in insulin-dependent diabetes mellitus (IDDM) mice in comparison to normal mice after application of an extract from *Siraitia grosvenori* fruits containing mogrosides, in particular, mogroside V. We hypothesized that mogroside extract (MG) attenuates the severity of alloxan-induced IDDM by effects on the immune system. Our data show that IDDM mice exhibited significant injury to pancreatic islets cells, which were atrophic. In addition, alloxan induced a notable increase in the expression of $CD8^+$ lymphocytes to form a dramatic decrease in $CD4^+/CD8^+$ ratio (while $CD4^+$ was unchanged). MG, administered to normal and experimental diabetic mice for 4 wk, effectively attenuated the early clinical symptoms, biochemical abnormalities, and pathological damages in pancreatic islets. Furthermore, at low dose, MG regulated the immune imbalance observed in alloxan-induced IDDM mice by up-regulating the $CD4^+$ T-lymphocyte subsets and CD4/CD8 ratio, and altering the intracellular cytokine profiles. The expression of the pro-inflammatory Th1 cytokines: IFN-γ, TNF-α in splenic lymphocytes was altered toward a beneficial Th2 pattern. MG therapy had no effect on normal mice, except that low dosage MG could up-regulate the IL-4 expression levels. The results revealed that MG exhibited antidiabetic effects presumably due to the presence of mogrosides.

Keywords: Cellular immune functions / Fruits of *Siraitia grosvenori* / Mogrosides / Type 1 diabetes mellitus

Received: December 2, 2005; revised: April 8, 2006; accepted: April 10, 2006

1 Introduction

Insulin-dependent diabetes mellitus (IDDM) is a chronic autoimmune disease characterized by islet β-cells adaptive destruction, which is mediated by T cells and induced by environmental factors [1].The proportional imbalance of T-helper cells Th1, Th2, and their cytokine productions plays a key role in the injury of islet β-cells and the incidence of IDDM. In order to prevent or alleviate the early stages IDDM, the use of immune interventions to prevent the islet autoimmune process has found increasing research interest. As some immune therapies, such as using cyclosporine and azathioprine, show serious side effects [2], dietary treatment with natural hypoglycemic products has become popular to prevent diabetes. Fruits of *Siraitia grosvenori Swingle*, a traditional medical plant with low caloric value, can serve as a sugar substitute for the obese and diabetic patients. *S. grosvenori* is used for many diseases such as high blood pressure, tuberculosis, and asthma *etc.* [3–5]. Triterpene glycosides, the so-called mogrosides (Fig. 1), are the main effective compounds of *S. grosvenori Swingle* [6, 7]. Recent research shows that mogrosides have many beneficial functions and effects on diabetes [8], cancer, and inflammation *etc.* [9, 10]. However, only few reports are available regarding the mechanisms of its antidiabetic effects [11]. In this paper, we compared the effects of an extract of *S. grosvenori* containing mogrosides (MG) on the splenic lymphocyte and cytokines expression levels between normal mice and diabetic mice induced by alloxan. The mechanisms of antidiabetic effects of MG were also investigated.

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Abbreviations: FCM, flow cytometer; **FITC,** fluoroisothiocyanate; **IDDM,** insulin-dependent diabetes mellitus; **MG,** mogroside extract; **PE,** phycoerytherin

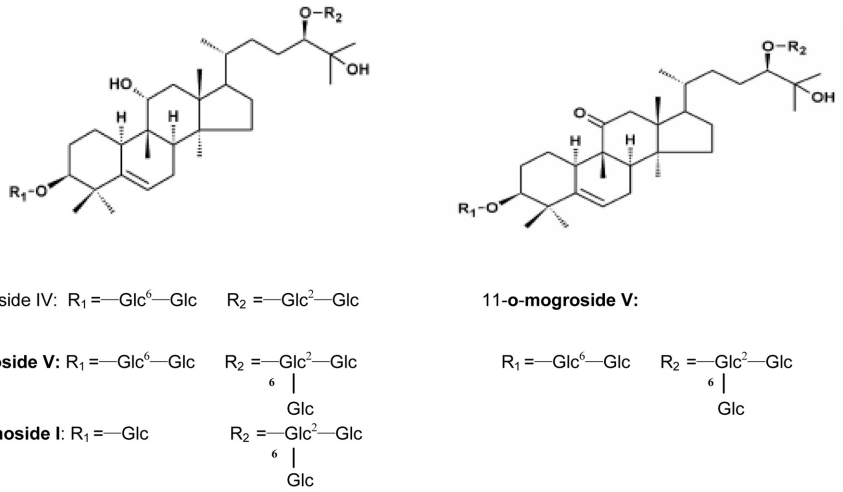

Figure 1. Structures of main mogrosides isolated from the fruits of *S. grosvenori.*

2 Material and methods

2.1 Chemicals

Alloxan, polyformaldehyde, Triton-X-100, and bovine serum albumin were purchased from Sigma Chemical (St. Louis, MO); phycoerytherin (PE) antimouse CD8a and IL-4 McAb, fluoroisothiocyanate (FITC) antimouse CD4, TNF-α and IFN-γ McAb (eBioscience Pharmingen); mogroside V, 11-*o*-mogroside V, mogroside IV, and siamenoside I were isolated from the fruits of *S. grosvenori* according to the reported method and identified with authentic samples [9].

2.2 Plant materials

Fruits of *S. grosvenori* were obtained from Laiyin Natural Ingredients (Guilin, China), and authenticated by Professor X. L., Chen at Hubei College of Traditional Chinese Medicine (Wuhan, China).

2.3 Animals

Male Balb/c mice with a body weight of 18∼20 g were obtained from Sipprai-bikai laboratory animal cooperation in Shanghai, China (the number of animals qualified:155). Animals were housed-in an animal facility at room temperature of (25 – 30°C) and at 45 – 55% relative humidity. The study was approved by the Animal Ethical Committee of the Institute.

2.4 Preparation of the plant extract

Fresh fruits of S. *grosvenori* (500 g) supplied by Laiyin Natural Ingredients, were ground and extracted at 80°C with 70% aqueous ethanol (3 × 2000 mL). The combined extract was concentrated on a rotatory evaporator at 40°C under low pressure, and the aqueous residue was concentrated and adjusted at a pH value to 9.0. With chromatographic separation, the concentrated extract was put on a D_{101} macroporous resin (Naikai University Chemical, Najing, China) column (2 × 80 cm^2 id) using pH 9.0 sodium hydroxide aqueous solution, distilled water, 30% aqueous ethanol, and 60% aqueous ethanol as eluting solvent successively. The column was eluted with 3.0 L of each solvent mixture. Fractions eluted with 60% ethanol (MG) were collected, concentrated at 40°C by a rotary evaporator (Heidolph, Germany), and freeze-dried to obtain 2.5 g of solid.

2.5 Induction of experimental diabetes

Male Balb/c mice were kept in-house for 1 wk before the initiation of the experiment. After 18 h fasting, diabetes was induced by a single intraperitoneal injection of alloxan, freshly prepared with sterile saline solution at a dose of 200 mg/kg. The same amount of saline solution was injected to control mice. The diabetic mice were randomized into three groups after confirming that blood glucose levels were more than 10 mmol/L.

2.6 Experimental design

After the confirmation of IDDM, the nondiabetic and diabetic mice were further subdivided into three subgroups. The first subgroup of nondiabetic mice were used as normal control (C) and received distilled water, the second and third subgroups received MG at low dose of 150 mg/kg (C-LMG) and high dose of 300 mg/kg (C-HMG), respectively. The IDDM group (n = 30) was divided into another three

more subgroups, the first subgroup was used as a diabetic control (D) and received distilled water, while the second and third received MG orally at lower dosage of 150 mg/kg (D-LMG) and higher dosage of 300 mg/kg (D-HMG), respectively. The animals received the above treatments by daily gavage over 4 wk. During the experiment, daily water intake was recorded, weekly body weight was measured. At the end of experiment, mice were killed. Blood samples were taken from the eyepit, and then centrifugated at 3500 rpm, 4°C for 15 min. The blood serum samples were kept at −20°C. At the same time, spleen tissues were taken to prepare spleen cells suspension.

2.7 Analysis of blood glucose

Blood glucose was measured by using the glucose oxidase method, following the protocol of the blood glucose reagent kit (Zhongsheng Beikong Biotechnology, Beijing, China).

2.8 Determination of T-lymphocyte subsets of mice spleen tissue

Fresh spleen tissue was placed into Petri dishes containing Hank's solution, and milled into unicellular suspension. The cell concentration was adjusted to 1×10^7/mL after lysing red blood cell. One μL of FITC antimice CD4 McAb and 2.5 μL of PE antimice CD8a McAb were added to 100 μL of splenocyte suspension. The mixture was vortexed well at room temperature and protected from light for 30 min. The mixture was washed twice with Hank's solution and centrifuged at 1000 rpm/min for 10 min each time. Finally, the mixture was resuspended to 500 μl with Hank's solution. The lymphocytes subpopulations CD4 and CD8 of splenocytes were detected by a flow cytometer (FCM).

2.9 Cytokine expression of the mice spleen lymphocyte

Intraprep Permeabilization Reagent (containing 0.1% Triton-X-100 and 0.3% BSA) was added to the splenocyte suspension and mixed at room temperature for 10 min. The mixture was washed twice with Hank's solution, followed by addition of 5 μL of PE antimice IL-4 McAb, 20 μL of FITC antimice TNF-α, and 20 μL of IFN-γ McAb *per* 100 μL of splenocyte suspension. Reaction was allowed to proceed for 30 min in the dark. To terminate the reaction, the mixture was washed twice with Hank's solution and cytokines' expression in lymphocytes (IFN-γ, TNF-α, and IL-4) were determined by using the FCM.

2.10 Histopathological examinations

At the end of experiment, mice were killed by cervical dislocation, tail of pancreas was rapidly soaked in 10% formaldehyde solution to fix for 12 h. After dehydration by ethanol, brightened by dimethylbenzene and embedded in paraffin, the samples were sliced into 5 μm thick pieces and set on silicon-coated glass slides. Then the samples were deparaffined and stained with Hematoxylin-Eosin using standard methods. The histopathological analysis of pancreatic islets was observed with light microscope.

2.11 HPLC analysis of mogrosides

The HPLC analysis was performed on a Varian chromatography instrument (Varian, USA) equipped with a prostar 335 photodiode array detector, a prostar 410 autosampler, a prostar dynamax system, and an LC workstation version 6.30 using an RP column (Phenomenex ODS C_{18}, 250×4.6 mm^2 id, 5 μm) with column temperature set at 30°C.The mobile phase of 23% aqueous ACN (v/v) was used with a flow-rate of 0.5 mL/min. A 10 μL sample was injected and the results were determined by UV absorption at 210 nm.

2.12 Statistical calculations

Statistical analysis was performed using the SPSS 11.5 statistical software package. Data were determined as $\overline{x} \pm s$ for all experiments. Differences among the control, diabetic, and treated groups were assessed by ANOVA.

3 Results

3.1 Water intake, body weight, and blood glucose

The diabetic mice exhibited hyperglycemic phenomenon in the form of polydipsia (their daily water intake was about three times as many as the normal mice, $P < 0.05$), polyphagia, polyuria (urine sugar+++ ∼ ++++), and body weight loss. At the end of the experiment, the body weights of IDDM mice were less than that of the normal mice, but the differences did not reach statistical significance ($P > 0.05$). The glucose concentration of diabetic mice increased significantly, about 6.7 times over the normal group ($P > 0.05$) (Table 1). After 4 wk treatment, water intake and blood glucose concentration of the diabetic mice groups with different doses of MG decreased toward baseline ($P < 0.01$, $P < 0.05$, respectively), but remained higher than nondiabetic control mice. MG, at all doses used, did not alter any of the parameters measured in normal mice ($P > 0.05$) (Table 1).

3.2 The detection of spleen lymphocyte subsets (CD4, CD8 positive lymphocytes)

The results are shown in Table 2 and Fig. 2. Compared to the normal group, injection of alloxan induced an increase

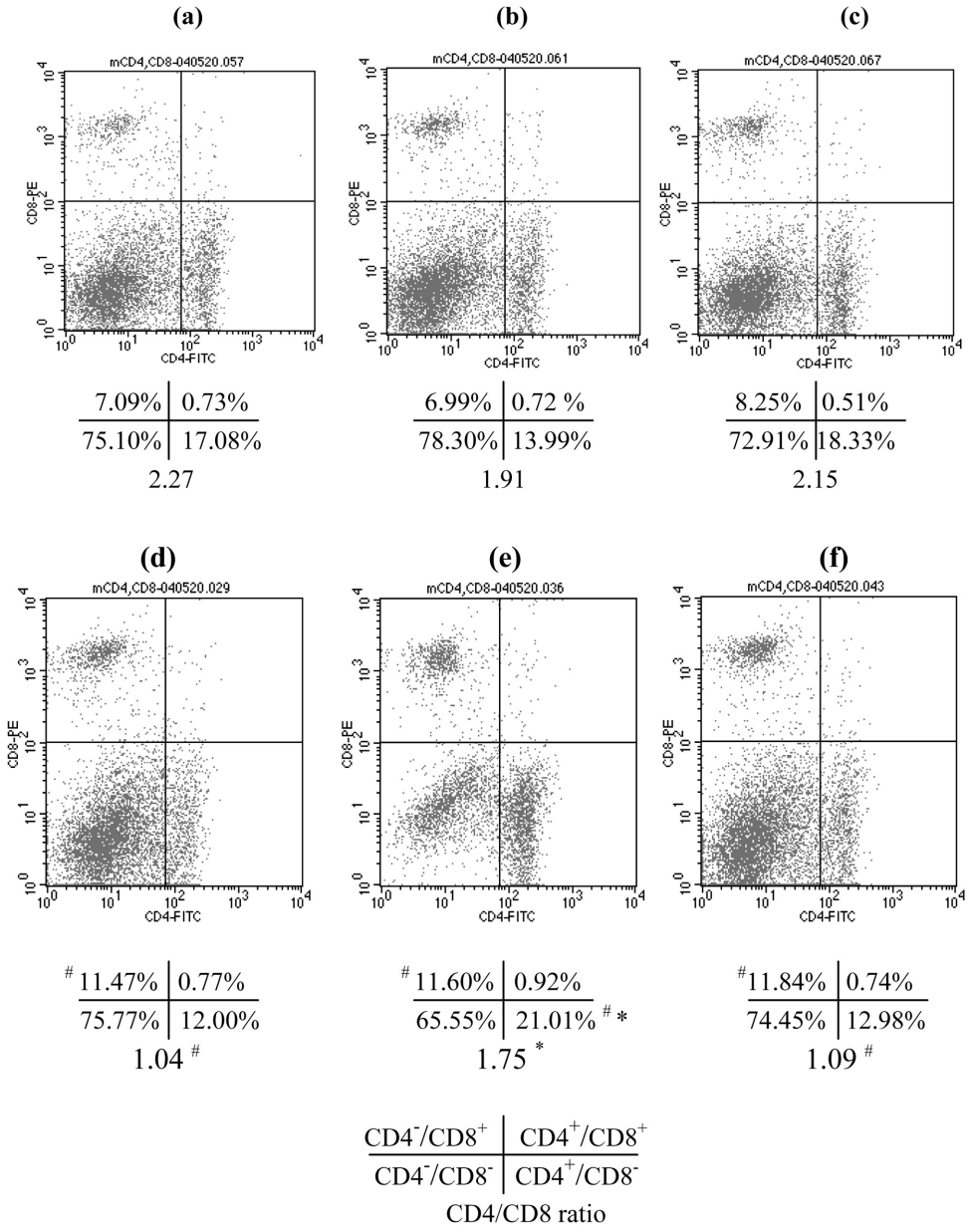

Figure 2. Representative two-color dot-plots of splenocyte subpopulation expression analyzed by FCM among all the experimental groups. (a) Control group, (b) C-LMG group, (c) C-HMG group, (d) diabetic group, (e) D-LMG group, and (f) D-HMG group. The percentage occurrence of $CD4^+$ T cells (lower right and upper right column) and $CD8^+$ T cells (upper left and upper right column) were calculated, and the CD4/CD8 balance was evaluated as the ratio of the percentage of $CD4^+$ T cells to the percentage of $CD8^+$ T cells. $^{\#}P < 0.05$ as compared with control group. $^{*}P < 0.05$ as compared with diabetic group. The superscripts ($^{\#}$ or *) located at the lower right corner of the cross represent the difference in the percentage of $CD4^+$ T cells, superscripts situated at the upper left corner of the cross denoted the difference in the percentage of $CD8^+$ T cells, while superscripts at the bottom of the cross indicated the CD4/CD8 ratio difference among all the experimental groups.

in splenic $CD8^+$ lymphocytes ($P < 0.05$), but not $CD4^+$ lymphocytes ($P > 0.05$). As a result, $CD4^+/CD8^+$ ratio was decreased ($P < 0.05$). Compared to the diabetic group, treatment with low dosage of MG raised the proportion of $CD4^+$ lymphocyte subsets of IDDM mice ($P < 0.05$) and had no influence on $CD8^+$ lymphocyte subsets. Therefore, the low dosage of MG altered the $CD4^+/CD8^+$ ratio toward normal values. However, no significant differences in the above

Table 1. Body weight, water intake, and blood glucose of experimental mice ($\bar{x} \pm s$)

Parameters	*n*	Body weight (g)	Water intake daily (mL)	Glucose (mmol/L)
C	10	21.84 ± 2.39	26.7 ± 3.35	3.29 ± 0.33
C-LMG	10	21.71 ± 2.83	26.8 ± 5.43[b)]	3.29 ± 0.49[b)]
C-HMG	10	22.29 ± 2.85	22.7 ± 3.12[b)]	4.37 ± 0.39[b)]
D	10	18.61 ± 2.34	79.8 ± 8.53[a)]	21.93 ± 1.50[a)]
D-LMG	10	18.76 ± 2.04	48.5 ± .11[a, b)]	10.51 ± 1.89[a, b)]
D-HMG	10	19.30 ± 3.12	64.3 ± 4.65[a, b)]	12.72 ± 1.45[a, b)]

a) $P < 0.05$ as compared with control group.
b) $P < 0.05$ as compared with diabetic group.

Table 2. Proportion of T-lymphocyte subsets in spleen ($\bar{x} \pm s$, $n = 6$)

Group	Spleen T-lymphocytes subsets (%)		
	$CD4^+$	$CD8^+$	$CD4^+/CD8^+$
C	15.16 ± 2.74	7.07 ± 1.06	2.13 ± 0.21
C-LMG	15.00 ± 1.86	7.88 ± 0.57	1.90 ± 0.14
C-HMG	16.58 ± 2.27	8.10 ± 1.88	2.10 ± 0.40
D	14.84 ± 2.64	12.22 ± 1.62[a)]	1.21 ± 0.11[a)]
D-LMG	22.81 ± 0.82[a, b)]	13.45 ± 0.45[a)]	1.74 ± 0.04
D-HMG	14.62 ± 3.12	11.93 ± 3.12[a)]	1.28 ± 0.34[a)]

a) $P < 0.05$ *vs.* control group.
b) $P < 0.05$ *vs.* diabetic group.

Table 3. Intracellular cytokines production by spleen lymphocytes detected by FCM ($\bar{x} \pm s$, $n = 3$)

Group	Splenic cytokine profile production (%)		
	IFN-γ	TNF-α	IL-4
C	0.93 ± 0.05	0.83 ± 0.25	0.73 ± 0.16
C-LMG	0.84 ± 0.15[b)]	1.23 ± 0.08[b)]	1.80 ± 0.18[a, b)]
C-HMG	1.09 ± 0.06	1.38 ± 0.11	0.85 ± 0.33
D	1.23 ± 0.17[a)]	2.02 ± 0.50[a)]	1.04 ± 0.25
D-LMG	0.79 ± 0.10[b)]	0.40 ± 0.19[b)]	3.09 ± 0.70[a, b)]
D-HMG	0.61 ± 0.16[b)]	0.58 ± .15[b)]	0.30 ± 0.06

a) $P < 0.05$ *vs.* control group.
b) $P < 0.05$ *vs.* diabetic group.

three parameters were observed in diabetic mice treated with a high dose of MG ($P > 0.05$). In addition, there were no changes of spleen T-lymphocyte subsets of normal mice for any of the given MG doses ($P > 0.05$).

3.3 Detection of intracellular cytokines produced by spleen lymphocytes (IFN-γ,TNF-α, and IL-4)

The expression levels of IFN-γ, TNF-α in IDDM mice spleen lymphocytes significantly increased compared to the normal group ($P < 0.05$), while the expression level of IL-4 was not different between the two groups (Table 3). Low and high doses of MG treatments reduced the expression of IFN-γ and TNF-α ($P < 0.05$); however, the low dose MG administration markedly up-regulated the expression of IL-4 in normal and IDDM mice ($P < 0.05$). Low and high doses of MG therapy had no effects on IFN-γ and TNF-α expression levels of normal mice.

3.4 Histology change of pancreatic islets in experimental mice

Compared to the normal mice (Fig. 3a), IDDM mice without MG treatment presented significant injury in pancreatic islets, which are in the form of degeneration and atrophy, including cell number loss, cytoplasm loss, and hyperplasia of blood vessels leading to the formation of considerable vacuoles (Fig. 3d–f), and the infiltration of a few pro-inflammatory cells was also observed (see arrows in Fig. 3d–f). On the other hand, the above pathological changes in diabetic mice treated with MG, especially low dose MG, improved while the number of pancreatic islet cells increased and its shape showed some improvement (Fig. 3g and h). There were no pathological changes in pancreatic islets of normal mice treated with MG (Fig. 3b, c).

3.5 Analysis of mogrosides extract

Table 4 shows that mogroside V, 11-*o*-mogroside V, mogroside IV, and siamenoside I were the main saponin components of the extract from fruits of *S. grosvenori.* The total mogroside content of these four components was above 94%, especially the mogroside V researched to 69.24%, this indicated that mogroside V was the main component of the extract.

4 Discussion

Alloxan is widely used as an animal model of IDDM [12]. Alloxan selectively destroys the islet β-cell. Our experiments show that blood glucose of IDDM mice was significantly elevated, and was associated with known symptoms such as polyuria and weight loss. MG treatment reduced the blood glucose, and effectively attenuated the early clinical symptoms, biochemical abnormalities, and pathological damages in pancreatic islet. These results indicate that MG could decrease the damage of the islet β-cells of mice induced by alloxan and improve metabolic disorder of IDDM mice. Therefore, we consider the MG may have beneficial effects in IDDM.

Recently, an immune mechanism of injury to pancreatic islet cell during IDDM has gained wide acceptance. This supports a relationship between an imbalance of cellular

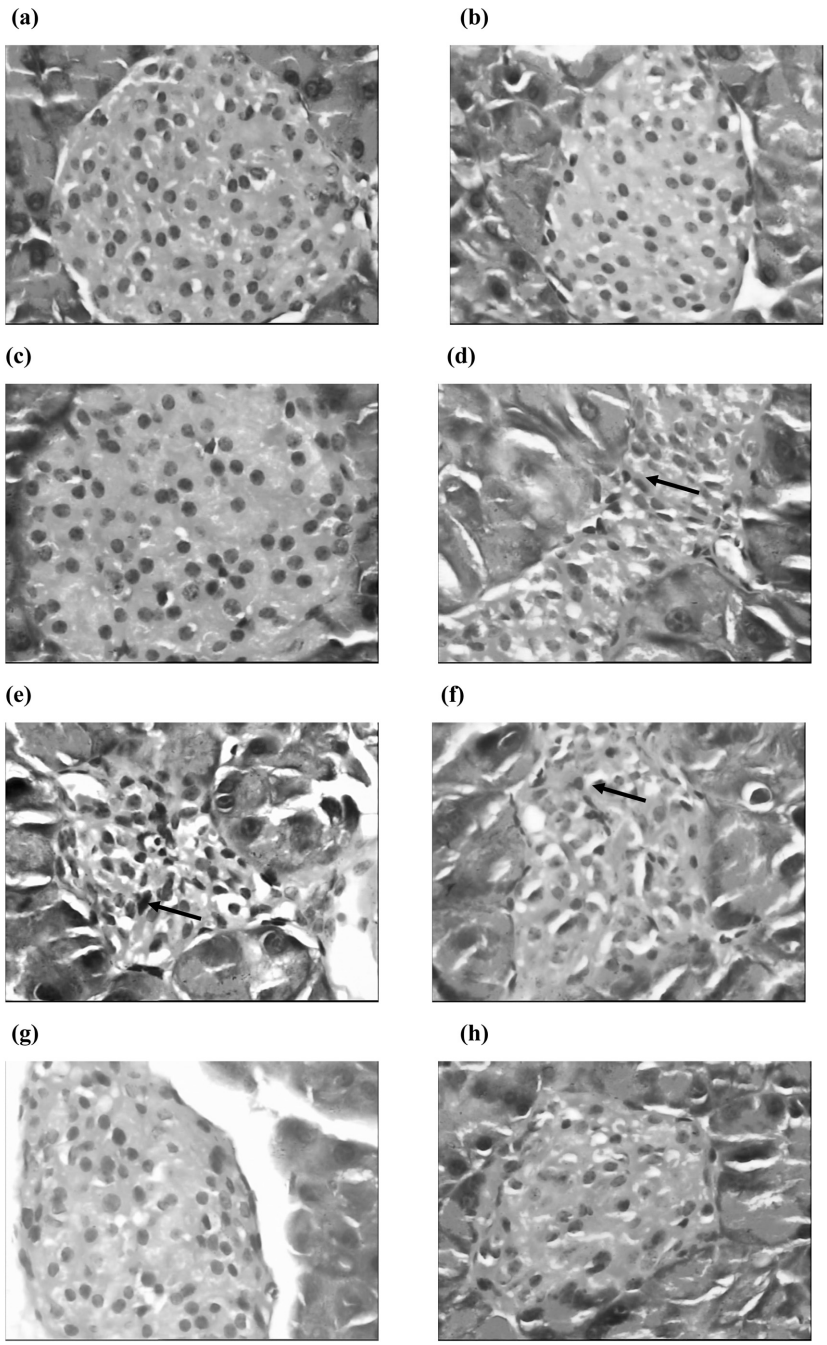

Figure 3. Representative photomicrographs of mice islets stained by haematoxylin and eosin (H&E × 400) among all experimental groups: (a) Control, (b) C-LMG, and (c) C-HMG group. (d – f) Diabetic group. (g) D-LMG group. (h) D-HMG group.

Table 4. The mogrosides contents of the extract

Composition	Mogroside V	11-*o*-Mogroside V	Siamenoside I	Mogroside IV
Content (%)	69.24	10.60	9.80	4.54

immunity, injury of the islet β-cell, and the generation of IDDM. It is generally accepted that functional imbalance of Th1 and Th2 cells plays a role in the genesis of IDDM. Th1 cells and their cytokines IFN-γ and TNF-γ cause damage to islet β-cells, whereas Th2 cell and its cytokines such as IL-4 and IL-10 have protective effects [1, 2]. Because the damage of islet β-cell during IDDM is a slow process, controversy exists regarding the main effects of different cytokines [13–15].

Wang *et al.* [16] reported that MG could enhance the functions of cellular immunity and humoral immunity of normal mice, and may suppress the decrease of the nonspecific immunity function induced by hydrocortisone. Our results indicate that MG may improve the cell immune imbalance in IDDM mice, thus favoring diabetes control. We conclude that one mechanism of MG is modification of lymphocyte subpopulations and cytokines' expression (IFN-γ, TNF-α, and IL-4) in splenocytes of IDDM mice.

Our data show that compared to the normal group, alloxan induced an increase in the number of $CD8^+$, but not $CD4^+$ lymphocyte subpopulation in the spleen of diabetic mice. This results in a decrease of the $CD4^+/CD8^+$ ratio. Moreover, the expression level of pro-inflammatory Th1 cytokines IFN-γ and TNF-α are also increased, without a signifinant change of Th2 cytokines in splenocytes. These results indicate a correlation between the IDDM generation and the up-regulating expression of $CD8^+$ lymphocyte antigen [17, 18]. Low MG doses restored the CD4/CD8 ratio toward normal level by increasing the number of $CD4^+$ lymphocyte subsets. MG, especially the low dosage MG, could down-regulate the expression of IFN-γ and TNF-γ while up-regulating the expression of beneficial cytokines IL-4 [19, 20]. Therefore, a predominance of Th2 cytokines may improve the disease manifestations in IDDM mice.

Previous studies exploring the relationship between peripheral blood, lymphocyte subsets, and cytokines expression in IDDM show inconsistent results [13, 15, 21]. Some investigators believe that the generation of IDDM only relates to partial invasion of lymphocytes in islet cells and cytokine expression [22]. The results of this paper provide evidence of altered lymphocytes subsets and cytokine expression in splenocytes of IDDM mice. Low dose MG treatment not only greatly improved the cell immune function and T cell imbalance, but also alleviated the degree of pathological islet cell injury. This indicate that the change in spleen cells, T cell, and cytokine expression may affect the immune system as a whole, and cause islet cell damage.

As a natural extract, MG shows high sweetness, low calories and, as far as it can be judged at present, harmlessness [4]. MG therapy has no effects on normal mice, except that low dose MG could up-regulate the expression levels of Th2 cytokines IL-4. At the same time, MG treatment could also regulate immune system disorder of diabetic mice induced by alloxan and reduce side effects of hypoglycemic medicines and immunotherapy. In summary, MG can be considered as a promising product both as sweetener and an oral hypoglycemic medicine. According to HPLC analysis, the mogrosides, especially mogroside V, were the main components of MG and they may be the main antidiabetic active compounds, but the structure-activity relationship needs to be further studied.

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