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Effect of *Momordica grosvenori* on oxidative stress pathways in renal mitochondria of normal and alloxan-induced diabetic mice

Involvement of heme oxygenase-1

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Abstract *Background* Oxidative stress plays an important role in the pathogenesis of diabetes and diabetic nephropathy. *Momordica grosvenori* (MG), a traditional medicinal herb used as substitute sugar for obese and diabetes, exhibits anti-oxidative activity in vitro. *Aim of the study* This study investigated the effect of MG on renal mitochondrial lipid peroxidation, anti-oxidative defense system, and a potent oxidative stress–responsive protein, heme oxygenase-1 (HO-1) of non-diabetic and alloxan-diabetic mice in different stages of diabetes. *Methods* Male Balb/c mice were rendered diabetic by a single intra-peritoneal injection of alloxan (200 mg/kg), while control mice received sham saline injection. Control and diabetic mice were further subdivided according to their treatments: control (saline), low dose MG (150 mg/kg) and high dose MG (300 mg/kg), which were administered immediately after confirmation of hyperglycemia by gavage daily over an 8-week period. Mice were killed by cervical dislocation at 4th and 8th week, respectively, and serum and renal tissues were harvested. Serum glucose, lipid profile and renal function were evaluated; renal homogenate were subjected to determination of malondialdehyde (MDA) and glutathione (GSH) concentration, manganese superoxide dismutase (Mn-SOD), glutathione peroxidase (GSH-Px) and HO-1 activities, together with Mn-SOD and HO-1 mRNA expression; paraffin-embedded renal tissues was used for routine histopathological examination. *Results* Short-term diabetes caused hyperglycemia and intensified oxidative stress in renal mitochondrial demonstrated by higher MDA and lower GSH levels than control group, accompanied by increased mRNA expression and activity of HO-1 and Mn-SOD, and augmented GSH-Px activity. Low dose of MG administration ameliorated hyperglycemia, inhibited HO-1 and Mn-SOD mRNA expression and reduced HO-1, Mn-SOD, GSH-Px activities. Diabetic mice did not demonstrate early symptoms of diabetic nephropathy until 8th week, characterized by hyperglycemia, hyperlipidemia, and renal damage. A progressive increment in MDA level and decrease in GSH level, as well as reduced mRNA expression and activity of Mn-SOD and HO-1 in the kidney were observed. Low dose of MG attenuated diabetic nephropa-

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thy symptoms partially, inhibited lipid peroxidation, up-regulated HO-1 and Mn-SOD mRNA expression, and increased HO-1 activity. *Conclusions* The study confirmed the involvement of oxidative stress in the development of diabetes mediated by the pro- and anti-oxidant role of HO-1, and pointed to the possible anti-oxidative mechanism of the anti-diabetic and nephroprotective action of MG.

Key words *Momordica grosvenori* – oxidative stress – alloxan – diabetes – heme oxygenase-1

Introduction

Diabetes is one of the most common chronic diseases with endocrine and metabolic disorders characterized by hyperglycemia and late micro- and macro-vascular complications, among which diabetic nephropathy is most deleterious. Though the precise underlying mechanisms are not completely understood, enhanced oxidative stress observed in both clinical and experimental diabetes has been proposed to be implicated in the etiology of diabetes and its complications [1, 2]. Hyperglycemia, a prominent clinical feature of diabetes, is a major cause responsible for the increased production of reactive oxygen species (ROS) and intensified oxidative stress [2, 3]. There is a strong belief renal glomeruli are particularly sensitive to oxidative stress [4], suggesting the involvement and participation of ROS in the pathogenesis of diabetic nephropathy. Anti-oxidants like vitamin E and C have been considered as new therapies for diabetes and diabetic nephropathy, but limited to experimental animals [5, 6]. Furthermore, the majority of currently available therapies for diabetes are insulin and oral hypoglycemic agents, which afford effective glycemic control but accompanied by serious adverse effects [7]. Therefore, searching new oral anti-diabetic drugs without side effects is still a great difficulty and challenge in medical field.

Increasing attention to dietary measures and traditional plant therapies in recent decades has ignited a new wave of research interest in plant kingdom. *Momordica grosvenori* (Cucurbitaceae), a traditional medicinal herb grown in Kwangshi, China, contains glycosides 200 times sweeter than sucrose. It is a kind of triterpene glycoside containing sapogenin with triterpenol structure, and the glucosidic bond is β-bond type like fiber, as is known not to be decomposed and digested by amylase in human body, and so cannot be absorbed and converted to energy, contributing to its lower calories. Therefore, glycoside has been authorized in China to be used as food additive (sweetener) in various foods for partial or complete substitution of sucrose, especially as substitute sugar of diabetes and obese. Some sweet triterpene glycosides have been reported to have anti-inflammatory [8], anti-carcinogenic [9], and anti-oxidative effects in vitro [10]. The study herein was undertaken to investigate the effect of *Momordica grosvenori* (MG) on oxidative stress and anti-oxidative defense system in renal mitochondria of non-diabetic and alloxan-diabetic mice in different stages of diabetes, and evaluate its potential anti-diabetic and anti-oxidative capacity.

Besides the known cytotoxic effects, oxidative stress condition or enhanced ROS production usually provokes transcriptional activation of specific genes encoding anti-oxidant proteins that participate in the defense against oxidative stress [11]. Heme oxygenase (HO) is a microsomal rate-limiting enzyme responsible for oxidation and degradation of heme into biologically active metabolites: biliverdin, which could be rapidly reduced to bilirubin by biliverdin reductase, carbon monoxide and iron [12]. HO-1, a redox-sensitive inducible isoform of HO superfamily ubiquitously distributed in mammalian tissues, could be induced by its substrate heme and non-heme stimulants such as hydrogen peroxide, ultraviolet light, heavy metals, and cytokines [13, 14]. Many studies have demonstrated the anti-oxidant, anti-inflammatory and anti-apoptotic effects of HO-1 [15, 16], and the induction of HO-1 is considered as an adaptive cellular defense reponse offering protection to cells in pathophysiological states [17–19]. Whereas a few in vivo studies [20, 21] reported the induction of HO-1 in short-term experimental diabetes and pointed out the pro-oxidant activity of HO-1 [21], leaving the biological significance of HO-1 in diabetes remain elusive. Thus, we further explored the role of HO-1 in the progression of diabetes in experimental animals, and evaluated the effect of MG on HO-1 system to elucidate the intrinsic mechanism for its postulated anti-oxidative ability.

Materials and methods

Reagents

Alloxan, standard enzymes: manganese superoxide dismutase (Mn-SOD), glutathione peroxidase (GSH-Px), and xanthine oxidase, 5,5′-dithio-bis-2-nitrobenzoic acid, thiobarbituric acid, 1,1,3,3-tetraethoxypropane, glutathione (GSH), NADPH, heme were procured from Sigma Co. (USA). Trizole Reagent was purchased from Invitrogen Life Technologies Co.

(USA). Moloney murine leukemia virus reverse transcriptase, random primers, RNasin ribonuclease inhibitor, Taq DNA polymerase, dNTPs, and 100 bp DNA ladder were provided by Promega Co. (USA). The primers for HO-1, Mn-SOD, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were synthesized and purified by Beijing AuGCT Biotech Co. (China).

Preparation of extracts

Samples of fruit of MG were provided by Guilin Laiyin Natural Ingredients Inc., China. Air-dried and powdered MG fruit (500 g) was dissolved into 3000 ml distilled water and extracted for 3three times at 70°C, 1 h every time. Subsequently, the water-soluble extracts were concentrated by vacuum, separated and purified using column chromatography, at last vacuum-dried to yield extracts of MG (50 g/kg), which were determined by HPLC, indicating the involvement of 80% glycosides, the majority of which is glycoside is V (28.12%). Solution of aqueous extract was prepared in saline for the experiment.

Animals

Male Balb/c mice (6–8 weeks) weighing 18–20 g were used in this experiment (Sino-British Sippr/BK Laboratory animal Ltd. Shanghai). All animals were housed in an air-conditioned animal room (22 ± 2°C) with a 12-h light/dark cycle. Rodent laboratory chow and tap water were available ad libitum except fasting periods. The animals were cared for in accordance with the *Guiding Principles in the Care and Use of Animals*. This protocol was approved by Tongji Medical College Council on Animal Care Committee, Huazhong University of Science & Technology, China.

Experimental design

Mice were rendered diabetic by a single intra-peritoneal injection of alloxan (200 mg/kg) dissolved in freshly prepared saline following an 18-h fast. Control mice received sham saline injection. Animals with a 5-h fasting blood glucose levels greater than 11.1 mM 2 days after alloxan injection were considered diabetic. Control and diabetic mice were further subdivided according to their treatments: control (saline), low dose MG (150 mg/kg) and high dose MG (300 mg/kg), which were administered immediately after the confirmation of hyperglycemia by gavage daily over an 8-week period. Mice were killed by cervical dislocation at 4th and 8th week, respectively. Blood were collected from the ocular vein after an overnight fasting for serum separation and stored at −20°C for biochemical analysis. The kidneys were quickly removed, rinsed in cold saline solution, blotted dry and stored at −80°C until analysed, and another small piece of renal tissue was subjected to routine histopathological examination.

Measurement of serum glucose, lipid profile and renal function

Serum glucose, triacylglycerols, and total cholesterol concentrations were estimated by commercially available kits (Zhongsheng Beikong Bio-Technology and Science Inc, Beijing, China) based on enzymatic methods. Renal function was assessed by measuring serum levels of urea nitrogen and creatinine using automatic biochemical analyzer (Sigma SD2000, Germany).

Preparation of tissue homogenate and subcellular fractionation

Mouse kidney tissues were homogenized in ice-cold homogenizing buffer (10 mM Tris-Base, 0.1 mM EDTA·2Na, 10 mM sucrose and 137 mM NaCl, pH 7.4) to yield a 10% (w/v) tissue homogenate. The homogenate was centrifuged at 1,000 × *g* at 4°C for 10 min to discard nuclei and cell debris. The post-nuclear supernatant was further centrifuged at 10,000 × *g* at 4°C for 10 min to obtain mitochondrial fraction, which was resuspended in the homogenizing buffer. The post-mitochondrial supernatant was aspirated and then re-centrifuged at 105,000 × *g* at 4°C for 60 min to obtain the cytosol fraction (supernatant) and microsomal pellet (resuspended in the aforementioned buffer).

Analysis of lipid peroxidation and anti-oxidant status in renal mitochondria

Lipid peroxidation was assayed by measurement of malondialdehyde (MDA) concentration following the method of Beuege and Aust [22]. GSH concentration was estimated by the method of Moron et al. [23]. Mn-SOD activity was determined according to the method of Kono [24], in the presence of potassium cyanide to ensure complete inactivation of copper/zinc superoxide dismutase. The method of Sazuka et al. [25] was employed to estimate GSH-Px activity.

Estimation of renal microsomal HO-1 activity

HO-1 activity was determined by the generation of bilirubin from heme metabolism as described by Tenhunen et al. [26] with slight modification.

Protein estimation

Protein of renal mitochondrial and microsomal was estimated with a standard method [27].

Analysis of gene expression by RT-PCR

cDNA was cloned by RT from total RNA, isolated from mouse kidney. The primers for PCR reaction were as following (sense and anti-sense, respectively). HO-1: 5′-GAAGGGTCAGGTGTCCAGAG-3′ and 5′-CCAGGTAGCGGGTATATGCGT-3′; Mn-SOD: 5′-GTTACAACTCAGGTCGCTCTTC-3′ and 5′-GTGCTGCAATGCTCTACACTAC-3′; GAPDH: 5′-TGGCCAAGGTCATCCATGAC-3′ and 5′-AGGCCATGCCAGTGAGCTTC-3′. PCR amplification was carried out for 35 (HO-1), 30 (Mn-SOD) and 27 (GAPDH) cycles, and the expected sizes of amplified HO-1, Mn-SOD and GAPDH cDNA fragment were 302 bp, 508 bp and 219 bp, respectively. The PCR reaction products were subjected to gel electrophoresis for separation and optical densities of DNA bands were quantified by Whatman Biometra BioDocAnalyze digital system (Germany). The amount of PCR products of target cDNA was normalized to that of GAPDH to exclude differences in reverse transcription efficiency and amount of template in the reaction.

Histopathological examination

After removal, the kidneys were subjected to routine histopathological examination process of haematoxylin and eosin staining for light microscopical examination.

Statistical analysis

The data were expressed as mean ± SD. Comparisons among groups were subjected to one-way analysis of variance followed by Student-Newman–Keuls multiple range test. A probability of $P < 0.05$ was considered significantly different.

Results

Clinical monitoring of animals

Mice demonstrated the typical symptoms of diabetes, including polydipsia, polyphagia, polyuria, and loss of body weight after alloxan induction. While treatment with low dose of MG for 8 week effectively ameliorated the polydipsia and polyuria symptom, and partially but significantly increased body weight of diabetes mice compared to diabetic control (data not shown).

Effects of MG on serum glucose, lipid profile and renal function

Diabetic mice suffered permanent hyperglycemia after alloxan injection, which increased to 6.7- and 5.7-fold of non-diabetic control at 4th week (Table 1) and 8th week (Table 2), respectively. High glucose levels were partially restored to basal values both after MG short-term (4 week) and long-term (8 week) administration, although a complete restoration to normalcy was not achieved.

Alloxan did not induce abnormal lipid profile until 8th week, confirmed by significantly increased total cholesterol and triacylglycerols concentration as compared with non-diabetic mice. The increased total cholesterol level was restored to normalcy after MG administration. Similarly, diabetic mice did not demonstrate renal dysfunction until 8th week, as could be seen from the significantly higher urea nitrogen and creatinine levels than that of non-diabetic mice. The increase in creatinine level was partially prevented by treatment with low dose of MG (Table 2).

Table 1 Effects of MG treatment for 4 weeks on serum glucose, lipid profile and renal function in non-diabetic and diabetic mice

Group	Glucose (mM)	Total cholesterol (mM)	Triacylglycerols (mM)	Urea nitrogen (mM)	Creatinine (μM)
C (10)	3.29 ± 0.33	2.91 ± 0.37	1.29 ± 0.25	3.78 ± 0.98	87.5 ± 13.1
C-LMG (10)	3.29 ± 0.49**	2.53 ± 0.56	1.19 ± 0.13**	4.68 ± 0.74	95.2 ± 14.3
C-HMG (10)	4.37 ± 0.39**	2.57 ± 0.33	1.37 ± 0.12	3.60 ± 1.07	99.0 ± 13.1
D (7)	21.9 ± 1.50*	3.23 ± 0.78	1.51 ± 0.25	4.41 ± 0.30	103 ± 10.1
D-LMG (8)	10.5 ± 1.89*,**	3.22 ± 0.68	1.38 ± 0.27	4.97 ± 1.58	93.3 ± 14.3
D-HMG (11)	12.7 ± 1.45*,**	3.03 ± 0.76	1.42 ± 0.22	5.01 ± 1.12	97.8 ± 13.8

Values are mean ± SD, and the number of observations is indicated in parentheses
Abbreviations: C: Control group, C-LMG: Control + low dose MG group (at a dose of 150 mg/kg), C-HMG: Control + high dose MG group (at a dose of 300 mg/kg); D: Diabetic group, D-LMG: Diabetic + low dose MG group (at a dose of 150 mg/kg), D-HMG: Diabetic + high dose MG group (at a dose of 300 mg/kg)
*$P < 0.05$ as compared with control group
**$P < 0.05$ as compared with Diabetic group

Table 2 Effects of MG treatment for 8 weeks on serum glucose, lipid profile and renal function in non-diabetic and diabetic mice

Group	Glucose (mM)	Total cholesterol (mM)	Triacylglycerols (mM)	Urea nitrogen (mM)	Creatinine (μM)
C (7)	4.94 ± 0.63	2.80 ± 0.50	0.89 ± 0.28	3.74 ± 1.20	81.4 ± 8.95
C-LMG (7)	4.78 ± 0.51**	3.24 ± 0.57**	1.28 ± 0.13**	4.01 ± 0.88	87.4 ± 10.7**
C-HMG (7)	4.30 ± 0.58**	2.99 ± 0.35**	0.71 ± 0.17**	3.20 ± 1.49	91.2 ± 8.87**
D (7)	28.1 ± 2.07*	4.43 ± 0.76*	2.03 ± 0.45*	5.53 ± 1.11*	123 ± 14.0*
D-LMG (8)	19.7 ± 4.17*,**	3.56 ± 0.70**	1.69 ± 0.28*	6.04 ± 1.16*	109 ± 11.6*,**
D-HMG (7)	20.2 ± 3.55*,**	3.09 ± 0.54**	1.69 ± 0.45*	6.00 ± 1.08*	113 ± 10.5*

Values are mean ± SD, and the number of observations is indicated in parentheses
Abbreviations are as in Table 1
*$P < 0.05$ as compared with control group
**$P < 0.05$ as compared with diabetic group

Effect of MG on lipid peroxidation and anti-oxidant status in renal mitochondria, and HO-1 activity in renal microsome

Renal mitochondrial MDA levels were increased between 1.51- and 3.07-fold in diabetic mice compared with non-diabetic control mice at 4th (Table 3) and 8th week (Table 4), respectively. Treatment with MG after 8 w completely abolished the accumulation of MDA. On the contrary, the level of GSH in diabetic group were 12 and 15% lower than the non-diabetic control group after short-term (Table 3) and long-term trial (Table 4), which was normalized after 8 w of MG administration. Besides, high dose of MG-treated control mice demonstrated a GSH level 18% higher than non-treated control mice at 8th week.

A significant increase in activities of renal mitochondrial Mn-SOD and GSH-Px was detected in diabetic mice 4 w after alloxan injection compared to non-diabetic mice, which was prevented by treatment with low dose of MG (Table 3). However, the activity of renal mitochondrial Mn-SOD was reduced significantly in diabetic mice at 8th week, which could not be significantly reversed by MG but a non-significant increase in Mn-SOD activity was found after low dose of MG administration, showing no significant difference with non-diabetic mice (Table 4). No significant differences were observed in the GSH-Px activity among the experimental mice (Table 4).

Table 3 Effect of MG treatment for 4 weeks on mitochondrial lipid peroxidation and anti-oxidant status, and microsomal HO-1 activity in the kidneys of non-diabetic and diabetic mice

Group	MDA (nmol/mg protein)	GSH (mg/g protein)	MnSOD (Nu/mg protein)	GSH-Px (U/mg protein)	HO-1 (pmol/h/mg protein)
C (10)	0.79 ± 0.12	80.7 ± 4.97	7.23 ± 1.34	39.6 ± 5.72	36.9 ± 4.06
C-LMG (10)	0.89 ± 0.11**	76.8 ± 8.28	5.71 ± 1.55**	38.7 ± 5.61**	36.3 ± 4.37**
C-HMG (10)	0.83 ± 0.12**	73.6 ± 7.04	6.61 ± 1.98**	41.2 ± 7.10	31.2 ± 6.91**
D (7)	1.19 ± 0.15*	70.9 ± 5.14*	10.6 ± 1.65*	52.2 ± 6.86*	48.5 ± 3.95*
D-LMG (8)	1.04 ± 0.29*	70.8 ± 6.68*	7.45 ± 1.56**	40.3 ± 6.44**	42.3 ± 6.57**
D-HMG (11)	1.37 ± 0.20*	64.8 ± 6.42*	10.2 ± 1.59*	49.5 ± 8.31*	37.1 ± 7.41**

Values are mean ± SD, and the number of observations is indicated in parentheses
Abbreviations are as in Table 1
*$P < 0.05$ as compared with control group
**$P < 0.05$ as compared with diabetic group

Table 4 Effect of MG treatment for 8 weeks on mitochondrial lipid peroxidation and anti-oxidant status, and microsomal HO-1 activity in the kidneys of non-diabetic and diabetic mice

Group	MDA (nmol/mg protein)	GSH (mg/g protein)	MnSOD (Nu/mg protein)	GSH-Px (U/mg protein)	HO-1 (pmol/h/mg protein)
C (10)	0.88 ± 0.16	72.9 ± 5.95	6.01 ± 0.65	38.6 ± 7.82	34.9 ± 3.79
C-LMG (10)	0.94 ± 0.18**	77.5 ± 4.38**	5.95 ± 0.72**	37.4 ± 8.63	36.8 ± 4.62**
C-HMG (10)	0.81 ± 0.21**	86.1 ± 8.20*,**	5.21 ± 0.83	29.6 ± 4.17	32.7 ± 3.53
D (7)	2.70 ± 0.27*	61.8 ± 6.78*	4.24 ± 0.86*	35.1 ± 4.40	26.6 ± 5.98*
D-LMG (8)	0.93 ± 0.14**	67.5 ± 4.57	5.24 ± 0.91	33.4 ± 7.83	36.5 ± 7.74**
D-HMG (11)	1.25 ± 0.17**	66.5 ± 7.27	4.78 ± 0.38*	31.5 ± 8.61	25.8 ± 4.94*

Values are mean ± SD, and the number of observations is indicated in parentheses
Abbreviations are as in Table 1
*$P < 0.05$ as compared with control group
**$P < 0.05$ as compared with diabetic group

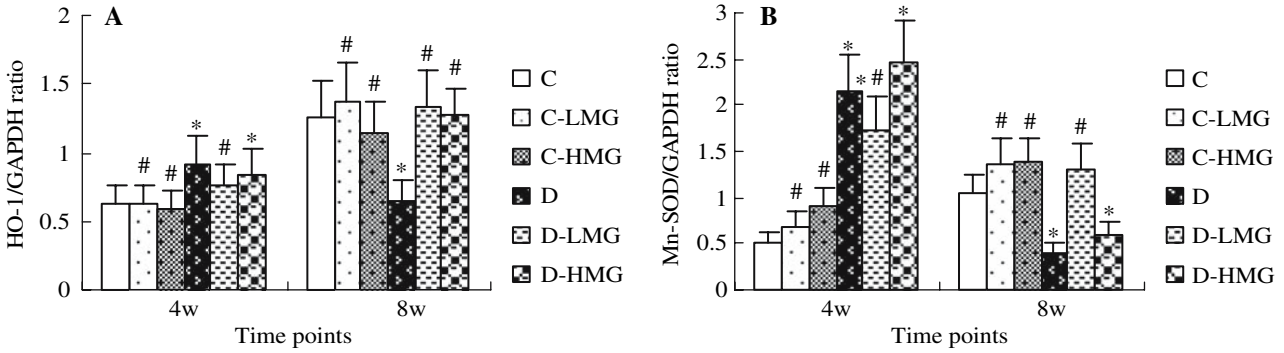

Fig. 1 Effects of MG treatment for 4 weeks and 8 weeks on mRNA expression of (**A**) HO-1 and (**B**) Mn-SOD of kidney in non-diabetic and diabetic mice (the blot photograph not shown). An optical density of the PCR electrophoresis band was measured quantitatively using Whatman Biometro BioDocAnalyze digital system and the ratio of the target mRNA to GAPDH mRNA was shown. Abbreviations are as in Table 1. Data are expressed as mean ± SD ($n = 5$). *$P < 0.05$ as compared with control group; $^{\#}$ $P < 0.05$ as compared with diabetic group

Diabetic mice demonstrated a significant higher and lower HO-1 activity 4 week (Table 3) and 8 week (Table 4) after alloxan injection with respect to the corresponding non-diabetic mice. Following 4 weeks of treatment, both dose of MG resulted in a significant decrease in HO-1 activity with respect to non-treated diabetic mice, whereas after 8 weeks of treatment, only low dose of MG increased HO-1 activity as compared to non-treated diabetic mice.

Effect of MG on HO-1, Mn-SOD mRNA expression in the kidney

Figure 1A, B showed mRNA expression of HO-1 and Mn-SOD were significantly enhanced by 1.5- and 4.2-fold, respectively in the kidney of diabetic mice at 4th week as compared with control mice. The enhancement of HO-1 mRNA expression was completely normalized by low dose of MG, and enhancement of Mn-SOD mRNA expression was significantly amelirated after treatment with low dose of MG, although a complete restoration to basal levels was not achieved.

Whereas alloxan markedly decreased mRNA expression of HO-1 and Mn-SOD by 1.9- and 2.6-fold, respectively in diabetic kidney compared to non-diabetic control at 8th week (Fig. 1A, B), which could be both returned to normalcy by low dose of MG. Moreover, treatment with high dose of MG also induced a higher mRNA expression level of HO-1 than that in non-treated diabetic mice.

Effect of MG on renal histomorphology

Diabetic mice did not demonstrate early pathological features of diabetic nephropathy until 8th week. As shown in Fig. 2D, E, alloxan caused significant morphological abnormalcy in the kidney characterized by glomerulus hypertrophy, expanded and congested glomerular capillaries, and hydropic degeneration in renal tubules in the form of swelling, necrosis and lysis in tubular epithelial cells and irregular tubular lumens, with respect to non-diabetic mice (Fig. 2A–C). Treatment with MG made the renal structures more similar to those from non-diabetic mice (when compared with Fig. 2A, D, E), though slight hydropic degeneration in renal tubules still existed (Fig. 2F, G).

Discussion

Numerous studies have demonstrated oxidative stress is a key pathological factor in the progression of diabetes and its late complications [1, 2]. Previously carried out studies have presented the particular sensitivity of kidney to oxidative stress, and mitochondria is believed to an important target suffering from ROS toxicity [4, 28]. Renal mitochondria MDA levels were already elevated in our short-standing diabetes cases. This revealed the involvement of intensified lipid peroxidation progress from early stage of diabetes [4, 29]. Concomitantly, short-term diabetes was associated with the loss of GSH in renal mitochondria, reflecting its increased utilization and consumption due to oxidative stress [30]. The so far carried out studies in clinical and experimental diabetes have reported alterations in the activities of anti-oxidative enzymes, known to largely rest on the duration of diabetes and the involving tissues [4]. With respect to kidney, most studies have obtained consistent results, i.e. SOD, GSH-Px were up-regulated in short-term diabetes [4, 29]. This study also observed up-regulated Mn-SOD mRNA expression and activity in renal mitochondria of short-term diabetic mice, suggesting increased production of

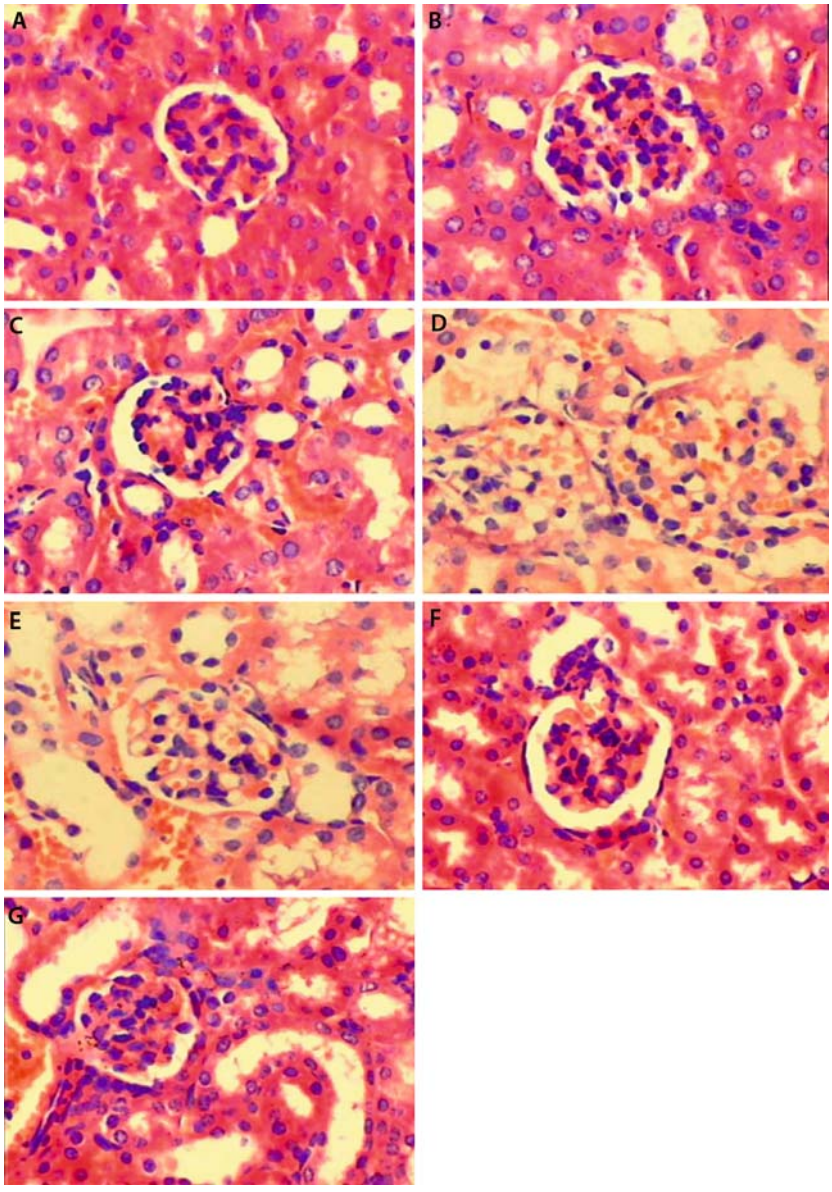

Fig. 2 Representative photomicrographs of kidney stained by haematoxylin and eosin (×400) in non-diabetic and diabetic mice after 8 weeks of MG treatment (**A**: C group; **B**: C-LMG group; **C**: C-HMG; D and **E**: D group; **F**: D-LMG group; **G**: D-HMG group). For abbreviations see captions in Table 1

superoxide anions. Meanwhile, there was a subsequent increase in GSH-Px activity due to its role in catalyzing excessive production of hydrogen peroxide converted from superoxide anions.

Though the hypothesis that HO-1 acts as an important component of cellular defense against oxidative stress has been identified by numerous studies [17], several in vitro studies has supported HO-1 exhibited either pro- or anti-oxidant activities, and such dual role may depend on particular condition [12, 21]. In early induction of HO-1, the non-destroyed heme and the transient iron accumulation are believed to lead to deleterious consequences with their known pro-oxidant effects, while the compensating up-regulation in ferritin after HO-1 induction is only available in the long-term to sequester cellular free iron and depress the cellular sensitivity to oxidant challenge [31]. Thus, the significant cytoprotective effects of HO-1 may occur only when free heme has been eliminated and free iron has been completely sequestered by ferritin [12]. We observed increased mRNA expression and activity of HO-1 were accompanied by concurrent changes in Mn-SOD and subsequently compensatory increase in GSH-Px activity in renal mitochondria of short-term diabetes, suggesting the presence of intra-mitochondria oxidative stress ascribed to pro-oxidant activity of HO-1. An in vitro study reported antecedent up-regulation of HO-1 is responsible for the subsequent induction of Mn-SOD in oxidatively-challenged astroglia [19], provid-

ing evidence for the implication of HO-1–Mn-SOD axis in the pathogenesis of some oxidative stress-related diseases, as demonstrated in the present study performed in diabetes.

Treatment with low dose of MG affected neither MDA nor GSH levels of renal mitochondria in short-term diabetic mice, while a non-significant decrease in MDA concentration was observed. At the same time, low dose of MG effectively abrogated the increase in mRNA expression and activity of HO-1 and Mn-SOD, and decreased GSH-Px activity in the kidney of diabetes. These results demonstrated renal mitochondria of low dose of MG-treated diabetic mice still suffered from oxidative stress due to the non-normalized blood glucose, which may be completely removed by depleting reduced GSH and make it unnecessary to induce HO-1, indirectly supporting the ROS-scavenging capacity of low dose of MG. Whereas, high dose of MG seemed less efficient in treating diabetes, which might deviate from the range of physiological effect of MG and become tolerated for mice, so further studies on the appropriate dose range of MG in preventing diabetes are needed.

The alterations in MDA and GSH levels were sustained and more pronounced following the next 4 weeks attributed to the chronic hyperglycemia [32, 33]. Oxidative stress in renal mitochondria at this time did not induce the expression of HO-1 as expected, on the contrary a decrease in mRNA expression and activity of HO-1 was found, bringing to light the anti-oxidative and protective activity of HO-1. The lost compensation in HO-1 system may be related to oxidative stress-induced pathological injury in the kidney. Therefore, the anti-oxidative defense enzymes were directively engaged with the removal of ROS, leading to decreased expression and activity of Mn-SOD, and a non-significant decrease in GSH-Px activity in renal mitochondria of mice with long-term diabetes [32, 33]. This finding indicated oxidative stress surpassed the capacity of anti-oxidative enzymes to detoxify the deleterious ROS, again supporting a close link between hyperglycemia, hyperlipidemia, renal damage, and oxidative stress among experimental and clinical diabetes [34].

Low dose of MG treatment in diabetic mice for 8 weeks of induced up-regulation in mRNA expression and activity of HO-1, as well as a striking up-regulation of Mn-SOD mRNA level but a non-significant increase in its activity, indirectly suggesting the role of HO-1 in the regulation of Mn-SOD gene expression, which could have a cumulative effect over time. Furthermore, low dose of MG treatment decreased MDA concentration and partially increased GSH level, indicating a weakened oxidative stress in renal mitochondria of diabetic mice, possibly ascribed to the induction of HO-1. Thus, low dose of MG-treated diabetic mice demonstrated a near-normal lipid profile and renal morphology. Similarly, high dose of MG was less effective.

The study herein strongly points to the participation of oxidative stress in the etiology of diabetic nephropathy, mediated by an important oxidative stress–responsive protein, HO-1, which has both pro- and anti-oxidant sequelae depending on cellular redox potential and metabolic fate of heme and iron. Treatment with MG can prevent the development of diabetic nephropathy through its anti-oxidative action relevant to inhibition/activation of HO-1 due to its dual roles, and exhibit no toxic effect on normal mice, considered promising as dietary supplement for treatment of diabetic nephropathy. Though in vitro study demonstrated the 11-oxo-function of B ring in the structure of 11-oxo-mogroside V may be relevant to its anti-oxidative activity [10], more extended research is necessitated to elucidate the bioactive elements and the structure–effect relationship responsible for the anti-oxidative effects of MG.

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