Protective Effects of 2-Dodecyl-6-Methoxycyclohexa-2,5-Diene-1,4-Dione Isolated from Averrhoa Carambola L. (Oxalidaceae) Roots on High-Fat Diet-Induced Obesity and Insulin Resistance in Mice

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Key Words
2-dodecyl-6-methoxycyclohexa-2,5-diene-1,4-dione • Obesity • Insulin resistance • TLR4 • Inflammatory cytokines

Abstract
Background/Aims: The roots of Averrhoa carambola L. (Oxalidaceae) have long been used as a traditional Chinese medicine for the treatment of diabetes and diabetes-related diseases. 2-dodecyl-6-methoxycyclohexa-2,5-1,4-dione (DMDD) has been isolated from A. carambola L. roots, and this study was carried out to investigate the potential beneficial effects of DMDD on obesity and insulin resistance induced by a high-fat diet (HFD) in mice. Methods: C57BL/6J mice were fed a HFD for 16 weeks and orally administered DMDD (12.5, 25, or 50 mg/kg of body weight per day) and metformin (280 mg/kg of body weight per day) for the last 4 weeks. Results: The body weights and adipose tissue weights as well as the serum levels of blood glucose, total cholesterol, triglycerides, free fatty acids, insulin, interleukin-6, and tumor necrosis factor-α were significantly decreased by DMDD, and the expression of Toll-like receptor 4 (TLR4) and myeloid differentiation factor (Myd88) in the epididymal adipose tissue was downregulated by DMDD. In contrast, insulin sensitivity was enhanced. The results of the glucose tolerance tests, insulin tolerance tests, and insulin release tests indicated that there was a marked improvement in insulin secretion, and the areas under the curve corresponding to the three tests were also significantly decreased by DMDD. The activities of superoxide dismutase and glutathione peroxidase were simultaneously enhanced, whereas the content of malondialdehyde was decreased by DMDD in the liver homogenates of the C57BL/6J mice. In addition, hepatic steatosis and adipocyte hypertrophy, as assessed by H&E staining of liver and adipose tissues, were significantly improved by DMDD. Conclusion: These data suggest that...
DMDD has potential benefits for the treatment of HFD-induced obesity and insulin resistance, and its effects may be associated with improvements in lipid metabolism and inhibition of the expression of TLR4 in adipose tissues.

Introduction

Obesity has become a common public health issue, and it leads to a multitude of metabolic disorders [1, 2]. Increasing evidence indicates that obesity is closely associated with many of chronic diseases, such as insulin resistance (IR), diabetes, and hypertension, and individuals die from complications of energy imbalance every year [3]. It is thought that in most instances, obesity results from a combination of genetic and environmental risk factors. As common environmental risk factors mainly include excessive caloric intake and a sedentary lifestyle, the establishment of animal models is indispensable for exploring the potential molecular mechanisms of obesity and their pathophysiological effects, as well as for researching new treatments for obesity [4]. Compared with the other strains of mice commonly used in our studies, C57BL/6J mice are more susceptible to diet-induced obesity [5], so they were chosen for the present study.

The improvement of IR plays a crucial role in the prevention of T2DM. Although DMDD exhibits favorable effects on hyperglycemia and improves lipid metabolism, its definitive mechanism remains largely unknown. Currently, the reduction of free fatty acids (FFAs) through the inhibition of the Toll-like receptor 4 (TLR4) signaling pathway has become an important means of improving IR. We discovered that DMDD improves hyperglycemia and hyperlipidemia and inhibits the expression of TLR4 [6]. Thus, we hypothesize that these effects may be associated with improvements in IR through the amelioration of lipid metabolism by restraining the activation of TLR4 and its downstream signal transduction pathway. Furthermore, accumulating evidence has demonstrated that obesity is closely linked to a state of systematic, low-grade inflammation that is characterized by activation of inflammatory signaling pathways and the generation of anomalous cytokines in adipose tissue [7, 8]. The levels of cytokines produced by adipocytes are generally elevated in patients with obesity. They include several inflammatory markers, such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and monocyte chemoattractant protein-1 [1, 9, 10], and these cytokines interfere with glucose and lipid metabolism. Therefore, to elucidate the explicit cellular mechanism of DMDD on TLR4, we investigated its effects on IR and the levels of serum lipids and inflammatory cytokines.

Our previous study demonstrated that the roots of *Averrhoa carambola L*. have beneficial anti-hyperglycemic effects [11], and the effective component (DMDD) that was extracted from the roots of *Averrhoa carambola L*. also has therapeutic potential for the treatment of diabetic nephropathy and inhibitory effects on the kidneys of diabetic mice [12]. Therefore, we hypothesized that DMDD plays a significant role in the prevention of HFD-induced obesity and insulin resistance in C57BL/6J mice, and we also compared the effects of DMDD with metformin. Metformin is used clinically for the treatment of obesity and diabetes, and its effects include the following: (1) reducing hepatic glucose production, (2) decreasing the intestinal absorption of glucose, and (3) improving insulin sensitivity by increasing peripheral glucose uptake and utilization [13].

Materials and Methods

Plant materials

DMDD was prepared according to the methods described in our previous study, and the chemical structure of DMDD is shown in Fig. 1. A powder from the air-dried roots of *A. carambola L.* was extracted with 60% aq. EtOH. The ethanol solution was then concentrated in a vacuum and extracted with cyclohexane,
followed by separation with an open silica gel CC. The compound was identified by FTIR spectroscopy using a Spectrum One spectrophotometer (PerkinElmer, America) and by 1H and 13C NMR analysis on a Bruker AV 600. The results were then compared with the literature [12, 14]. Furthermore, the purity of DMDD was determined by HPLC (MeOH/H2O 25:75, 8 mL/min, 203 nm), and the purity was ≥ 95% [11]. DMDD was dissolved in distilled water (2.5 mg/L) before being administered to the mice.

**Animals**

The study was approved by the Ethical Committee of Experimental Use of Animals at Guangxi Medical University (Guangxi, China). Eight-week-old, male C57BL/6J mice were obtained from the Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). All mice were housed in individual cages with sufficient bedding (5 mice/cage) at a temperature of 25 ± 1 °C and a humidity of 60 ± 5% on a 12-hour light/dark cycle. The bedding was changed often, and the cages were cleaned frequently to keep the mice dry and clean. The body weights were monitored weekly.

**Animal Experiments**

After an acclimatization period of 7 days, 120 mice were randomly divided into normal diet (ND) and HFD groups. Both the ND and HFD were provided by the Guangxi Institute of Chinese Medicine. Starting on the 16th week of the experimental period, the ND group received the ND (consisting of 20% crude protein, 10% crude fat, and 70% carbohydrates), and the HFD group received the HFD (consisting of 20% crude protein, 35% crude fat, and 45% carbohydrates). The HFD contained 60% ND, 8% lard, 20% sucrose, 10% egg yolk powder, and 2% cholesterol. Twelve weeks later, FBG testing was conducted, and the body weights were measured. The mice were considered obese and insulin resistant when their body weights and fasting blood glucose (FBG) levels were obviously different from the ND group (body weight > 20% ND, FBG > 7.8 mmol/L). The mice were then assigned to the following groups:

- **Group I (n = 10):** healthy C57BL/6J mice that were administered distilled water: normal control.
- **Group II (n = 10):** obese and insulin-resistant mice that were administered distilled water: model control.
- **Group III (n = 10):** obese and insulin-resistant that were mice administered metformin (280 mg/kg of body weight) by gastric perfusion once a day for 28 days: positive control.
- **Group IV (n = 10):** obese and insulin-resistant mice that were administered DMDD (12.5 mg/kg of body weight) by gastric perfusion once a day for 28 days.
- **Group V (n = 10):** obese and insulin-resistant mice that were administered DMDD (25 mg/kg of body weight) by gastric perfusion once a day for 28 days.
- **Group VI (n = 10):** obese and insulin-resistant mice that were administered DMDD (50 mg/kg of body weight) by gastric perfusion once a day for 28 days.

**Intraperitoneal Glucose Tolerance Test (IPGTT)**

The IPGTT was performed at the beginning of week 15. After a 15-hour fast (5:00 pm—8:00 am), the mice were injected intraperitoneally with glucose (2 g/kg of body weight). The blood glucose levels at 0, 15, 30, 60, and 120 min were measured using an accurate blood glucose meter (Accu-check Performa, Roche, Germany) [15-18]. The area under the curve (AUC) was calculated using the trapezoid rule.

**Insulin Tolerance Test (ITT)**

After 4 hours of fasting during the middle of week 15, the mice were injected intraperitoneally with 0.75 U/kg of insulin (Wanbang medicine, Jiangsu, China), and blood glucose levels were measured after 0, 5, 15, 30, and 60 min using an accurate blood glucose meter (Accu-check Performa) [19, 20]. The AUC was calculated using the trapezoid rule.

**Insulin Release Test (IRT)**

After 15 hours of fasting at the end of week 15, the mice were injected intraperitoneally with glucose (3 g/kg of body weight). Blood samples were collected 0, 5, 15, 30, and 60 min after glucose injection, and...
the insulin levels were measured by enzyme-linked immunosorbent assay (ELISA) [21, 22]. The AUC was calculated using the trapezoid rule.

Collection of the Blood and Tissues

After 28 days of treatment, the mice were fasted overnight, and blood was then collected from the retro-orbital venous sinus and centrifuged at 4000 x g for 10 min at 4°C for serum collection. Meanwhile, the liver and deposits of body fat (epididymal fat, perirenal fat, and abdominal fat) were removed and washed with cold saline. Portions of the tissues were fixed in 10% formaldehyde, and the serum and remaining tissues were stored at -80°C for further analysis.

Biochemical index assays

The serum levels of total cholesterol (TC) and triglycerides (TGs) and the malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px) content in the liver homogenates were measured using commercially available kits (Nanjing Jiancheng Bioengineering Research Institute, Nanjing, China) according to the manufacturer’s instructions. The serum concentrations of insulin, FFAs, TNF-α, and IL-6 were detected using ELISA kits (Beijing Yonghui Biological Technology Co, Beijing, China) according to the manufacturer’s instructions. The insulin sensitivity index (ISI) and insulin resistance index (IRI) were calculated using the following formulas: ISI = ln (FBG × FINS)⁻¹ and IRI = (FBG × FINS) / 22.5 [23].

Histopathological examination

The livers and epididymal fat pads were fixed in 10% formaldehyde for 24 hours and then embedded in paraffin. The slices were stained with H&E (hematoxylin and eosin) and observed using a light microscope at 400X. The mean diameter and mean area of the adipocytes were calculated using Image-Pro Plus 6.0 [24, 25].

The criteria for the hepatic steatosis score were as follows [26]:

Grade 0 – Normal lobular architecture with central veins and no obvious steatosis, inflammation or necrosis was observed.

Grade 1 – Clear lobular architecture with central veins, a small accumulation of fat droplets, and no obvious steatosis, inflammation or necrosis was observed.

Grade 2 – Unclear lobular architecture, increasing accumulation of fat droplets and slight steatosis, and inflammation or necrosis was observed.

Grade 3 – Lobular architecture was no longer present, the structure of the hepatic cord was deranged, a large accumulation of fat droplets was observed, and various degrees of diffuse hepatic steatosis were prominent.

Real-time polymerase chain reaction (RT-PCR)

Total RNA was obtained from the epididymal adipose tissue using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Complementary DNA (cDNA) was synthesized using the PrimeScript RT reagent kit with gDNA Eraser. RT-PCR was conducted using a 7300 real-time PCR detection system (Applied Biosystems, Foster City, CA, USA). cDNA samples (equivalent to 1 μg of total RNA) were used as templates with the corresponding gene primers (Table 1). The real-time PCR results were calculated by the \( \frac{\Delta \Delta C_t}{} \) method and normalized to β-actin with arbitrary units [27].

Statistical Analysis

All the experimental data were analyzed by SPSS 16.0 software (SPSS Inc., USA) and are presented as the mean ± SD. Statistical significance was assessed using one-way analysis of variance (ANOVA), and count data were analyzed by a nonparametric test. P-values less than 0.05 between groups were considered to be statistically significant.

Results

Effects of the HFD on body weight, and the effects of DMDD on food intake and adipose tissue weight

Body weight was monitored weekly, as it is an important characteristic of obesity. Compared to the ND, HFD feeding resulted in significantly more weight gain throughout the
12-week molding period (Fig. 2A). Compared to the untreated HFD mice, the administration of DMDD to HFD-fed mice resulted in a significant decrease in adipose tissue weight (Fig. 2C). However, food intake (grams of food) was not obviously different (Fig. 2B).

**Effects of DMDD on body weight and blood glucose**

The body weights and blood glucose levels are shown in Fig. 3A and 3B, respectively. The body weights and blood glucose levels of the treated groups were significantly different from the ND group at the beginning of the treatment. Compared to the untreated HFD mice, DMDD treatment significantly decreased the body weights and blood glucose levels ($P < 0.01$). However, there were no significant differences among the treated groups.

**Effects of DMDD on fasting insulin, IRI, and ISI**

As shown in Fig. 4, the insulin resistance index (IRI) (Fig. 4C) and serum insulin levels (Fig. 4A) were significantly elevated, and the insulin sensitivity index (ISI) (Fig. 4B) was decreased in the HFD-induced mice. Interestingly, the groups treated with metformin and DMDD exhibited effective reductions in the IRI and a downregulation of serum insulin levels, as well as enhanced insulin sensitivity.

**Effects of DMDD on glucose regulation, insulin secretion capacity, and insulin sensitivity**

According to the IPGTT testing conducted after 15 weeks of continuous HFD feeding, the blood glucose levels of the HFD groups at 5, 30, and 60 min were significantly higher and declined more slowly after the glucose load compared with the ND group (Fig. 5A). Furthermore, the blood glucose levels after 120 min were still above the baseline. Interestingly, the blood glucose levels of the DMDD and metformin groups at 15, 30, and 60 min were obviously lower, but there were no significant differences among the DMDD and ND groups. These results imply that after the same HFD stimulation, the glucose regulation capabilities of the DMDD groups were more powerful than those of the HFD group.

The fasting insulin content of the HFD group was notably higher than that of the ND group before receiving the glucose load, although the insulin levels at 5, 15, 30, and 60 min
remained higher than those of the ND group (Fig. 5B). Five minutes after the ND group was injected with glucose, its peak secretion was three to four times higher than that of
the baseline; whereas in the HFD group, not only was the peak delayed but there was also a decline in the glucose-stimulated acute insulin secretion reactions. In contrast, these conditions were improved by DMDD and metformin to some degree. These results suggest that after the mice were stimulated continuously with a HFD, hyperinsulinemia emerged and acute islet insulin secretion was obviously impaired, but it was improved by treatment with DMDD and metformin.
An ITT was performed to assess insulin sensitivity (Fig. 5C). Compared with the HFD group 30 min after insulin load, the blood glucose levels of the ND group were reduced significantly, and the blood glucose levels began to rise at 90 min. The DMDD groups showed an obvious improvement in insulin intolerance, and the differences were statistically significant ($P < 0.01$). Sustained HFD feeding resulted in the deterioration of insulin sensitivity [22].

The AUCs corresponding to the three tests were calculated using the trapezoid rule, as they may reflect the function of the pancreatic $\beta$ cells and insulin sensitivity. This parameter has been widely applied in basic research of experimental diabetes as an evaluation index of IR. As shown in Fig. 5D, the AUCs were also significantly decreased by DMDD treatment compared with the HFD group ($P < 0.01$).

Effects of DMDD on serum lipids and inflammatory cytokines

Hyperlipidemia is a characteristic of HFD-induced obesity in mice. It has long been recognized that serum TC, TGs, and FFAs levels are generally elevated in obese individuals. As shown in Fig. 6A, the serum levels of TC, TGs, and FFAs in the DMDD- and metformin-treated mice were markedly lower compared with the HFD group ($P < 0.01$).

The serum IL-6 and TNF-α levels in the mice with HFD-induced obesity and IR were higher than those of the ND group ($P < 0.01$). The levels of these cytokines were decreased by DMDD, but there were no significant differences among the treated groups (Fig. 6B).

Effects of DMDD on oxidant and antioxidant enzyme levels in the liver

Oxidative stress was observed in our study. The model group exhibited a notable elevation in MDA and a reduction in SOD and GSH-Px activity in the liver homogenates (Fig. 7A and 7B). Administration of different doses of DMDD (12.5, 25, or 50 mg/kg of body weight per day) for 4 weeks resulted in an increase in antioxidant activity in the liver homogenates compared with the untreated HFD group.

Effects of DMDD on hepatic steatosis and adipose histology

The liver is also a major target for HFD-induced obesity. As shown in Fig. 8 and Table 2, hepatic steatosis was remarkably improved by treatment with metformin and DMDD.

H&E staining also demonstrated that the adipose cells were enlarged, as there were more fat deposits in the adipocytes of the HFD-fed mice (Fig. 8). However, the mean diameter (Fig. 9A) and mean area (Fig. 9B) of the adipocytes were obviously lowered by metformin and DMDD treatment, and the size of adipocytes in the metformin and DMDD (50 mg/kg) groups was similar to that of the ND group.

Effects of DMDD on the expression of TLR4 and myeloid differentiation factor 88 (Myd88) mRNA

PCR analysis revealed that the mRNA expression levels of TLR4 and Myd88 were upregulated in the adipose tissue of the HFD group compared with the ND group. Following
DMDD treatment, the mRNA expression levels of TLR4 and Myd88 were significantly reduced \( (P < 0.01) \) (Fig. 10).

**Discussion**

T2DM has become a global disease that absorbs major public health resources and places a heavy burden on both industrialized and developing countries [28-30]. Insulin resistance is a prominent feature that is central to the development of T2DM. It decreases the ability of insulin to interact with insulin-sensitive tissues (especially muscle, liver, and fat), impairs glucose utilization, and induces hepatic glucose output [31, 32].

In the present study, our results showed that the administration of DMDD effectively suppressed weight gain and glucose/insulin intolerance induced by HFD, suggesting a protective role of DMDD in diet-induced obesity and insulin resistance. This is in accordance with the previous finding that DMDD could significantly improve blood glucose levels in KKAy mice, a model of type 2 diabetes mellitus.

In addition to the effects on weight gain and glucose/insulin intolerance, DMDD treatment also markedly improved hyperlipidemia. Obesity and IR in animals are thought to be commonly accompanied by increases in TC, TGs, and FFAs [33, 34]. Early studies have shown that FFAs play a crucial role in the development of obesity and IR [35]. FFAs are thought to inactivate insulin receptors in target cells and inhibit the binding of insulin to its receptor [11, 36]. The hypoglycemic effects of DMDD may be related to reductions in lipotoxicity or lipogenesis. As metformin is specialized for the treatment of obesity and diabetes, we speculated that treatment with a combination of metformin and DMDD may have a significant impact on diabetes management.

Previous studies have demonstrated that oxidative stress is also an essential pathogenic factor in the evolution of obesity and IR [37, 38]. Excessive levels of active oxygen are
produced in hyperglycemic conditions, and the levels of MDA are a good indicator of enhanced oxidative stress in tissues because they reflect the process of peroxidation. Among the important antioxidative enzymes, SOD catalyzes the dismutation of harmful superoxide anion radicals into hydrogen peroxide, and GSH-Px both detoxifies hydrogen peroxide and converts lipid hydroperoxides to nontoxic alcohols. In this study, the content of MDA was obviously increased, demonstrating a state of oxidative stress in the mice with HFD-induced obesity and IR. Simultaneously, SOD and GSH-Px activity were significantly decreased in the HFD-fed mice compared with the ND mice, whereas this oxidative injury was inhibited by DMDD treatment. These results suggest that DMDD could be used to protect insulin-resistant mice from oxidative damage.

Furthermore, H&E staining was used to examine the histopathological changes in the liver and adipose tissues. The integrity and lack of lipid deposition in the liver tissue structures were reflected by the morphology of the liver cells. The adipocytes of the ND group were suitable and uniform in size, whereas the adipocytes of the model group varied greatly in size. Nevertheless, the condition was improved by treatment with DMDD and metformin.

More attention has been given to the exploration of the potential mechanisms correlated with the development of obesity and IR. Increasing evidence has shown that excessive glucose and fat, commonly referred to as glucotoxicity and lipotoxicity, are the most important causes of IR. FFAs and adipokines, such as TNF-α, IL-6, and IL-8, play a key role in the development of IR [39, 40]. Furthermore, these inflammatory cytokines activate serine kinases in the insulin signaling pathway to phosphorylate the threonine and serine residues on the insulin receptor substrate (IRS) and PI3K pathway proteins, which restrains insulin-stimulated IRS and PI3K tyrosine phosphorylation and impedes insulin signal transduction leading to IR [28, 41, 42]. Our data demonstrate that DMDD can reduce the activity of serum TNF-α and IL-6 in obese and IR mice and that DMDD inhibits inflammatory cytokine secretion. Nevertheless, it did not affect cytokine activity in healthy mice. Generally, glucose tolerance tests, ITTs, and IRTs are performed to evaluate the function of pancreatic β cells and insulin sensitivity. Previous studies have demonstrated that chronic inflammation suppresses insulin sensitivity via activation of the TLR4 signaling pathways, which directly disturbs the normal function of crucial components of the insulin signaling pathways. Members of the Toll-like receptor (TLR) family, particularly TLR4, play important roles in mediating the deleterious effects that occur during IR. TLR4 is widely expressed in a variety of tissues, such as adipose, liver, muscle, and the pancreas [36, 43]. TLRs have been shown to expedite harmful responses through signaling cascades that involve Myd88. Hence, we hypothesized that the effects of DMDD on obesity and insulin resistance involve the TLR4/Myd88 pathway. Our results indicated that the expression of TLR4 mRNA in adipose tissue was at the baseline level in the ND group, and it was upregulated in the HFD group. In contrast, the expression of TLR4 mRNA was markedly reduced by DMDD treatment compared with the untreated HFD group. The expression of Myd88 signaling pathway mRNA corresponded to the TLR4 mRNA levels. We found that with the decline in the serum levels of FFAs, TNF-α, and IL-6, the activity of TLR4 was suppressed in the IR mice. These results suggest that TLR4 is likely an ideal therapeutic target for IR. Nevertheless, our studies are still preliminary. Therefore, further studies are required to elucidate the TLR4-mediated inflammation signaling pathways in HFD-induced obesity and IR.

Conclusions

In summary, our study demonstrates that DMDD plays a crucial role in improving HFD-induced obesity and insulin resistance. Reductions in the levels of FFAs and inflammatory cytokines can inhibit the TLR4 signaling pathway, which may be an essential modulator of the connection between the inflammatory and metabolic pathways. Furthermore, DMDD may be an attractive choice for the treatment of human obesity and IR.
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Disclosure Statement

The authors declare that there are no conflicts of interest.

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