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Barbaloin Treatment Contributes to the Rebalance of Glucose and Lipid Homeostasis of Gestational Diabetes Mellitus Mice

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Abstract

Aloe vera L has been shown to possess hypoglycemic and hypolipidemic effects on type 2 diabetic patients, and its major benefits may be linked to barbaloin, which is a major component of Aloe vera L. This study focused on investigating the potential effects and underlying mechanisms of barbaloin on gestational diabetes mellitus (GDM). The db/+ diabetic mice with GDM were daily orally administered with barbaloin or metformin during the gestational period. The results demonstrated that administration of barbaloin significantly reduced blood glucose levels and increased insulin levels in GDM mice. We further found that barbaloin treatment reduced inflammatory response and ROS levels in the liver. Finally, we revealed that the AMP-activated protein kinase (AMPK) / peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) signaling pathway was involved in BAT-mediated beneficial effects on mice with GDM. Our study suggested that barbaloin exerted hypoglycemic and hypolipidemic effects on GDM mice, via, at least in part, modulation of AMPK/ PGC-1α signaling in GDM mice.

Keywords

gestational diabetes mellitus, barbaloin, glucose, inflammation, oxidative stress

Introduction

Gestational diabetes mellitus (GDM) is a type of diabetes only diagnosed during the pregnancy of women who do not have a previous diabetes diagnosis.[1] GDM is generally caused by insufficient insulin production in pregnancy of women. It is reported that more than 50% of GDM women might develop type 2 diabetes mellitus in the following 10 years.[2] GDM has adverse impacts on the health of both the mother and infant. Women with GDM are highly associated with maternal birth complications such as gestational hypertension, and pre-eclampsia, as well as increased risk for fetal loss or congenital disabilities.[3] The most recent updated definition of GDM is that diabetes diagnosed in the second or third trimester of pregnancy that was not clearly overt diabetes prior to gestation.[4]

Currently, the hypoglycemic agents, glyburide, and metformin have been approved to be used for GDM treatment. Pregnant women are recommended to minimize their exposure to various medications to avoid potentially harmful side effects to the fetus. However, pharmacologic therapies with metformin (Met), glyburide, or insulin are necessary to reduce the blood glucose level for women with uncontrolled GDM. Nevertheless, daily insulin administration may increase appetites and body weight as well as the risk of hypoglycemia for GMD women.[5,6] The use of metformin or glyburide may cause dermatological and gastrointestinal side effects on GDM women.[7] Thus, alternative safe and effective GDM treatment strategies are urgently needed.

Herbal medicines provide potential safe and valuable sources of therapeutic substances.[8] The plant-based medicines have always been used as natural first aid remedies for thousands of years in China and ancient Egypt and have gained

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growing attention worldwide in new drug research and application fields.[9] Aloe vera L is a medicinal plant that has been shown to exhibit no toxicity to tested subjects and multiple medicinal properties, including anti-bacterial, anti-inflammatory, hypoglycemic and hypolipidemic effects.[10-12] The barbaloin is a natural bioactive anthracycline extracted from Aloe vera L. Similar to Aloe vera L, barbaloin also displays numerous pharmacological activities, such as anti-cancer, anti-inflammation, anti-oxidant and anti-microbial.[13-15] More importantly, several publications have demonstrated that administration of Aloe vera gel exerts hypoglycemic and hypolipidemic effects on mouse models of non-insulin dependent diabetics.[16-18] Although the hypoglycemic and hypolipidemic effects of barbaloin on GDM have never been studied, the barbaloin is a major compound of Aloe vera and we hypothesized that barbaloin may be beneficial for GDM management.

In the current study, we aimed to investigate the potential pharmacological effects of barbaloin on GDM using a well-established db/+ mice model. Because C57BL/KsJ-Lepdb/+ (db/+) mouse can mimic GDM phenotypes and is considered to be an important GDM animal model. The C57BL/KsJ-Lepdb/+ (db/+) mouse, harboring a heterozygous mutation in the leptin receptor gene Lepr, closely mimicked GDM symptoms observed in human patients. At the non-pregnant state, they exhibited largely normal glucose and insulin tolerance until gravidity. During pregnancy, the db/+ females presented typical GDM symptoms including hyperglycemia, insulin resistance and obesity. In our experiment design, we incorporated a metformin treatment group as a positive reference group. Metformin is an anti-hyperglycemic medicine that is used for the treatment of patients with type 2 diabetes and has also been recommended for use in the management of GDM patients.[19] The results of this study may contribute to a better understanding of the effects and underlying mechanism of barbaloin on GDM and may further beneficial for the development of new strategies for the management of GDM patients.

Methods

GDM Mice Model

Barbaloin (purity > 98%) was obtained from J&K Scientific Ltd (Beijing, China), and metformin (Met, purity > 98%) was purchased from Sigma Aldrich (St. Louis, MO, USA). The chemical structure of barbaloin was presented in Figure 1A. Six to eight weeks old C57BL/KsJ+/+ (wild type) and 64 C57BL/KsJ-Lepdb/+ (db/+) mice were purchased from Nanjing Model Animal Institute (Nanjing, China). All mice were retained in specific pathogen free cages with free access to clean water and food. The pregnant wild type (+/+) mice were used as a normal control group (8 rats were used). Female mice were individually mated with males of the same genotype, and mating was confirmed by the presence of a copulatory plug the next morning, which was designated gestation day (GD) 0. Pregnant db/+ mice were randomly divided into 4 groups, and each group contained 8 db/+ mice. The 4 groups were listed as follows: group 1. GDM, group 2. GDM+barbaloin (20 mg/kg),[20] group 3. GDM+barbaloin (50 mg/kg),[15] and group 4. GDM+Met (metformin). Mice from groups 1 to 4 were treated with daily oral gavage administration of DMSO, barbaloin (20 mg/kg), barbaloin (50 mg/kg), and Met (10 mg/kg), respectively. The GDM mice with Met treatment was utilized as a positive treatment control. Because Met is an effective insulin-sensitizing medicine and has been used for GDM management. Thus, GDM mice treated with Met would display reduced GDM phenotypes. GDM mice treated with barbaloin (20 and 50 mg/kg) were our targeted group. The potential beneficial effects of barbaloin and Met on alleviation of GDM phenotypes on db/+ mice were analyzed based on the comparison results between treatment groups and normal or positive control groups.

The mouse non-fasting blood samples were collected on GD 0 and 10 from dorsal pedal vein with a 27G needle. On GD 20, all mice were sacrificed under deep anesthesia (100 mg/kg ketamine, 10 mg/kg acepromazine and 100 mg/kg xylazine) and blood were collected from orbital sinus with capillaries. Liver tissues were also collected and kept in liquid-nitrogen for future analysis. The period for treatment was from the day of pregnancy to the end of the experiment. The body weight of mice from each group was measured on gestation day (GD) 20 using an electronic scale. The animal experimental protocol was approved by the Ethics Committee of Cangzhou Central Hospital.

Enzyme-Linked Immunosorbent Assay (ELISA) and Colorimetric Assay

For mouse blood sample preparation, the non-fasting blood samples collected and were allowed to clot for 2 h at room temperature before centrifuging for 20 minutes at 2000 × g. Remove serum and assay immediately.

The mouse blood glucose level and serum insulin level on GD 0, 10, and 20 were determined via a mouse glucose assay kit (81692) from Crystal Chem (IL, USA), and Insulin mouse ELISA Kit (EMINS) from ThermoFisher Scientific (Waltham, MA, USA) according to manufactory's instruction, respectively.

The mouse serum TNF-α, IL-6, and MCP-1 levels on GD 20 were measured by TNF-α (MTA00B, Mouse TNF-alpha Quantikine ELISA Kit), IL-6 (M6000B, Mouse IL-6 Quantikine ELISA Kit), and MCP-1 (MJE00B, Mouse CCL2/JE/MCP-1 Quantikine ELISA Kit) ELISA kit from R&D Systems (Norcross, GA, USA) according to manufactory's instruction, respectively.

For mouse liver protein sample preparation, the mouse liver tissues were minced to small pieces, and the tissues were homogenized with phosphate-buffered saline (PBS) (10 mg tissue in 100 µl PBS). The homogenate was centrifuged at 1000 × g for 20 minutes. The supernatant was collected carefully. The samples were assayed immediately.

The levels of malondialdehyde (MDA) (ab118970, Abcam, USA), superoxide dismutase (SOD) (ab65354, Abcam, USA), glutathione peroxidase (GPx) (ab102530, Abcam, USA), and glutathione (GSH) (NBP2-68015, NOVUS Biologicals,

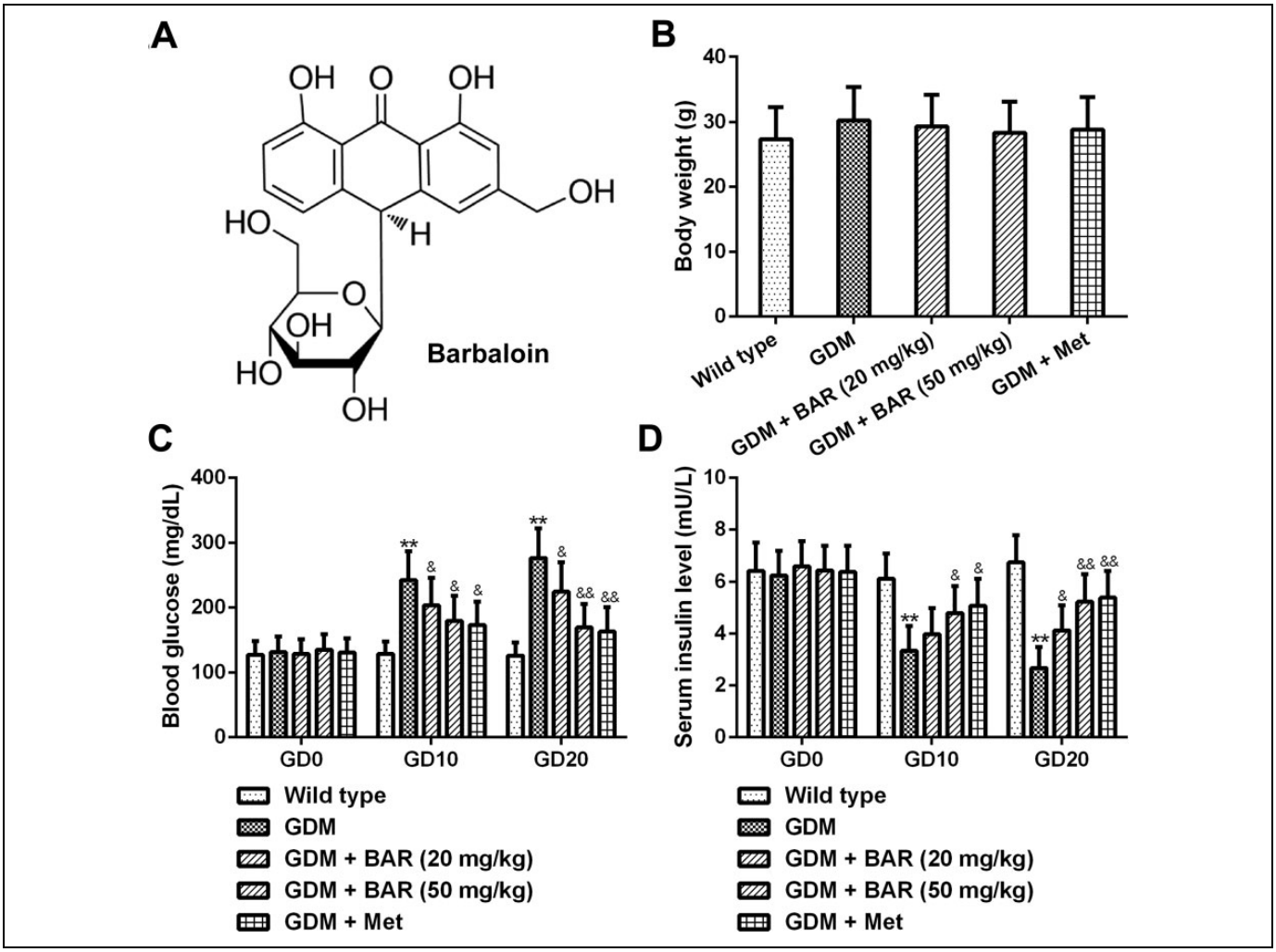

Figure 1. Barbaloin reduces gestational diabetes mellitus phenotypes in pregnant mice model of GDM. (A) Chemical structure of barbaloin. Maternal body weight (B) was measured on gestation day (GD) 20 among different groups. Blood glucose (C) and serum insulin (D) were measured on gestation day (GD) 0, 10 and 20. Data are presented as mean $\pm$ SD. n = 8 for each group. $^{**}p < 0.01$ compared to wild type group, $^{\&}p < 0.05$, $^{\&\&}p < 0.01$ compared to GDM mice.

Littleton, CO, USA) in liver tissue on GD 20 were measured by colorimetric kit according to manufactory's instruction.

Serum Lipid Level Measurement

The non-fasting blood samples collected and were allowed to clot for 2 h at room temperature before centrifuging for 20 minutes at 2000 $\times$ g. Remove serum and assay immediately. The serum levels of cholesterol, triglycerides, low density lipoproteins (LDL), and high-density lipoproteins (HDL) were measured by ILab Chemistry Analyzer 300 PLUS (Instrumentation Laboratory, Bedford, MA, USA).

Quantitative Reverse Transcriptase PCR (qRT-PCR) Analyses

Mouse liver tissue was snap-frozen in liquid-nitrogen and ground tissue into small pieces with 1 ml TRIZOL reagent. Total mouse liver RNA was extracted using the TRIZOL reagent (Invitrogen, Waltham, MA, USA) according to manufactory's instruction. The RNA was reverse transcribed into cDNA using the SuperScript II Reverse Transcriptase Kit (Invitrogen, USA). For gene expression analysis, the qRT-PCR were performed using SYBR Green PCR mastermix (ThermoFisher Scientific, Waltham, MA, USA) and analyzed with an ABI 7500 instrument (Life Technology, Pleasanton, CA, USA). The relative expression levels of target genes were normalized to GAPDH. The primer sequences are shown in Table 1.

Western Blot

The frozen mouse liver tissue was placed into a tube and was ground in the cell lysis buffer with protein inhibitors. After centrifugation, the total protein from the liver tissue was kept in the upper level supernatant. The protein concentration was

Table 1. Primer Sequences Used in Real-Time PCR.

Gene	Primer direction	Sequence (5′-3′)
IL-6	Forward	TCCAGTTGCCTTCTTGGGAC
	Reverse	GTGTAATTAAGCCTCCGACTTG
TNF-α	Forward	CATCTTCTCAAAATTCGAGTGACAA
	Reverse	TGGGAGTAGACAAGGTACAACCC
MCP-1	Forward	GCTCAGCCAGATGCAGTTAA
	Reverse	TCTTGAGCTTGGTGACAAAAACT
GAPDH	Forward	TTCACCACCATGGAGAAGGC
	Reverse	GGCATGGACTGTGGTCATGA

determined using a NanoDrop UV-Vis spectrophotometer (ThermoFisher Scientific). An equal amount of protein samples was loaded on an 8% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel and separated via electrophoresis. The separated proteins were transferred to a PVDF (polyvinylidene difluoride) immobilon-P membrane (Millipore, Billerica, MA, USA). After blocking with 5% non-fat milk, the membranes were probed with primary antibodies in cold-room overnight and followed by incubated with second antibodies. The target protein bands were visualized using an iBind western system (ThermoFisher Scientific). The antibodies against AMPKα (1:1000, #2532, Cell Signaling Technology, Danvers, MA, USA), PGC-1α (1:1000, ab54481, Abcam, USA), and GAPDH (1:1000, #5174) were from Cell Signaling Technology (Danvers, MA, USA).

Statistical Analysis

Data were expressed as mean ± SD. Difference between multiple groups were analyzed by 1-way analysis of variance (ANOVA) followed by Tukey–Kramer test or 2-way analysis of variance (ANOVA) followed Tukey's multiple comparisons test. The $P < 0.05$ was statistically significant.

Results

Barbaloin Decreases Blood Glucose, Increases Insulin Levels, but No Effect on Body Weight of GDM Mice

As illustrated in Figure 1B-D, GDM mice exhibited significantly increased levels of blood glucose, accompanied with an evidently decreased levels of serum insulin as compared to wild type mice (Figure 1C, D). As expected, administration of Met effectively reduced the blood glucose levels, and enhanced serum insulin level in GDM mice on both gestation day (CD) 10 and CD 20. Oral administration of barbaloin markedly reduced glucose level, and increased insulin level in a dose-dependent manner in GDM mice (Figure 1C, D). Noticeably, the high dose of barbaloin (50 mg/kg) treatment exhibited comparable effects with Met treatment on the changes on glucose and insulin levels. The body weight of mice from 5 groups showed no significant difference (Figure 1B). In addition, barbaloin treatment also significantly decreased the level of HOMA-IR (Figure S1a), while significantly increased HOMA-β (Figure S1b), in the GDM mice.

Barbaloin Reduces TCh, TG, and LDL Levels, and Enhances HDL Levels in GDM Mice

Dyslipidemia is defined as serum levels of cholesterol (Ch) higher than 200 mg/dL, triglyceride (TG) and Low-density lipoprotein (LDL) higher than 130 mg/dL, and HDL lower than 40 mg/dL, and is reported to be associated with GMD.[21,22] Upregulation of cholesterol (Ch), triglyceride (TG), and Low-density lipoprotein (LDL) was commonly observed in pregnant women with GDM.[23,24] Indeed, 2-3 folds elevation of TCh, TG, and LDL levels were identified in GDM mice as compared to wild type mice. Similarly, administration of Met or barbaloin exerted strong inhibition effect on GDM-induced upregulation of TCh, TG, and LDL levels in GDM mice (Figure 2A-D). On the contrary, the HDL level was less than 50% in GMD mice compared with that in wild type mice. Met or barbaloin treatment enhanced the HDL level in GMD mice in a dose-dependent manner (Figure 2A-D).

Barbaloin Inhibits Inflammatory Markers Levels in GDM Mice

The disruption of several inflammatory factors (e.g., TNF-α, IL-6, and MCP-1) in the circulation system, placental, and liver compartments are reported to be associated with GDM.[25] Not surprisingly, we detected a upregulation of TNF-α, IL-6, and MCP-1 in the serum and liver of GDM mice when compared to wild type mice. Met or barbaloin treatment significantly reduced the levels of TNF-α, IL-6, and MCP-1 in the serum and liver of GDM mice (Figure 3A-F). We found that the high-dose of barbaloin treatment caused a stronger anti-inflammatory effect than Met treatment (Figure 3A-F).

Barbaloin Decreases Reactive Oxygen Species (ROS) Levels in the Liver of GDM Mice

Elevated levels of ROS are key features of GDM.[26] The levels of MDA, SOD, GPx, and GSH are markers of ROS status. High level of MDA indicates high ROS levels, whereas, high levels of SOD, GPx, and GSH mean sufficient ROS elimination ability and low ROS levels.[27] GDM mice exhibited substantially elevated MDA level, and declined SOD, GPx, and GSH levels. Administration of Met or barbaloin significantly reduced the ROS levels in the liver of GDM mice, as demonstrated by considerably decreasing MDA level and increasing SOD, GPx, and GSH levels in the liver of GDM mice (Figure 4A-D).

Barbaloin Activates AMPKα Signaling in GDM Mice

To investigate whether AMPKα/PGC-1a signaling pathway participates in the barbaloin-mediated beneficial effects on GDM mice. We compared the expression levels of AMPKα and PGC-1α in the liver-tissues from wild type, GDM, and

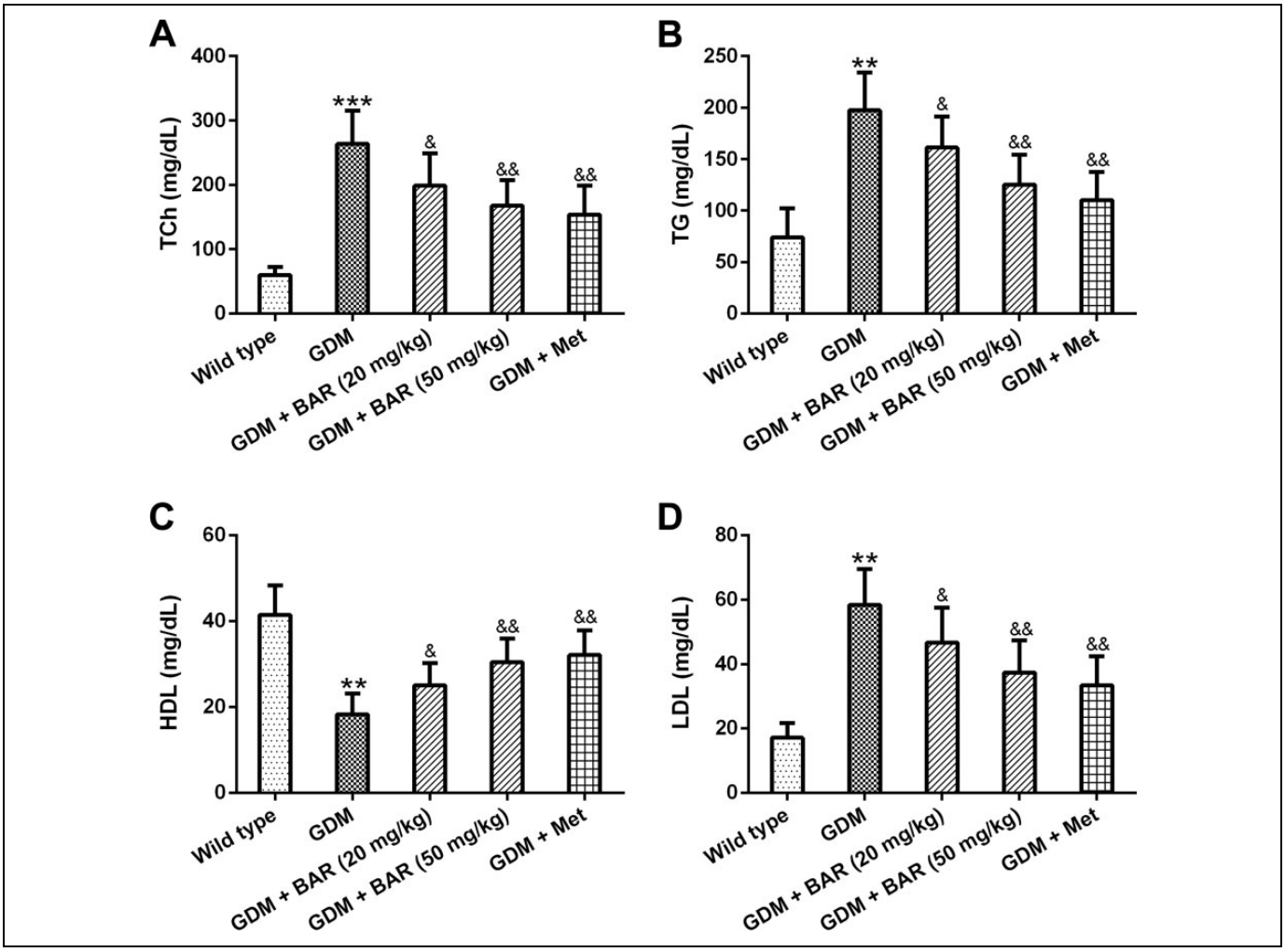

Figure 2. Barbaloin ameliorated biochemical indexes in gestational diabetes mellitus mice in the late stage of pregnancy. Total serum cholesterol (TCh) (A), serum triglyceride (TG) (B), serum high-density lipoprotein (HDL) (C), serum low-density lipoprotein (LDL) (D) were tested on GD 20 among different groups. Data are presented as mean ± SD. n = 8 for each group. $^{**}p < 0.01$, $^{***}p < 0.001$ compared to wild type group, $^{\&}p < 0.05$, $^{\&\&}p < 0.01$ compared to GDM mice.

GDM+barbaloin (50 mg/kg) mice. As shown in Figure 5A, B, the AMPKα and PGC-1α, levels were all markedly suppressed in the liver of GDM mice when compared with those in wild type mice. barbaloin treatment partially enhanced the expression of AMPKα and PGC-1α in the liver of GDM mice (Figure 5A, B). These results suggested that the AMPKα/PGC-1α signaling pathway may play an important role in barbaloin-mediated beneficial effects on GDM mice.

Discussion

The current study was developed to explore the effects of oral administration of barbaloin on the GDM phenotypes in the db/+ mice model of GDM.[28] We, for the first time, found that barbaloin exerted its beneficial effects on mitigation of the GMD phenotypes in db/+ mice through 2 mechanisms. Barbaloin treatment, on the one hand, significantly reduced blood glucose and lipids levels, as well as decreased inflammatory response and ROS levels in the liver of GMD mice, and on the other hand, markedly enhanced insulin secretion, ROS-scavenging proteins expression, and AMPK/PGC-1α signaling activation in the liver of GMD mice.

The db/+ mice are heterozygous mutant for leptin receptor. The leptin receptor plays a critical role in islet growth and beta-cell function. Partially loss of leptin receptor may impair the development and insulin secretion function of beta-cell. In the non-pregnant state, the female db/+ mice exhibit unperturbed glucose homeostasis with normal blood glucose and insulin levels. After pregnancy, the female db/+ mice display hyperphagic feeding behavior leading to the development of GDM phenotypes featured by glucose intolerance and insulin resistance.[29,30]

Indeed, marked upregulation of blood glucose and downregulation of serum insulin level were observed in the bd/+ mice after pregnancy. Numerous studies have revealed that metformin works on reducing intestinal absorption of glucose,

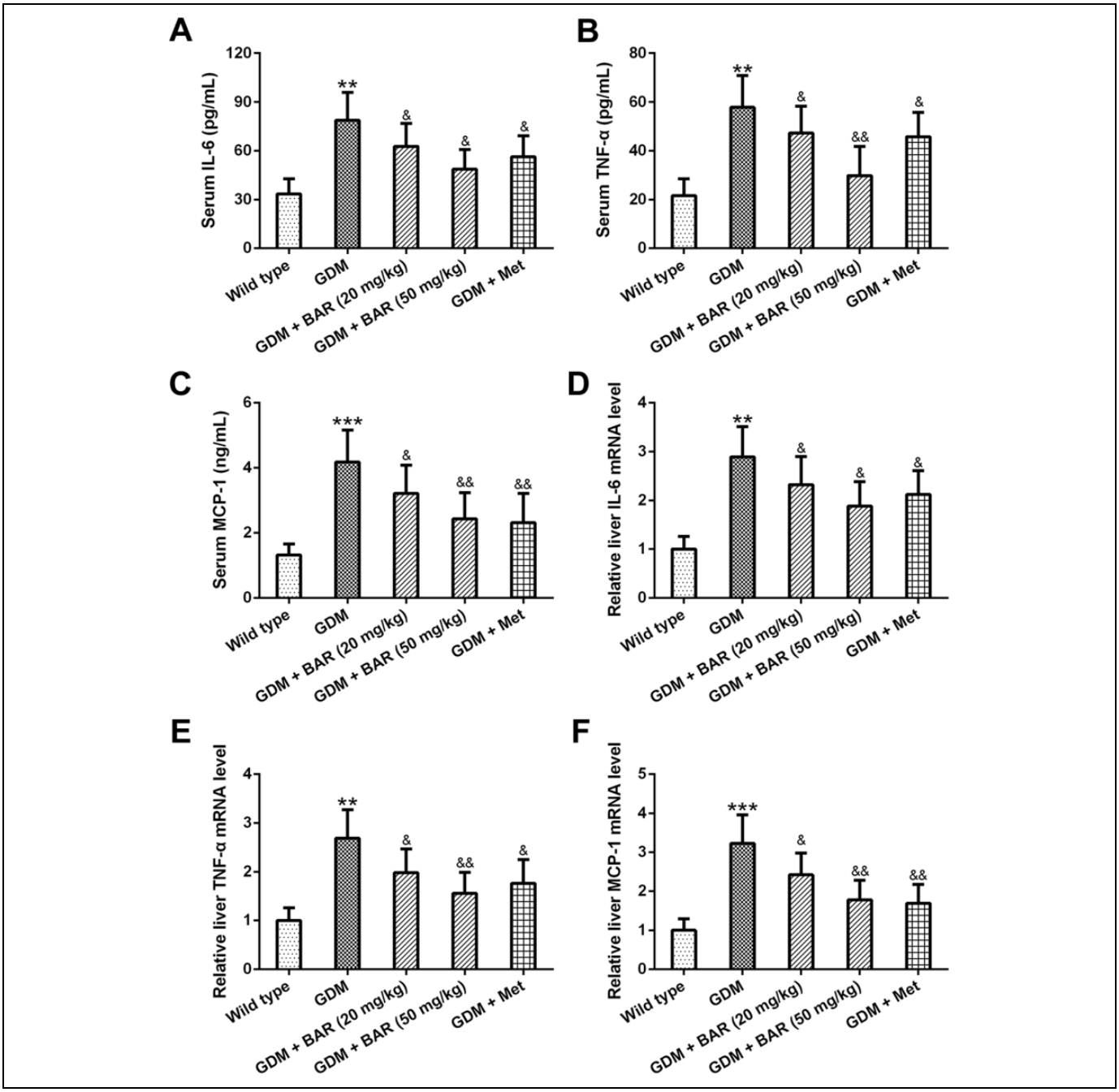

Figure 3. Barbaloin decreased the inflammatory response in gestational diabetes mellitus mice. Serum IL-6 (A), TNF-α (B) and MCP-1 (C) concentrations were measured by ELISA on GD 20 among different groups. qRT-PCR was used to analyzed the mRNA levels of IL-6 (D), TNF-α (E) and MCP-1 (F) in liver tissue on GD 20 among different groups. GAPDH was set as a loading control and the relative expressions were normalized to wild type. Data are presented as mean ± SD. n = 8 for each group. $^{**}p < 0.01$, $^{***}p < 0.001$ compared to wild type group, $^{\&}p < 0.05$, $^{\&\&}p < 0.01$ compared to GDM mice.

promoting peripheral glucose utilization, and enhancing insulin sensitivity.[31,32] Oral administration of barbaloin or Met during the gestation potently alleviated the phenotypes of glucose intolerance and improved insulin resistance through the rebalancing of blood glucose level and stimulation of insulin secretion. Barbaloin or Met treatment did not change the body weight of mice or cause death suggesting barbaloin or Met might be a safe and effective agent for GMD management.

Normal lipid metabolism is important for the health of maternity and fetus. However, maternal glucose and lipid metabolism were usually impaired in women with GDM.[33] Thus, the elevation of lipids (e.g., cholesterol, triglycerides, and LDL), and reduction of HDL are commonly observed in GDM

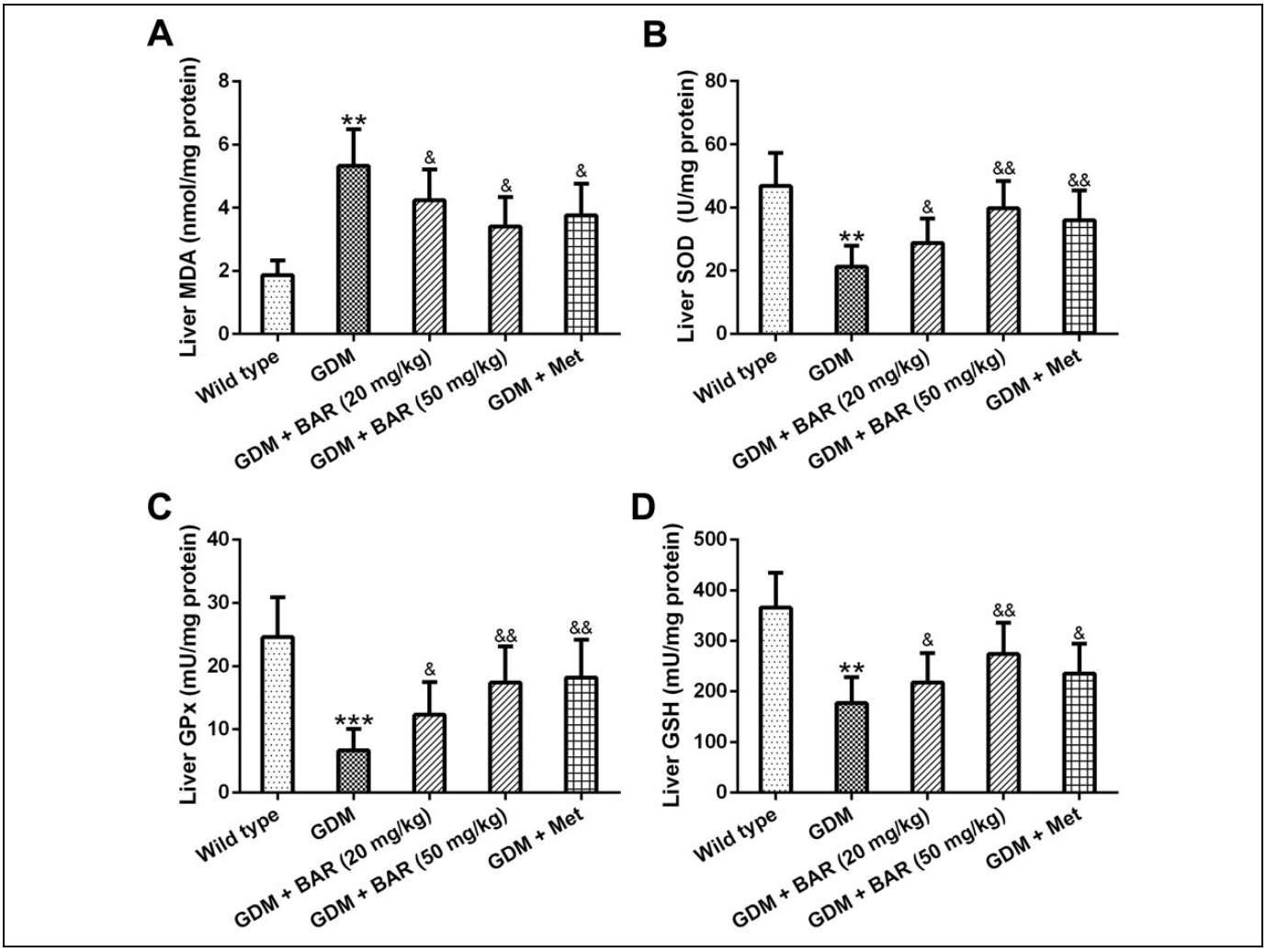

Figure 4. Barbaloin decreased the ROS levels in gestational diabetes mellitus mice. Malondialdehyde (MDA) (A), superoxide dismutase (SOD) (B), glutathione peroxidase (GPx) (C), glutathione (GSH) (D) in liver tissue were measured by ELISA on GD 20 among different groups. Data are presented as mean $\pm$ SD. n = 8 for each group. $^{**}p < 0.01$, $^{***}p < 0.001$ compared to wild type group, $^{\&}p < 0.05$, $^{\&\&}p < 0.01$ compared to GDM mice.

women.[34] Our bd/+ mice model also presented maternal dyslipidemia with increased cholesterol, triglycerides, and LDL levels and decreased HDL level. Oral administration of barbaloin can alleviate dyslipidemia in GDM mice through the downregulation of TCh, TG, and LDL levels as well as upregulation of HDL level.

In addition, GDM is reported to be associated with chronic and low-grade inflammation and oxidative stress.[25,26] Inflammation is a mediator of the programming of maternal glucose and lipid metabolism and also involved in the development of pregnancy complications. Studies have shown that GDM is linked to the upregulation of proinflammatory cytokines, such as TNF-1α, IL-6, and chemokines, such as MCP-1. For example, TNF-α is correlated with insulin resistance, and TNF-α antagonist contributes to reverse insulin sensitivity.[35] Similarly, enhanced IL-6 production, particularly by placenta, is also linked to insulin resistance.[36] The MCP-1 is a key chemokine that promotes the migration and infiltration of macrophages. The increased infiltration of inflammatory M1-type of macrophages in the pancreas and adipocytes during pregnancy also cause an increase in TNF-α, IL-6, and MCP-1 production.[37]

Oxidative stress is to describe the oxidative state in cells, tissues, or organs, which is raised by reactive oxygen species. The production of ROS comes from the metabolic reaction of glucose and lipid within mitochondria. GDM women with metabolic perturbations experienced increased ROS production and anti-oxidant defense system depletion. SOD, GPx, and GSH are the central endogenous enzymatic systems that can directly scavenge those harmful free radicals, which include singlet oxygen, hydrogen peroxide, superoxides, and hydroxyl anions.[38] Consistent with the typical GDM phenotypes, we also observed excessive inflammatory markers (TNF-α, IL-6, and MCP-1) expression as well as the exhausted anti-oxidant system, as demonstrated by markedly reduced levels of SOD, GPx, and GSH in the liver of GDM mice. We found that barbaloin-treated GDM mice exhibited a reduction of

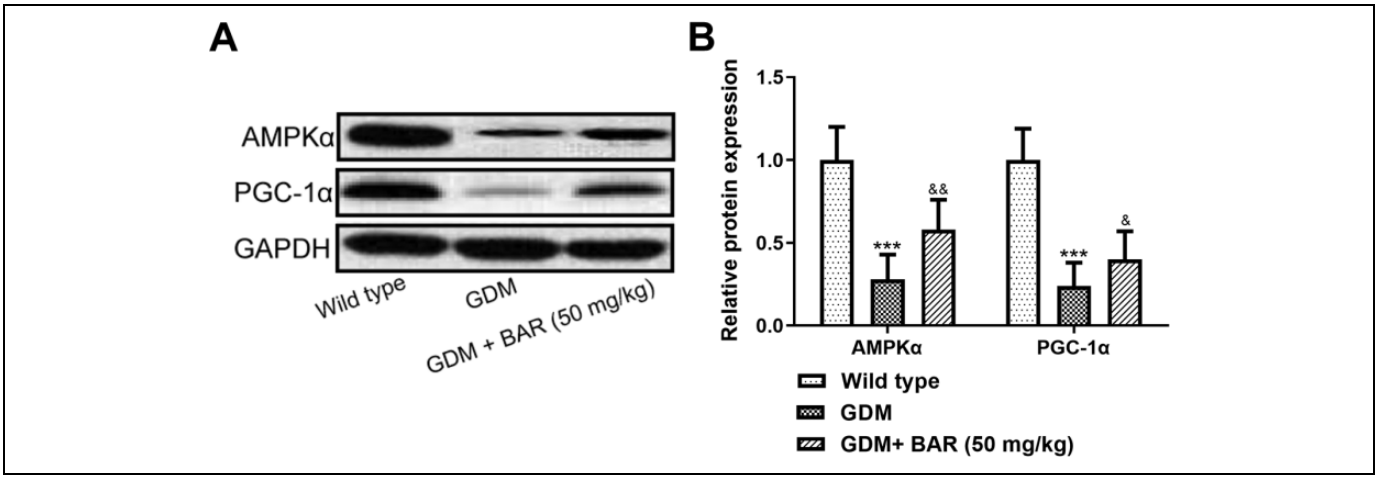

Figure 5. Barbaloin activates hepatic AMPK pathway in gestational diabetes mellitus mice. A, the expressions of AMPKα, PGC-1αin liver tissue were measured by Western blot on GD 20. GAPDH was set as a loading control and the relative expressions were normalized to wild type (B). Data are presented as mean ± SD. n = 8 for each group. $^{**}p < 0.01$, $^{***}p < 0.001$ compared to wild type group, $^{\&}p < 0.05$, $^{\&\&}p < 0.01$ compared to GDM mice.

pro-inflammatory markets (e.g., IL-6, TNF-α, and MCP-1), and MDA level as well as a marked increase of SOD, GPx, and GSH levels in the liver of GDM mice.

Studies have reported that activation of the AMPKα signaling pathway was an important compensatory mechanism to protect the heart against I/R-induced cardiomyocytes injury.[39] Knockdown of AMPKα resulted in depletion of ATP homeostasis in cardiomyocytes and transformed it more sensitive to myocardial injury.[40] Moreover, AMPK/PGC-1α signaling pathway activation has been shown to play an essential role in some Chinese herbal agents regulated recovery of glucose homeostasis and metabolism of diabetes mellitus.[41-43] Indeed, a recent study showed that pretreatment of barbaloin enhanced AMPK signaling activation resulted in cardio-protection effect against ischemia/reperfusion-induced injury.[20] Consistent with these findings, we observed that barbaloin treatment partially enhanced the expression levels of AMPK and PGC-1α in the liver of GDM mice.

Conclusion

Our results demonstrated that administration of barbaloin contributed to the rebalance of glucose and lipid homeostasis of GMD mice (Figure S2), suggesting that barbaloin might serve as a potential medicine for management of patients with GMD.

Declaration of Conflicting Interests

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Supplemental Material

Supplemental material for this article is available online.

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