Original Paper

Therapeutic Effects of *Coccomyxagloeobotrydiformis* on the Metabolic Syndrome in Rats

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Key Words

Metabolic Syndrome • Cardiovascular Disease • Mitochondrial Respiratory Chain • Oxidative Stress

Abstract

Background/Aims: The metabolic syndrome (MS) is a cluster of metabolic changes that carry a high risk of cardiovascular disease (CVD). A newly discovered microalga, *coccomyxagloeobotrydiformis* (CGD), has been reported to improve ischemic stroke and metabolism-related indicators. We observed the therapeutic effects of CGD on MS and postulated the underlying mechanism. **Methods:** A diet-induced MS model in rats was used to observe the therapeutic effects of CGD on MS. Blood-glucose and lipid indices were measured using enzymatic colorimetric kits. A biologic data acquisition and analysis system (BL-420F) was used to evaluate cardiac function. Expression of mitochondrial respiratory chain (MRC) enzymes was measured by immunofluorescence staining. The proteins associated with oxidative stress, apoptosis and inflammation were detected by western blotting. **Results:** Body weight, abdominal circumference, fasting blood glucose, blood pressure as well as serum levels of total cholesterol, triglycerides and low-density lipoprotein-cholesterol were decreased whereas serum levels of high-density lipoprotein-cholesterol was increased in CGD-treated MS rats. CGD increased left-ventricular systolic pressure, left-ventricular end-diastolic pressure, left-ventricular systolic pressure maximum rate of increase and left-ventricular diastolic pressure maximum rate of decrease in MS rats with cardiovascular complications. CGD up-regulated expression of adenosine monophosphate-activated protein kinase and peroxisome proliferator activated receptor gamma coactivator 1-alpha in the heart, adipose

tissue and skeletal muscle. Expression of the MRC subunits of ATPase 6, cytochrome b and succinate dehydrogenase complex, subunit-A was increased whereas that of uncoupling protein-2 decreased in different tissues. CGD showed anti-oxidation effects by increasing expression of superoxide dismutase and decreasing that of malondialdehyde. High expression of Bcl-2 and low expression of Bax and caspase-3 supported the anti-apoptotic effect of CGD on the cardiovascular complications of MS. **Conclusion:** CGD has a therapeutic effect on MS and associated cardiovascular complications by eliciting mitochondrial protection and having anti-oxidation and anti-apoptosis effects. CGD could be used for MS treatment.

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Introduction

An indolent lifestyle and excessive intake of calories make the metabolic syndrome (MS) a truly global epidemic [1]. There are several definitions of MS, but they mostly describe it as a cluster of metabolic changes such as abdominal obesity, dyslipidemia, hypertension, hyperglycemia, insulin resistance and cardiovascular disease (CVD) [2–4]. The morbidity and mortality of the MS has increased gradually over recent years, and it causes ≈17.3 million deaths per year worldwide. By 2030, this number is expected to be over 23.6 million [5, 6]. Molecular biologic alterations in the mitochondrial genome, tissue damage due to oxidative stress, apoptotic stimuli, and low-grade inflammation have been shown to be important mechanisms of MS [7–9]. The most direct consequence of MS is the increased morbidity and mortality of CVD, which is the primary cause of death for people suffering from obesity and type-2 diabetes mellitus (T2DM) [10–12]. CVD is also the leading cause of death worldwide [13]. Studies aimed at discovering compounds that could be useful in the prevention/treatment of MS and its cardiovascular complications have been carried out. *Coccomyxa gloeobotrydiformis* (CGD) is species of green algae containing ≈24% α-linolenic acid (ALA). Increased intake of ALA has been demonstrated to show improvements in reperfusion and a reduced risk of middle cerebral artery occlusion (MCAO)-induced mortality and infarct volumes [14]. It has been reported that CGD can relieve the risk factors for stroke, including hypertension, DM and hyperlipidemia [15]. Moreover, previously we revealed that CGD could lower the prevalence of ischemic stroke and aging-related memory impairment, possibly by inducing mitochondrial protection and having anti-inflammatory and anti-apoptotic effects, which are the primary mechanisms of MS [16, 17]. If its cardiovascular-protective effects could be confirmed further, CGD could be worthy of further investigation for the prevention/cure of the MS and its cardiovascular complications. We adopted a well-established method of diet-induced MS to observe the therapeutic effects of CGD on MS and cardiovascular complications. We attempted to ascertain the underlying mechanism of action of CGD on MS and cardiovascular complications from the viewpoints of cell metabolism as well as anti-inflammatory, anti-oxidation and anti-apoptosis properties.

Materials and Methods

Ethical approval of the study protocol

This animal study was approved by the Animal Care and Use Committee of China Medical University (Shenyang, China). All animal experiments were carried out in accordance with the *Guide for the care and use of laboratory animals* (National Institutes of Health, Bethesda, MD, USA). All surgical procedures were undertaken under anesthesia, and all efforts were made to minimize pain and suffering to animals.

Animals

A diet-induced MS model in rats was employed. Sprague–Dawley rats (8 weeks; 100–110 g; Experimental Animal Center of China Medical University) were maintained in cages at 21–22°C on a 12-h light–dark cycle. Sixty rats were classified into two groups randomly. Ten rats (controls) were given a diet of standard laboratory chow. The remaining 50 rats were fed a high-energy diet consisting of 20% lard,

10% sugar, 10% dry milk solution, 2% cholesterol, 0.5% cholate, 5% salt, and 52.5% standard laboratory chow for 16 weeks. Abdominal circumference (AC) and body weight (BW) were measured every day and water was available continually. The 50 rats were divided into three groups: negative control (NC), MS, and MS with cardiovascular complications (MS+CVD). CGD (100 mg/kg body weight) was supplied by Nikken Sohonsa (Tokyo, Japan) and administered by gavage for 12 weeks; an identical volume of physiologic (0.9%) saline was administered as control.

Biochemical assays and sample preparation

Whole blood was collected from the tail vein of rats at the end of the 16th week in the fasting state. Serum was obtained by centrifugation at 1000 ($\times g$) for 15 min at room temperature. Levels of fasting blood glucose (FBG), total cholesterol (TC), triglycerides (TG), low-density lipoprotein-cholesterol (LDL-C) and high-density lipoprotein-cholesterol (HDL-C) were measured using enzymatic colorimetric kits (BHKT Clinical Reagents, Beijing, China). Blood pressure (BP) was also measured in the resting state. Then, all rats were anesthetized using 10% chloral hydrate (300 mg/kg, i.p.) and killed by decapitation. The liver, heart, adipose tissue and leg muscles were removed and divided into four sections. One-quarter was frozen immediately on dry ice, and then stored at -80°C for extraction of protein and RNA. One-quarter was stored in formalin for immunohistochemical analyses. One-quarter was cut into 1-mm³ cubes and fixed in 2.5% glutaraldehyde for observation by electron microscopy. The remaining one-quarter was stored at -80°C as a backup sample.

Cardiac function test

The indices of cardiac function, left-ventricular systolic pressure (LVSP), left-ventricular diastolic end pressure (LVEDP), maximal rate of increase of left-ventricular pressure ($+dp/dt_{\max}$) and minimal rate of decrease of left-ventricular pressure ($-dp/dt_{\max}$), were measured using a biologic data acquisition and analysis system (BL-420F; Cheng Du TaiMeng, Beijing, China).

Western blotting

Western blotting was done as described previously [18]. The antibodies used for western blotting were: phosphor-5'-adenosine monophosphate-activated protein kinase (AMPK; Thr172; 1:500 dilution); peroxisome proliferator activated receptor gamma coactivator 1-alpha (PGC-1 α ; 1:500); tumor necrosis factor (TNF)- α ; 1:1000; superoxide dismutase (SOD; 1:1000); malondialdehyde (MDA; 1:1000); B-cell lymphoma (Bcl)-2; 1:500; Bax (1:500), which were all from Cell Signaling Technologies (Danvers, MA, USA), and caspase-3 (1:500), which was from Abcam (Cambridge, UK).

Immunofluorescence staining

Formalin-fixed, paraffin-embedded tissues were cut into 5 μm -thick sections, placed on Superfrost™ glass slides (Fisher Scientific, Pittsburgh, PA, USA) and heated at 56°C overnight. Tissues were deparaffinized in xylene for 30 min and rehydrated in ethanol, then incubated in 0.3% H_2O_2 in methanol for 20 min. To unmask antigens, slides were incubated in 1 mM EDTA (pH 8.0), heated to $98-100^{\circ}\text{C}$ for 15 min, and then cooled down slowly to room temperature. Tissues were incubated in 5% goat serum for 30 min at room temperature to block non-specific-binding sites followed by incubation with the respective primary antibodies (ATPase 6, 1:500 dilution; cytochrome b, 1:500; uncoupling protein (UCP)2, 1:500; succinate dehydrogenase complex, subunit-A (SDHA), 1:500; tropomodulin (TMOD)1, 1:500; Abcam) in 5% goat serum at 4°C overnight. The primary antibody was washed thrice in phosphate-buffered saline (PBS), followed by incubation with Cy3-fluorescent secondary antibody (1:100 dilution in PBS) for 60 min at room temperature, washed in PBS and incubated with 4',6-diamidino-2-phenylindole. Tissues were washed in PBS and coverslips mounted. Finally, images were taken with a fluorescence microscope (BX50F; Olympus, Tokyo, Japan) using a XC30 camera and cellSens™ software (Olympus).

Statistical analyses

Data are the mean $\pm$ SD of at least three independent experiments. Statistical analyses were carried out by analysis of variance. $p < 0.05$ was considered significant.

Results

Variations in the biochemical parameters of MS rats

A diet-induced MS model in rats was created. Levels of FBG, TC, TG, HDL-C and LDL-C were measured using enzymatic colorimetric kits. The physiologic indicators of this MS model are listed in Fig. 1. At 16 weeks, 40 rats that met the criteria of MS were chosen for subsequent experiments.

Effects of CGD on variations in biochemical parameters on MS

BW, AC, BP, FBG levels and blood-lipid parameters were measured accordingly in NC, MS and MS+CGD groups. BW, AC, FBG level and BP were decreased in the CGD-treated group compared with the MS group ($p < 0.05$). The level of TC, TG and LDL-C decreased whereas that of HDL-C increased in the CGD-treated group compared with the MS group ($p < 0.05$), which suggested that CGD had therapeutic effects on MS (Fig. 2).

Expression of the AMPK signaling pathway in different tissues

To investigate the underlying mechanism of the therapeutic effects of CGD on MS, we measured expression of AMPK and PGC-1 α in the heart, liver, adipose tissue and skeletal muscle in MS rats. The AMPK/PGC-1 α pathway serves as a "sensor" that has a critical role in regulating mitochondrial function and energy metabolism. Reduced activity of AMPK has been noted in obese mice and is associated with diminished uptake of glucose into skeletal muscle cells [19]. We found that CGD could up-regulate expression of p-AMPK and PGC-1 α in the heart, adipose tissue and skeletal muscle of MS rats ($p < 0.05$) (Fig. 3). Such increased expression could be an underlying mechanism of the therapeutic effects of CGD on MS.

Expression of mitochondrial respiratory chain (MRC) enzymes

The function of the MRC is biologic oxidation coupled with phosphorylation and then complete oxidative phosphorylation (OXPHOS) to produce adenosine triphosphate (ATP). A deficiency or defect of the coenzymes that participate in OXPHOS reduces the efficiency of mitochondrial OXPHOS and results in disorders of the metabolism of glucose and lipids as well as ATP insufficiency. Expression of the MRC subunits ATPase 6, cytochrome b and SDHA in the heart, liver and muscles was increased whereas that of UCP2 was decreased in the CGD-treated group. There were no obvious changes in levels of these coenzymes in adipose tissue (Fig. 4).

Fig. 1. The physiological indicators of metabolic rats model. A. Body weight (g); B. Abdominal circumference (cm); C. Systolic pressure (mmHg); D. Fasting blood glucose (mmol/L); E. Blood lipid (mmol/L). All of these indicators showed significant difference in 16-week compared with 0-week ($*P < 0.05$).

Fig. 2. The variations of physiological and biochemical criteria of MS rats by CGD treatment. A~D. Body weight, abdominal circumference, systolic pressure and fasting blood glucose decreased in the CGD treated MS rats compared with MS group ($*P < 0.05$); E. TC, TG and LDL-C decreased while the HDL-C increased in the CGD treated MS rats compared with MS group ($*P < 0.05$).

Fig. 3. Western blot analysis of AMPK signaling pathway in each group. P-AMPK and PGC-1 α both increased in heart, adipose and skeletal muscle and no obvious change in liver in CGD treatment MS rats compared with untreated MS rats. β -actin was used as protein loading control (* $P < 0.05$).

Fig. 4. Immunofluorescence staining analyses of mitochondrial respiratory chain enzymes in each group. A,C,D. Fluorescence intensity of ATPase 6, cytochrome b and SDHA increased in the heart, liver and muscle in the CGD treatment group; B. Fluorescence intensity of UCP2 decreased in the heart, liver and muscle in the CGD treatment group. There was no significant change of them in the adipose tissue. The original magnification: $\times 400$. Scale bar = 200 μ m.

Cardiac function in the MS with CGD treatment group

Twenty MS rats were diagnosed with cardiovascular complications. Here, we had four groups of 10: NC, MS, MS+CVD, and MS+CVD with CGD treatment. Indicators of cardiac function were measured. CGD increased the LVSP, LVDEP and $\pm dp/dt_{max}$ in the MS+CVD group ($p < 0.05$), which suggested that CGD improved the cardiac function of MS rats with cardiac complications (Fig. 5).

Expression of TMOD1 in the hearts of MS rats

TMOD1 is a member of the TMOD family located in skeletal and cardiac muscle cells [20]. It regulates the thin filament lengths and dynamics of actin, and is a major isoform essential for normal cardiac function [21–23]. Studies have revealed the antagonistic function of TMOD1 in modulating thin filament lengths in cardiomyocytes [24, 25]. Analyses of several TMOD1-knockout mouse lines have revealed that targeted deletion of TMOD1 is embryonic-lethal, with embryos dying due to defects in the development and function of the heart [26, 27]. We examined the effect of CGD on TMOD1 in the myocardial tissues of MS+CVD rats by immunofluorescent staining. TMOD1 expression was increased greatly in the CGD-treated group compared with the control (Fig. 6). Hence, CGD may have protective effects on the cardiovascular complications of MS.

Fig. 5. The alterations of cardiac function indicators. LVSP, LVDEP and $\pm dp/dtmax$ were all decreased in MS with CVD group compared with MS rats ($*P < 0.05$) and increased in MS with CVD and CGD group compared with MS with CVD rats ($\#P < 0.05$).

Fig. 6. The expression of TMOD1 in the heart of MS with cardiovascular complications rats. Fluorescence intensity of TMOD1 was increased in the CGD treatment group. The original magnification: $\times 400$. Scale bar = $200\mu m$.

Expression of TNF- α , SOD and MDA in rat hearts

We measured expression of TNF- α , SOD (a free radical-scavenger) and MDA (an index for lipid peroxidation in myocardial tissues) of MS+CVD rats by western blotting. Application of physiologic saline (NS group) was used as the control of CGD treatment. CGD could increase expression of SOD and reduce that of MDA and TNF- α ($p < 0.05$). CGD could defend against oxidative damage and inflammation in the cardiomyocytes of MS rats with cardiovascular complications (Fig. 7). This result suggested that CGD might protect cardiomyocytes as an antioxidant and anti-inflammatory agent.

Expression of Bcl-2, Bax, and caspase-3 in rat hearts

We measured expression of Bcl-2, Bax, and cleaved caspase-3 in the myocardial tissues of MS+CVD rats by western blotting. Application of physiologic saline (NS group) was used as the control of CGD treatment. The anti-apoptotic protein Bcl-2 and pro-apoptotic protein Bax are crucial determinants of the apoptotic response and also control caspase-3 release. Expression of Bcl-2 was higher and that of Bax was lower. Expression of cleaved caspase-3 decreased significantly in the CGD-treated group ($p < 0.05$) (Fig. 8).

Fig. 7. Western blot analysis of TNF- α , SOD and MDA in the heart of MS with cardiovascular complications rats. CGD decreased the expression of TNF- α and MDA and increased the level of SOD1 and SOD2 in heart tissue of MS with CVD. β -actin was used as protein loading control (* $P < 0.05$).

Fig. 8. Western blot analysis of Bcl-2, Bax, caspase-3 and cleaved caspase-3 in the heart of MS with cardiovascular complications rats. CGD increased the expression of Bcl-2 and decreased the level of Bax, and cleaved caspase-3 in heart tissue of MS with CVD. There was no obvious change of caspase-3. β -actin was used as protein loading control (* $P < 0.05$).

Discussion

MS, as a major challenge to public health, has attracted considerable attention in recent years [28]. The main consequence of MS is that it increases the risk of CVD and T2DM. Adequate control of MS might reduce the risk of morbidity and mortality of these diseases, and thereby extend the lifespan of the general population. Therefore, a safe and efficacious agent for the prevention and treatment of MS is needed.

The present study showed that CGD improved all biochemical parameters and had a therapeutic effect on MS rats due (at least in part) to its effect on cellular metabolism. Expression of three key MRC coenzymes, ATPase 6, cytochrome b and SDHA, was up-regulated by CGD. ATPase 6 is a mitochondrial gene encoding ATP synthase, which is a subunit of MRC complex V [29]. Cytochrome b is a component of complex III and SDHA acts as a major catalytic subunit of complex II of the MRC [30, 31]. They are all key components of the mitochondrial electron transfer system, participate in OXPHOS, and contribute to ATP production. Expression of UCP2, a member of a large family of mitochondrial anion carrier proteins, was down-regulated by CGD. It has been reported that UCP2 can induce deficiency of ATP synthesis by reducing the mitochondrial membrane potential and uncoupling OXPHOS [32]. Our results suggested that CGD could improve energy metabolism in mitochondria, the center of cellular metabolism. To further clarify the therapeutic mechanisms of CGD on MS, we also measured expression of AMPK and the transcriptional co-activator PGC-1 α . AMPK can regulate mitochondrial biogenesis and mitophagy [33–36]. AMPK activation helps to restore homeostasis of physiologic energy [37]. PGC-1 α is a potent regulator of several metabolic pathways including, in particular, activation of OXPHOS and mitochondrial biogenesis. Recent evidence has suggested that increasing PGC-1 α activity might have beneficial effects in various disorders, including MS [38]. In the present study, expression of p-AMPK and PGC-1 α was up-regulated in the heart, adipose tissue and skeletal muscle. Therefore, CGD could have more extensive application prospects for other metabolism-related diseases.

MS can trigger the onset and development of CVD through low-grade inflammation, oxidative stress, apoptotic stimuli and mitochondrial damage. Based on our previous studies, we investigated the underlying mechanism of the cardiovascular-protective effects of CGD. Three mechanisms could be postulated.

First, CGD could decrease expression of the pro-inflammatory cytokine TNF- α in the cardiomyocytes of MS rats with cardiovascular complications. Experimental, epidemiologic and clinical evidence in the past decade has shown that metabolic diseases and their complications are closely associated with chronic inflammation [39]. Adipose tissue has emerged as a major player in regulation of the inflammatory response [40]. TNF- α is highly expressed in the adipose tissue of obese mice and humans, which provides the first clear link between obesity, DM and chronic inflammation [41]. TNF- α can cause the production of pro-inflammatory cytokines but also trigger cell signaling that may lead to arteriosclerosis and lipid peroxidation [42]. In the present study, CGD decreased TNF- α expression and acted as an anti-inflammatory agent to protect cardiomyocytes.

Second, oxidative stress, along with chronic inflammation, can contribute to the development of metabolic disorders and lead to CVD. We showed that CGD increased expression of SOD (free radical-scavenger) in the cardiomyocytes of MS rats with cardiovascular complications. Simultaneously, CGD could defend against oxidative damage by reducing expression of MDA, which suggested that CGD might protect cardiomyocytes as an antioxidant agent.

Third, CGD had a therapeutic role by regulating cardiomyocyte apoptosis. Studies have suggested that the apoptotic network is incorporated into multiple physiologic processes in cells, including metabolism [43]. Bcl-2 family members maintain mitochondrial respiration by controlling the integrity of the outer and inner membranes of mitochondria. High expression of Bcl-2 can protect against oxidative stress [44]. Members of the Bcl-2 and Bax families localized or targeted to the outer mitochondrial membrane can promote or prevent permeabilization of the mitochondrial outer membrane [45, 46]. Caspase-3 is a

cysteine protease that mediates apoptosis. We demonstrated that CGD promoted expression of the anti-apoptotic factor Bcl-2, inhibited expression of the pro-apoptotic factor Bax, and decreased expression of cleaved caspase-3, which suggested that CGD could inhibit cardiomyocyte apoptosis.

The three possible mechanisms mentioned above are related to the mitochondrion, the hub of cell metabolism, inflammation, oxidative stress and apoptosis. We found that CGD could also protect mitochondrial function by optimizing expression of several key coenzymes of OXPHOS in cardiomyocytes.

Conclusion

CGD could improve most of the metabolic parameters and cardiac function of MS rats due (at least in part) to its effects on energy metabolism, anti-inflammation, anti-oxidation, anti-apoptosis and mitochondrial protection. In clinical practice, most MS patients are treated with a combination of drugs targeting a single component of MS. Clinical efficacy is blunted due to the inevitable drug toxicity and side effects. As a naturally produced drug with minimal side-effects, CGD might be a promising candidate for MS considering its multiple-target effects achieved by the multiple mechanisms mentioned above. Even though the present study was based on rats, it could provide clues for the clinical prevention and control of MS and its cardiovascular complications.

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Disclosure Statement

None of the authors has any conflicts of interest with regard to this research.

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