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Corosolic acid improves erectile function in metabolic syndrome rats by reducing reactive oxygen species generation and increasing nitric oxide bioavailability

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Abstract

To investigate the effect of corosolic acid treatment on erectile function in metabolic syndrome induced rat model. Fifty male 3-week-old SD rats were fed a high fat and high sugar diet. Six months later, metabolic variables were determined. Metabolic syndrome induced erectile function (MED) rats were confirmed by an apomorphine test. Then MED rats were treated with corosolic acid daily by oral gavage for 4 weeks. To evaluate erectile function, intracavernosal pressure (ICP)/mean arterial blood pressure (MAP) ratio was measured. Thiobarbituric acid reactive substances assay and dihydroethidium staining were used to assess reactive oxygen species (ROS) level. Protein expressions of $gp91^{phox}$ and eNOS were examined by western blotting and immunohistochemistry. Fasting blood glucose, body weight, total cholesterol and insulin were markedly increased in metabolic syndrome rats compared with those of the control rats ($p < 0.05$). The ratios of max ICP/MAP and area under curve (AUC)/MAP was markedly reduced in MED rats compared with the control rats ($p < 0.05$). The concentration of cyclic guanosine mono-phosphate (cGMP) and the expression of eNOS were significantly decreased in MED rats compared with the control group ($p < 0.05$). Moreover, ROS level and the expression of $gp91^{phox}$ were significantly increased in MED rats. Treatment with corosolic acid reversed these changes (each $p < 0.05$). Corosolic acid reduces the level of ROS, ameliorating endothelial dysfunction and improvement of erectile function in MED rats.

Keywords: corosolic acid; MED; ROS; bioavailability.

Practical Application: MetS, a cluster of metabolic derangements that are major risk factors for vascular disease, is one of the important threats to individuals with erectile dysfunction (ED). We investigated the mechanism of MED and the effect of corosolic acid treatment on erectile function in the MED rat model. We found that erectile function in MED rats was markedly reduced and corosolic acid treatment attenuated the reduction, while ROS were upregulated and that eNOS expression was obviously reduced. The corpus cavernosum cGMP concentration was consistently decreased in MED rats. These data show that upregulation of ROS is involved in MED and that downregulation of ROS might be an appropriate therapeutic option for ED patients with MetS. This study determines the underlying mechanisms of MetS related ED (MED) and may provide novel therapeutic options to the clinical treatment of patients with MED.

1 Introduction

Metabolic syndrome (MetS) is defined as a pathologic condition characterized by abdominal obesity, insulin resistance, high blood pressure, and high total cholesterol. Studies have found that about one third of US adults have MetS, and the prevalence of MetS in China would be about 15.5% (Alberti et al., 2005). MetS, a cluster of metabolic derangements that are major risk factors for vascular disease, is one of the important threats to individuals with erectile dysfunction (ED). Phosphodiesterase type 5 inhibitor (PDE5i) therapy is currently the first-line treatment for ED, but some patient groups are either unresponsive to or contraindicated for PDE5i treatment (Shabsigh & Mattern, 2016). Hence, the aims of this study were to determine the underlying mechanisms of MetS related ED (MED) to provide novel therapeutic options.

Reactive oxygen species (ROS) play an important role in the body. A lower level of ROS promotes cellular proliferation, differentiation, tissue regeneration and metabolism, high concentrations of ROS induce cell apoptosis and necrosis by oxidative stress. NADPH oxidase family of protein are main sources of ROS, consisting of cytosolic regulatory subunits ($p67^{phox}$, $p47^{phox}$, $p40^{phox}$) and catalytic subunits ($gp91^{phox}$ and $p22^{phox}$) (Drummond et al., 2011) (Bedard & Krause, 2007). Recently studies have found that ROS play a major role in a rat model of hypertension-induced ED, diabetes-induced ED and hypercholesterolemia-induced ED pathogenesis (Gonzaga et al.,

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2020; Long et al., 2016). During the process of ED, increased ROS leads to oxidative stress, which induces endothelial dysfunction, reduces the levels of eNOS and the concentration of cGMP, and causes pathological ED. However, the role of ROS remains to be elucidated in MED.

Corosolic acid is one of the pentacyclic triterpenoids that has been used as a folk medicine to treat many diseases. Studies have found that corosolic acid demonstrates anti-oxidative, anti-inflammatory, anti-obesity and anti-diabetic activities in rats (Horlad et al., 2013) (Fukushima et al., 2006) (Yamada et al., 2008) (Yamaguchi et al., 2006). Corosolic acid regulates dynamin related protein 1 phosphorylation (Ser637) in an AMPK-dependent manner, and this action contributes to anti-oxidative activity in the endothelium (Li et al., 2016a). Despite the numerous pharmacological activities identified for corosolic acid, little is known about its molecular targets in the protection against ED. The purpose of the study was to investigate the effect of corosolic acid on the erection process in MED rats.

2 Materials and methods

2.1 Animal treatments

In the experiment, 50 male 3-week-old SD rats were randomly divided into two groups: MetS group (n = 41) and the control group (n = 9). MetS rats were received a high sugar and high fat diet, and control rats were given a regular diet. Six months later, plasma insulin, body weight, fasting blood glucose, blood pressure and lipids were tested. MetS rats were selected from rats that were given a high sugar and high fat diet (three or more MetS characteristics) (Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults, 2001). Then, MED rats were chosen using an apomorphine test. Finally, 20 MED rats were divided into MED group (n = 10) and MED+CA group (n = 10). Rats in the MED+CA group were treated with corosolic acid (20 mg/kg) daily by oral gavage for 4 weeks. All animal experiments were approved by the Committees on Animal Experiments at Taizhou Affiliated Hospital of Nanjing University of Chinese Medicine, and all procedures complied with the Chinese Council on Animal Care regulations for the care and use of laboratory animals.

2.2 Measurement of metabolic variables

Fasting blood glucose levels were determined using a blood glucose meter through obtaining blood samples from the tail vein. A photoelectric tail-cuff system was used to detect rat blood pressures as described previously (Xiao et al., 2010). An ELISA kit was used to assess plasma lipid and insulin levels.

2.3 Assessment of erectile function

The intracavernous pressure (ICP) and mean arterial blood pressure (MAP) were determined as previously described (Li et al., 2017). Briefly, the cavernous nerve was identified and mounted onto stainless steel bipolar wire electrodes, which were connected to an electrical stimulator. Then, the carotid artery was cannulated (a PE-50 cannula) to monitor MAP. Finally, a 25-gauge needle catheter was inserted into right penile crus to monitor ICP. ICP and systemic blood pressure were determined continuously using a data acquisition system. The ratio of area under curve (AUC)/MAP and max ICP/MAP was recorded for each rat.

2.4 Detection of ROS, MDA and cGMP

After the assessment of erectile function, corpora cavernosa was quickly frozen and then made into frozen sections. Fresh dihydroethidium solution was prepared at 2 $\mu mol \cdot L^{-1}$ (Vigorous Biotechnology Beijing Co., Ltd, Beijing, China), and the slices were incubated with the solution for 20 min in the dark. Fluorescence microscope was used to capture the images and to assess the fluorescence intensities (Olympus Corporation, Tokyo, Japan). MDA levels were analyzed using the TBARS assay kit. The rat corpus cavernosum cGMP concentrations were determined using an ELISA kit. The analyses were performed in duplicate, and the data were normalized to the corpus cavernosum protein concentrations.

2.5 Western blot analysis

Protein lysates (40 mg) were loaded onto SDS-PAGE for electrophoretic analysis and transferred to PVDF membranes (Millipore, Billerica, Massachusetts, USA). The PVDF membranes were incubated with antibodies against $gp91^{phox}$ (1:500, Affinity, Zhenjiang,

Jiangsu, China), eNOS (1:1000, Abcam, Cambridge, MA, USA), or β-actin (1:500, Affinity, Zhenjiang, Jiangsu, China). Finally, they were analyzed and quantified using an chemiluminescence detection system (Pierce, Rockford, IL, USA).

2.6 Immunohistochemistry and immunofluorescence

Sections were adhered to charged slides, dewaxed with xylene, hydrated with gradient ethanol, and treated with hydrogen peroxide/methanol to quench endogenous peroxidase activity (immunofluorescence did not require this step). The sections were incubated with anti-$gp91^{phox}$ (1:50, Affinity, Zhenjiang, Jiangsu, China), anti-eNOS (1:50, Abcam, Cambridge, MA, USA) overnight at 4°C. The sections were incubated with Cy3-conjugated rabbit antibody against IgG or Cy3-conjugated mouse antibody against IgG for 60 min after several washes with PBS. Antigen-antibody reactions were performed using diaminobenzidine. Fluorescence microscope was used to capture the images and Image-Pro Plus was used to analyze the images.

2.7 Statistical analysis

Data were analyzed using SPSS 20.0 software and are presented as the mean±SD. All statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison posttest to determine the significance of differences observed ($p < 0.05$).

3 Results

3.1 Metabolic variables

6 months later, MetS rats had a higher fasting blood glucose, weight, total cholesterol and plasma insulin levels compared to the age-matched controls (Table 1).

Table 1. Effect of feeding high fat and high sugar diet on general paraments.

	CO	MED	CA
Fasting blood glucose (mmol/L)	6.1 ± 0.6	7.8 ± 0.9*	7.9 ± 1.1*
Weight (g)	566.0 ± 24.0	638.0 ± 36.0*	639.0 ± 42.0*
Total cholesterol (mmol/L)	1.93 ± 0.08	4.52 ± 1.25*	2.98 ± 0.27*#
Insulin level (mIU/L)	16.9 ± 1.2	21.6 ± 1.5*	22.5 ± 1.5*

Data are shown as the means±SD of 7~9 rats. CO, control; MED, metabolic syndrome related erectile dysfunction; CA, MED rats that received corosolic acid treatment. #p < 0.05 compared with the MED group. *p < 0.05 compared with the CO group.

3.2 Effects of corosolic acid treatment on erectile function

The AUC/MAP and max ICP/MAP ratios were markedly lower in the MED group than those in the other groups. Corosolic acid therapy caused a substantial increase in the AUC/MAP and max ICP/MAP ratios compared with those of the MED group (Figure 1, $p < 0.05$). However, these ratios were still lower than those of the control rats ($p < 0.05$).

3.3 Effects of corosolic acid treatment on ROS level

As shown in Figure 2A, B, the level of ROS (detected by dihydroethidium fluorescence) was severely increase in the MED group than those in the other groups, corosolic acid therapy reduced the ROS level. Moreover, MDA levels were markedly increases in the corpora cavernosa of MED rats when compared to those of normal control rats. Treatment with corosolic acid significantly reduced MDA levels in the corpora cavernosa of MED rats ($p < 0.05$, Figure 2C). In addition, to determine whether MetS-induced ROS is related to NADPH oxidase, the expression of the NADPH oxidase subunits was tested. Western blot and immunohistochemistry analysis showed that $gp91^{phox}$ was markedly increased in the MED rats and that expression greatly reduced by corosolic acid treatment ($p < 0.05$, Figure 3A, B, C).

3.4 Effects of corosolic acid treatment on the eNOS/cGMP signaling pathways

The eNOS expression was greatly reduced in MED rats than those of normal control rats. Treatment with corosolic acid markedly increased the expression of eNOS in MED rats (Figure 4A, B, D). Further, ELISA was performed to determine the cGMP concentration of corpus cavernosum. As shown in Figure 4C, the cGMP concentration of corpus cavernosum was greatly reduced in the MED rats compared with those of the normal control rats ($p < 0.05$). Corosolic acid treatment markedly attenuated the MED-induced reduction in the cGMP concentration of corpus cavernosum ($p < 0.05$).

4 Discussion

In the current study, we investigated the mechanism of MED and the effect of corosolic acid treatment on erectile function in the MED rat model. We found that erectile function in MED rats was markedly reduced and corosolic acid treatment attenuated the reduction. Then, we carried out molecular mechanism studies to demonstrate that ROS were upregulated in MED rats and that eNOS expression was obviously reduced in MED rats. The corpus cavernosum cGMP concentration was consistently decreased in MED rats. These data show that upregulation of

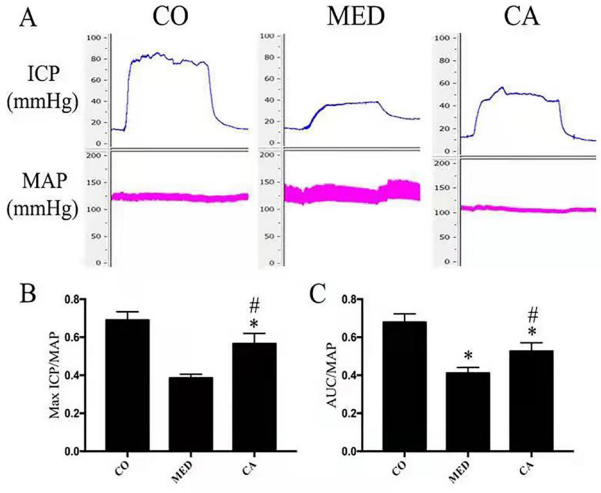

Figure 1. Evaluation of erectile function in three group. (A) Representative carotid artery pressure and ICP traces (stimulation parameters: 5 V, 1 min) in three group. (B, C) Max ICP/MAP and AUC/MAP ratios after cavernous nerve stimulation was applied to the cavernous nerve in the three groups. The data are expressed as the means ± SD. *p < 0.05 compared with CO group, #p < 0.05 compared with MED group. CO, control; MED, metabolic syndrome associated erectile dysfunction; CA, MED rats that received corosolic acid treatment.

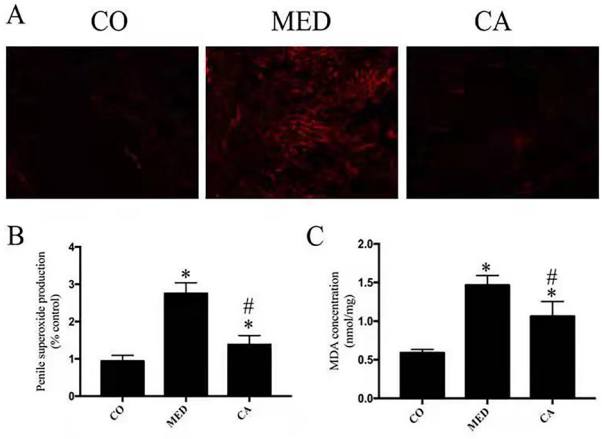

Figure 2. ROS level in three groups. (A, B) Representative images of dihydroethidium in the three groups. (C) Concentrations of MDA in the three groups. *p < 0.05 vs CO group, #p < 0.05 compared with MED group. CO, control; MED, metabolic syndrome associated erectile dysfunction; CA, MED rats that received corosolic acid treatment.

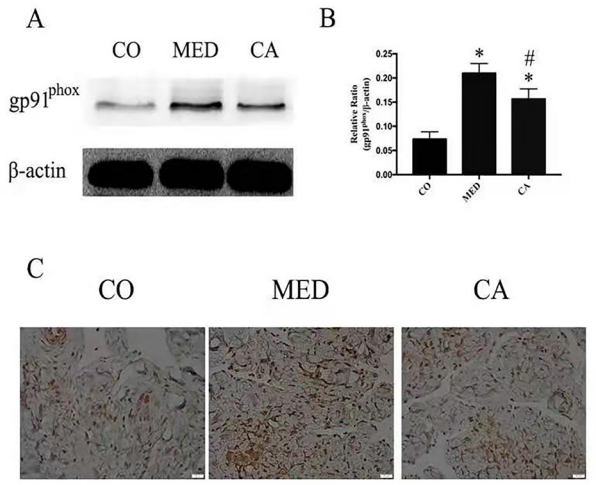

Figure 3. The expression of gp91^{phox} in three groups. (A, B) Western blot analysis of the expression of gp91^{phox} in the three groups. (C) Immunohistochemistry staining in the three groups. *p < 0.05 vs the CO group, #p < 0.05 compared with the MED group. CO, control; MED, metabolic syndrome associated erectile dysfunction; CA, MED rats that received corosolic acid treatment.

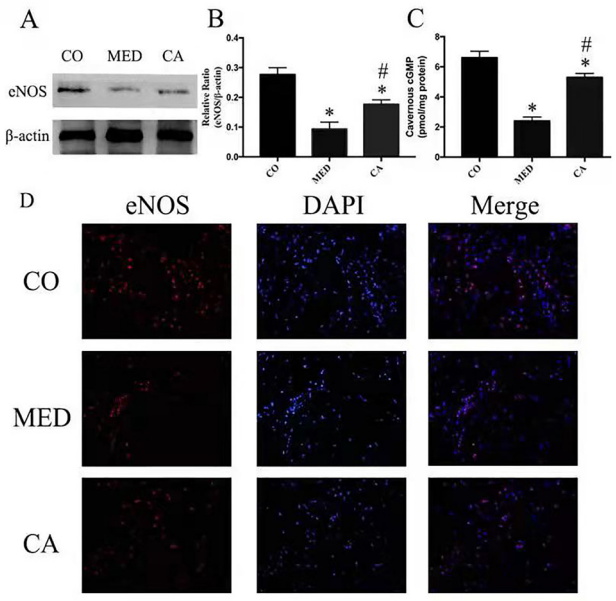

Figure 4. eNOS staining of cavernosal sinusoid and cGMP concentration in three groups. (A, B) Western blot analysis of the expression of eNOS in the three groups. (C) Concentrations of cGMP in the three groups. (D) Immunofluorescence staining in the three groups. *p < 0.05 vs the CO group, #p < 0.05 compared with the MED group. CO, control; MED, metabolic syndrome associated erectile dysfunction; CA, MED rats that received corosolic acid treatment.

ROS is involved in MED and that downregulation of ROS might be an appropriate therapeutic option for ED patients with MetS.

Oxidative stress is one of the important mechanisms in various diseases. Oxidative stress occurs when ROS accumulates excessively and/or defense mechanisms fail. In diabetes, long-term hyperglycemia induces advanced glycation end formation, that causes ROS accumulation. The latter could reduce the activity of eNOS/cGMP signal pathway. However, until now, it was unclear whether increasing ROS is a prerequisite for MED. In this study, we determined the changes in oxidative stress in the corpus cavernosum of MED rats. We demonstrated that ROS production and the expression of gp91^{phox} were significantly increased in the MED rats. Consistent with these, the higher MDA activity existed in MED rats. Therefore, ROS accumulation excessively, that are activated by NADPH oxidase, might be an important molecular mechanism underlying MED.

Corosolic acid is a compound that is extracted from the Lagerstroemia speciosa tree in Banaba. It is found in many weight-loss supplements, and it is also known as lagerstroemia speciosa or 2-alpha-hydroxyursolic acid. Recent studies have showed that corosolic acid is crucial for exerting antioxidant effects through reducing ROS and acts as an antioxidant in the body (Woo et al., 2018) (Guo et al., 2016) (Li et al., 2016a) (Peng et al., 2019). The study found that corosolic acid inhibits GMC proliferation mediated through the p38 MAPK and NADPH/ERK1/2 signal pathways. Corosolic acid exerts a protective effect on diabetic nephropathy through inhibiting proliferation resulting from inhibition of inactivation of ROS (Li et al., 2016b). In addition, Guo et al showed that corosolic acid might be a promising agent in the treatment of alcoholic liver diseases by reducing ROS levels. However, the influence of corosolic acid on oxidative stress in the corpora cavernosa of MED rats is still unclear and needs to be clarified. In this study, we demonstrated that corosolic acid treatment decreased the production of ROS, the level of MDA, the expression of gp91^{phox} and improved erectile function. After corosolic acid treatment, oxidative stress was ameliorated accompanied by the downregulation of gp91^{phox} expression. These preliminarily indicated that corosolic acid ameliorated ED by preventing the generation of ROS.

The nitric oxide (NO)/cGMP pathway, which is the pivotal mechanism of erectile pathway, has been shown to be related to ROS (Vrankova et al., 2019). In current study, we demonstrated that the expression of eNOS and the concentration of cGMP were decreased in MED rats and that treatment with corosolic acid restored the reduction. Many studies have showed that increased ROS production is one of the important causes of reduced NO bioavailability. Hence, according to our findings regarding NADPH oxidase and ROS, we believe that treatment with corosolic acid ameliorates erectile function of MED rats through decreasing the expression of gp91^{phox}. These reductions subsequently trigger a decrease in ROS, improving endothelial cell function, increasing the concentration of cGMP.

Conflict of interest

Authors declared no conflict of interest.

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Authors' contributions

Bi-Bo Li study concept and design. Kun Pang, Guang-Hui Zang, Xi-Tao Wang, Jian-Jun Zhang acquisition of data. Kun Pang, Lin Hao, Jian Wang, Chen-Di Yang analysis and interpretation of data. Bi-Bo Li, CongHui Han drafting of the manuscript. Bi-Bo Li, Cong-Hui Han critical revision of the manuscript for important intellectual content. Lin Hao, Guang-Hui Zang statistical analysis. Chen-Di Yang, Cong-Hui Han administrative, technical, and material support. Bi-Bo Li, Kun Pang, Long-Jun Cai study supervision.

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