The mechanistic study on Wa medicine Niang-Mu-Liang medicinal liquor mitigating diabetes mellitus erectile dysfunction in rats by inhibiting ferroptosis

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Author contributions
Wang YM carried out the experiments and manuscript writing. Qian R provided experimental help. Xie XH performed data analysis and result interpretation. Cui Y provided technical guidance. HT and Wei WB provided ideas and manuscript review & editing. Zhao J provided technical guidance and funding acquisition. All authors contributed to the article and approved the submitted version.

Competing interests
The authors declare no conflicts of interest.

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Abbreviations
ED, erectile dysfunction; DM, diabetes mellitus; DMED, diabetes mellitus erectile dysfunction; TCM, traditional Chinese medicine; NO, nitric oxide; cGMP, cyclic guanosine monophosphate; NML, Niang-Mu-Liang medicinal liquor; APO, apomorphine; FTH, ferritin heavy chain; FTL, ferritin light chain; GPX4, glutathione peroxidase 4.

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Abstract
Background: This study aims to investigate the therapeutic effect of Wa medicine Niang-Mu-Liang medicinal liquor (NML) on rats with diabetes mellitus erectile dysfunction (DMED) and its impact on the ferroptosis signaling pathway. Methods: Thirty Sprague-Dawley rats were randomly divided into three groups: Control, DMED, and NML. After establishing the DMED model, treatments were administered for 8 weeks. After the administration, apomorphine hydrochloride tests were conducted to measure the mass and organ index of testes and epididymides, sperm concentration and viability in each group. Penile corpus cavernosum tissues were stained with hematoxylin and eosin. Nitric oxide and cyclic guanosine monophosphate levels in the penile corpus cavernosum tissues were determined using biochemical kits and enzyme-linked immunosorbent assay, while the expression of proteins related to the ferroptosis signaling pathway was measured by Western blot. Results: Compared to the DMED group, the DMED rats treated with NML showed significantly increased erection frequency, testicular and epididymal mass and index, sperm count and viability, along with noticeable improvement in the pathological morphology of penile corpus cavernosum. The content of nitric oxide and cyclic guanosine monophosphate, and the expression of ferritin heavy chain, ferritin light chain, and glutathione peroxidase 4 proteins in penile corpus cavernosum tissue were elevated, while the expression of transferrin and STEAP3 proteins was reduced. Conclusion: NML can improve erectile function in DMED rats by inhibiting the ferroptosis signaling pathway.

Keywords: Niang-Mu-Liang medicinal liquor; diabetes mellitus erectile dysfunction; nitric oxide/cyclic guanosine monophosphate; ferroptosis
Introduction

Erectile dysfunction (ED) is a common male sexual dysfunction characterized by the inability to achieve and maintain an erection sufficient for satisfactory sexual intercourse, significantly affecting the quality of life of patients and their partners [1]. ED can be a complication of many diseases or even an initial symptom, including diabetes mellitus (DM) [2]. Epidemiological surveys indicate that China has the highest number of diabetic patients globally, with 75% of male diabetic patients experiencing varying degrees of ED [2-4]. The current first-line treatment for ED, phosphodiesterase type 5 inhibitors, shows poor efficacy in patients with diabetes mellitus erectile dysfunction (DMED) and is associated with side effects like headaches, back pain, and facial flushing [1, 5]. Hence, there is an urgent need to explore new therapeutic drugs for DMED.

Traditional Chinese medicine (TCM) has shown unique advantages in preventing and treating DMED, with definite efficacy, high safety, and few side effects. Studies have indicated that Shaofu Zhiyu decoction might inhibit inflammation in diabetic rats through the PI3K-AKT signaling pathway, promoting erectile function [6]. Huxuexie Tongluo Qiwei decoction enhances antioxidative capabilities and prevents platelet activation by inhibiting the PKC β signaling pathway, thus improving erectile function in DMED rats [7]. Resveratrol recipe can improve DMED by activating the nitric oxide (NO)-cyclic guanosine monophosphate (cGMP) pathway [8].

Traditional medicinal liquor, as excellent food-medicine products in traditional medicine for disease prevention, body strengthening, and health improvement, have been widely used. Niang-Mu-Liang (transliteration of Nya Miex Tiang in the Wa language of Yunnan), a rare medicinal herb from the Wa ethnic group of Yunnan, has been extensively used in Wa medical clinical practice. Its medicinal liquor is known for significantly enhancing kidney function, calming the mind, nourishing the brain, and promoting pregnancy [9]. However, its mechanism of action is not well understood. This article establishes a DMED rat model to analyze the effects of Niang-Mu-Liang medicinal liquor (NML) on DMED rats and explores its mechanism of action based on the ferropotosis pathway.

Methods

Animals

Specific pathogen-free male Sprague-Dawley rats, 30 in total, 6–8 weeks of age, weighing 200–220 g, were acquired from Beijing Huafukang Bioscience Co., Inc. (Beijing, China), with the animal license number SCXK (Jing) 2019-0008. Rats were housed five per cage, kept at a room temperature of 21 ± 2 °C, relative humidity of 50%-60%, with a 12 h light/12 h dark cycle, and had free access to food and water. This study was conducted by the National Institutes of Health Guide for the Care and Use of Laboratory Animals and was approved by The Experimental Animal Ethics Committee in Yunnan University of Chinese Medicine (ethics approval number: R-072023LH300).

Drugs and reagents

NML (200 g Niang-Mu-Liang + 4000 mL 53% vol Chinese liquor), Apomorphine (APO) hydrochloride (Cat: P92621, Merek, Shanghai, China) and biocinchoninic acid protein assay kit (Cat: A045-4-2), NO assay kit (Cat: A013-2-1) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Rat cGMP enzyme-linked immunosorbent assay kit (Cat: ml003133, enzyme-linked biotech) and antibodies for ferritin heavy chain (FTH, Cat: #3998) from cell signaling technology, ferritin light chain (FTL, Cat: 10727-1-AP), glutathione peroxidase 4 (GPX4, Cat: 30388-1-AP), transferrin (Cat: 17435-1-AP), and STEAP3 (Cat: 17186-1-AP) antibodies were all from Proteintech Group, Inc. (Wuhan, China).

Establishment of DMED model

After a 16 h fast, 20 rats were randomly selected and injected intraperitoneally with 1% streptozotocin at a dose of 60 mg/kg to establish the DM model. The remaining 10 rats were injected with 0.1 mol/L citrate-phosphate buffer (pH 4.2) as the Control group. Fasting blood glucose was tested one week after injection, and a blood glucose level ≥ 16.7 mmol/L was considered a successful DM model. Treatment began 4 weeks after model establishment [6].

The model was validated using the APO test [10]: 100 μg/kg of APO was injected into the loose skin of the rat’s neck, and the number of penile erections within 30 minutes was recorded, with one erection being counted each time there was an increase in penile size or the tip of the penis became exposed. The absence of penile erection indicated successful model construction.

Grouping and administration

Rats were divided equally into three groups (10 rats per group): Control, DMED, and NML. The NML group was administered 6 mL/kg of NML medicinal liquor daily by gavage, while the Control and DMED groups received an equivalent volume of saline as a control. All groups were treated for 8 weeks, followed by APO erection experiments, after which the rats were euthanized, weighed, and samples collected.

Testicular and epididymal index measurement

Rat testes were collected, weighed, and fixed in 4% paraformaldehyde. The organ index was calculated as organ mass/body mass × 100%.

Sperm count and viability measurement

The right epididymis of rats was soaked in 37 °C saline, sperm suspension prepared, and 10 μl of the mixed sperm suspension was dropped onto a hemocytometer for counting under a microscope. The sperm activity rate was calculated based on the motility of the sperm.

Measurement of bioactive factors related to penile erection

After the above experiments, the rats’ lower abdomen and perineum were disinfected, the penis was cut from the base with sterile surgical instruments, and the glans and prepuce tissues were quickly removed and washed with sterile saline. The penis was then fixed in 4% paraformaldehyde. NO and cGMP levels were measured according to the biochemical kit and enzyme-linked immunosorbent assay kit instructions.

Histological staining of penile corpus cavernosum tissue

Penile tissues fixed in 4% paraformaldehyde were dehydrated, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for microscopic observation of pathological changes in the corpus cavernosum.

Western blot analysis of ferropotosis-related protein expression in penile tissue

Proteins were extracted from penile tissues, separated by electrophoresis, transferred to prepare polyvinylidene fluoride membranes, and blocked with 5% skim milk at room temperature for 2 hours. Membranes were then incubated with primary antibodies overnight at 4 °C. Secondary antibodies were diluted in blocking solution and incubated at room temperature for 2 hours before exposure in a chemiluminescence imager.

Statistical methods

SPSS 25.0 software was used for statistical analysis. Results are expressed as mean ± standard deviation. The independent sample t-test was used for comparisons between two groups, while one-way analysis of variance was utilized for multiple groups.

Results

Erectile function comparison among groups

The APO test results showed that the erectile frequency in the DMED group was significantly lower than in the Control group. In contrast,
the NML group showed a significant increase in erectile frequency compared to the DMED group (Figure 1).

Comparison of epididymal and testicular mass and index among groups

Compared to the Control group, the DMED group showed a significant decrease in epididymal and testicular mass and index, which were significantly increased in the NML group (Figure 2).

Sperm quality comparison among groups

The DMED group exhibited a significant reduction in sperm count and viability compared to the Control group, while these parameters significantly improved in the NML group (Figure 3).

Figure 1 The results of the APO test for each group of rats. **: P < 0.01, compared to the control group; ##: P < 0.01, compared to the DMED group. APO, apomorphine; DMED, diabetes mellitus erectile dysfunction; NML, Niang-Mu-Liang medicinal liquor.

Figure 2 The mass and index of the epididymis and testes in each group of rats. (a) Epididymal weight, (b) testicular weight, (c) epididymal index, (d) testicular index. **: P < 0.01, compared to the control group; ##: P < 0.01, compared to the DMED group. DMED, diabetes mellitus erectile dysfunction; NML, Niang-Mu-Liang medicinal liquor.

Figure 3 The quality of sperm in each group of rats. (a) Sperm concentration, (b) sperm motility. **: P < 0.01, compared to the control group; ##: P < 0.01, compared to the DMED group. DMED, diabetes mellitus erectile dysfunction; NML, Niang-Mu-Liang medicinal liquor.
Changes in penile corpus cavernosum pathology among groups
Hematoxylin and eosin staining results indicated that the DMED group experienced pathological changes such as reduced and disordered blood sinusoids and damaged endothelial cells, which gradually returned to Control in the NML group (Figure 4).

Changes in NO/cGMP signaling among groups
NO and cGMP levels were significantly lower in the DMED group compared to the Control group but were significantly increased in the NML group (Figure 5).

Changes in ferroptosis-related proteins among groups
Western blot results showed that, compared to the Control group, FTH, FTL, and GPX4 protein expressions were decreased in the DMED group, while transferrin and STEAP3 expressions were increased. In the NML group, FTH, FTL, and GPX4 expressions increased, and transferrin and STEAP3 levels significantly decreased (Figure 6).

Figure 4 H&E staining of the penile corpus cavernosum in each group of rats. The arrow indicates vascular endothelial cells, which are primarily located on the walls of the cavernous sinuses. H&E, hematoxylin and eosin; DMED, diabetes mellitus erectile dysfunction; NML, Niang-Mu-Liang medicinal liquor.

Figure 5 Detection of the NO/cGMP signaling pathway in each group of rats. (a) NO, (b) cGMP. *P < 0.05, compared to the control group; **P < 0.01, compared to the DMED group. DMED, diabetes mellitus erectile dysfunction; NML, Niang-Mu-Liang medicinal liquor; NO, nitric oxide; cGMP, cyclic guanosine monophosphate.

Figure 6 Detection of the ferroptosis signaling pathway in each group of rats. (a) Western blot, (b–f) relative protein expression. ***P < 0.01, compared to the control group; *P < 0.05, compared to the DMED group; **P < 0.01, compared to the DMED group. DMED, diabetes mellitus erectile dysfunction; NML, Niang-Mu-Liang medicinal liquor; FTH, ferritin heavy chain; FTL, ferritin light chain; GPX4, glutathione peroxidase 4.
Discussion

TCM attributes ED to imbalances among organs, meridians, and bodily fluids. NML is praised for its benefits in kidney support, calming the mind, and relieving phlegm and spasms. It’s clinically used for conditions like kidney deficiency, premature ejaculation, and symptoms of palpitations and asthma due to kidney deficiency. The Wa people have long recognized its strength-enhancing and fatigue-fighting properties, making it a natural tonic. NML, formulated on these principles, is said to invigorate the body, improve blood circulation, and support kidney Yang (in traditional Chinese medicine, kidney Yang refers to the activating, impelling and warming aspects of the kidney’s functions) [9].

This study was based on a diabetic rat model induced by streptozotocin, using APO testing to confirm model success. It was found that diabetic rats exhibited limited erectile function, which was restored following intervention with NML. ED was associated with reduced sperm quality and changes in the morphology of penile corpus cavernosum and testicular tissue, which improved after treatment with the NML. The NO/cGMP signaling pathway plays a crucial role in erection, with NO stimulating soluble guanylate cyclase to convert GTP to cGMP, thus facilitating the relaxation of cavernosal smooth muscle and enhancing penile blood flow [11]. The study noted impaired NO and cGMP production in ED rats, which was significantly ameliorated following intervention with NML.

Ferroptosis is an iron-dependent form of programmed cell death, distinct from apoptosis, necrosis, and autophagy [12]. Its main mechanism involves reduced cellular antioxidant capacity and imbalanced production and degradation of lipid reactive oxygen species, leading to accumulation and oxidative cell death [13]. High glucose can trigger oxidative stress and subsequent ferroptosis in various organs and cells [14]. In physiological conditions, Fe²⁺ enters cells via transferrin and is reduced to Fe³⁺ by STEAP3, which can then be stored with ferritin or exported to maintain iron homeostasis [15, 16]. GPX4, the only enzyme known to directly remove lipid peroxides, plays a central role in regulating ferroptosis [17]. This study found that NML medicinal liquor upregulates GPX4 expression, inhibits lipid peroxidation, reduces cell ferroptosis, and alleviates diabetic nephropathy kidney damage.

In conclusion, NML can enhance NO and cGMP production and inhibit ferroptosis, thereby improving erectile function in DMED rats. This study provides a preliminary exploration of the effects and mechanisms of NML in treating ED in rats, offering a basis for its clinical application. While our experiments offer valuable insights, they also reveal certain limitations. The deeper mechanisms of action by which NML affects DMED remain to be further explored. In future studies, we plan to introduce a positive control group and combine both in vivo and in vitro research with clinical trials. By employing experimental methods such as immunofluorescence and immunohistochemistry, we aim to analyze the mechanisms related to DMED and conduct an in-depth examination of NML’s precise therapeutic targets within DMED. This comprehensive approach will allow us to explore the mechanisms of DMED more thoroughly.

References