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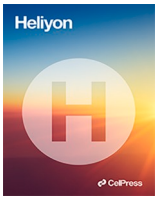

Research article

NLRP3 inhibitor combined with Yimusake improves erectile dysfunction in rats with diabetes mellitus through the attenuation of pyroptosis

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ABSTRACT

Erectile dysfunction (ED) is a prevalent complication associated with diabetes mellitus (DM), yet pharmacological treatments for diabetes-related erectile dysfunction (DMED) continue to be inadequate in clinical settings. Our previous studies have indicated that there is a close correlation between ED and pyroptosis, but the specific mechanism remains unclear. In this study, we sought to explore the therapeutic effects of DMED through the modulation of NLRP3, aiming to elucidate its potential molecular mechanisms. The DMED rat model was established via intraperitoneal injection of streptozotocin. The rats were randomly assigned to the control group, the DMED group, the Yimusake group, the MCC950 (NLRP3 inhibitor) group, and the MCC950+Yimusake group. Erectile function of rats was observed by measuring intracavernosal pressure (ICP) and mean arterial pressure (MAP). HE staining was performed to observe the histopathological changes in penile; immunofluorescence was performed to measure the level of CD31 (Platelet endothelial cell adhesion molecule-1) in penile. Besides, immunohistochemistry, RT-qPCR and Western blot were performed to demonstrate the expression of NLRP3, caspase-1, IL-1β and eNOS. After treatment with the MCC950 and Yimusake, the number of blood sinusoids and small vessels significantly reduced in penile tissue; NLRP3, caspase-1, IL-1β proteins and mRNA expression decreased, eNOS protein and mRNA expression increased. Compare with the Y group and the MCC950 group, MCC950+Yimusake group had a more significant effect. MCC950 and Yimusake might potentially suppress pyroptosis in the penile tissue of DMED rats by modulating the NLRP3/caspase-1 pathway, thus enhancing erectile function. This discovery could offer a promising therapeutic approach for individuals with DMED.

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1. Introduction

Erectile dysfunction (ED) denotes the persistent inability to attain or sustain an adequate penile erection for satisfactory sexual activity over a period of no less than three months, and is a prevalent clinical condition [1]. With changes in lifestyle habits and dietary structure, the incidence of ED is increasing year by year and there will be approximately 322 million ED patients worldwide by 2025 [2,3]. As a frequent complication of diabetes mellitus (DM), the prevalence of ED among individuals with diabetes is threefold greater compared to that observed in the non-diabetic population [4,5]. It was estimated that, by 2045, the prevalence will rise to 10.9 % (700 million) [6]. In addition, the age of onset for ED in diabetic patients is earlier compared to non-diabetic patients, and the symptoms are more severe. According to research, the primary cause of DMED is the excessive loss of endothelial cells in the penile corpus cavernosum [7]. The current first-line treatment drugs for ED include phosphodiesterase-5 inhibitors (PDE5). However, it has been shown that the inhibitors are still ineffective in 30%–40 % of the ED patients. Therefore, it is important to explore new pathophysiological mechanisms for DMED.

Pyroptosis is a form of programmed cell death that relies on NLRP3 inflammasome-mediated inflammation response. Pyroptosis, a process that is crucial in many disorders, differs from apoptosis in that it can lead to cell enlargement, membrane rupture that creates membrane holes, the release of pro-inflammatory cytokines and cell contents including interleukin-1β (IL-1β), and an increase in the inflammatory cascade reaction [8,9]. Research has shown that pyroptosis increases the expression of inflammatory factors in the blood of patients with vascular diseases, thereby exacerbating their condition [10,11]. Pyroptosis plays a crucial role in inflammatory responses and may be closely associated with inflammation-mediated ED. Therefore, inhibiting the pyroptosis pathway can effectively suppress the release of inflammatory factors, slow down vascular stenosis, sclerosis, damage, and improve endothelial function.

Yimusake is a medicinal remedy employed in the treatment of impotence, premature ejaculation, spermatorrhea, and related conditions. It is composed of a variety of component, including opoponax, musk, ambergris, saffron, strychnine, frankincense, bovine spurge, nutmeg, clove, poppy husk and galangal, which has the effect of tonifying the kidney, strengthening the yang, benefiting the essence and fixing astringency [12–16]. In a previous study, we found that inflammation caused by pyroptosis is closely related to ED, and Yimusake improved erectile function in ED rats by attenuating inflammation [17–20]. However, it is not clear what the relationship between the three. Elucidating the specific pathogenesis of ED will be beneficial for a deeper understanding of the physiology and pathology of penile erection, and for identifying potential molecular targets for the treatment of ED.

MCC950 is a selective small molecule inhibitor that shows remarkable effectiveness in treating a variety of inflammatory disorders by blocking the NLRP3 inflammasome's activation, reducing the maturation and release of downstream IL-1β. Research has shown that MCC950 can inhibit the levels of inflammatory proteins in cardiac inflammation and feature anti-inflammatory and vascular protective effects [21]. MCC950 is considered as a promising treatment approach for ED [22–25]. Elucidating the specific pathogenesis of ED is of importance to improve the understanding of the physiology and pathology of penile erection, and to identify potential molecular targets of ED.

According to these findings, we created an animal model of DMED rats to investigate the potential therapeutic advantages of MCC950 and Yimusake on DMED, as well as the underlying processes.

2. Materials and methods

2.1. Experimental materials

Yimusake tablets were purchased from Xinjiang Hotan Uygur Pharmaceutical Company (Z65020144), composition of Yimusake tablets is given in supplementary material. MCC950 was purchased at Med Chem Express (HY-12815A), NLRP3 antibody was purchased from Bosterbio (BA3677), caspase-1 antibody was purchased from Proteintech (DF7438), IL-1β antibody was purchased from Affinity Biosciences (207701-AP), eNOS antibody was purchased from Proteintech (AF3000), CD31 antibody purchased from Abcam (210663-1-AP).

2.2. Experimental animals

40 SPF-grade male Sprague-Dawley (SD) rats, weighing 200 ± 10g at three weeks of age, were purchased from the Animal Experimental Center of Xinjiang Medical University (SCXK 2018-0003). This study was approved by the Animal Experimental Medicine Ethics Committee of the First Affiliated Hospital of Xinjiang Medical University (IACUC-20230503-24).

SD rats were randomly assigned into the control group (n = 6) and the model groups (n = 34). The model rats were intraperitoneally injected with streptozotocin solution on the first and second day (45 mg/kg, 2 times, 10:00 a.m.) [26–29]. The control group was injected with saline. Model rats with random blood glucose consistently greater than 16.7 mmol/L were considered diabetes mellitus (DM) rats. An apomorphine (APO) represents the apomorphine experiment, in which the ability of rats to achieve penile erection was observed. After 8 weeks, APO solution (90 μg/kg) was subcutaneously injected into DMED rats, and the erectile latency and number of erections in rats were observed for 1800 s immediately after injection. The APO experiment was performed to screen the rats with DMED. DMED rats were separated into four groups, DMED group (n = 6), Yimusake group (Y, n = 6), MCC950 group (n = 6), MCC950+Yimusake group (MCC950+Y, n = 6). The Yimusake dose was 250 mg/kg/day, while the MCC950 dose was 10 mg/kg/day, and the DMED and control groups got the same amount of saline therapy (orally 1 time/week, treatment 2 weeks). The weights of the rats were recorded at the start and finish of the therapy.

2.3. Blood glucose assay

Random blood glucose was tested in rats during the construction of DM rats. After disinfection, the rat tail tip was clipped about 3 mm and squeezed out the blood, and the blood was detected by a glucometer, and the blood glucose value was displayed after 5 s. The blood glucose value was measured by a glucose meter.

2.4. Erectile function evaluation

The common carotid artery was cut at its proximal end. A PE-50 catheter needle was inserted into the common carotid artery to observe the changes in Mean arterial pressure (MAP). The cavernous nerve was applied electrical stimulation (intensity, 7.5 V; frequency, 15 Hz; pulse width, 1 ms; duration,1 min). The ratio of Maximum intracavernous pressure (Max ICP) to MAP was used to determine erectile function. After the rats were euthanized, the penis was divided into two parts. The distal tissues were fixed in 4 % paraformaldehyde and then routinely embedded in paraffin, while the proximal tissues were immediately preserved in liquid nitrogen.

2.5. Hematoxylin–eosin (H&E) staining

The penile tissue was fixed in a 4 % formaldehyde solution, and dehydrated with different gradients of alcohol, embeded, and then cut section. Hematoxylin-eosin staining was conducted on paraffin slices after being put in an electrically heated incubator at 60 °C for 1 h. We observed the pathological morphological changes in the rat penile corpus cavernosum under a bright—field optical microscope (Nikon Corporation of Japan, Eclipse Ni-U).

2.6. Immunofluorescence

Penile tissue paraffin sections (4 μm thickness) were prepared for the procedure of immunofluorescence. The primary antibody CD31 was incubated overnight at 4 °C on paraffin slices. Following incubation with secondary antibody at 37 °C for 1 h, the nucleus was stained with DAPI. The samples were observed and photographed using an inverted fluorescence microscope (Leica, DMi8), and Image J software is used for analysis.

2.7. Immunohistochemistry

Penile tissues paraffin sections were dehydrated and transferred to H_2O_2. After sections were blocked with BSA, primary antibodies against NLRP3(1:200), caspase-1 (1:100), IL-1β (1:100), eNOS (1:150) were added and incubated. The normal goat IgG (1:200) was used as a negative control. Use a light microscope to capture images, and analyze the area of positive staining using Image J software.

2.8. Real time quantitative PCR (RT-qPCR)

Part of the penile tissue was minced, added to RLA lysis buffer, and lysed on ice. The tissue was then homogenized, and proteinase K was added. The total RNA was extracted from the penile tissue by centrifugation, and the RNA concentration was determined using a UV spectrophotometer. Reverse transcription reaction was performed according to the instructions of the reagent kit. $2^{-\triangle\triangle}$Ct was calculated based on the obtained Ct values. The primers were shown as followed: NLRP3 (forward 5′-GATAGGTTTGCTGGGATA; reverse 3′-GGTGTAGCGTCTGTTGAG), caspase-1 (forward 5′-AGGAGGGAATATGTGGG; reverse 3′-AACCTTGGGCTTGTCTT); IL-1β (forward 5′-AGAGTGTGGATCCCAAACAAATACCCA; reverse 3′-GCTCTGCTTGAGAGGTGCTGATGTA); eNOS (forward 5′-GCTGTGAGCAGCCCCC; reverse 3′-GCCGGCTCTGTAACTTCCTT), β-actin (forward 5′-CTGGAGAAACCTGCCAAGTATG; reverse 3′-GGTGGAAGAATGGGAGTTGCT)

2.9. Western blotting (WB)

Part of the penile tissue was placed in RIPA lysis buffer, minced, and incubated with proteinase on ice for lysis. The supernatant was collected after centrifugation, and protein extraction was performed. The BCA technique for protein quantification was used to determine the samples' protein content. Primary antibodies, including NLRP3 (1:2000), caspase-1 (1:1000), IL-1β (1:10000), eNOS (1:3000) were added and incubated at 4 °C overnight. Subsequently, secondary antibodies (1:8000) were added and incubated at room temperature for 2 h. After color development, images were captured, and the target protein's and internal reference protein's absorbance values were analyzed to determine the ratio.

2.10. Statistical analysis

The statistical analysis of the relevant data was conducted using SPSS 26.0 software. One-way analysis of variance (ANOVA) was used for multiple group comparisons, and pairwise comparisons were performed using the LSD-t test. The results are presented as mean ± standard deviation ($\bar{x}\pm s$). A significance level of $P < 0.05$ indicates a statistically significant difference.

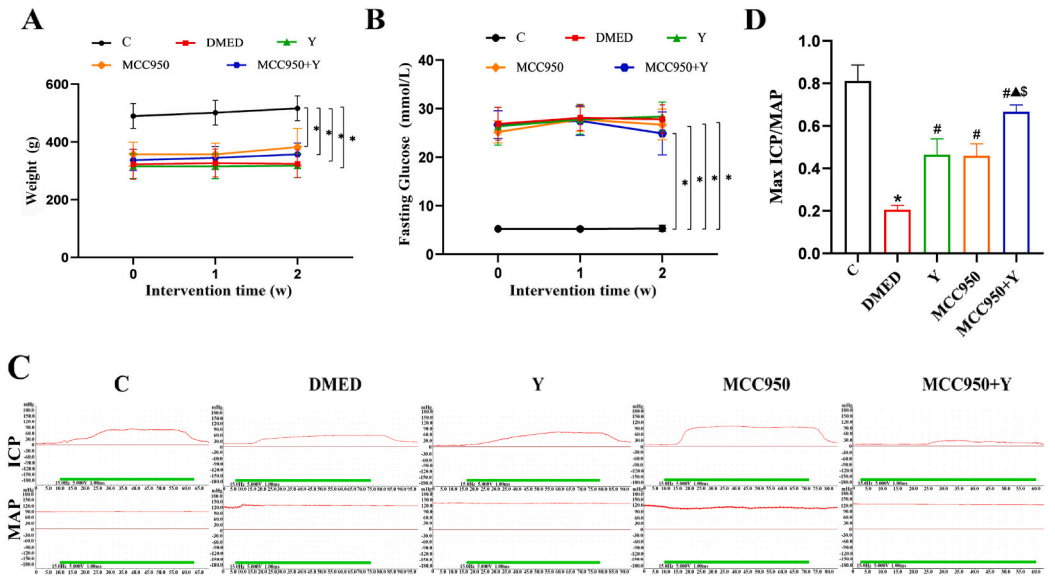

Fig. 1. Assessment of metabolic and physiological parameters of rats. (A) Body weights. (B) Blood glucose levels. (C) Representative traces of ICP and MAP in rats. (D) ICP/MAP ratios. The data are presented as means ± SD. N = 6 per group. $^{*}P < 0.05$ compared with the control group. $^{\#}P < 0.05$ compared with the DMED group. $^{\blacktriangle}P < 0.05$ compared with the Yimusake group. $^{\$}P < 0.05$ compared with the MCC950 group. C, control; DMED, diabetes mellitus-induced erectile dysfunction; Y, Yimusake (250 mg/kg/d); MCC950, NLRP3 inhibitor (10 mg/kg/d); MCC950+Y, NLRP3 inhibitor and Yimusake.

3. Results

3.1. Metabolic and physiological parameters

As shown in Fig. 1A and B, in period during intervention, compared with the control group, the DMED group, the Y group, the MCC950 group, the MCC950+Y group had higher blood glucose and lower body weight after streptozotocin induction ($P < 0.05$). Compared with the DMED group, there were no significant difference in the body weight and fasting blood glucose after MCC950 and Yimusake interventions ($P > 0.05$). These results indicated that the construction of DM rats was successful. The ratio of ICP to MAP reflected erectile function. As shown in Fig. 1C and D, compared with the control group, the ICP/MAP indicator was severely lowered in the DMED group, whereas it was greatly improved after treatment in the MCC950 and Y groups ($P < 0.05$). It is worthy pointing out that the MCC950+Y group exhibited a more pronounced therapy effect than the MCC950 and Y groups.

3.2. Inhibition of NLRP3 improve endothelial function in vivo

HE staining showed that the structure of the corpus cavernosum was irregularly arranged in the DMED group compared with the control group (Fig. 2A). This finding demonstrated that increased levels of glucose caused endothelial cell dysfunction in DMED rat penile tissue. However, after treatment of the MCC950 and Y groups, the number of blood sinusoids and small vessels were significantly increased. It was important that the structure of the corpus cavernosum in the MCC950+Y group was similar to the control group ($P < 0.05$) (Fig. 2A). Additionally, it was observed from the result of immunofluorescence staining that CD31 in the endothelial cells of the corpus cavernosum of the penis showed red fluorescence in all groups (Fig. 2B). The finding that compared with the control group, the level of CD31 was significantly decreased in DMED rats indicated that more serious damage was present within penile tissue in this group. Compared with the DMED group, the expression of CD31 protein in the MCC950+Y group was increased, with statistical significance ($P < 0.05$) (Fig. 2B and C). These findings consistently demonstrated that MCC950 has the ability of reducing endothelial cell death in DMED rats.

3.3. Inhibition of NLRP3 decreased inflammation via NLRP3/caspase-1 pathway in DMED rats

To assess potential mechanism in penile tissue of DMED rats after MCC950 treatment, we examined changes in pyroptosis pathway. Immunohistochemical and WB showed that compare with the control group, the protein levels of NLRP3 and caspase-1 were higher, but the protein level of eNOS was significantly lower in the DMED group ($P < 0.05$). The protein levels of NLRP3 and caspase-1 were reduced following treatment with the MCC950 and Y groups, whereas the protein level of eNOS was dramatically higher ($P < 0.05$). The MCC950+Y group showed a more pronounced effect to inhibit NLRP3/caspase-1 pathway ($P < 0.05$). Inflammation is considered a critical risk factor in the pathogenesis of DM. Result revealed that the protein level of proinflammatory cytokine IL-1β was

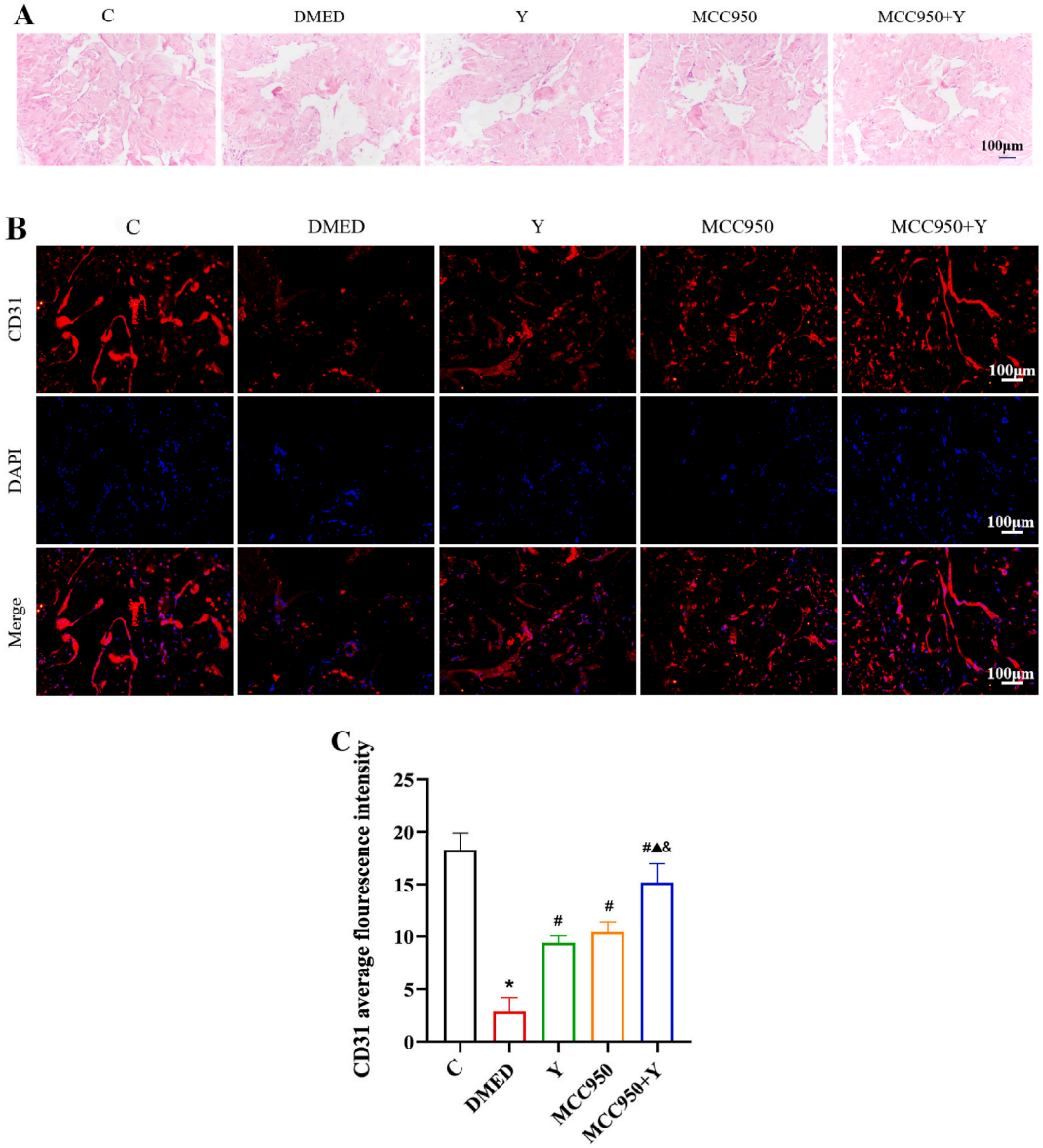

Fig. 2. Hematoxylin-eosin staining and Immunofluorescence staining of penile tissues. (A) Transverse portions of all rats in all groups penile tissues. The length of scale bars 100 μm. (B) Representative immunofluorescent staining of CD31 in penile tissues and quantification (C). The length of scale bars 100 μm. Nuclei (blue) are stained with DAPI. The data are presented as means ± SD. N = 6 per group. $^{*}P < 0.05$ compared with the control group. $^{\#}P < 0.05$ compared with the DMED group. $^{\blacktriangle}P < 0.05$ compared with the Yimusake group. $^{\$}P < 0.05$ compared with the MCC950 group. C, control; DMED, diabetes mellitus-induced erectile dysfunction; Y, Yimusake (250 mg/kg/d); MCC950, NLRP3 inhibitor (10 mg/kg/d); MCC950+Y, NLRP3 inhibitor and Yimusake.

significantly increased in the DMED group as compared with that observed in the control group. However, the protein level of the IL-1β was significantly decreased in the MCC950 and Y groups versus the DMED group. The MCC950+Y group showed a more pronounced effect to inhibit inflammation response ($P < 0.05$) (Fig. 3A–J). These results revealed that MCC950 could alleviate inflammation by suppressing the NLRP3-mediated pyroptosis pathway.

As shown in Fig. 4, compared with the control group, we discovered that the mRNA levels of NLRP3, and caspase-1 were all significantly higher, whereas the mRNA level of eNOS was significantly lower ($P < 0.05$) (Fig. 4A and B). However, compared with the DMED group, the mRNA levels of NLRP3 and caspase-1 were lower in the MCC950 and Y groups, butthe mRNA level of eNOS was significantly higher ($P < 0.05$) (Fig. 4D). The effect was particularly significant in the MCC950+Y group compare with the MCC950 and Y groups ($P < 0.05$). The mRNA level of IL-1β was increased in DMED rats, which indicates an increase in inflammatory responses in the penis tissue. However, in the MCC950 and Y groups, the mRNA level of IL-1β was reduced than that in the DMED group ($P < 0.05$). The mRNA level of IL-1β in the MCC950+Y group was more significantly decreased ($P < 0.05$) (Fig. 4C).

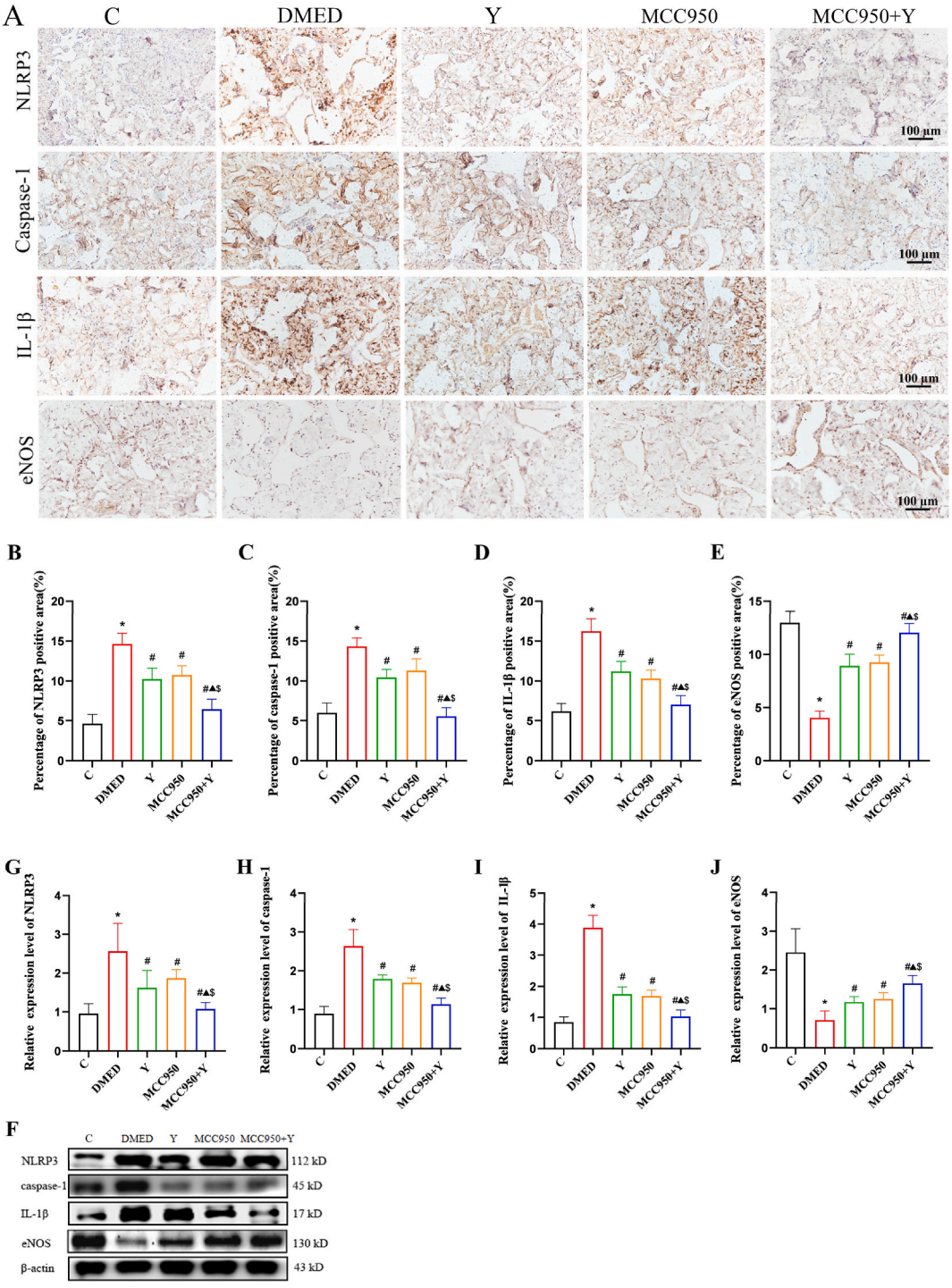

Fig. 3. Immunohistochemical staining and Western Blot detected the protein expression of pyroptosis factors of penile tissue. (A) Representative mmunohistochemistry of NLRP3, caspase-1, IL-1β and eNOS in penile tissues and quantification (B–E). The length of scale bars 100 μm. (F) Representative Western Blot of NLRP3, caspase-1, IL-1β, and eNOS in penile tissues and quantification (G–J). The data are presented as means ± SD. N = 6 per group. *$P < 0.05$ compared with the control group. #$P < 0.05$ compared with the DMED group. ▲$P < 0.05$ compared with the Yimusake group. $$P < 0.05$ compared with the MCC950 group. C, control; DMED, diabetes mellitus-induced erectile dysfunction; Y, Yimusake (250 mg/kg/d); MCC950, NLRP3 inhibitor (10 mg/kg/d); MCC950+Y, NLRP3 inhibitor and Yimusake.

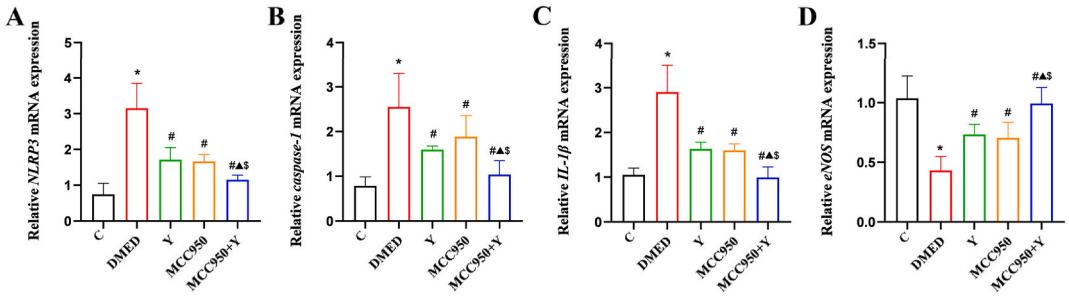

Fig. 4. RT-qPCR detected the mRNA expression of pyroptosis factors penile tissue. (A–D) Representative RT-qPCR quantification of NLRP3, caspase-1, IL-1β, and eNOS in the corpus cavernosum of DMED rats. The data are presented as means ± SD. N = 6 per group. $^{*}P < 0.05$ compared with the control group. $^{\#}P < 0.05$ compared with the DMED group. $^{\blacktriangle}P < 0.05$ compared with the Yimusake group. $^{\$}P < 0.05$ compared with the MCC950 group. C, control; DMED, diabetes mellitus-induced erectile dysfunction; Y, Yimusake (250 mg/kg/d); MCC950, NLRP3 inhibitor (10 mg/kg/d); MCC950+Y, NLRP3 inhibitor and Yimusake.

4. Discussion

The occurrence of DMED is a complex process. Recent studies have found that long-term hyperglycemia in diabetes patients leads to cell death of nerve, vascular endothelium, cavernous smooth muscle, testis and other tissues, which is closely related to erectile function, and then to the occurrence of ED [30]. As penile neurovascular lesions give rise to a poor response to oral PDE5 inhibitors in DMED patients, the options of an appropriate treatment is crucial for these patients [31].

Pyroptosis is a type of inflammation-related cell death. When pyroptosis occurs, NLRP3 acts as an intracellular pattern recognition receptor and recruits pro-caspase-1 to form a protein complex (called the inflammasome) [32]. When cells are stimulated, these inflammasomes induce the activation of caspase-1, which can promote the maturation and release of IL-1β [33] and trigger inflammatory cell death [34]. However, the role of pyroptosis in DMED remains unclear. Researches have suggested that hyperglycemia can promote NLRP3 inflammasome formation, which leads to inflammation and pyroptosis [35,36]. We speculate that the knockdown of NLRP3 may inhibit inflammasome-mediated pyroptosis and inflammation in endothelial cells of the penis in a high glucose environment. In this work, we focus on investigating changes within the corpus cavernosum microenvironment of DMED rats after treatment. Giving the regulatory capacity of NLRP3 in pathological settings, we inhibit the expression of NLRP3 in penile corpus cavernosum endothelial cells through MCC950 and Yimusake to achieve a better therapeutic effect on DMED. In recent years, the treatment of erectile dysfunction by Yimusake has been studied, providing a approach for the diagnosis and treatment of patients with ED [37–39]. We investigate the effect of NLRP3 inhibitor and Yimusake on erectile function based on the construction of DMED model. With this model, we find that the ICP/MAP ratio in the MCC950 and Yimusake combined treatment group increase and is close to that in the control group. HE staining show that the penile corpus cavernosum tissue structure is fuller and clearer and the smooth muscle content is higher in the MCC950 and Yimusake combined treatment group than the DMED group. As a marker of endothelial cells, CD31 maintains the vessel structural integrity and is crucial to endothelial cell-to-cell adhesion and signaling. The immunofluorescence results indicate that the expression of CD31 in the MCC950 and Yimusake combined treatment group increase than the DMED group. Downregulation of NLRP3 can inhibit endothelial cells death and reduce endothelial dysfunction and smooth muscle cell damage, thereby improving the effect of MCC950 and Yimusake on DMED. Thus, knocking down the NLRP3 gene would potentially block pyroptosis-related signaling pathways, thereby inhibiting inflammation and alleviating the disease.

According to our research, DMED rats have activated NLRP3 inflammasomes, which triggers caspase-1 and releases IL-1β. After the combined treatment with MCC950 and Yimusake, the expression of NLRP3, caspase-1, and the inflammatory cytokines IL-1β significantly decreased. The above results suggest that MCC950 and Yimusake may reduce the inflammatory response and improve erectile function in DMED rats by inhibiting pyroptosis. These changes in inflammation responses within the local microenvironment of the penis may be critical factor causing ED. Further immunohistochemical analysis and WB reveal that the NLRP3 expression in the cavernous tissue of the penis of DMED rats increases. We detected caspase-1 and other pyroptosis-related factor proteins, and found that pyroptosis indeed occurs in the penis cavernous tissue of the DMED. Our study demonstrates that MCC950 and Yimusake improve DMED by suppressing NLRP3 Inflammasome-mediated pyroptosis and inflammation in corpus cavernosum tissue. In addition, the expression of eNOS is higher in the MCC950 and Yimusake combined treatment group than in the DMED group, which indicates that MCC950 and Yimusake significantly increase the eNOS level in the DMED rats with sexual dysfunction, and improve mating ability.

In summary, through preventing NLRP3 activation, MCC950 and Yimusake improve DMED and decrease inflammation. This research demonstrates that by regulating the NLRP3-mediated pyroptosis pathway, MCC950 and Yimusake intervention provide protection against DMED penile damage. However, this study is an animal experiment, which confirms that pyroptosis is involved in the process of DMED, but there are still limitations and deficiencies. We need to explore the previously mentioned limitations in more detail in our upcoming studies.

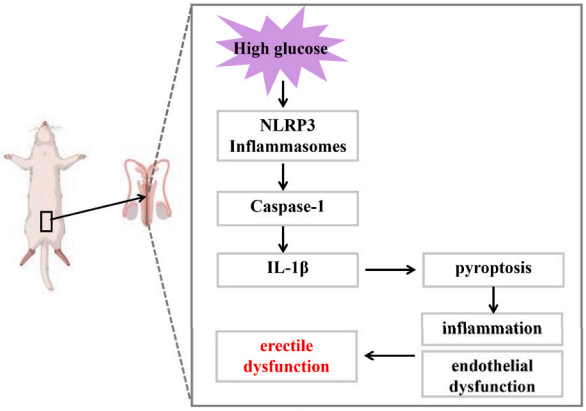

Fig. 5. Potential mechanism of MCC950 and Yimusake in the treatment of DMED rats. MCC950 improves erectile function in DMED rats. This mechanism involves the regulation of inflammation and endothelial dysfunction. (Figure was created with biorender.com).

5. Conclusions

MCC950 and Yimusake are confirmed to alleviate DMED rats by inhibits NLRP3-mediated pyroptosis pathway. The possible therapeutic effects of MCC950 and Yimusake were associated with the regulation of inflammation and endothelial function (Fig. 5). This process also includes the restoration of normal function to smooth muscle cells and endothelial cells. MCC950 and Yimusake may provide DMED patients a new and effective treatment approach.

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Patient consent for publication

Not applicable.

Ethics declarations

This study was reviewed and deemed exempt from ethics approval by Experimental Animal Ethics Committee of Xinjiang Medical University with the reference number: IACUC-20230503-24, dated 2023-5-3.

Data availability statement

The data included in article and supplementary material in article.

CRediT authorship contribution statement

Bingbing Zhu: Writing – original draft. **Xijia Zhang:** Writing – review & editing, Data curation. **Lipan Niu:** Methodology, Formal analysis. **Chengxia Yang:** Writing – review & editing, Data curation. **Xiufang Jin:** Supervision, Data curation. **Fengxia Liu:** Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Not applicable.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e38626.

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