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Pretreatment with kuanxiong aerosol protects against coronary microvascular dysfunction

[El pretratamiento con aerosol de kuanxiong protege contra la disfunción microvascular coronaria]

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Abstract: This study investigates the efficacy and mechanisms of Kuanxiong Aerosol (KXA) on coronary microvascular dysfunction (CMD) in rats. Thirty-two Sprague-Dawley rats were divided into control, model, KXA, and nicorandil groups, receiving respective treatments for three weeks. The CMD model was established by injecting lauric acid into the left ventricle. Compared to the model group, the KXA group showed significant reductions in serum CK-MB, LDH, cTnI, ET-1, TNF-α, IL-6, MDA, and ROS (p<0.01) and increased NO and SOD levels (p<0.01). KXA mitigated apoptosis and ameliorated CMD-associated pathological alterations. Pretreatment with KXA improves endothelial function and microvascular structure by counteracting inflammation, oxidative stress, and apoptosis, thereby improving CMD.

Keywords: Endothelial function; Inflammatory response; Oxidative stress; Apoptosis; Traditional Chinese medicine.

Resumen: Este estudio investiga la eficacia y los mecanismos del Aerosol de Kuanxiong (KXA) sobre la disfunción microvascular coronaria (CMD) en ratas. Treinta y dos ratas Sprague-Dawley se dividieron en grupos de control, modelo, KXA y nicorandil, recibiendo los respectivos tratamientos durante tres semanas. El modelo de CMD se estableció inyectando ácido láurico en el ventrículo izquierdo. En comparación con el grupo modelo, el grupo KXA mostró reducciones significativas en los niveles séricos de CK-MB, LDH, cTnI, ET-1, TNF-α, IL-6, MDA y ROS (p<0.01) y un aumento en los niveles de NO y SOD (p<0.01). KXA mitigó la apoptosis y mejoró las alteraciones patológicas asociadas con la CMD. El pretratamiento con KXA mejora la función endotelial y la estructura microvascular al contrarrestar la inflamación, el estrés oxidativo y la apoptosis, mejorando así la CMD.

Palabras clave: Función endotelial; Respuesta inflamatoria; Estrés oxidativo; Apoptosis; Medicina Tradicional China

INTRODUCTION

Coronary Microvascular Dysfunction (CMD) has garnered heightened scrutiny for its potential contribution to the etiology of ischemic cardiomyopathy. CMD manifests through derangements in the structure and function of coronary microcirculation, resulting in obstruction of coronary blood flow, oxygen supply unable to meet the need of myocardial cells, and myocardial ischemia as the ultimate outcome (Del Buono *et al*., 2021). Recent findings have implicated CMD in a notably elevated incidence of cardiovascular events, including heart failure, myocardial infarction, and even sudden cardiac death (Ayub & Kalra, 2020).

Currently, CMD is perceived as a complex pathophysiological process involving multiple pathways. It is generally accepted that the onset and progression of CMD are associated with the abnormal structure or function of the coronary microvasculature, along with specific molecular mechanisms. This process is closely related to oxidative stress and the ensuing inflammatory response (Masi *et al*., 2021). In terms of functional mechanisms, CMD may involve impaired vasodilation and excessive constriction of coronary microvessels. The impairment of vasodilation can originate from both endothelium-dependent and endothelium-independent mechanisms, with the former being the predominant one (Pries *et al*., 2015). Normal endothelial cells play a crucial role in regulating vasodilatory activity through the release of vasoactive substances such as the vasodilator nitric oxide (NO) and the vasoconstrictor endothelin-1 (ET-1) (Xu *et al*., 2021). A reduction in production or an increase in degradation of NO and other endothelial-derived vasodilatory factors can lead to the inhibition of endothelial-mediated vasodilatory capacity. Furthermore, an increased release of vasoconstrictor substances, such as ET-1, enhanced sensitivity of microcirculating vessels to normal vasoconstrictor substances, and abnormal sympathetic nerve activity are also potential mechanisms contributing to microvascular spasm (Del Buono *et al*., 2021). CMD is often detected in patients with myocardial infarction. As treatment strategies for myocardial infarction improve, the myocardial damage caused by ischemia is significantly decreased. However, microvascular endothelial cell injury may precede myocardial injury and be more severe. The microvascular damage caused by myocardial ischemia reperfusion injury can't be overlooked. Additionally, sustained myocardial ischemia can lead to elevated markers of myocardial injury, such as lactate dehydrogenase (LDH), creatine kinase-MB (CK-MB), and cardiac troponin I (cTnI) (Mair 1997). In CMD patients, inflammation or oxidative stress may damage coronary microvascular endothelial cells, leading to microvascular spasm or occlusions. Concurrently, literatures reveal that the pathogenesis of Coronary Microvascular Dysfunction (CMD) is associated with elevated levels of IL-6, TNF-α, and MDA. (Wang *et al*., 2020a; Chang *et al*., 2021; Godo *et al*., 2021). Structural abnormalities of CMD are mainly characterized by intramural arteriole and capillary lumen stenosis, perivascular fibrosis, and diminished capillary density (Camici & Crea, 2007). In coronary CMD, the impaired function and structural damage of coronary microvessels may lead to alterations in the intracellular environment, such as increased oxidative stress and intracellular calcium imbalance, thus promoting cell apoptosis. Additionally, the ischemic and hypoxic conditions caused by CMD can activate the apoptotic pathways, leading to apoptosis and death of cardiomyocytes (Li *et al*., 2023). It has been shown that CMD leads to increased expression of apoptotic proteins in cardiomyocytes, such as Bax, while the level of anti-apoptotic protein, such as Bcl-2, is reduced (Liu *et al*., 2016; Wang *et al*., 2016).

Treatment strategies for CMD primarily consist of vasodilation, endothelial protection, and anti-arteriosclerosis, employing common drugs such as nicorandil, statins, nitrates, angiotensin-converting enzyme inhibitors, and phosphodiesterase inhibitors. Among the drugs mentioned above, nicorandil is considered to be the first-line drug. As an ATP-sensitive potassium channel opener, nicorandil has been demonstrated to treat or prevent CMD by dilating coronary microvessels (Tsuchida *et al*., 2002). Reports suggest that nicorandil can improve CMD stemming from a variety of pathogeneses (Hirohata *et al*., 2014; Zhan *et al*., 2020). However, these medications typically operate via a single mechanism. Given the multifactorial nature of CMD, clinical treatment often employs a polypharmacy approach, potentially leading to issues such as oversubscription, increased side effects, and poor medication compliance (Bairey Merz *et al*., 2020). In this context, traditional Chinese medicine compound preparations offer advantages by exerting effects through diverse pathways. Kuanxiong aerosol (KXA) is comprised of five Chinese herbal medicines: *Asarum sieboldii* Miq, *Santalum album* L, *Alpinia officinarum* Hance, *Piper longum* L. and borneol

(Zhang *et al*., 2021). Long used in the treatment of angina pectoris, KXA has shown significant efficacy and minimal side effects. In angina patients, KXA is known to decrease serum levels of ET-1 and C-reactive protein while increasing NO levels (Zhuang *et al*., 2020). Its pharmacological actions in treating angina pectoris relate to non-hemodynamic mechanisms, including anti-myocardial ischemia, anti-inflammatory, and antioxidation (Wu *et al*., 2020). Yet, the impact of KXA on endothelial function, oxidative stress, and inflammatory response in CMD remains unreported. Our study aims to pretreat rats with KXA and establish a CMD model by injecting sodium laurate into the left ventricle to determine whether KXA can improve CMD in rats and elucidate the underlying mechanism.

MATERIAL AND METHODS

Drugs and reagents

KXA was purchased from Hangzhou Supor Nanyang Pharmaceutical Co., Ltd. (Hangzhou, China). Nicorandil was purchased from Tohoku Nipro Pharmaceutical Corporation (Japan). Sodium laurate and sodium carboxymethyl cellulose (CMC) were purchased from MedChemExpress LLC (Shanghai, China). Enzyme-linked immunosorbent assay ELISA kits for ET-1, interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α) were purchased from Cloud-Clone Technology (Wuhan, China). Malondialdehyde (MDA), NO, superoxide dismutase (SOD) assay kit and blocking buffer for Western blot were purchased from Beyotime Biotechnology (Shanghai, China). Reactive oxygen species (ROS) assay kit was purchased from Shanghai Enzyme-linked Biotechnology Co., Ltd. (Shanghai, China). B-cell lymphoma (Bcl)-2-associated X protein (Bax) monoclonal antibody, Bcl-2 antibody, TNF-α antibody, IL-6 antibody, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody and second antibody were all purchased from Proteintech (ProteinTech Group, Wuhan, China).

Preparation of KXA

The preparation of KXA adhered to a standardized protocol. A mixture containing *Asarum sieboldii* Miq. oil (23 mL), *Santalum album* L. oil (70 mL), *Alpinia officinarum* Hance oil (32 mL), and *Piper longum* L. oil (25 mL) was homogenized and subjected to a water bath at 40°C. The mixture's volume was then precisely adjusted to 625 mL with absolute ethanol. The final KXA solution was acquired post meticulous mixing and filtration (Lu *et al*., 2021).

Animals and treatment

Thirty-two male Sprague-Dawley (SD) rats, weighing between 250-300 g and sourced from the Laboratory Animal Research Center of Zhejiang Chinese Medical University, Hangzhou, China, were housed in cages, with 2-3 rats per cage. All the rats were provided with ample water and food daily, under optimal living conditions featuring a comfortable temperature (23 ± 2°C) and humidity (55 ± 5%), to facilitate normal growth. Following a week of acclimatization, the rats were randomly divided into four groups (n=8): control, model, KXA and nicorandil. Both the KXA and nicorandil groups were administered KXA intragastrically (1 mL/kg, dissolved in 0.5% CMC, 10% v/v) once per day and nicorandil (1.6 mg/kg, dissolved in 0.5% CMC, 16% w/v) once per day for 3 weeks. Calculation of the equivalent dose of KXA in rats was based on pharmacokinetics and conversion by body surface area, while calculation of the equivalent dose of nicorandil in rats was based solely on conversion by body surface area (Nair *et al*., 2018). Rats in the control group and the model group were administered intragastrically with 0.5% CMC (10 mL/kg) once per day for 3 weeks. After the last treatment, all rats were fasted for 12 h, then they were anesthetized with an intraperitoneal injection of pentobarbital sodium (40 mg/kg). Each rat was positioned supine and the chest was opened at the midpoint of the left second and third ribs to fully expose the heart and aortic arch. The rats in the model group, KXA group, and nicorandil group were injected with sodium laurate (2 g/L, 0.2 mL, dissolved in 0.5% CMC) into the left ventricle to induce a microvascular injury model (Liu *et al*., 2021; Sawuer *et al*., 2021). The aortic arch of the rat was clamped from the time of injection until 30 sec after the injection. The control group rats also underwent the same surgical procedure, receiving an equivalent volume of 0.5% CMC as a vehicle control. All rats were euthanized 24 h later by cervical dislocation. Blood was collected from the rat abdominal aorta for serological analysis. Hearts from all rats were collected for subsequent Western blot analysis and histopathology.

Measurement of myocardial ischemia related indexes

The serum levels of lactate dehydrogenase (LDH), creatine kinase-MB (CK-MB), and cardiac troponin I (cTnI) were measured using an automated biochemical analyzer (Abbott Aeroset, USA).

Detection of vascular endothelial function
NO assay kit and ET-1 ELISA kit were used to detect the levels of NO and ET-1 in rat serum, following the provided instructions.

Detection of oxidative stress levels
ROS, SOD and MDA levels were measured using their respective detection kits, in accordance with the manufacturer's instructions.

Detection of inflammation cytokines
Serum levels of IL-6 and TNF-α were measured using the respective ELISA detection kits, following the provided instructions.

Western blotting for expression of Bax and Bcl-2
Rat heart tissue with a mass of about 20 mg was cut with scissors. Approximately 20 mg of heart tissue was mixed in RIPA lysis buffer containing protease inhibitors, and the tissue was homogenized with a tissue grinder until fully dissolved. After centrifugation at 14000 g for 5 min at 4℃, the supernatant was collected. The protein concentration of the supernatant was determined by the Bicinchoninic Acid Assay. Next, the protein was fully mixed with 5× loading buffer, then heated at 100℃ for 5 min for denaturation. Subsequently, equal amounts of protein (30 μg) were separated from different samples for 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were transferred to polyvinylidene fluoride membranes (Merck Millipore Ltd, IRL, UK) using a transblot apparatus. The membranes were blocked in a blocking buffer for Western blot for 30 min, then placed on a shaker, and incubated with specific primary antibodies: Bax rabbit polyclonal antibody (1:5000), Bcl-2 rabbit polyclonal antibody (1:500) and GAPDH rabbit polyclonal antibody (1:5000), to incubate overnight at 4℃. The membranes were then incubated with an anti- rabbit secondary antibody (1:2000) for 2 h. After being washed with TBST solution, the membranes were treated with a luminescent solution and observed under the ECL system.

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) assay
Apoptosis was evaluated using TUNEL assay kit (Beyotime Biotechnology, Shanghai, China) following the manufacturer’s instructions and analyzed with a Confocal laser scanning microscope (Leica, DE).

Myocardial pathological staining
The myocardium tissue of the rats was fixed, dehydrated, embedded in paraffin, and sectioned into 5 μm thick sections parallel to the coronary sulcus. Histopathological analysis of myocardial tissue was performed using both hematoxylin-eosin (HE) staining and Masson's trichrome staining, provided by Senbeijia Biotechnology, Nanjing, China.

Immunohistochemistry (IHC) staining
The expression of both TNF-α and IL-6 in myocardial tissue of rats was detected using IHC. Myocardium tissue slices were incubated with the primary antibody (1:100) overnight at 4°C, followed by incubation with the secondary antibodies (1:200). Next, the slices were stained with a DAB chromogenic reagent kit (Gene Tech, Shanghai, China) and with hematoxylin staining solution. Finally, the slices were visualized under the microscope. The extent of immunoreactivity was quantified as the proportion of positive areas, analyzed using the ImageJ software.

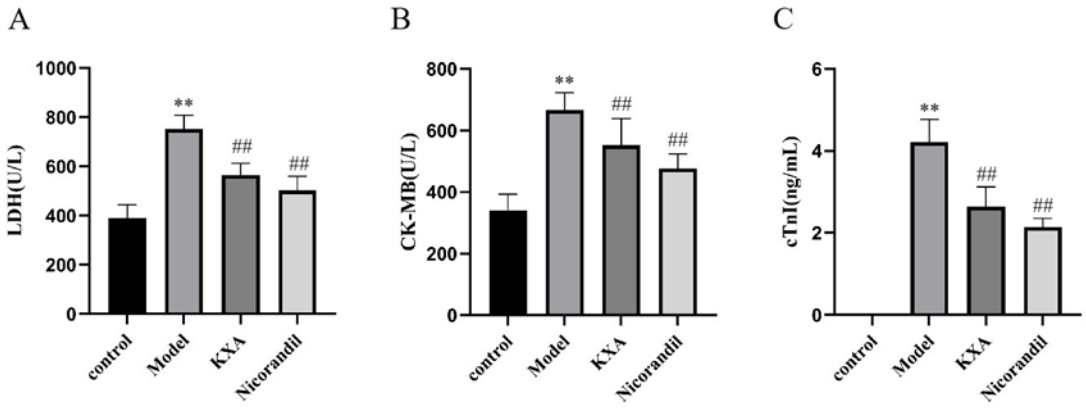
Statistical analysis
All data were analyzed using SPSS statistical software (version 19.0, IBM, Shanghai, China). All values are e reported as the mean ± standard deviation. The parameters were evaluated by One-way analysis of variance, and Scheffe's post- hoc test was used if there were significant differences. In this study, the significance threshold is established at $\alpha = 0.05$.

RESULTS

KXA protects myocardium in CMD rats
The concentrations of CK-MB, LDH and cTnI in the model group were significantly elevated compared to those in the control group ($p<0.01$). Additionally, LDH, CK-MB and cTnI levels demonstrated a notable reduction in both the KXA and nicorandil groups when contrasted with the model group ($p<0.01$) (Figure No. 1A and Figure No. 1C).

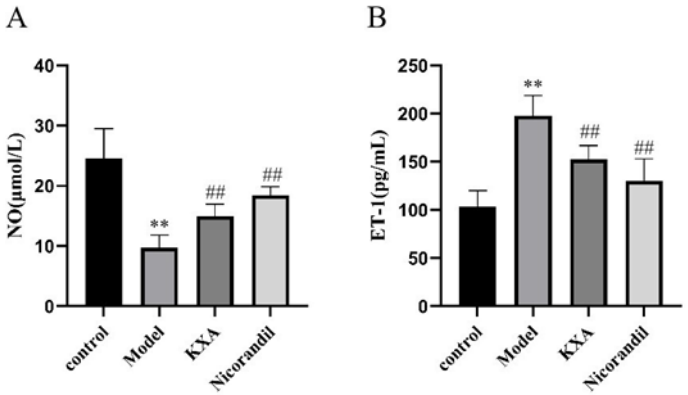
The effect of KXA on vascular endothelium
We observed a significant increase in ET-1 levels in the model group, accompanied by a decrease in NO concentration compared to the control group ($p<0.01$). However, in both the KXA and nicorandil groups, this trend was less pronounced than in the model group ($p<0.01$) (Figure No. 2A and Figure No. 2B).

Figure No. 1



Effects of KXA on serum LDH (A), CK-MB (B) and cTnI (C) levels in CMD rats. Data are the mean ± standard error of mean (n=8). *****p*<0.01 vs control. ##*p*<0.01 vs model

Figure No. 2

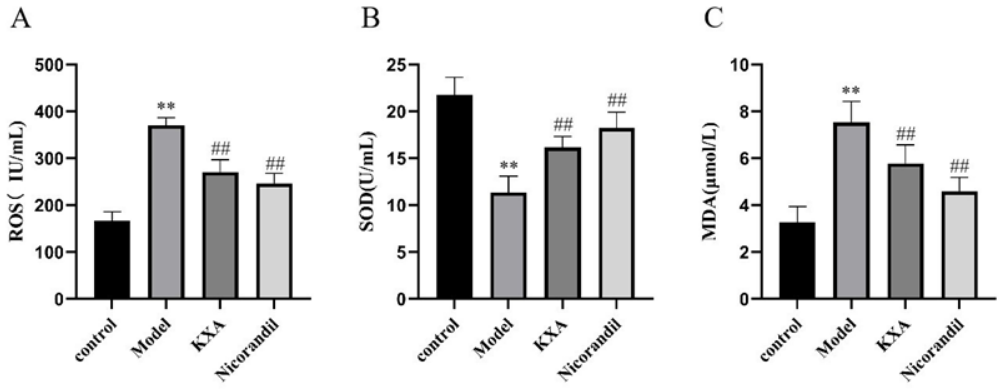


Effects of KXA on serum NO (A) and ET-1 (B) levels in CMD rats. Data are the mean ± standard error of mean (n=8). *****p*<0.01 vs control. ##*p*<0.01 vs model

Effects of KXA pretreatment on oxidative stress in CMD rats

In comparison to the control group, the serum levels of ROS and MDA increased in the model group, while the SOD level decreased (p<0.01). Conversely, in both the KXA and nicorandil groups, the serum levels of ROS and MDA significantly decreased, accompanied by an increase in the SOD level when compared to the model group (p<0.01) (Figures No. 3A, 3B and 3C).

Figure No. 3

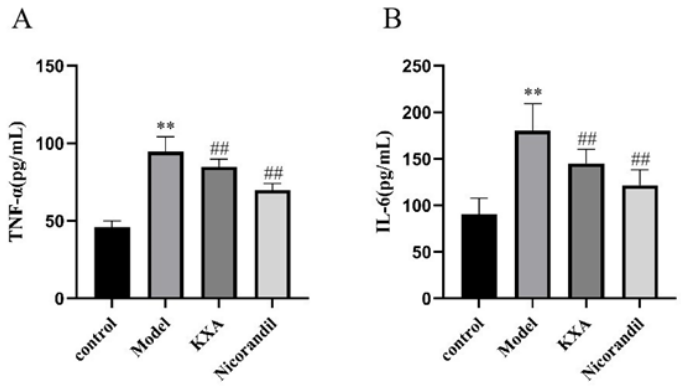


Effects of KXA on serum ROS (A), SOD (B) and MDA (C) levels in CMD rats. Data are the mean ± standard error of mean (n=8). **p<0.01 vs control. ##p<0.01 vs model

KXA pretreatment can reduce inflammatory factors in CMD rats

The serum levels of TNF-α and IL-6 in the model group exhibited a significant increase compared to those in the control group (p<0.01). Conversely, in both the KXA and nicorandil groups, the serum levels of TNF-α and IL-6 were markedly decreased when compared to the model group (p<0.01) (Figures No. 4A and 4B).

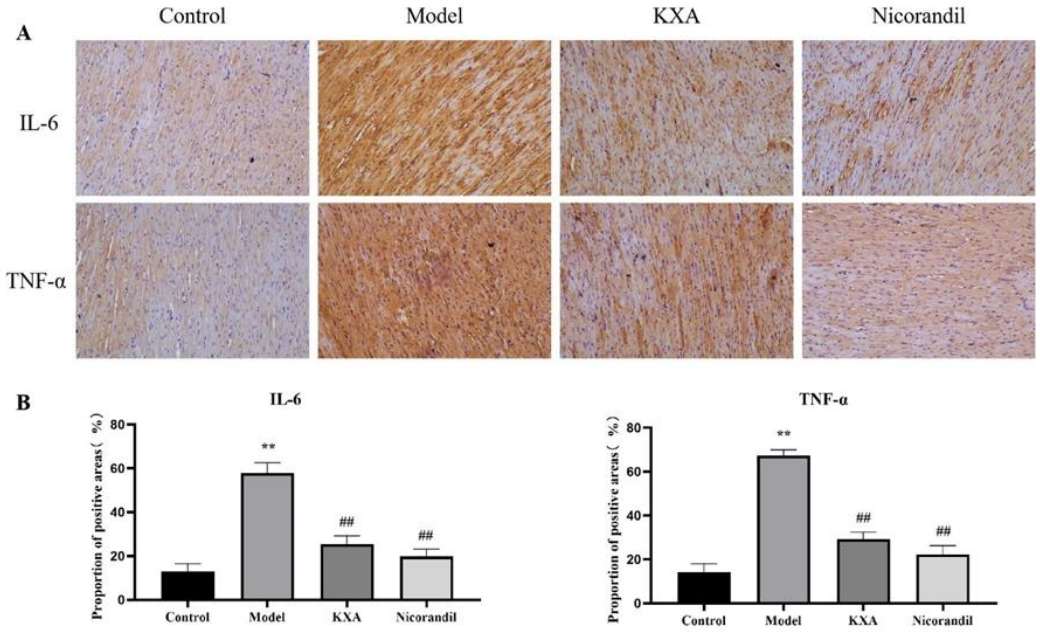
Figure No. 4



Effects of KXA on serum TNF-α (A) and IL-6 (B) levels in CMD rats. Data are the mean ± standard error of mean (n=8). **p<0.01 vs control. ##p<0.01 vs model

IHC was conducted to assess the impact of KXA on TNF-α and IL-6 expression. The IHC results indicated a significant increase in the expression of TNF-α and IL-6 in myocardial tissue following modeling (p<0.01). Furthermore, the expression of TNF-α and IL-6 in both the KXA and nicorandil groups exhibited a notable reduction compared to the model group (p< 0.01). Therefore, KXA demonstrates an inhibitory effect on inflammatory reactions in both serum and myocardial tissue of rats (Figures No. 5A and 5B).

Figure No. 5

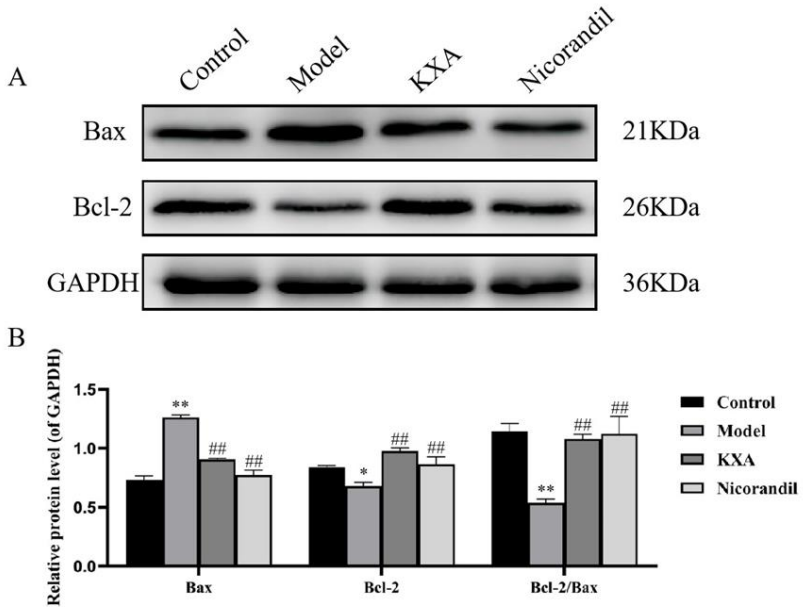


Effects of KXA on expression of TNF-α and IL-6. (A) Images of IHC (magnification × 200). (B) Immunohistochemical analysis of TNF-α and IL-6 in in myocardial tissues (n=5). **p<0.01 vs control; ##p<0.01 vs model

KXA Inhibits apoptosis of rat cardiomyocytes

We assessed the expression of Bax and Bcl-2 in cardiac tissue to gain deeper insights into the effect of KXA on CMD-related apoptosis. Compared to the control group, the model group exhibited elevated expression of Bax (p<0.01) and decreased expression of Bcl-2 (p<0.05). Additionally, the Bcl-2/Bax ratio was lower in the model group (p<0.01). Following treatment with KXA and nicorandil, both the KXA and nicorandil groups demonstrated reduced expression of Bax and increased expression of Bcl-2 (p<0.01). Moreover, the Bcl-2/Bax ratio was higher in both the KXA and nicorandil groups (p<0.01) (Figures No. 6A and 6B).

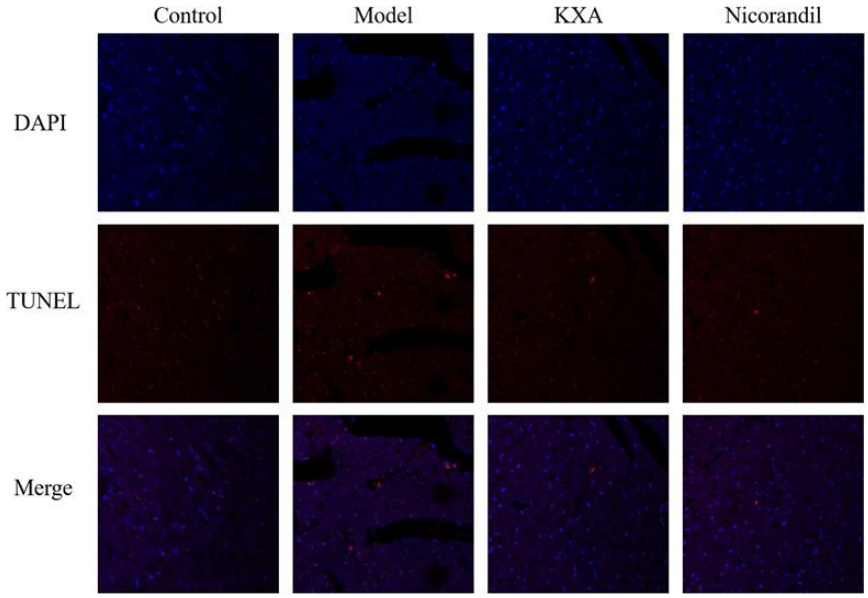
Figure No. 6



Western blots showing expression of Bcl-2 and Bax in myocardial tissues. (A) bands correspond to protein expression of Bax and Bcl-2; (B) Quantified protein expression of Bax and Bcl-2. Protein levels were determined by normalisation to GAPDH levels. Bcl-2: B-cell lymphoma-2; Bax: Bcl-2-associated X protein; GAPDH: glyceraldehyde phosphate dehydrogenase. Densitometric values are the mean ± standard error of mean (n=4). *p<0.05 vs control; **p<0.01 vs control; ##p<0.01 vs model

TUNEL staining revealed an increased rate of TUNEL positive cells in model group, whereas only a few TUNEL positive cells were detected in the control group. Furthermore, the rate of cell apoptosis significantly decreased in both the KXA and nicorandil groups (Figure No. 7).

Figure No. 7

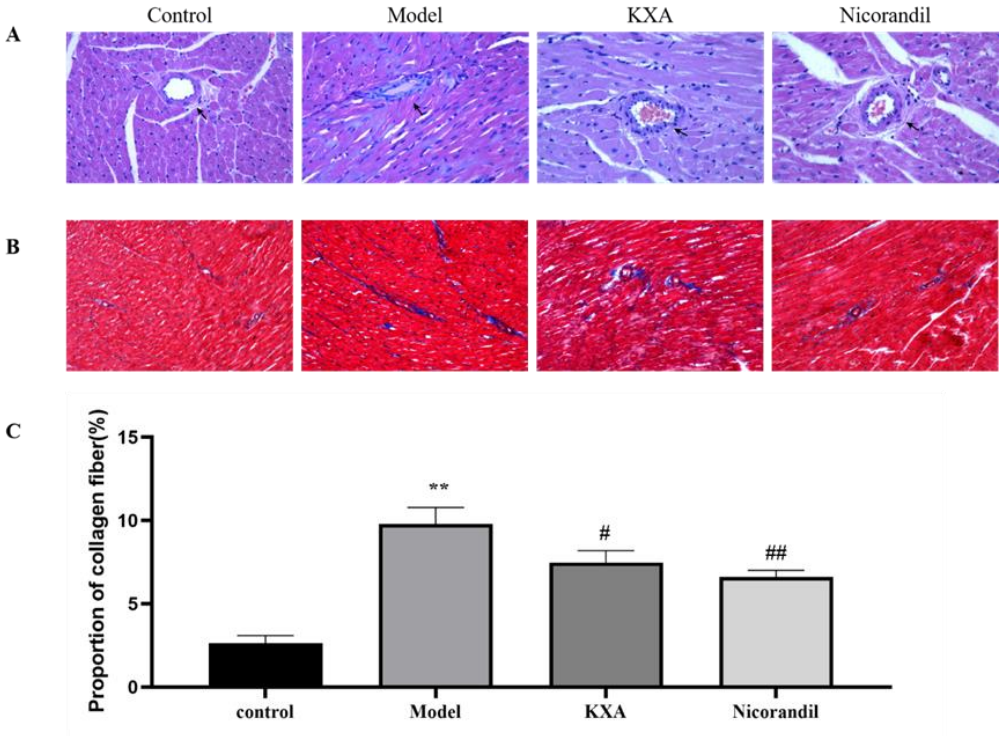


TUNEL staining of myocardial tissue in CMD rats (magnification × 200)

KXA can prevent the injury of coronary microvessels and the proliferation of collagen fibers in CMD rats

Histopathological examination was conducted using HE and Masson's trichrome staining to visually depict the microvascular structure and myocardial injury in rats. HE staining revealed myocardial microvascular stenosis, microthrombus formation within the microvessels, and disorganized myocardial cell arrangement in the modeled rats. These pathological changes were significantly ameliorated in both the KXA and nicorandil groups (Figure No. 8A). The results of Masson's trichrome staining exhibited significant proliferation of collagen fibers around the microvessels, which was effectively alleviated by intervention with KXA and nicorandil (Figures No. 8B and 8C).

Figure No. 8



HE pathological staining and Masson's trichrome staining of myocardial tissue in CMD rats. (A)The black arrows indicate normal or diseased microvessels (magnification × 400). (B) The representative images of Masson's trichrome staining (magnification × 200). (C) Quantitative analysis of Masson's trichrome staining (n=5). **p<0.01 vs control; #p<0.05 vs model; ##p<0.01 vs model

DISCUSSION

The coronary microcirculation comprises arterioles and anterior arterioles less than 500 μm in diameter. The onset and progression of CMD are attributed to structural and functional alterations within these vessels, culminating in hindered coronary blood flow and resulting in myocardial ischemia (Ong *et al*., 2018). Pathologically, CMD is typified by microvascular lumen stenosis, intravascular microthrombosis and perivascular fibrosis. In our study, pathological staining revealed the formation of microthrombus, increased deposition of perivascular collagen fibers, and the presence of microvascular stenosis in the model group. Furthermore, pyknosis of myocardial nuclei, chromatin aggregation, and disorganized arrays of myocardial cells proximal to the obstructed microvessels were also notable in the model group specimens. In contrast, the KXA group exhibited a marked reduction in intravascular microthrombi and perivascular collagen fibril

deposition. Additionally, the previously disturbed arrangement of cardiomyocytes and the observed microvascular stenosis were ameliorated. The results of IHC showed that the expression of IL-6 and TNF-α in the myocardial tissue were increased significantly after modeling. Furthermore, in the KXA group, the expression of IL-6 and TNF- α were significantly decreased. These results suggest that KXA can inhibit inflammatory reaction, reduce perivascular fibrosis, and improve the pathological changes of microvascular structure. *Piper longum* L. and *Alpinia officinarum* Hance, both components of KXA, have been reported in modern pharmacological studies to possess multiple effects, such as anti-platelet aggregation, anti-inflammatory, and anti-hyperlipidemia effects (Abubakar *et al*., 2018; Biswas *et al*., 2022). Additionally, *Piper longum* L. can inhibit collagen-induced platelet activation, aggregation, and thrombosis (Yuan *et al*., 2015). Alpinia officinarum Hance is effective in anti-inflammation, antioxidation, anti-fibrosis, and lipid-decreasing (Zhang *et al*., 2019). Curcumin, the primary extract of *Alpinia officinarum* Hance, has been shown to exert anti-fibrotic effects on the heart by reducing collagen deposition and extracellular matrix accumulation, as well as inhibiting the proliferation and migration of cardiac fibroblasts (Xiao *et al*., 2016). Regarding KXA, the possible mechanism for pathological improvement in CMD involves KXA's capacity to inhibit platelet activation and prevent thrombosis. Besides, KXA may exert anti-fibrotic effects by regulating collagen deposition, extracellular matrix accumulation, and the proliferation and migration of cardiac fibroblasts. When compared with the model group, we observed that the serum levels of LDH, CK-MB and cTnI, which usually respond to myocardial ischemia, were significantly decreased in the KXA group. These findings indicate that pretreatment with KXA can effectively ameliorate the structural changes of coronary microvessels and preserve the myocardial microcirculation's blood supply, thereby mitigating myocardial damage caused by ischemia.

Besides the characteristic changes in pathological structure, endothelial dysfunction is an important functional alteration in CMD progression. ET-1 and NO are crucial indicators of vascular endothelial function. Consequently, we assessed the serum concentrations of these markers in all rat groups. Endothelial cells play a crucial role in regulating vasodilatory activity through the release of vasoactive substances such as NO and ET-1 (Xu *et al*., 2021). NO also protects endothelial integrity through the angiogenesis effects and its anti-inflammatory, anti-fibrotic, anti-platelet aggregation, and anti-apoptosis effect (Vancheri *et al*., 2020). When endothelial cells are injured, the NO release decreases and the ET-1 release increases, leading to microvascular constriction. In addition, the decreased release of NO also promotes collagen deposition and the conversion of endothelial cells to mesenchymal cells, reducing angiogenesis and collateral development, with fibrosis, hypertrophy and even the loss of perfused microvessels as the outcome (O'Riordan *et al*., 2007; Goligorsky, 2010). In our study, we found that compared with the model group, the serum NO level was increased and the ET-1 level was decreased in the KXA group, indicating that KXA protects endothelial function and prevents microvascular spasm, promoting elevated NO levels and enhancing the anti-inflammatory and anti-fibrotic functions of the vessels themselves. These results were consistent with the outcome of pathological staining of rats in the KXA group.

Currently, oxidative stress caused by excessive production and accumulation of cellular ROS and the consequent inflammatory response is considered to be one of the fundamental pathogenic mechanisms driving the development of CMD (Masi *et al*., 2021). Mitochondria play an important role in ROS production. The dysfunction of mitochondria caused by various reasons can induce oxidative stress, disturb metabolism, and activate endothelial cell apoptosis, thus exacerbating the progression of coronary artery microvascular disease. When stimulated by ROS, cardiomyocytes can utilize antioxidant factors to neutralize ROS produced by mitochondria, reducing the damage to cardiomyocytes and stabilizing the mitochondrial state (Wang & Zhou, 2020; Wang *et al*., 2020b; Sun *et al*., 2022). ROS include superoxide anion, hydrogen peroxide, hydroxyl radicals and singlet oxygen (Borisov *et al*., 2021). Among them, the binding between superoxide anion and NO can lead to a decrease in NO bioavailability and a reduction in the antiproliferative, anticoagulant, anti-inflammatory and vasodilatory effects (Förstermann, 2010; Drummond *et al*., 2011; Li & Pagano, 2017). Peroxynitrite is a product of this binding reaction, and has strong oxidizing properties. It can cause damage to endothelial cells by the peroxidation of DNA, lipids and proteins along with superoxide anion (Laursen *et al*., 2001). The MDA is the end product of lipid peroxidation, which can exacerbate

cell membrane damage. Therefore, the level of MDA can reflect lipid peroxidation in vivo and indirectly reflect the cell damage (Tsikas, 2017). In contrast, SOD is a vital part of the antioxidant defense system against superoxide anion. Superoxide anion can react with SOD to form H_2O_2, then H_2O_2 can be catalyzed by catalase, peroxidase or glutathione peroxidase to form H_2O (Fukai & Ushio-Fukai, 2011). Through these reactions, the oxidative stress of the superoxide anion is eliminated. It has been shown that components such as *Alpinia officinarum* Hance, *Santalum album* L. and borneol in KXA have antioxidant effects (Liu *et al*., 2011; Zhang *et al*., 2019; Younis, 2020). Our research showed that the CMD rats pretreated with KXA had elevated SOD activity and a decreased level of MDA in serum, suggesting that KXA has antioxidant effects. And the possible mechanisms are that KXA can enhance SOD activity in vivo and strengthen the antioxidant defense system, reducing the lipid oxidation reaction and thereby protecting the endothelial cells.

Existing literature indicates that the inflammatory response is a contributing factor in the incidence and progression of CMD (Sagris *et al*., 2021). Endothelial cells are activated in response to the stimulation of injury or oxidative stress, producing various inflammatory factors. The activated endothelial cells attract monocytes and neutrophils to aggregate, which triggers inflammatory reaction (Chistiakov *et al*., 2018). Especially the IL-6 levels seem to be directly associated with increased vascular inflammation and endothelial damage (Walter *et al*., 2019). TNF-α can promote the apoptosis of endothelial and smooth muscle cells by regulating the formation of ROS (Zhang, 2008). In our study, the serum levels of IL-6 and TNF-α were decreased in CMD rats pretreated with KXA, suggesting that KXA could exert an anti-inflammatory effect by reducing inflammatory factors and inhibiting the aggregation of inflammatory cells, thereby preventing further damage to endothelial cells by the inflammatory reaction. Furthermore, we propose that IL-6 and TNF-α may contribute to the development of vascular fibrosis through three mechanisms: stimulation of fibroblast proliferation and collagen synthesis, directly leading to fibrosis; increasing vascular permeability resulting in the extravasation of inflammatory cells, which can release cytokines that further promote fibrosis; and activating signaling pathways, such as those involving TGF-β and PDGF, which enhance perivascular fibrosis (Antar *et al*., 2023). Perivascular fibrosis refers to the proliferation of fibrous tissue occurring around vascular structures, often accompanied by alterations in the extracellular matrix and inflammatory responses under pathological conditions. This fibrotic process can affect the microvascular architecture, potentially leading to thickening of the vessel wall, narrowing of the lumen, or even complete vessel occlusion (Wynn, 2008). Reducing the levels of IL-6 and TNF-alpha may slow the progression of perivascular fibrosis. This could be achieved by diminishing inflammatory response, inhibiting fibroblast activity and synthesis of collagen, as well as modulating signaling pathways associated with fibrosis.

Both oxidative stress and the inflammatory response can eventually lead to apoptosis. Apoptosis is generally regarded as one of the most common myocardial cell deaths. Apoptosis is regulated by the outer mitochondrial membrane (OMM) permeability, mitochondrial membrane potential reduction and caspase-9 activation. Bax is an important inducer of OMM permeability, and Bcl-2 can prevent Bax-induced OMM damage. Therefore, both of the Bcl-2 and Bax levels and the Bcl-2/Bax ratio can be used to monitor apoptosis (Wang & Zhou, 2020). In our findings, the results of the Western Blot showed that KXA pretreatment decreased the expression of Bax and increased the expression of Bcl-2, which prevented apoptosis in cardiomyocytes. In addition, the results of the TUNEL also showed that KXA had an effect on inhibiting cell apoptosis. The mechanism may involve KXA inhibiting the apoptosis of cardiomyocytes by regulating the expression of apoptotic proteins involved in the pathways of apoptosis.

CONCLUSION

KXA can improve CMD by protecting vascular endothelial function and resisting oxidant stress, inflammation and cell apoptosis. This finding shows that KXA, one of the popular traditional Chinese medicines for the treatment of angina pectoris, holds promise for improving CMD.

Limitations of the study

There are still some shortcomings in this study. Firstly, the sample size of the study is limited, and an expanded sample size is necessary to further corroborate the results of this experiment. Secondly, since KXA is an aerosol, sublingual spray should be the most appropriate method of administration. However, due to limited experimental conditions, the

administration method adopted in this study was by gavage. Moreover, due to limitations in the administration method, we cannot determine the optimal dose of the drug for rats, which may have a certain impact on bringing the effect of KXA into full play. Thirdly, in clinical practice, there is still no specific indicator to evaluate and diagnose CMD. In this study, we were not able to identify methods that directly measure the microvascular function of rats. To evaluate whether the model was established successfully, we opted to observe CMD-related pathological changes in the myocardial tissue of the rats in the model group.

Ethics statement

This animal experiment was reviewed and approved by the Ethics Committee of Shaoxing People's Hospital [SYXK (Zhe) 2022-0022; Yuecheng, China] and all methods were carried out according to relevant guideline and regulations. We followed the ARRIVE guidelines throughout the entire animal experiment process.

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Data Availability

All datasets presented in this study are available from the corresponding authors on reasonable request.

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