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Protective effects of matrine on cardiomyocytes infected with coxsackievirus B_3 via modulation of the calpain-2/caspase-12 signaling pathway

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Abstract

Viral myocarditis (VMC) presents a substantial threat, especially for children, often leading to cardiogenic shock and fulminant myocarditis. Our study aimed to evaluate the role of calpain-2 and caspase-12, which were involved in the endoplasmic reticulum apoptosis pathway, and the influence of Matrine on these proteins during Coxsackie virus B_3 (CVB_3)-induced acute VMC mice in vitro and in vivo, shedding light on the potential cardioprotective effects. We first performed primary cultured cardiomyocytes, which were infected with CVB_3in vitro. We observed cell viability, the beating of cardiomyocytes and cytopathic effects. And we utilized Balb/c mice to establish the VMC animal model and determined viral titers, histopathological changes, and myocardial pathological scores. Furthermore, we detected CK-MB levels and myocardial cell apoptosis in vitro and in vivo. In order to further explore the possible mechanisms, the protein expression of calpain-2 (by immunohistochemistry and Western blot) and caspase-12 activity (by fluorescence assay for substrate cleavage) were detected in vitro and in vivo. Our findings indicated that, in comparison to the normal control group, the virus-infected group exhibited increased injured myocardial cells, virus titer, CK-MB levels, and apoptotic cells ($P<0.05$). Matrine treatment groups significantly reduced CK-MB levels, myocardial cellular damages and apoptosis in vitro and in vivo, with Matrine notably suppressing calpain-2 protein expression and Caspase-12 activity compared to the virus-infected group ($P<0.05$). In conclusion, our study revealed that calpain-2 and caspase-12 played roles in CVB_3-induced myocardial cell apoptosis. Matrine effectively mitigated myocardial cell injury and reduced apoptosis, thereby providing substantial protection against CVB_3infection in vitro and in vivo, which may be related to the down-regulation of calpain-2/caspase-12 signaling pathway.

Keywords: Matrine, Coxsackievirus B_3, Cardiomyocytes, Calpain-2, Caspase-12.

1. Introduction

Matrine is a naturally occurring tetracyclic quinolizidine alkaloid predominantly found in leguminous plants such as *Sophora flavescens* and *Sophora alopecuroides* [1,2]. Its chemical formula is $C_{15}H_{24}N_2O$, with a molecular weight of 248.36 g/mol [2-5]. The well-defined and relatively simple chemical structure of matrine as a monomer facilitates its use as a scaffold in the development of novel therapeutic agents [3,4]. As the principal bioactive metabolite of *Sophora flavescens*, matrine has been extensively studied for its diverse pharmacological properties, including anti-inflammatory, antiviral, anticancer, and cardioprotective effects [1-5].

Previous studies have demonstrated that matrine exhibits protective effects on cardiomyocytes infected with Coxsackievirus B_3 (CVB_3), a common etiological agent of viral myocarditis (VMC). Despite these promising findings, the detailed molecular mechanisms underlying matrine's cardioprotective actions, particularly in the context of virus-induced myocardial injury, remain largely unexplored. Moreover, the in vivo effects of matrine on acute viral myocarditis and its modulation of intracellular signaling pathways have not been fully elucidated [6].

Apoptosis, or programmed cell death, plays a pivotal role in the pathogenesis of myocarditis, contributing to cardiomyocyte loss and cardiac dysfunction [7]. One key apoptotic pathway implicated in this process is endoplasmic reticulum stress (ERS)-induced apoptosis, which can be triggered by various cellular stressors. Calpains, a family of neutral cysteine proteases, have been shown to mediate cellular damage and apoptosis through proteolytic cleavage of target substrates. Among them, calpain-2 is particularly important in regulating apoptotic signaling cascades. Caspase-12, a member of the cysteine protease family with specificity for aspartate residues, is closely associated with ERS-mediated apoptosis and inflammatory responses and serves as a critical effector molecule within this pathway [8-12].

Given the significance of calpain-2 and caspase-12 in ERS-induced apoptosis and myocardial injury, this study

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aimed to investigate their roles in the development of viral myocarditis. Furthermore, we seek to elucidate the potential protective mechanisms of matrine on cardiomyocytes infected with CVB_3, both in vitro and in vivo, focusing on the modulation of the calpain-2/caspase-12 signaling pathway. Understanding these mechanisms may provide valuable insights into matrine's therapeutic potential for the treatment of viral myocarditis and related cardiac diseases.

2. Materials and Methods

2.1. Materials

2.1.1. Major reagents

The major reagents used in this study included: Matrine, with a purity of ≥ 98%, batch number 040102, was provided by the Institute of Regenerative Medicine, Jilin University. Ribavirin injections, batch number: 061027, were purchased from Suzhou (China).

2.1.2. Cells and virus

Human amniotic cell line (FL) and CVB_3 (Nancy strain) were obtained from the Virus Research Laboratory, Institute of Regenerative Medicine, Jilin University.

2.1.3. Experimental animals

The experimental animals used in this study included 1-3-day-old neonatal Wistar rat pups and 4-week-old Balb/c mice (male, weight: 16±2 g). All animals were specific pathogen-free grade and certified with the qualification number SCXK (Ji) 2003-0001. The animals were purchased from the Experimental Animal Center of the Basic Medical School at Jilin University.

2.2. Methods

2.2.1. In Vitro experiments

2.2.1.1. Preparation and culture of primary cardiomyocytes

Neonatal Wistar rats were disinfected with 75% ethanol, and the apex portion (2/3 of the heart) was extracted and placed in IMDM medium. The tissue was washed to remove blood, minced, and digested with 0.25% trypsin at 37°C for 15 minutes with agitation. This process was repeated 3-4 times. The supernatants were combined and centrifuged at 1200 rpm for 15 minutes. The cell pellet was resuspended in IMDM containing 15% fetal bovine serum (FBS-IMDM) and seeded into culture flasks. After 1-2 hours, unattached cells were collected, diluted to a concentration of 2.0×10^5 cells/mL, and seeded at 0.2 mL/well in 96-well plates. Cells were cultured at 37°C in a 5% CO_2 humidified incubator for 48 hours, then the medium was changed. Once cardiomyocyte beating was observed, the cells were used for experiments.

2.2.1.2. Experimental groups

The cytotoxicity of Matrine on primary cardiomyocytes was first assessed. Groups were then formed based on the TC_{50} and TC_0 values of the drug. Seven groups were established: normal control (NC), virus (VK), ribavirin treatment (RBV at maximum non-toxic concentration), and Matrine treatment at four concentrations (600 $mg\cdot L^{-1}$, 300 $mg\cdot L^{-1}$, 150 $mg\cdot L^{-1}$, and 75 $mg\cdot L^{-1}$). Each group had eight replicates.

To investigate the effects of Matrine on CVB_3-infected cardiomyocytes, three distinct administration protocols were employed:

Type I (Simultaneous Administration):

Matrine and CVB_3 virus were added to the cardiomyocyte cultures simultaneously and allowed to adsorb for 1.5 hours. Following adsorption, the medium was replaced with fresh maintenance medium containing Matrine.

Type II (Pre-treatment with Matrine):

Matrine was added to the cultures and incubated for 1.5 hours. After removal of Matrine, CVB_3 virus was added and adsorbed for 1.5 hours. Subsequently, the medium was replaced with maintenance medium containing Matrine.

Type III (Post-infection Treatment):

CVB_3 virus was added to the cultures and adsorbed for 1.5 hours. After removal of the virus, the medium was replaced with maintenance medium containing Matrine.

All protocols ensured that after the initial adsorption period, the cells were maintained in a medium supplemented with Matrine for the remainder of the experiment. This design allowed for the assessment of Matrine's protective effects when administered at different stages relative to viral infection.

2.2.1.3. Inhibition assay of Matrine on CVB_3-infected cardiomyocytes

Cardiomyocytes cultured for 48 hours were used. Each group had eight replicates. All groups except NC were infected with 100 $TCID_{50}$ of CVB_3 virus. After 1.5 hours, the virus was removed, and the medium containing the respective treatments was added. After 48 hours, cardiomyocyte morphology, cytopathic effects (CPE), and beating were observed. When the CPE in the virus control group reached the maximum level (++++), the experimental outcomes were recorded. Cell viability in each well was then assessed using the MTT assay, with optical density (OD) measured at 570 nm.

2.2.1.4. Measurement of myocardial enzymes

Supernatants from infected cardiomyocyte cultures were collected after 48 hours and immediately tested for creatine phosphokinase isoenzyme-MB (CK-MB) levels by Beckman fully automatic biochemical analyzer (LX20, USA).

2.2.1.5. Detection of CVB_3-induced apoptosis in cultured cardiomyocytes by flow cytometry

The experiment was conducted with the following three groups: Normal control group (NC), Virus group (VK) and Matrine treatment group (Mat 300 $mg\cdot L^{-1}$). Cardiomyocytes were cultured in 24-well plates, with six replicates for each group. The cells were incubated at 37°C, and apoptosis percentages were assessed using flow cytometry (BD, FACS Aria, USA) at two time points: 24 hours and 36 hours. The percentage of apoptotic cells was measured according to the Annexin V-EGFP/PI apoptosis detection kit manual (KeyGen, Nanjing, China). Flow cytometry was performed with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

2.2.1.6. Immunocytochemistry for detecting Calpain-2 protein

The myocardial cells were prepared for immunocytochemistry staining according to standardized protocol and incubated with antibody (Santa Cruz) subsequently.

Images were visualized under a microscope. The Luzex-F image analysis system (Japan) was used to measure the grayscale value of staining.

2.2.1.7. Western blotting for Calpain-2 protein expression in cardiomyocytes

The cells were fully lysed to extract the protein. Total protein quantification was performed using a spectrophotometer (Toshiba, Japan). Samples were subjected to SDS-PAGE for separation and then transferred onto nitrocellulose membrane under controlled conditions.

The primary antibody was incubated overnight at 4 °C, the secondary antibody was incubated at room temperature for 2 h, and the protein bands were colored and actin was used as the internal reference. Analyze the images using a computer-based image analysis system.

2.2.1.8. Caspase-12 activity assay

Caspase-12 activity was measured using a fluorescent assay kit (Biovision), according to the manufacturer's protocol. Briefly, 2–5 × 10^6 cells were harvested and processed as instructed in the kit manual. The resulting reaction mixture was transferred to a micro quartz cuvette, and fluorescence was measured using a fluorometer with an excitation wavelength of 400 nm and an emission wavelength of 505 nm.

2.2.2. Animal experiments

2.2.2.1. Virus virulence determination

Four groups of Balb/c♂ mice (10 mice per group) were intraperitoneally infected with CVB_3 virus 7 days prior to the experiment. The virus stock solutions were diluted to 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} dilutions, and each mouse was injected intraperitoneally with a daily dose of 0.2ml for 3 consecutive days. Mortality rates were recorded 8 days post-infection, and LD_{50} was calculated using the Reed-Muench method.

2.2.2.2. Experimental grouping

Balb/c mice were randomly divided into 6 groups: normal control group (NC, n=25), virus infection group (VK, n=45), ribavirin treatment group (RBV, 80mg·kg^{-1}, n=25), high-dose Matrine group (80mg·kg^{-1}, n=25), medium-dose Matrine group (40mg·kg^{-1}, n=30), and low-dose Matrine group (20mg·kg^{-1}, n=25).

2.2.2.3. Animal model establishment and drug administration

The VMC animal model was established by intraperitoneal injection of CVB_3 virus. Except for the normal control group, all groups received intraperitoneal injections of 100$TCID_{50}$ of CVB_3 virus at a daily dose of 0.2ml for 3 consecutive days. Drug administration started 60 minutes after virus injection and continued with intraperitoneal injections for 15 days.

2.2.2.4. Effect of Matrine on survival rate of Balb/c mice infected with CVB_3

Same grouping as above, with 15 mice per group. All animals were euthanized on the 15th day. The general condition of mice was observed, and survival rates of each group were calculated.

2.2.2.5. Serum myocardial enzyme detection

On the 10th day after initial virus injection, blood was collected from euthanized animals, centrifuged at 2000r·min^{-1} for 5 minutes, serum was collected and tested for CK-MB.

2.2.2.6. Myocardial virus titer determination

Conducted according to the research [15]. ①Hearts were aseptically removed on day 10 and homogenized using a glass tissue grinder. The homogenates underwent three freeze-thaw cycles, followed by centrifugation to collect the supernatant. Each sample was then serially diluted 10-fold in IMDM culture medium containing 2% fetal bovine serum, resulting in dilutions from 10^{-1} to 10^{-6}. ②FL cells were seeded as monolayers in 96-well culture plates and grown to confluence. After removing the culture medium and washing once with PBS, each dilution of the virus was added to 12 wells per dilution, starting from the highest dilution, at 0.1 ml per well. Adsorption occurred at 37°C for 1.5 hours. ③The viral suspension was removed, and the monolayer was washed once with PBS to remove unabsorbed virus. ④IMDM culture medium containing 2% fetal bovine serum was added. ⑤Cells were cultured for 24-72 hours with daily observation of CPE. ⑥The lg$TCID_{50}$ of the virus in each supernatant was determined.

2.2.2.7. Morphological examination

All heart histological samples were fixed in 4% paraformaldehyde. Histological sections were stained with hematoxylin and eosin (HE) for pathological examination. Pathological scores were assigned based on the percentage of myocardial necrosis and inflammation areas according to Rezkalla's method [16].

2.2.2.8. TUNEL assay

Three groups of mice were used: normal control group (CN), virus infection group (VK), and medium-dose Matrine treatment group (M, 40mg·kg^{-1}). Heart samples were sectioned to detect apoptosis according to manufacturer's instructions of a commercial TUNEL detection kit (Roche).

2.2.2.9. Immunohistochemical detection of Calpain-2 protein

The above 3 groups of specimens were used for calpain-2 protein detection using immunohistochemistry.

2.2.2.10. Western blot analysis of Calpain-2 protein expression

The above 3 groups of specimens were used for Western blot analysis of calpain-2 protein expression.

2.2.2.11. Caspase-12 activity assay

Hearts were quickly homogenized after removal, and protein extraction was performed according to the kit instructions to measure caspase-12 activity.

2.3. Statistical analysis

Statistical analysis was performed using GraphPad Prism 9.0 software. Numerical variables obtained from the experiment were presented as the mean ± standard deviation (SD), and multiple group comparisons were performed using one-way analysis of variance (ANOVA). A significance level of $p< 0.05$ was considered statistically

significant.

3. Results

3.1. *In Vitro* experiments

3.1.1. Protective effect of Matrine on cardiomyocytes infected with CVB3 in Vitro

As shown in Figure 1A, the OD570nm values of the first three concentrations of Matrine (600mg·L^{-1}, 300mg·L^{-1}, 150mg·L^{-1}) and the positive drug RBV group, when compared with the virus infection group, were significantly different (all $P < 0.05$). This indicated that these three doses of Matrine and the RBV group had significant inhibitory effects on CVB3, thereby improving the survival rate of cardiomyocytes. However, the effect of Matrine at 75mg·L^{-1} was not significant ($P > 0.05$ compared with the virus group). Among the three administration methods (simultaneous addition of drug and virus, drug added before virus, and drug added after virus), the effects of the same dose under different administration methods were similar (inter-group comparison, $P > 0.05$).

3.1.2. Impact of Matrine on Cytopathic Effect (CPE) in CVB3-infected rat cardiomyocytes

As demonstrated in Figure 1B, effects of Matrine on CPE of Wistar rat cardiomyocytes infected with 100 $TCID_{50}$ CVB3 were observed at 48 hours in vitro culture. Panel 1Ba showed the virus group VK, where cells exhibited clumping, aggregation, increased refractivity, widened spacing, and were in a state of independent dispersion; some cells ruptured or detached, resulting in clear plaques (CPE++++). Panel 1Bb represented the 300 mg·L^{-1} Matrine treatment group, where the number of shrunken and ruptured cells was significantly reduced, and most cells communicated through filopodia, showing moderate CPE (CPE++). Panel 1Bc depicted normal pulsating cardiomyocytes in vitro culture, showing cells that were overall transparent under low magnification, with normal morphology, a tightly packed arrangement without spacing, visible filopodia, and interconnected networks of communication, indicating no CPE (CPE-).

3.1.3. Cardiomyocyte contractility

After 18-24 hours of cardiomyocyte culture, cells began to divide and proliferate, forming clusters and initiating contractions. As culture time extended and cell division continued, cell clusters grew larger, and contractions progressed from irregular to rhythmic, occurring at a rate of (60-100) beats per minute.

At 24 hours post-infection with CVB3 virus, cells still exhibited contractions, albeit slower and irregularly, with no significant CPE observed. By 48 hours post-infection, cardiomyocyte contractions slowed down, eventually ceasing, and CPE began to appear. Cardiomyocytes gradually became rounder and darker in color, aggregating, exhibiting increased birefringence, accumulating intracellular particles, undergoing shrinkage and leading to fragmentation. Concurrently, CPE rapidly progressed from + to ++++ during this period.

In the normal control group, cells exhibited regular and vigorous contractions with no evidence of CPE. Compared to the virus-infected group, all treatment groups with Matrine or ribavirin showed significantly increased numbers of contracting cells ($P<0.05$) and reduced CPE. Among the Matrine-treated groups (600mg·L^{-1},300mg·L^{-1}, and 150mg·L^{-1}), improvements were similar (no significant differences observed among groups, $P>0.05$). See Figure 1C for details.

3.1.4. Effect of Matrine on myocardial enzyme (CK-MB) levels In Vitro

As shown in Figure 1D, the myocardial enzyme CK-MB in the virus group was significantly higher than that in the normal control group ($P < 0.05$). Compared with the virus group, the concentrations of Matrine at 600 mg·L^{-1}, 300 mg·L^{-1}, 150 mg·L^{-1}, and 75 mg·L^{-1}, as well as the ribavirin group (all $P < 0.05$), significantly reduced CK-MB. However, these concentrations still remained higher than those in the normal control group ($P < 0.05$), indicating that Matrine at various concentrations can significantly decrease CK-MB released from primary cultured Wistar rat myocardial cells infected with CVB3, but cannot reduce it to normal levels.

3.1.5. Detection of CVB3-induced apoptosis in cultured cardiomyocytes by flow cytometry

Significant apoptosis was observed in cardiomyocytes of the virus group, while the normal group showed very few apoptotic cells. Compared with the virus group, the apoptosis of cells treated with Matrine decreased significantly ($P < 0.05$), indicating that Matrine has a significant inhibitory effect on CVB3-induced apoptosis in cultured cardiomyocytes. For detailed apoptosis rates (Annexin V+), please refer to Table 1 in reference [17].

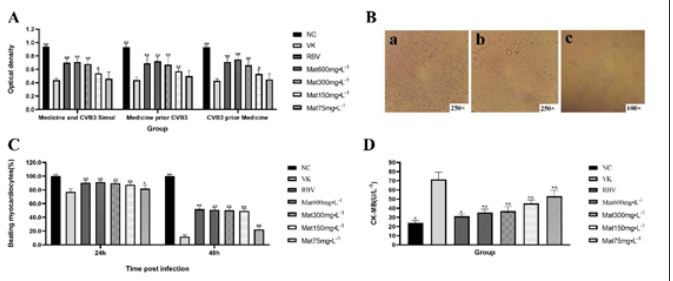

Fig. 1. In vitro effects of Matrine on CVB3-infected cardiomyocytes. (A) Cell viability (OD570nm, n=8) of cardiomyocytes infected with CVB3 following three different drug administration protocols. (B) Representative images showing cytopathic effects (CPE) in cardiomyocytes at 48 hours post-infection: a: Virus control (VK); b: Matrine 300 mg·L^{-1}; c: Normal control (NC). (C) Percentage of beating cardiomyocytes following CVB3 infection and Matrine treatment (n=8). (D) CK-MB levels in culture supernatants of CVB3-infected cardiomyocytes at 48 hours post-infection (n=6). Statistical significance: *P<0.05, **P<0.01 vs. VK group; ^P<0.05 vs. NC group.

Table 1. Effects of Matrine on viral titers in VMC mice myocardium (n=6).

Group	lgTCID50
VK	-3.39±0.63
RBV	-1.43±0.32*
Mat 80mg·kg^{-1}	-1.78±0.41*
40mg·kg^{-1}	-2.33±0.50*$^{\Delta}$
20mg·kg^{-1}	-2.20±0.79*$^{\Delta}$

vs VK group: *P<0.01; vs RBV group: $^{\Delta}$ P<0.05.

3.1.6. Immunocytochemistry to detect Calpain-2 protein

Figure 2Ba showed the immunocytochemical analysis of calpain-2 protein in normal group cells, where a small amount of DAB-stained granule was visible in the cytoplasm of myocardial cells, indicating low expression of calpain-2 in the normal group. Figure 2Bb illustrates the analysis of the 36-hour virus group, displaying a large amount of unevenly dispersed DAB-stained granules in the cytoplasm with deeper staining, indicating increased expression of calpain-2 protein. Figure 2Bc represents the analysis of the Matrine group, where fewer cytoplasmic DAB-stained cells were observed with lighter staining, suggesting lower expression of calpain-2 in the Matrine group (see Figure 2B). Gray scale analysis results (Figure 2A) demonstrated a significant difference between the 36-hour Matrine group and virus group ($P < 0.05$), indicating Matrine may inhibit calpain-2 protein expression induced by the virus.

3.1.7. Effect of Matrine on Calpain-2 protein expression In Vivo

In the virus group, calpain-2 protein expression was significantly higher compared to the control group, while Matrine treatment group showed lower expression levels compared to the virus group, with statistically significant differences ($P < 0.05$). This indicated that Matrine can inhibit calpain-2 protein expression in CVB_3-infected myocardial cells (see Figure 2C).

3.1.8. Effect of Matrine on Caspase-12 activity in Vitro

Protein was extracted from CVB_3-infected myocardial cells at 24h, 36h, and 48h post-infection, and caspase-12 activity was measured using specific fluorescent substrates. Results indicated that compared to the normal control, caspase-12 was activated at 24h, peaked at 36h, and remained present at 48h post-CVB_3 infection ($P<0.05$). Treatment with Matrine significantly reduced caspase-12 activity in CVB_3-infected myocardial cells compared to the virus group ($P<0.05$), although at 24h, it decreased to normal levels compared to the control group ($P>0.05$), while at 36h and 48h, it did not decrease to normal levels ($P<0.05$). See Figure 2D for details.

3.2. In Vivo experiments

3.2.1. Effect of Matrine on the general condition and survival rate of animals

Three days after virus injection, animals showed reduced activity, dull and curled fur, refusal to eat, and some developed diarrhea. They exhibited significantly lower weight loss than the normal control group. All groups except the normal control group experienced deaths: animals in the virus control group and the low-dose treatment group began to die from the 4th day. In the ribavirin group, one animal died on the 5th and 7th days respectively; in the high-dose treatment group, one animal died on the 5th day; in the medium-dose treatment group, two animals died on the 5th day and one on the 6th day; in the low-dose treatment group, one animal died on the 4th day, two on the 6th day, and one on the 7th day; in the virus group, two animals died on the 4th day, two on the 5th day, two on the 6th day, one on the 7th day, and one on the 10th day.

Survival rates of animals in each group were as follows: NC group 100% (15/15), RBV group 86.7% (13/15), high-dose Matrine group 93.3% (14/15), medium-dose Matrine group 80% (12/15), low-dose Matrine group 73.3% (11/15), and VK group 46.7% (7/15). Compared with the virus control VK group, the survival rates of animals in the high, medium, and low-dose Matrine treatment groups were significantly higher than those in the virus-infected group, and the survival rates of the high and medium-dose groups were close to the positive drug ribavirin RBV group.

3.2.2. Effect of Matrine on serum myocardial enzyme (CK-MB) levels In Vivo

As shown in Figure 3A, on the 10th day after virus injection, the CK-MB levels in the virus group were significantly higher than those in the normal control group ($P<0.01$). Treatment with 80 $mg \cdot kg^{-1}$, 40 $mg \cdot kg^{-1}$, and 20

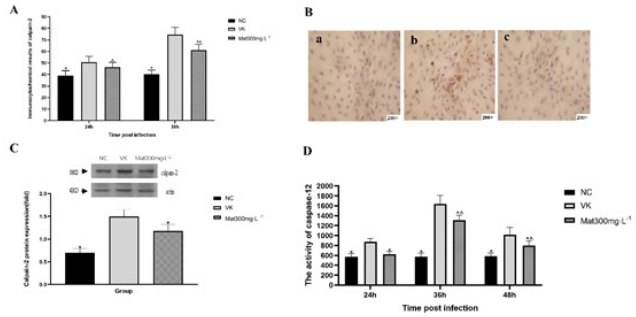

Fig. 2. Matrine modulates the calpain-2/caspase-12 signaling pathway in vitro. (A, B) Quantification and representative immunocytochemical images of calpain-2 protein expression in cardiomyocytes infected with CVB_3 at 36 hours (n=6 per group). a: Normal control (NC); b: Virus control (VK); c: Matrine 300 $mg \cdot L^{-1}$. (C) Calpain-2 protein expression in cardiomyocytes at 36 hours post-infection, assessed by Western blotting (n=3 per group). (D) Caspase-12 activity in CVB_3-infected cardiomyocytes (n=3 per group). Statistical significance: *P < 0.05 vs. VK group; ^P < 0.05 vs. NC group.

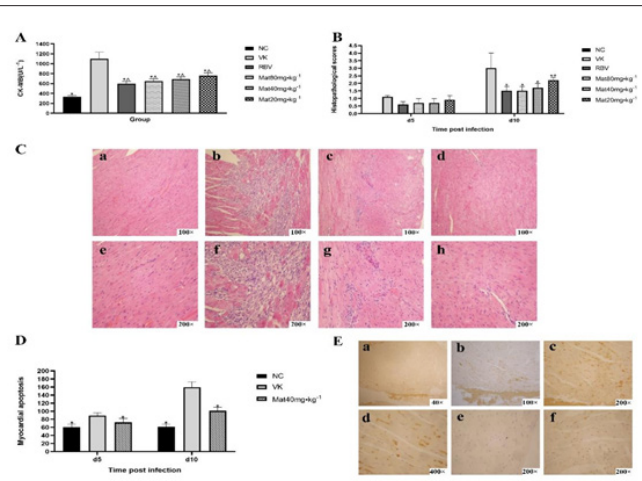

Fig. 3. In vivo experiments. (A) Effect of Matrine on serum CK-MB levels in VMC mice (n=6 per group). (B) Myocardial histopathological scores in each group of VMC mice (n=6 per group). (C) Representative histopathological images of myocardial tissue from each group of VMC mice at day 10 post-CVB_3 infection. a, e: NC (normal control); b, f: VK (virus control); c, g: Matrine 40 $mg \cdot kg^{-1}$; d, h: RBV (ribavirin). (D, E) Quantification (n=6, at days 5 and 10 post-CVB_3 infection) and representative images of myocardial apoptosis in VMC mice at day 10 post-CVB_3 infection. a–d: VK; e: NC; f: Matrine 40 $mg \cdot kg^{-1}$. Statistical significance: *P<0.05 vs. VK group; ^P<0.05 vs. NC group; +P<0.05 vs. RBV group.

mg·kg^{-1} of Matrine, as well as ribavirin, resulted in a significant decrease in CK-MB levels compared to the virus group (P<0.01). However, these levels remained higher compared to the normal control group (P<0.05), indicating that various concentrations of Matrine and ribavirin can significantly reduce serum CK-MB levels in VMC mice, though they do not reach normal levels.

3.2.3. Effect of Matrine on myocardial virus titer

The results showed that the high, medium, and low doses of Matrine, as well as the ribavirin group, significantly reduced myocardial virus titers (P < 0.01). The inhibitory effect of 80 mg·kg^{-1} Matrine treatment on myocardial virus replication in VMC mice was similar to that of the ribavirin group (no significant difference between groups, P > 0.05). (Table 1).

3.2.4. Histopathological examination and pathological scores

In the normal control group (Figure 3Ca), myocardial fibers were neatly arranged with clear striations, rich and uniform cytoplasm, and no inflammatory cell infiltration, edema, or necrosis in the interstitium. By the 5th day post-CVB3 infection, extensive inflammatory lesions were widely distributed throughout the hearts of model group mice, affecting various layers of the ventricular wall. Some myocardial areas showed focal degenerative and necrotic changes. Extensive inflammatory lesions were observed beneath the epicardium and around blood vessels, characterized by lymphocytic infiltration. However, in mice treated with high, medium, and low doses of Matrine and ribavirin, the area of inflammatory lesions in the myocardium is significantly reduced. Although some areas showed interstitial inflammatory edema dominated by lymphocytic infiltration, the severity is notably decreased, presenting as focal lesions without myocardial cell necrosis. When compared with the model group, the extent of myocardial necrosis and inflammation was reduced in the high, medium, and low dose groups of Matrine and the group of ribavirin, but the differences were not significant among the groups of Matrine and ribavirin (P > 0.05). By the 10th day post-infection, lesions were predominantly located beneath the epicardium and around blood vessels in the virus group, with a detection rate of 100% (6/6). The severity of necrosis and inflammation was higher compared to the 5th day, featuring patchy or focal necrosis, myocardial fiber dissolution and fracture, disappearance of cell structure, and remaining myocardial cells within inflammatory lesions, maintaining partial cell structure. Trends in treatment with each drug (Figure 3Cc,d) were similar to those observed on the 5th day. Pathological scores for Ribavirin and Matrine in high, medium, and low doses were significantly lower than those in the model group (P < 0.05) (See Figure 3B for details). Morphological observations indicated that high, medium, and low doses of Matrine can significantly reduce inflammatory cell infiltration and myocardial tissue necrosis. Compared with the positive drug ribavirin, the low-dose group was slightly less effective (P < 0.05), while the other two groups exhibited similar efficacy.

3.2.5. Effect of Matrine on apoptosis of myocardial cells (TUNEL assay)

The TUNEL staining results showed that apoptotic positive cells appeared dark brown, with condensed nuclei, chromatin margination, and unstained cytoplasm. Adjacent sections of each animal's heart were stained with TUNEL and compared with HE staining results. In the virus group, a small number of apoptotic cells appeared on day 5, with rare individual apoptotic myocardial cell nuclei observed. Animals with severe myocardial lesions showed significant apoptosis on day 10 (Figure3Ea-d), with apoptotic cells primarily concentrated in areas of myocardial inflammation and necrosis (Figure3Ca-d), corresponding to the distribution of fiber dissolution and necrosis in HE-stained sections. The myocardial cells appeared mostly regular in shape, but numerous brown-stained nuclei indicated the presence of scattered apoptotic cells in myocardial tissue sections. The normal control group (Figure3Ee) showed no apoptotic cells, with regular myocardial cell shapes and all nuclei stained blue, indicating no occurrence of apoptotic myocardial cells. Apoptosis in the Matrine treatment group (Figure3Ef) correlated with the extent of lesions, showing few brown-stained nuclei in myocardial tissue sections and rare individual apoptotic myocardial cell nuclei observed. In summary, Matrine significantly inhibited myocardial cell apoptosis. Apoptosis results were analyzed using the Luzex-F imaging analysis system [18].

3.2.6. Effect of Matrine on Calpain-2 protein expression *In Vivo*

3.2.6.1. Immunohistochemical detection of calpain-2 protein

In the normal control group, minimal expression of calpain-2 was observed. By day 5 post-virus injection, expression was detected in the virus control group, with increased levels compared to the normal control group. By day 10, expression levels further increased in severely affected sections compared to day 5, with calpain-2 observed to be membrane-bound. Treatment with Matrine significantly reduced the expression of calpain-2 compared to the virus group (P<0.05), indicating a notable inhibitory effect of Matrine on calpain-2 protein expression (See Figure 4AB for details).

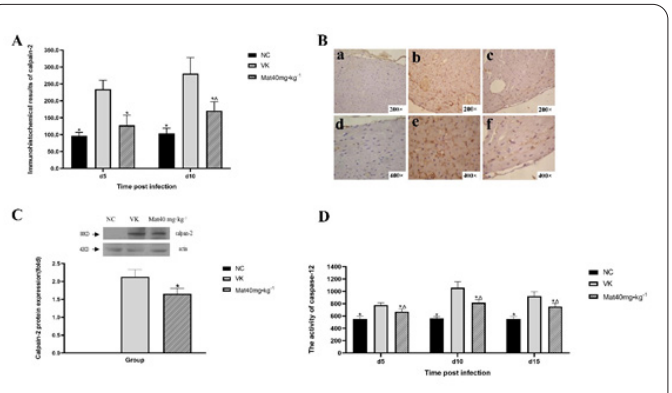

Fig. 4. Matrine downregulates the calpain-2/caspase-12 signaling pathway in vivo. (A, B) Quantification and representative immunohistochemical images of calpain-2 protein expression in myocardial tissue from VMC mice at day 10 post-CVB3 infection (n=6 per group). a: Normal control (NC); b: Virus control (VK); c: Matrine 40 mg·kg^{-1}. (C) Calpain-2 protein expression in myocardial tissue at day 10 post-infection, assessed by Western blotting. (D) Caspase-12 activity in myocardial tissue of VMC mice (n=3 per group). Statistical significance: *P < 0.05 vs. VK group; ^P < 0.05 vs. NC group.

3.2.6.2. Western blotting method to detect the expression of calpain-2 protein

In the normal control group, calpain-2 expression was not detected. In the virus-infected group, calpain-2 expression increased, while in the Matrine treatment group, expression was reduced compared to the virus-infected group, showing significant differences ($P < 0.05$). It was inferred that Matrine had an inhibitory effect on calpain-2 protein expression in the myocardial tissue of VMC mice. (Figure 4C)

3.2.7. Effect of Matrine on Caspase-12 activity In Vivo

Mice were intraperitoneally injected with CVB_3 for 5, 10, and 15 days, and myocardial protein was extracted for caspase-12 activity detection using specific fluorescent substrates. Compared to the normal control group, CVB_3 infection activated caspase-12 at 5, 10, and 15 days ($P < 0.05$). Caspase-12 activity began to increase at day 5, peaked at day 10, and remained detectable at day 15. The treatment group with Matrine showed a significant decrease in myocardial caspase-12 activity in VMC mice compared to the virus group ($P < 0.05$), but did not decrease to normal levels compared to the control group ($P < 0.05$) (See Figure 4D for details).

4. Discussion

In the past three years, the novel coronavirus (COVID-19) has ravaged globally, claiming millions of lives. In recent months, influenza A virus outbreaks have occurred on a large scale in certain regions of our country, afflicting children with influenza virus torment. Besides the novel coronavirus, influenza viruses A and B, as well as classic enteroviruses, parvovirus B19, Epstein-Barr virus (EBV), human herpesvirus 6 (HHV6), among others, can affect the heart [19-21], potentially inducing myocarditis with similar inflammatory characteristics. The severity of the disease varies; it can be asymptomatic or severe. Severe cases can lead to cardiogenic shock and fulminant myocarditis, posing a serious threat to children's health. Severe cases may require mechanical ventilation or extracorporeal membrane oxygenation (ECMO) therapy [22,23], and some may progress to dilated cardiomyopathy, ultimately requiring heart transplantation.

For pediatric cases, aside from influenza viruses, most viral infections lack specific therapeutic methods and drugs. Scholars have been conducting ongoing research on viral myocarditis (VMC). However, the pathogenesis of pediatric VMC remains incompletely understood, and specific therapeutic methods and drugs are still lacking [24].

This study explored the protective effects of Matrine on CVB_3-infected mouse myocardial cells and investigated the relevant mechanisms.

4.1. Antiviral activity of Matrine and its protection of cardiomyocytes

This study observed that Matrine, administered both before and after viral infection, as well as simultaneously with the virus, increased the survival rate of primary cultured rat cardiomyocytes compared to the virus infection group. The mechanism of Matrine's antiviral action remains unclear; however, it has been reported that Matrine may exert its effects by inhibiting viral replication and modulating the type I interferon antiviral signaling pathway [25].

The study also noted that Matrine not only possessed antiviral activity but also enhanced the percentage of beating cardiomyocytes and reduced CPE in primary cultured rat cardiomyocytes infected with the virus. In vivo and in vitro studies further demonstrated that Matrine reduced the activity of the cardiac enzyme CK-MB. CK-MB is a crucial biomarker for myocardial injury, inflammation, and heart failure [26], potentially indicating a risk factor for acute myocarditis [27]. Most CK-MB is present in the cytoplasm of cardiomyocytes; viral infection damaged cell ultrastructure and integrity, causing cellular degeneration, insufficient energy supply, damaged cell membranes, and altered permeability, leading to increased levels of cardiac enzymes in the culture supernatant and serum of viral myocarditis mice. Studies have also confirmed the presence of anti-endothelial antibodies in the serum of VMC patients, with enteroviruses capable of infecting vascular endothelial cells [28], leading to coronary artery lesions, exacerbating myocardial ischemia, damaging cardiomyocyte membranes, and leaking cardiac enzymes. Matrine significantly inhibited the release of CK-MB from virus-infected cardiomyocytes, possibly related to its antiviral properties, reducing cell damage, and maintaining cell membrane integrity.

Histological examination of tissue morphological changes is the most direct and objective indicator of disease severity. This study showed that mice began to die from day 4, peaking in deaths between days 6-10, with typical clinical symptoms. By day 5 of viral infection, focal necrotic changes could be observed in individual mouse myocardia, peaking in severity around day 10, predominantly affecting the epicardial myocardium and surrounding vessels. The detection rate of lesions in the virus control group reached 100% (6/6). The mortality rate and detection rate of myocardial lesions in the treatment group and positive drug control group were significantly lower than those in the model group, showing better outcomes in terms of myocardial necrosis and inflammatory cell infiltration compared to the virus control group. Pathological scoring also indicated a treatment effect similar to that of the positive drug ribavirin, suggesting that Matrine protects the myocardium from viral infection and improves survival rates in virus-infected mice.

In the early stages of VMC, direct viral damage to myocardium predominated, making determination of viral titers in the myocardium reflective of the extent of myocardial damage. Results of viral titer analysis in the viral replication inhibition experiment confirmed the presence of virus in cardiac tissues, with significant differences observed in viral titers between the Matrine group and the virus group, indicating that Matrine may inhibit CVB_3 replication by affecting viral RNA transcription, protein synthesis, or virus particle assembly. The protective effects of Matrine observed in this study may be associated with its inhibitory effects on the virus.

4.2. Matrine and apoptosis of myocardial cells infected with virus

Apoptosis, also known as programmed cell death, is genetically regulated and associated with the pathological damage of many diseases. Apoptosis represents the final stage of cellular life. In this study, Annexin V/PI staining via flow cytometry was utilized to detect apoptosis in myocardial cells. Results showed that apoptosis

appeared in the virus group at 24 hours, with an apoptosis rate (Annexin V+) of 39.7±5.4%, significantly increasing to 80.3±5.8% at 36 hours. However, Matrine significantly inhibited virus-induced apoptosis in cultured myocardial cells in vitro ($P<0.05$).

In animal experiments, myocardial cell apoptosis in mice infected with CVB_3 began on the 5th day, with a substantial increase observed on the 10th day, mainly concentrated in the lesion and surrounding areas. No apoptosis was observed in the normal control group. These findings indicate that CVB_3 infection was a trigger for myocardial cell apoptosis. Apart from causing myocardial inflammatory damage, CVB_3 induced apoptosis to participate in the occurrence and development of viral myocarditis (VMC). Early apoptosis induced by virus infection is considered a host defense mechanism, restricting virus replication and spread [29]. However, some viruses encode anti-apoptotic factors that block apoptosis induction and/or execution, thereby promoting viral replication [29-31] and resulting in persistent infection and compensatory hypertrophy of cells. Conversely, late-stage infection-induced apoptosis may benefit viral replication by facilitating the infection of progeny viral particles and their spread to adjacent cells, evading host immune defenses [32].

Thus, apoptosis is not only a physiological process but also a pathological injury process. For myocardial cells lacking effective regeneration capacity, virus-induced apoptosis will inevitably impair cardiac function and may progress to dilated cardiomyopathy [33-35]. Morphological observations and grayscale analysis demonstrated that Matrine treatment significantly reduced apoptosis compared to the virus group ($P<0.05$), indicating that Matrine can effectively inhibit myocardial cell apoptosis in CVB_3-induced myocarditis mice.

4.3. Matrine and calpain-2

The relationship between some important proteases in the apoptosis process, such as calpain-2, and viral myocarditis (VMC) remains unclear. Calpain is a calcium-dependent neutral cysteine protease. Calpain-2, also known as m-calpain, primarily exists in an inactive proenzyme form within cells and is activated when micromolar levels of free calcium are present. As a proteolytic enzyme, calpain-2 undergoes autolysis in the presence of phosphatidylinositol, leading to activation. Once activated, it can degrade protein scaffolds, protein kinases, receptor proteins, and more than a dozen other substances, playing crucial roles in cellular apoptosis and signal transduction [36]. A sustained increase in calpain-2 may be detrimental to the heart [37].

Calpain is predominantly located in the cytoplasm and, upon activation, can translocate to specific substrates such as intracellular membrane structures to exert its effects. Animal experiments have clearly demonstrated this phenomenon. In this study, virus-infected group showed significant expression of calpain-2 by day 5, which increased further by day 10 with increased apoptosis. This suggested that calpain-2 may be associated with cellular apoptosis; once activated, it excessively degrades downstream substrates uncontrollably, leading to severe structural and functional damage of cardiomyocytes and subsequent cell death. Compared to the virus-infected group, Matrine-treated groups exhibited significantly reduced expression of calpain-2 ($P < 0.05$), indicating that Matrine may reduce the expression of the calpain-2 protein within cardiomyocytes, consistent with levels of apoptosis inhibition.

4.4. Matrine and caspase-12

The endoplasmic reticulum (ER) pathway is involved in various cellular apoptotic processes, with caspase-12 being a specific molecule in the apoptosis pathway induced by ER stress. ER stress is caused by the accumulation of excessive and misfolded proteins within the ER lumen. When the load of misfolded proteins exceeds capacity, ER stress induces cell death through the caspase-12 signaling pathway [13,14]. The inactive precursor form of caspase-12 is present on the cytoplasmic surface of the endoplasmic reticulum (ER) [38]. As a specific marker of ER stress [39], it can be selectively cleaved and activated by the ER signaling pathway. Activated caspase-12 subsequently triggers downstream effectors such as Caspase-9 and executioner molecule Caspase-3, leading to cell apoptosis [14].

Reportedly, caspase-12 is widely expressed in various cells of mice at 4 days postnatal, including muscle, heart, liver, kidney, and eyes [40-42]. Caspase-12 plays a crucial role in ER stress-induced apoptosis and is associated with diabetes-induced cardiomyocyte apoptosis [43] and oxidative stress-induced cardiomyocyte apoptosis [44,45]. Caspase-12 participates in protein hydrolysis and activation, coordinating central events of mammalian cell apoptosis. However, whether caspase-12 mediates CVB_3-induced cardiomyocyte apoptosis is currently underreported.

Therefore, we conducted cellular-level experiments to verify this. The results showed that caspase-12 activity began to increase 24 hours after viral infection, peaked at 36 hours, and remained elevated at 48 hours, consistent with the apoptosis process, indicating that caspase-12 was involved in CVB_3-induced cardiomyocyte apoptosis. Compared with the virus group, the group treated with Matrine showed a significant inhibition of caspase-12 activity ($P < 0.05$), suggesting a clear suppressive effect of Matrine on caspase-12 activity.

In VMC experimental mice, caspase-12 activity was significantly increased in the virus group compared to the normal control group ($P < 0.05$). Increased caspase-12 activity was detected in the virus group starting from day 5, peaked on day 10, and began to decline by day 15. Compared with the virus group, Matrine treatment group showed a significant difference in caspase-12 activity ($P < 0.05$), indicating that Matrine markedly reduced caspase-12 activity in VMC mouse myocardial tissue, although it remained higher than the normal control group ($P < 0.05$).

In summary, this study demonstrated that Matrine exerts significant protective effects against Coxsackievirus B_3 (CVB_3)-induced myocardial injury both in vitro and in vivo. Matrine treatment not only improved cardiomyocyte viability and reduced the release of myocardial enzymes, but also attenuated histopathological damage and decreased apoptosis in infected myocardial tissue. Mechanistically, these cardioprotective effects are closely associated with the downregulation of the calpain-2/caspase-12 signaling pathway, suggesting that inhibition of endoplasmic reticulum stress-mediated apoptosis is a key component of Matrine's action. These findings highlighted the therapeutic potential of Matrine as a promising candidate for the prevention and treatment of viral myocarditis

and provide new insights into the molecular mechanisms underlying its protective effects. Further studies are warranted to explore the clinical applicability and safety of Matrine in the management of viral myocarditis.

Conflict of interests

The author has no conflicts with any step of the article preparation.

Ethics approval and consent to participate

The study was conducted according to the Declaration of Helsinki principles and was approved by the Ethics Committee of Jilin University.

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions

Y.S. contributed to experimental design, supervision and performed experiments. Z.M. participated in experimental execution and performed data analysis and drafted sections of the manuscript. J.L. participated in collecting data. Y.G. did editing and formal analysis. All authors have read and approved the final manuscript for publication.

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