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In vitro and *in vivo* antiviral activity of a fluoronucleoside analog, NCC, against Coxsackie virus B3

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Summary. – Coxsackie virus B3 (CVB3) is believed to be a major cause of viral myocarditis, with virus-induced apoptosis playing an important role in pathogenesis. The purpose of this study was to characterize the antiviral activity of a novel fluoronucleoside analogue, N-cyclopropyl-4'-azido-2'-deoxy-2'-fluoro-β-D-cytidine (NCC), against CVB3 *in vitro* and *in vivo*, and to establish whether NCC inhibits apoptosis in infected cells. In this study, HeLa cells infected with CVB3 were treated with NCC. Cell viability and apoptosis were examined. Caspase-3 and Bcl-2 levels were monitored by real-time RT PCR and Western blot analysis. For *in vivo* studies, BALB/c mice infected with CVB3 were treated with NCC daily. Serum markers of myocardial injury and histological studies were measured to examine myocardial injury on day 8 post-infection. To measure apoptosis, levels of Bcl-2 and caspase-3 were examined by immunohistochemistry and real-time RT-PCR. We found that NCC inhibited virus-mediated cytopathic effects in HeLa cells with an EC_{50} of 116.60 ± 0.32 μM. In infected mice, administration of NCC (2 mg/kg) decreased the activities of serum creatine kinase and lactic dehydrogenase, inhibited the replication of CVB3 and alleviated damage to the heart. Importantly, NCC suppressed CVB3-induced apoptosis in HeLa cells and affected the expression of apoptosis-related factors in infected mice. Together, our results demonstrate that NCC exerts significant antiviral activities against CVB3. We conclude that NCC is a potential therapeutic agent for the treatment of viral myocarditis.

Keywords: coxsackie virus B3; viral myocarditis; N-cyclopropyl-4'-azido-2'-deoxy-2'-fluoro-β-D-cytidine; apoptosis

Introduction

Coxsackie virus B3 (CVB3) is a non-enveloped, single-stranded, positive-sense RNA virus belonging to the *Enterovirus* genus of the *Picornaviridae* family. CVB3 is considered to be the most common pathogenic cause of viral myocarditis (VMC) (Wang *et al*., 2010; Lasrado *et al*., 2020), the prevalence of which is continuously increasing

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Abbreviations: CVB3 = Coxsackie virus B3; NCC = N-cyclopropyl-4'-azido-2'-deoxy-2'-fluoro-β-D-cytidine; TI = therapeutic index; VMC = viral myocarditis

in children and young people (Massilamany *et al*., 2014; Cabrerizo *et al*., 2017). Studies have shown that virus-induced injury and the ensuing inflammatory response can result in serious damage to the myocardium, leading to acute heart failure and dilated cardiomyopathy (Garmaroudi *et al*., 2015). Virus-specific prophylactic and therapeutic options to protect humans against CVB3-induced heart diseases are not well developed (Baggen *et al*., 2018).

The pathogenic mechanisms of CVB3-induced myocarditis are not fully understood; however, the damage to the heart resulting from CVB3 infection is commonly believed to be caused by several factors, including direct CVB3-induced myocardial injury (McManus *et al*., 1993; Pauschinger *et al*., 1999) and immune and autoimmune processes associated with viral infection (Zhang *et al*., 2017; Respondek *et al*., 2017). Recently, an increasing number of studies have shown that virus-induced apoptosis occurs frequently during CVB3 infection *in vitro* and *in vivo* (Feuer *et al*., 2003; Chau *et al*., 2007; Cai *et al*., 2015; Peischard *et al*., 2019), and plays an important role in CVB3-induced pathogenesis. In the early stages of myocarditis, apoptosis is triggered by viral replication (Peischard *et al*., 2019). Although some pathogenic pathways of CVB3 are well characterized, details regarding CVB3 involvement in the apoptotic process remain unclear.

NCC, N-cyclopropyl-4'-azido-2'-deoxy-2'-fluoro-β-D-cytidine, is a novel fluoronucleoside analog synthesized at the College of Chemistry at Zhengzhou University (Henan province, China) and its biological effects have not been widely studied. In this study, we performed *in vitro* and *in vivo* assays to examine the anti-CVB3 activity of NCC and establish possible pharmacological mechanisms.

Materials and Methods

Reagent. NCC was synthesized at the College of Chemistry, Zhengzhou University and its chemical structure is shown in Fig. 1. As determined by high-performance liquid chromatography, NCC has at least 95% purity. The 4 mM NCC stock solution was dissolved in phosphate-buffered saline.

Virus and cell cultures. CVB3 Nancy strain, which was generously provided by the Jinan University, was maintained by passage through HeLa cells and stored at -80°C. Viral titers were determined by a 50% tissue culture infectious dose ($TCID_{50}$) assay in a HeLa cell monolayer according to the Reed and Muench method (Bopegamage *et al*., 2005), and infections with CVB3 were performed at 100× the determined $TCID_{50}$. HeLa cells were obtained from the American Type Culture Collection and cultured in complete Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 0.1% 200 mM L-glutamine. Cells were grown at 37°C in a humidified atmosphere with 5% CO_2.

Animals. Male BALB/c mice were purchased from Hunan SJA Laboratory Animal Co., Ltd (Hunan. China) and housed at the Institute of Medicine, Zhengzhou University. The mice were housed in pathogen-free conditions at a temperature of 23 ± 1°C, a humidity level of 40 ± 5% and on a 12 h light /12 h dark cycle. They had free access to pellet feed and tap water. The study was approved by Ethical Committee of Zhengzhou University (number of approval: 2020-08).

Cytotoxicity assay. To determine cell viability, an MTT-based assay was performed (Mosmann *et al*., 1983). HeLa cells were seeded in 96-well plates at a density of 3×10^4 cells per well and grown to monolayers. Four parallel wells were treated with serial 2-fold dilutions (100 and 200 μM) from 2 mM NCC for 72 h. Then, 20 μl of MTT (5 mg/ml, Amresco, USA) were added and incubated for 4 h. The precipitated formazan was dissolved in 100 μl of dimethyl sulfoxide. Cell viability was assessed at the dual wavelength of 570 and 630 nm and the 50% cytotoxic concentration (CC_{50}) was calculated.

In vitro antiviral activity of NCC in CVB3-infected HeLa cells. HeLa cells were seeded in 96-well plates at a density of 3×10^4 cells per well and were allowed to form monolayers. Fifty microliters of viral suspensions containing 100 $TCID_{50}$ of the virus stock and an equal volume of medium containing various concentrations of 100 and 200 μM NCC were added to each well. Ribavirin (Shanghai Furen Medicine R&D Co., China, lot. No. 61805051) was used as a positive inhibition control. Uninfected and infected cells without the test compound served as normal and infected controls, respectively. About 24 h after infection, when infected control cells showed the maximum cytopathic effect, the antiviral effect was measured using the MTT assay following the protocol described in the cytotoxicity assay section. Data were analyzed and the half maximal effective concentration (EC_{50}) was calculated. The therapeutic index (TI) was determined by the CC_{50}/EC_{50} ratio.

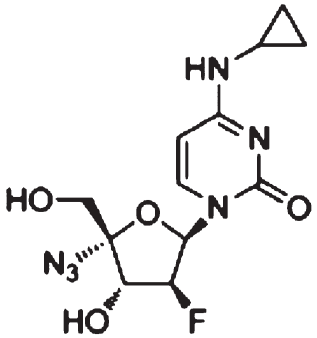

Fig. 1

The chemical structure of NCC

Table 1. RT-PCR primers

Primers	Sequences	
VP-1	forward	5'-CATTCAAGGTCCGAGTCAACCATAG-3'
	reverse	5'-CTCCTAAGTTGTGCTGCTTGTCGTG-3'
caspase-3 (human)	forward	5'-TGAGGCGGTTGTAGAAGAG-3'
	reverse	5'-TAATGAGAATGGGGGAAGA-3'
caspase-3 (mouse)	forward	5'-CCCCACTCATGTTAAGAATGTTGT-3'
	reverse	5'-CCCCACTCATGTTAAGAATGTTGT-3'
Bcl-2 (human)	forward	5'-GATTGATGGGATCGTTGCCTTA-3'
	reverse	5'-CCTTGGCATGAGATGCAGGA-3'
Bcl-2 (mouse)	forward	5'-CTTCCAGCCTGAGAGCAACC-3'
	reverse	5'-CACCACCAACTGCTTAGCC-3'
GAPDH (human)	forward	5'-AACGGATTTGGTCGTATTGGG-3'
	reverse	5'-T CGCTCCTGGAAGATGGTGAT-3'
GAPDH (mouse)	forward	5'-CATTCAAGGTCCGAGTCAACCATAG-3'
	reverse	5'-CTCCTAAGTTGTGCTGCTTGTCGTG-3'

Fluorescence-activated cell sorting (FACS) for detection of apoptosis. The apoptosis percentage was determined by using the AnnexinV-FITC apoptosis detection kit (KeyGEN Biotech, China) according to the manufacturer's instruction. HeLa cells were seeded in 6-well plates at a density of 2.5×10^5 cells per well and were allowed to form monolayers. Then the cells infected with CVB3 were treated with 100 μM and 200 μM NCC for 6 h, 12 h and 24 h. At the different time point, after removing the supernatant, cells were digested by trypsin, washed in ice-cold phosphate buffered saline (PBS) and stained. The apoptosis ratio of fresh samples was analyzed by using a BD FACScan flow cytometer (BD Biosciences, USA).

RNA isolation and real-time RT-PCR. Total RNA was extracted from cell culture using TRIzol reagent (TaKaRa, Japan) following the manufacturer's instructions. Subsequently, 1 μg of cellular RNA was reverse-transcribed into cDNA by using PrimeScript™ RT reagent kit with gDNA eraser (Perfect Real Time) reverse transcriptase kit (TaKaRa, Japan) and stored at -20°C. To examine the relative mRNA levels of indicated genes, real-time PCR with SYBR Premix Ex Taq™ (TaKaRa, Japan) was used. The primers are shown in Table 1. The relative expression levels of the target genes were normalized to the housekeeping gene GAPDH and were calculated using the ΔΔCt method (Livak and Schmittgen, 2001). Each real-time PCR reaction was performed in triplicate.

Western blot analysis. HeLa cells were seeded in 6-well plates at a density of 2.5×10^5 cells per well and were allowed to form monolayers. Cells infected with CVB3 were treated with 100 μM and 200 μM NCC for 24 h. Then cell samples, either untreated or treated, were lysed in 400 μl of RIPA lysis buffer on ice for 30 min. The lysates were centrifuged at 13,000 x *g* for 5 min, and 22.5 μg of denatured proteins per well were loaded on 12% SDS-polyacrylamide gels (Leagene, China). Samples were separated and transferred onto a PVDF membrane (Millipore, Ireland). The membranes were blocked with 5% non-fat dry milk solution to avoid non-specific binding. The membranes were subsequently incubated overnight at 4°C overnight with caspase-3, 1:3000 (Santa Cruz Biotechnology), Bcl-2, 1:2,000 (Santa Cruz Biotechnology) and β-actin, 1:2,000 (Santa Cruz Biotechnology) antibodies. The membranes were washed and incubated with secondary antibody with dilution 1:7,000 (CWBio, China) for 2 h at room temperature. Protein bands were developed with enhanced chemiluminescence (ECL) detection system according to the manufacturer's protocol (Genview, China). Then, the differences in protein loading were normalized to corresponding levels of β-actin control.

Experimental design and antiviral therapy. In this study, a total 85 BALB/c mice were used. At day 0, 75 male mice were infected by intraperitoneal injection with 0.2 ml of physiological saline containing 10^4 $TCID_{50}$ of CVB3 and were randomly divided into five groups. According to the results of toxicity testing (data not shown), NCC was administered to 45 inoculated mice by oral gavage at a dose of 0.5 $mg \cdot kg^{-1} \cdot d^{-1}$ (n = 15), 1 $mg \cdot kg^{-1} \cdot d^{-1}$ (n = 15), or 2 $mg \cdot kg^{-1} \cdot d^{-1}$ (n = 15). Fifteen mice were injected intravenously with Ribavirin at a dose of 125 $mg \cdot kg^{-1} \cdot d^{-1}$. The remaining 15 inoculated mice were administered with 0.9% NaCl solution by oral gavage daily and served as infected controls. Ten uninfected mice served as uninfected and untreated controls. These treatments were performed from day1 to 7 post-infection (pi) and mice were carefully observed. At day 8 pi, blood was collected from the orbital plexus and all mice were sacrificed, and heart samples were obtained.

We detected the activities of several biochemical markers in the serum, including creatine kinase (CK) and lactic dehydrogenase (LDH) using commercially available kits (Nanjing Jiancheng Biology Engineer Institute, China) according to the manufacturers' protocols. The heart was divided into two parts, one part was to determine viral gene VP-1, caspase-3 and bcl-2 expression levels by real-time RT-PCR method, and the other part was fixed for further histological and immunohistochemical examinations.

Histology. Histology was performed to examine myocardial injury and inflammatory lesions. Mice hearts were removed and fixed in 10% formalin solution. The tissues were embedded in paraffin, sectioned serially to a thickness of 3 μm, and stained with hematoxylin-eosin (HE) for histological examination. HE-stained sections were blindly and independently examined under a light microscope for signs of myocarditis.

Immunohistochemistry. Paraffin-embedded sections 3 μm thick were deparaffinized through a graded series of alcohol, heat-treated in a microwave oven for citrate antigen retrieval, and blocked in 5% bovine serum albumin for 30 min. Next, the sections were incubated at 4°C overnight with the primary

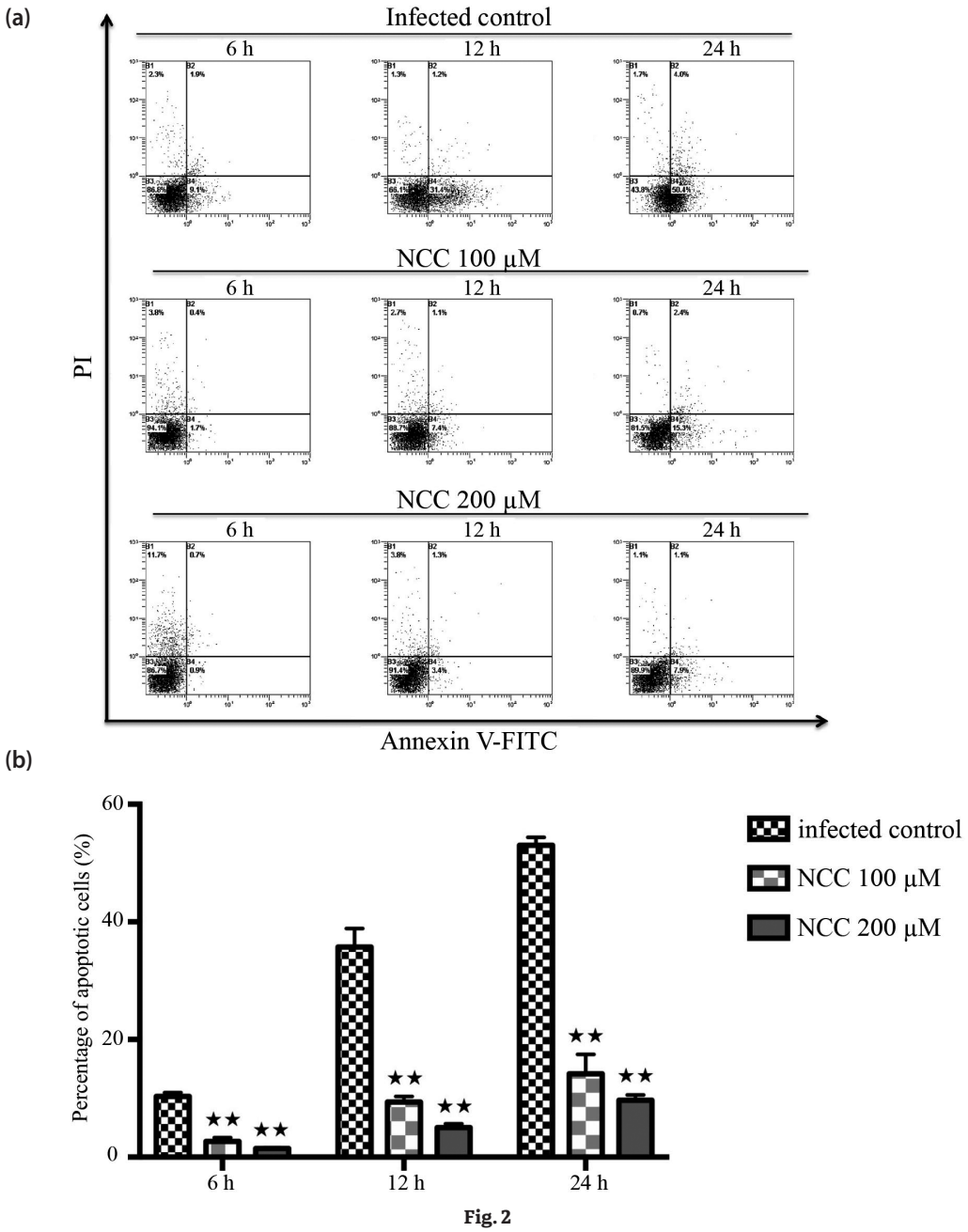

Fig. 2

Annexin V/PI analysis of apoptosis in NCC treated cells

The apoptosis ratio of fresh CVB3 infected NCC treated or untreated HeLa cells were analyzed by FACScan flow cytometer. HeLa cells infected with CVB3 were treated with 100 μM and 200 μM NCC for 6 h, 12 h, 24 h, and analyzed by FACS **(a)**. 100 μM and 200 μM NCC decreased the proportion of apoptotic cells at different time points **(b)**. The percentage of apoptotic cells. ** stand for $p < 0.01$, compared with the infected control.

caspase-3 (Wuhan Boster Biological Technology, China) or Bcl-2 (Wuhan Boster Biological Technology) antibodies. After washing three times with PBS, slides were incubated with the secondary antibody (Wuhan Boster Biological Technology, China). Bound immunoglobulins were detected by the avidin biotin complex method using commercially available kits (Wuhan Boster Biological Technology), according to the manufacturer's instructions.

Statistical analysis. Statistical analyses were carried out using SPSS19.0 software (SPSS Inc., Chicago, IL). All values are presented as mean ±SD and statistical significance was determined by one-way analysis of the *t*-test.

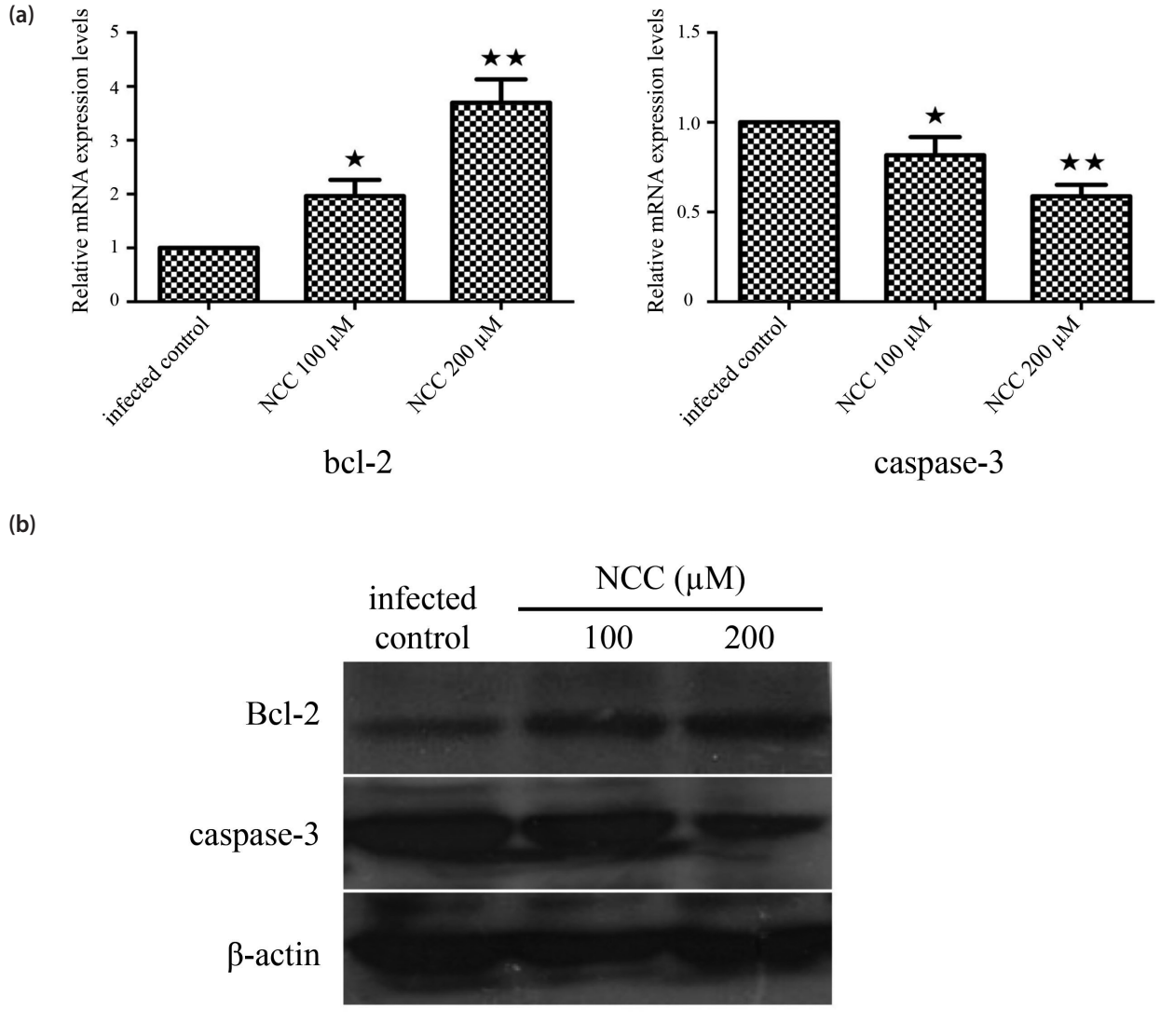

Fig. 3

The mRNA and protein expression of caspase-3 and bcl-2 in NCC treated and infected control groups

The mRNA expression levels of caspase-3 and bcl-2 in untreated or treated with 100 μM and 200 μM NCC HeLa cells were analyzed by real-time RT-PCR **(a)**. Caspase-3 and bcl-2 mRNA levels expressed as a fraction of the reference gene GAPDH. Where ** stand for $p < 0.01$, * stands for $p < 0.05$, compared with the infected control. The protein expression levels of caspase-3 and bcl-2 were detected by Western blot analysis **(b)**. β-actin was examined to normalize any differences in loading.

Results

NCC has potent activity against CVB3 replication in vitro

The cytotoxicity and inhibitory effects of NCC on CVB3 were examined in infected HeLa cells, and the results are presented in Table 2. In NCC treated cells, the CC_{50} and EC_{50} were found to be 993.34±0.18 μM and 116.60±0.32 μM, respectively. The TI of NCC and Ribavirin were determined to be 8.56±0.15 and 3.81±0.11 respectively. These results indicate that NCC exerts good antiviral activities against CVB3.

Table 2. Inhibitory effects of NCC in infected HeLa cells

Drug	CC_{50} (μM)	EC_{50} (μM)	TI
NCC	993.34±0.18	116.60±0.32	8.56±0.15
Ribavirin	2923±19	768±7.81	3.81±0.11

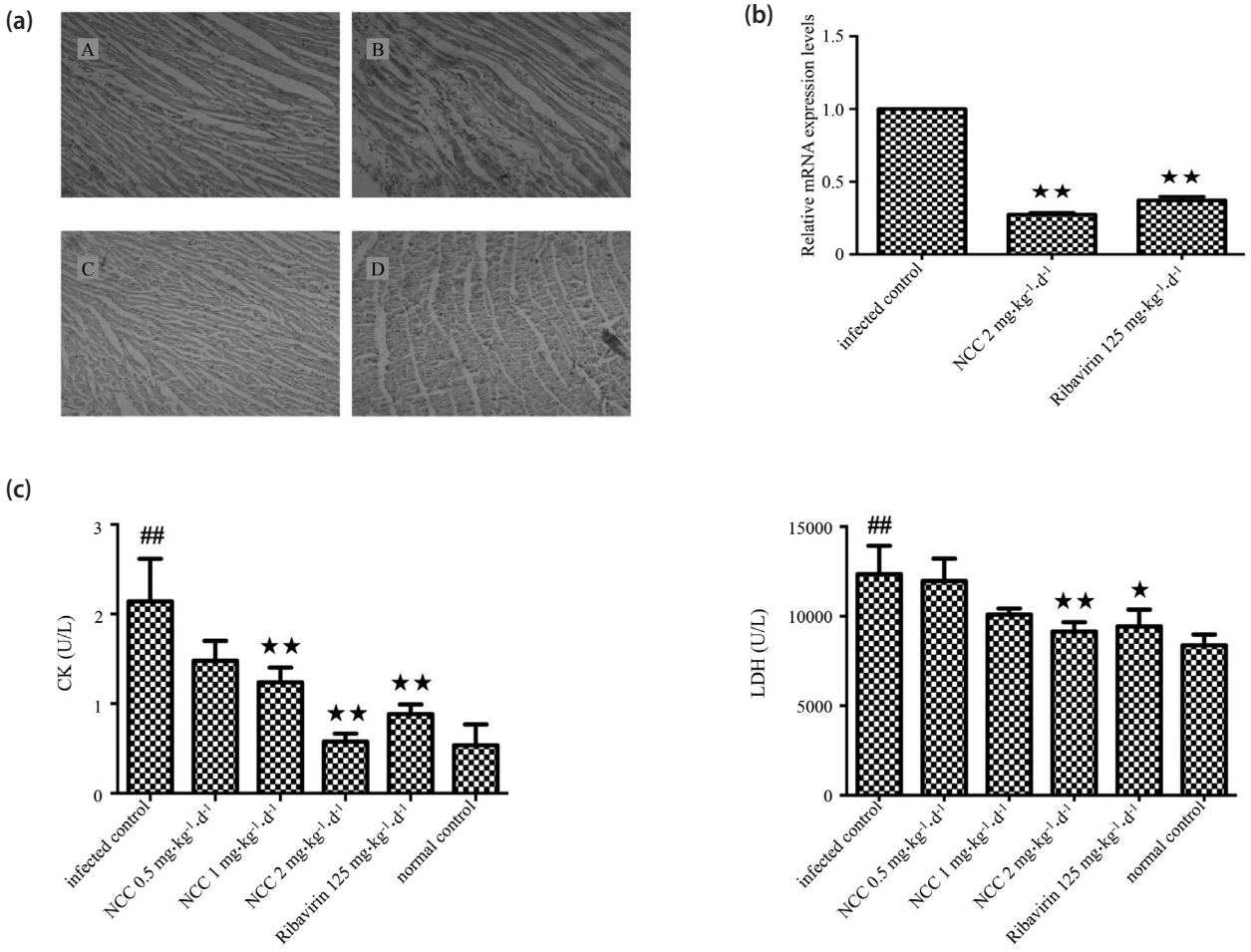

Fig. 4

Treatment with NCC can improve cardiac function and reduce the levels of serum CK and LDH

To evaluate the effects of NCC in a VMC mouse model, on day 8 pi, we studied pathological changes in mouse hearts and the activity of CK and LDH in serum. The mRNA expression levels of viral gene VP-1 in different groups were also checked by real-time RT-PCR. HE-stained sections of hearts from different groups (200×) **(a)**. (A) normal control, (B) infected control, (C) infected with CVB3 and treated with NCC (2 mg·kg^{-1}·d^{-1}), (D) infected with CVB3 and treated with Ribavirin (125 mg·kg^{-1}·d^{-1}). Detection of viral expression in treated and control groups **(b)**. Where ** stand for $p < 0.01$, compared with the infected control. Detection of activity of CK and LDH after NCC treatment in infected mice serum at day 8 pi **(c)**. Where ** stand for $p < 0.01$, * stand for $p < 0.05$, compared with the infected control, $^{\#\#}$ stand for $p < 0.01$, compared with the normal control.

NCC inhibits CVB3-induced apoptosis in vitro

It has been reported that cell apoptosis occurs frequently during CVB3 infection (Ammer *et al.*, 2015), so firstly we have analyzed whether NCC had an effect on apoptosis. HeLa cells infected with CVB3 were incubated with 100 and 200 μM NCC for 6 h, 12 h and 24 h. The cells were then stained with Annexin V/PI and harvested to evaluate apoptotic changes. Compared to the infected control, at different time points the ratio of apoptotic cells treated with NCC groups were significantly lower (Fig. 2). At 24 h, the apoptosis rate in cells treated with 200 μM NCC was 9.67% ± 0.91, while the apoptosis rate in the infected control was 53.00%±1.35. These results suggested that NCC could strongly inhibit apoptosis induced by CVB3.

NCC treatment alters expression levels of apoptosis-related genes in vitro

We next analyzed the expression levels of Bcl-2 and caspase-3 by real-time RT-PCR and Western blot analysis to further determine the effects of NCC on apoptosis induced by CVB3. As shown in Fig. 3a, compared with the infected control, expression of bcl-2 is up-regulated and caspase-3 is down-regulated after treatment with NCC. Western blot analysis confirmed the results of real-time

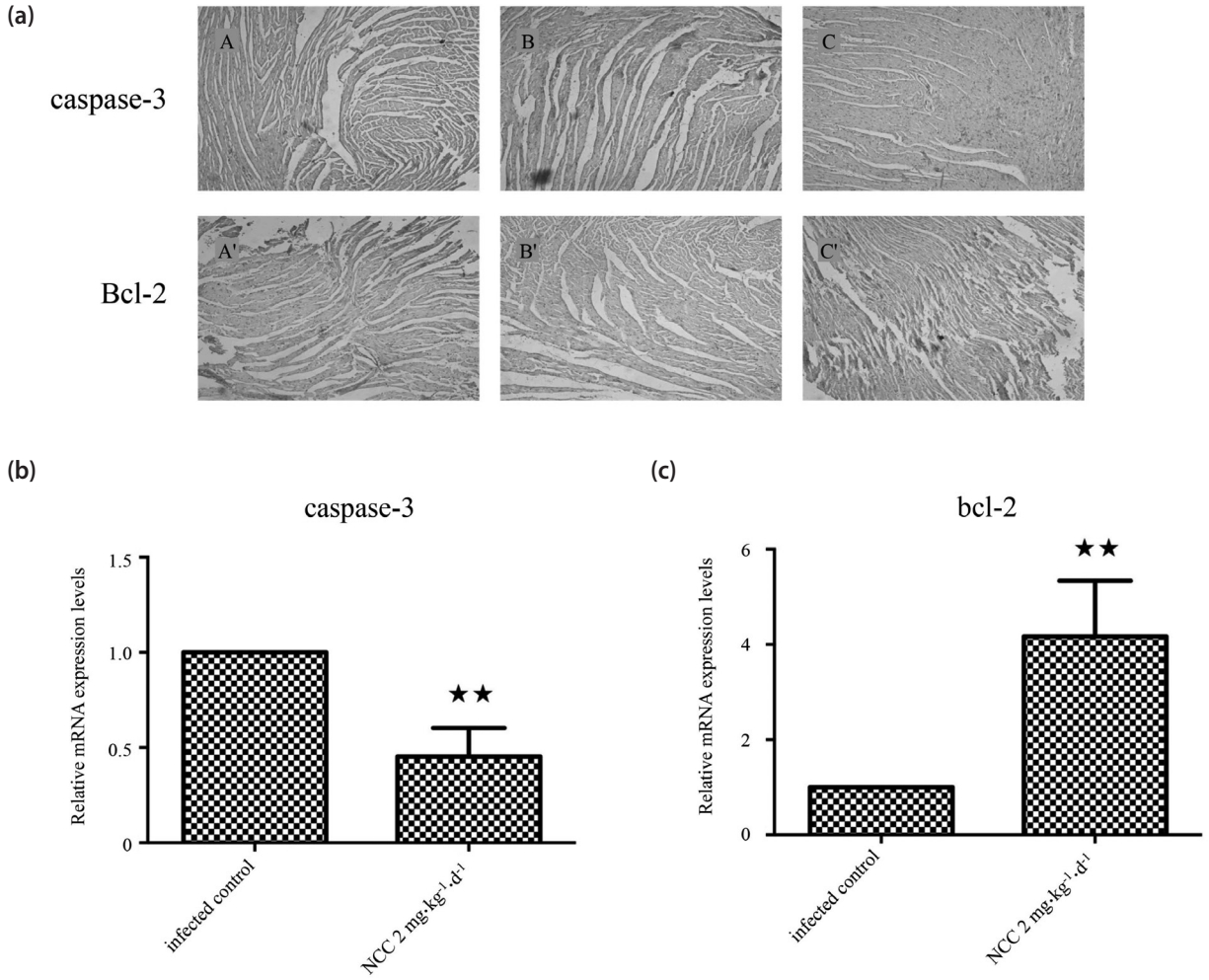

Fig. 5

Caspase-3 and Bcl-2 expression after NCC treatment in murine myocarditis

Expression of caspase-3 and bcl-2 were checked by real-time PCR and immunohistochemistry on day 8 pi. Histological appearance of CVB3-induced murine myocarditis (200×) **(a)**. (A and A') normal control, (B, B') infected control, (C, C') infected with CVB3 and treated with NCC (2 mg·kg^{-1}·d^{-1}). The mRNA expression of caspase-3 in mice heart **(b)**; ** stand for p <0.01, compared with the infected control. The mRNA expression of bcl-2 in mice heart **(c)**; ** stand for p <0.01, compared with the infected control.

RT-PCR, showing that expression of Bcl-2 expression significantly increased and caspase-3 greatly decreased after treatment with NCC, compared with the infected control (Fig. 3b). These results indicate that NCC inhibits CVB3-induced apoptosis.

NCC can improve cardiac function and reduce the levels of serum CK and LDH in CVB3-infected mice

To evaluate the effects of NCC in a VMC mouse model, we studied pathological changes in mouse hearts. Although CVB3 infection did not result in death or obvious weight loss in mice, the infection caused severe myocarditis. On day 8 pi, hematoxylin-eosin (HE) staining of the heart showed apparent injury in the infected control group, significant mononuclear cell infiltrations, and the appearance of multiple foci in necrotic cardiomyocytes. In mice treated with NCC and Ribavirin, the damage to the myocardium was alleviated and the area of necrosis and inflammatory infiltrates was decreased (Fig. 4a), compared with the infected control group. Especially in the NCC-treated group (2 mg·kg-1·d-1), there was no necrosis and inflammatory infiltrates. To examine the effects of NCC directly on CVB3, we measured the expression levels of the CVB3 VP1 gene in mice hearts. As shown in Figure 4b, administration of NCC significantly decreased expression of the CVB3 VP1 gene compared with the infected control group. To further investigate the therapeutic

effects of NCC against cardiac injury induced by CVB3, we detected the activity of CK and LDH in mouse serum. Compared with the healthy control group, the activity of CK and LDH were clearly enhanced at day 8 pi following CVB3 infection. In the NCC-treated group, activities of CK and LDH were clearly decreased compared with the infected control group (Fig. 4c).

NCC can reduce the expression of apoptosis-related factors in infected mice

On day 8 pi, we examined expression of apoptosis-related factors. As shown in Figure 5, treatment with NCC notably decreased the levels of caspase-3 gene and protein expression while increasing the levels of Bcl-2 gene and protein expression compared with the infected control group.

Discussion

NCC is a novel fluoronucleoside analog. In this paper, we found that NCC also has potent antiviral activity against CVB3 *in vitro* and *in vivo* and can dramatically inhibit CVB3-induced apoptosis and myocarditis.

CVB3 is the pathogen most commonly associated with acute and chronic myocarditis. Myocarditis is usually a mild disease that often goes undiagnosed (Friman *et al*., 1995; Garmaroudi *et al*., 2015). Persistent CVB3 infection, however, can eventually lead to irreversible myocardial injury and progressive cardiac dysfunction, which can result in dilated cardiomyopathy, heart failure and sudden unexpected death. An effective antiviral therapy is important for improving the outcomes of CVB3 infection, and there is a significant need for the development of antiviral drugs for the treatment of the various diseases caused by CVB3. Significant efforts over the years have led to the discovery and development of many antiviral drugs (Kytö *et al*., 2002; Fechner *et al*., 2011; Song *et al*., 2014; Kang *et al*., 2015). Despite the therapeutic potential of these promising drug candidates, viral resistance, severe side effects and toxicity still limit their use in treatment of CVB3 infection in humans. Therefore, further investigation is necessary and new, safe therapeutic candidates need to be developed quickly.

CVB3 infection of mouse hearts is routinely detected by the activities of biochemical markers, virus expression levels and histological changes on the heart on day 8 pi. CK and LDH are the most commonly used biochemical markers (Ghormade *et al*., 2014; Ahmad *et al*., 2017). In this study, mice were inoculated with CVB3 to induce myocarditis, and then received NCC by oral gavage daily. The results show that, in comparison with the infected controls, oral administration of NCC significantly decreased activities of CK and LDH in serum of infected mice. Treatment also reduced viral expression levels in the heart, as detected by real-time PCR. Pathological examination showed that the areas of necrosis and inflammatory infiltrates in cardiac muscles were much lower in the NCC treated groups. Especially in the high dose group (2 $mg \cdot kg^{-1} \cdot d^{-1}$), there was no necrosis and inflammatory infiltrates. Together, these data indicate that NCC has an inhibitory effect on CVB3 *in vivo* and protects myocardial cells against virus-induced harmful effects.

Both direct CVB3-induced myocardial injury (McManus *et al*., 1993; Pauschinger *et al*., 1999) and the inflammatory response (Wang *et al*., 2016) are commonly believed to contribute to the development and progression of myocarditis. Recently, some studies have suggested that apoptosis plays an important role in the pathogenesis and development of viral myocarditis and is associated with fatal heart failure (Esfandiarei *et al*., 2008; Zhou *et al*., 2015). CVB3 is known to induce apoptosis in host cells. In the early stages of myocarditis, apoptosis is triggered by viral replication (Saraste *et al*., 2003; Peischard *et al*., 2019). In murine models, apoptotic lesions peak at four days post-infection, and Bax expression is significantly enhanced (Joo *et al*., 2003). In humans, cardiomyocyte apoptosis has been found in myocardial biopsies of patients with chronic myocarditis or dilated cardiomyopathy (Alter *et al*., 2001). In fatal acute myocarditis, high rates of apoptosis and activation of caspase-3 have been observed in autopsy samples and endomyocardial biopsies from patients (Kytö *et al*., 2004). Studies show that CVB3-induced apoptosis is triggered by many signal pathways (Xin *et al*., 2015; Hanson *et al*., 2016; Nie *et al*., 2020), with the mitochondrion-mediated pathway being believed to be particularly important (Lin *et al*., 2017). In this pathway, cytochrome c is released from the mitochondria into the cytosol and initiates a downstream caspase cascade, activating caspase-9 and subsequently caspase-3 (Li *et al*., 2019a). The mitochondrial membrane protein Bcl-2, expressed prior to irreversible cellular damage, is a key anti-apoptosis factor (Scorrano *et al*., 2003; Li *et al*., 2019b).

In the present study, apoptosis was measured by FACS assay *in vitro*. The rate of apoptosis in the NCC treated groups was markedly lower than in the infected group. We further detected caspase-3 and bcl-2 mRNA and protein expression levels in CVB3-infected HeLa cells and murine hearts. These results indicated that, after NCC treatment, the expression of caspase-3 at the mRNA and protein levels decreases. In contrast, the expression of Bcl-2 is much higher in the treated group. Taken together, these results suggest that NCC can significantly inhibit apoptosis induced by CVB3.

In summary, the present study for the first time reports that NCC has potent activity in inhibiting CVB3 replication and CVB3-induced apoptosis *in vitro* and *in vivo*, and significantly reduces CVB3-induced myocardial injury. These findings suggest that NCC may be a potential therapeutic agent against CVB3 infection and associated myocarditis.

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References

Ahmad S, Panda BP, Kohli K, Fahim M, Dubey K (2017): Folic acid ameliorates celecoxib cardiotoxicity in a doxorubicin heart failure rat model. Pharm. Biol. 55, 1295–1303. https://doi.org/10.1080/13880209.2017.1299768

Alter P, Jobmann M, Meyer E, Pankuweit S, Maisch B (2001): Apoptosis in myocarditis and dilated cardiomyopathy: does enterovirus genome persistence protect from apoptosis? An endomyocardial biopsy study. Cardiovasc. Pathol. 10, 229–234. https://doi.org/10.1016/S1054-8807(01)00077-1

Ammer E, Nietzsche S, Rien C, Kühnl A, Mader T, Heller R, Sauerbrei A, Henke A (2015): The anti-obesity drug orlistat reveals anti-viral activity. Med. Microbiol. Immunol. 204(6), 635–645. https://doi.org/10.1007/s00430-015-0391-4

Baggen J, Thibaut HJ, Strating JRPM, van Kuppeveld FJM (2018): The life cycle of non-polio enteroviruses and how to target it. Nat. Rev. Microbiol. 16, 368–381. https://doi.org/10.1038/s41579-018-0005-4

Bopegamage S, Kovacova J, Vargova A, Motusova J, Petrovicova A, Benkovicova M, Gomolcak P, Bakkers J, van Kuppeveld F, Melchers WJG, Galama JM (2005): Coxsackie B virus infection of mice: inoculation by the oral route protects the pancreas from damage, but not from infection. J. Gen. Virol. 86, 3271–3280.

Cabrerizo M, Díaz-Cerio M, Muñoz-Almagro C, Rabella N, Tarragó D, Romero MP, Pena MJ, Calvo C, Rey-Cao S, Moreno-Docón A, Martínez-Rienda I, Otero A, Trallero G (2017): Molecular epidemiology of enterovirus and parechovirus infections according to patient age over a 4-year period in Spain. J. Med. Virol. 89, 435–442. https://doi.org/10.1099/vir.0.81249-0

Cai Z, Shen L, Ma H, Yang J, Yang D, Chen H, Wei J, Lu Q, Wang DW, Xiang M, Wang J (2015): Involvement of endoplasmic reticulum stress-mediated C/EBP homologous protein activation in Coxsackievirus B3-induced acute viral myocarditis. Circ. Heart Fail. 8, 809–818. https://doi.org/10.1161/CIRCHEARTFAILURE.114.001244

Chau DH, Yuan J, Zhang H, Cheung P, Lim T, Liu Z, Sall A, Yang D (2007): Coxsackievirus B3 proteases 2A and 3C induce apoptotic cell death through mitochondrial injury and cleavage of eIF4GI but not DAP5/p97/NAT1. Apoptosis 12, 513–524. https://doi.org/10.1007/s10495-006-0013-0

Esfandiarei M, McManus BM (2008): Molecular biology and pathogenesis of viral myocarditis. Annu. Rev. Pathol. 3, 127–155. https://doi.org/10.1146/annurev.pathmechdis.3.121806.151534

Fechner H, Pinkert S, Geisler A, Poller W, Kurreck J (2011): Pharmacological and biological antiviral therapeutics for cardiac coxsackievirus infections. Molecules 16, 8475–8503. https://doi.org/10.3390/molecules16108475

Feuer R, Mena I, Pagarigan RR, Harkins S, Hassett DE, Whitton JL (2003): Coxsackievirus B3 and the neonatal CNS: the roles of stem cells, developing neurons, and apoptosis in infection, viral dissemination, and disease. Am. J. Pathol. 163, 1379–1393. https://doi.org/10.1016/S0002-9440(10)63496-7

Friman G, Wesslén L, Fohlman J, Karjalainen J, Rolf C (1995): The epidemiology of infectious myocarditis, lymphocytic myocarditis and dilated cardiomyopathy. Eur. Heart J. 16, 36–41. https://doi.org/10.1093/eurheartj/16.suppl_O.36

Garmaroudi FS, Marchant D, Hendry R, Luo H, Yang D, Ye X, Shi J, McManus BM (2015): Coxsackievirus B3 replication and pathogenesis. Future Microbiol. 10, 629–653. https://doi.org/10.2217/fmb.15.5

Ghormade PS, Kumar NB, Tingne CV, Keoliya AN (2014): Distribution and diagnostic efficacy of cardiac markers CK-MB & LDH in pericardial fluid for postmortem diagnosis of ischemic heart disease. J. Forensic Leg. Med. 28, 42–46. https://doi.org/10.1016/j.jflm.2014.09.011

Hanson PJ, Ye X, Qiu Y, Zhang HM, Hemida MG, Wang F, Lim T, Gu A, Cho B, Kim H, Fung G, Granville DJ, Yang D (2016): Cleavage of DAP5 by coxsackievirus B3 2A protease facilitates viral replication and enhances apoptosis by altering translation of IRES-containing genes. Cell Death Differ. 23, 828–840. https://doi.org/10.1038/cdd.2015.145

Joo CH, Hong HN, Kim EO, Im JO, Yoon SY, Ye JS, Moon MS, Kim D, Lee H, Kim YK (2003): Coxsackievirus B3 induces apoptosis in the early phase of murine myocarditis a comparative analysis of cardiovirulent and noncardiovirulent strains. Intervirology 46, 135–140. https://doi.org/10.1159/000071453

Kang H, Kim C, Kim DE, Song JH, Choi M, Choi K, Kang M, Lee K, Kim HS, Shin JS, Kim J, Han SB, Lee MY, Lee SU, Lee CK, Kim M, Ko HJ, van Kuppeveld FJ, Cho S (2015): Synergistic antiviral activity of gemcitabine and Ribavirin against enteroviruses. Antiviral Res. 124, 1–10. https://doi.org/10.1016/j.antiviral.2015.10.011

Kytö V, Saraste A, Fohlman J, Ilbäck NG, Harvala H, Vuorinen T, Hyypiä T (2002): Cardiomyocyte apoptosis after antiviral WIN 54954 treatment in murine coxsackievirus B3 myocarditis. Scand. Cardiovasc. J. 36, 187–192. https://doi.org/10.1080/cdv.36.3.187.192

Kytö V, Saraste A, Saukko P, Henn V, Pulkki K, Vuorinen T, Voipio-Pulkki LM (2004): Apoptotic cardiomyocyte death in fatal myocarditis. Am. J. Cardiol. 94, 746–750. https://doi.org/10.1016/j.amjcard.2004.05.056

Lasrado N, Gangaplara A, Arumugam R, Massilamany C, Pokal S, Zhou Y, Xiang SH, Steffen D, Reddy J (2020): Identification of immunogenic epitopes that permit the detection of antigen-specific T cell responses in multiple serotypes of group B Coxsackievirus infections. Viruses 12, 347. https://doi.org/10.3390/v12030347

Li W, Liu M, Zhao C, Chen C, Kong Q, Cai Z, Li D (2019a): MiR-1/133 attenuates cardiomyocyte apoptosis and electrical remodeling in mice with viral myocarditis. Cardiol. J. doi: 10.5603/CJ.a2019.0036. [Epub ahead of print] https://doi.org/10.5603/CJ.a2019.0036

Li X, Yang Z, Nie W, Jiang J, Li S, Li Z, Tian L, Ma X (2019b): Exosomes derived from cardiac progenitor cells attenuate CVB3-induced apoptosis via abrogating the proliferation of CVB3 and modulating the mTOR signaling pathways. Cell Death Dis. 10, 691. https://doi.org/10.1038/s41419-019-1910-9

Lin L, Zhang M, Yan R, Shan H, Diao J, Wei J (2017): Inhibition of Drp1 attenuates mitochondrial damage and myocardial injury in Coxsackievirus B3 induced myocarditis. Biochem. Biophys. Res. Commun. 484, 550–556. https://doi.org/10.1016/j.bbrc.2017.01.116

Livak KJ, Schmittgen TD (2001): Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) method. Methods 25, 402–408. https://doi.org/10.1006/meth.2001.1262

Massilamany C, Gangaplara A, Reddy J (2014): Intricacies of cardiac damage in coxsackievirus B3 infection: implications for therapy. Int. J. Cardiol. 177, 330–339. https://doi.org/10.1016/j.ijcard.2014.09.136

McManus BM, Chow LH, Wilson JE, Anderson DR, Gulizia JM, Gauntt CJ, Klingel KE, Beisel KW, Kandolf R (1993): Direct myocardial injury by enterovirus: a central role in the evolution of murine myocarditis. Clin. Immunol. Immunopathol. 68, 159–169. https://doi.org/10.1006/clin.1993.1113

Mosmann T (1983): Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55–63. https://doi.org/10.1016/0022-1759(83)90303-4

Nie J, Ta N, Liu L, Shi G, Kang T, Zheng Z (2020): Activation of CaMKII via ER-stress mediates coxsackievirus B3-induced cardiomyocyte apoptosis. Cell Biol. Int. 44, 488–498. https://doi.org/10.1002/cbin.11249

Pauschinger M, Phan MD, Doerner A, Kuehl U, Schwimmbeck PL, Poller W, Kandolf R, Schultheiss HP (1999): Enteroviral RNA replication in the myocardium of patients with left ventricular dysfunction and clinically suspected myocarditis. Circulation 99, 889–895. https://doi.org/10.1161/01.CIR.99.7.889

Peischard S, Ho HT, Theiss C, Strutz-Seebohm N, Seebohm G (2019): A kidnapping story: how Coxsackievirus B3 and its host cell interact. Cell Physiol. Biochem. 53, 121–140. https://doi.org/10.33594/000000125

Respondek D, Voss M, Kühlewindt I, Klingel K, Krüger E, Beling A (2017): PA28 modulates antigen processing and viral replication during coxsackievirus B3 infection. PLoS One 12, e0173259. https://doi.org/10.1371/journal.pone.0173259

Saraste A, Arola A, Vuorinen T, Kytö V, Kallajoki M, Pulkki K, Voipio-Pulkki LM, Hyypiä T (2003): Cardiomyocyte apoptosis in experimental coxsackievirus B3 myocarditis. Cardiovasc. Pathol. 12, 255–262. https://doi.org/10.1016/S1054-8807(03)00077-2

Scorrano L, Korsmeyer SJ (2003): Mechanisms of cytochrome c release by ptoapoptotic BCL-2 family members. Biochem. Biophys. Res. Commun. 304, 437–444. https://doi.org/10.1016/S0006-291X(03)00615-6

Song JH, Choi HJ, Song HH, Hong EH, Lee BR, Oh SR, Choi K, Yeo SG, Lee YP, Cho S, Ko HJ (2014): Antiviral activity of ginsenosides against coxsackievirus B3, enterovirus 71, and human rhinovirus 3. J. Ginseng Res. 38, 173–179.

Wang JP, Cerny A, Asher DR, Kurt-Jones EA, Bronson RT, Finberg RW (2010): MDA5 and MAVS mediate type I interferon responses to coxsackie B virus. J. Virol. 84, 254–260. https://doi.org/10.1016/j.jgr.2014.04.003

Wang T, Zhang J, Xiao A, Liu W, Shang Y, An J (2016): Melittin ameliorates CVB3-induced myocarditis via activation of the HDAC2-mediated GSK-3β/Nrf2/ARE signaling pathway. Biochem. Biophys. Res. Commun. 480, 126–131. https://doi.org/10.1016/j.bbrc.2016.09.135

Xin L, Ma X, Xiao Z, Yao H, Liu Z (2015): Coxsackievirus B3 induces autophagy in HeLa cells via the AMPK/MEK/ERK and Ras/Raf/MEK/ERK signaling pathways. Infect. Genet. Evol. 36, 46–54. Epub 2015 Aug 22. https://doi.org/10.1016/j.meegid.2015.08.026

Zhang H, Yue Y, Sun T, Wu X, Xiong S (2017): Transmissible endoplasmic reticulum stress from myocardiocytes to macrophages is pivotal for the pathogenesis of CVB3-induced viral myocarditis. Sci. Rep. 7, 42162. https://doi.org/10.1038/srep42162

Zhou L, He X, Gao B, Xiong S (2015): Inhibition of histone deacetylase activity aggravates Coxsackievirus B3-induced myocarditis by promoting viral replication and myocardial apoptosis. J. Virol. 89, 10512–10523. https://doi.org/10.1128/JVI.01028-15