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Lithium chloride confers protection against viral myocarditis via suppression of coxsackievirus B3 virus replication

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ABSTRACT

Viral myocarditis (VMC) is a type of inflammation affecting myocardial cells caused by viral infection and has been an important cause of dilated cardiomyopathy (DCM) worldwide. Type B3 coxsackievirus (CVB3), a non-enveloped positive-strand RNA virus of the *Enterovirus* genus, is one of most common agent of viral myocarditis. Till now, effective treatments for VMC are lacking due to lack of drugs or vaccine. Lithium chloride (LiCl) is applied in the clinical management of manic depressive disorders. Accumulating evidence have demonstrated that LiCl, also as an effective antiviral drug, exhibited antiviral effects for specific viruses. However, there are few reports of evaluating LiCl's antiviral effect in mice model. Here, we investigated the inhibitory influence of LiCl on the CVB3 replication *in vitro* and *in vivo* and the development of CVB3-induced VMC. We found that LiCl significantly suppressed CVB3 replication in HeLa via inhibiting virus-induced cell apoptosis. Moreover, LiCl treatment *in vivo* obviously inhibited virus replication within the myocardium and alleviated CVB3-induced acute myocarditis. Collectively, our data demonstrated that LiCl inhibited CVB3 replication and negatively regulated virus-triggered inflammatory responses. Our finding further expands the antiviral targets of LiCl and provides an alternative agent for viral myocarditis.

1. Introduction

Viral myocarditis (VMC) is a disorder that affects the myocardiocytes following a viral infection and can progress into dilated cardiomyopathy and heart failure in young adults [1]. Coxsackievirus B3 (CVB3), a member of the *Enterovirus* genus of Picornaviridae, is well-identified as the dominant etiological factor causing viral myocarditis. It was reported that about 25–27% cases of dilated cardiomyopathy and VMC in young adults and children were caused by CVB3 [2,3]. Although type-I interferon is reported to have certain therapeutic effect, the treatment or vaccine has been successful in treating CVB3 infection and myocarditis [4,5].

Lithium chloride (LiCl) is applied as a psychotropic anti-depressant agent in the clinical management of manic depressive disorders since its approval by the FDA in 1949 [6]. Cumulative evidence suggests that LiCl participates in many cellular processes, such as cell gene expression, apoptosis, proliferation, oncogenesis, glycogen synthesis and inflammation. So LiCl has been explored for treating diabetes, Alzheimer's disease and ovarian cancer, as well as antimicrobial infection [7]. Prior investigations have also revealed that LiCl exhibits antiviral effects on certain viruses, e.g., coronavirus, herpes simplex, infectious bronchitis virus, porcine reproductive and respiratory syndrome (PRRS) and feline calicivirus [8–15]. Given that there are few reports about the influence of LiCl on the enteroviruses infections and it's rarely studied to evaluate the antiviral potential of LiCl in viral infection models based on animals, it's of high significance to examine the effect of LiCl on CVB3 infections and virus-induced viral myocarditis in mice.

Therefore, we studied the antiviral effects of LiCl in HeLa cells and in CVB3-infected mouse. The results indicate that LiCl acts as a potential antiviral agent to significantly inhibit CVB3 replication through decreasing virus-induced cell apoptosis. Moreover, LiCl treatment could markedly suppress the cardiac virus replication and alleviate the progress of myocarditis in mice, which might expand LiCl's new application in virus-induced inflammatory diseases.

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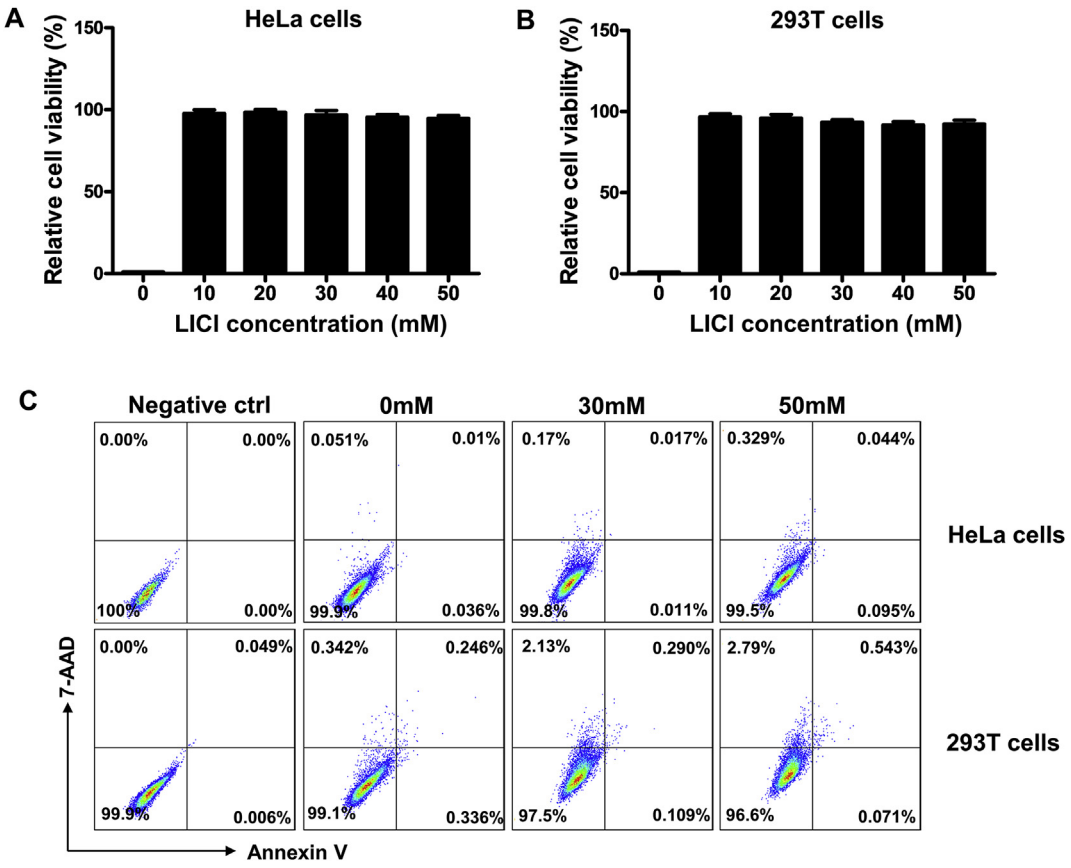

Fig. 1. Cytotoxic effect of LiCl administration on cell lines. HeLa or 293T cells were added to a various LiCl doses (0,10, 20, 30, 40, 50 mM) for 72h, after being cultured for 24h in 96-well plates with DMEM with 10% FBS. (A–B) Comparison of the cell viability by CCK8 assay. Each value was standardized to that of 0 mM group (set at 100%). (C)AnnexinV-FITC and 7-AAD were stained on HeLa or 293T cells, after being treated with LiCl. The cellapoptosis was detected by flow cytometry.

2. Materials and methods

2.1. Mice, viruses, cells and reagents

About 6–8 weeks-old male BALB/c mice (16–18g) were obtained from (Shanghai Slac Animal Inc.). All mice were handled in conformity with the institutional guidelines of Soochow University were approved by the Ethics Committee. Professor Yingzhen Yang (Key Laboratory of Viral Heart Diseases, Zhongshan Hospital, Shanghai Medical College of Fudan University) provided the CVB3-eGFP and CVB3 (Nancy strain). They were tittered by $TCID_{50}$ assay on HeLa cells. HeLa and 293T cell-lines were bought from the Cell Bank of Chinese Academy of Sciences (Shanghai, China) and maintained as detailed before [17]. LiCl (Sigma, St. Louis, MO, USA) was dissolved in DMEM and sterilized through a 0.22 μm filter.

2.2. Cytotoxicity tests

HeLa or 293T cells were added into 96-well plate with LiCl which had been diluted with DMEM (without serum) for 48 h. Six wells were mock treated as control. At the end of the incubation period, 20 μl of CKK8 solution and 80 μl of DMEM were put into every well, followed by incubation for 4 h at 37 ˚C. OD_{450} was calculated using a microplate reader and the relative cell viability values were calculated as (mean OD_{450} LiCl)/(mean OD_{450} control) × 100%.

2.3. RNA extraction and RT-PCR

The detailed protocol of RNA isolation, RT-PCR and primer sequences used were detailed in a previous report [16].

2.4. Assessment of apoptosis

Cell apoptosis analysis was performed by staining cells with staining Annexin V-FITC kit (BioVision, Palo Alto, CA, USA). Briefly, cells were infected CVB3 (MOI = 10) and then were treated with LiCl for 24 h. They were then recovered and washed two times using cold PBS and were staining with Annexin V for 15min. 7-AAD was then added for 5 min at room temperature (RT). The number of Annexin V^+ apoptotic cells was analyzed with FlowJo v10.0 software (Tree Star) after processing on a Canto II flow cytometer (BD Bioscience).

2.5. Western blot

SDS-PAGE and immunoblot analysis were performed as described previously [16,17]. The proteins extracted from HeLa cells or heart tissues were detected with anti-VP1 (Clone 5-D8/1, DAKO, Denmark).

2.6. Establishment of mice model of viral myocarditis

About 6–8 weeks-old male BALB/c mice (16–18g) received an intraperitoneal injection of 1500 $TCID_{50}$ (1050 pfu) dose of CVB3. The body weight and survival of mice were measured at the end of the test. After 3 and 7 days following infection, heart tissues were obtained for the detection of histology analysis, virus RNA and cytokines. The details of histology tests are as described before [17]. Severity of myocarditis was evaluated from five sections per heart by using a 1 to 5 scoring system: grade 0, no inflammation; grade 1, less than 10% of the heart

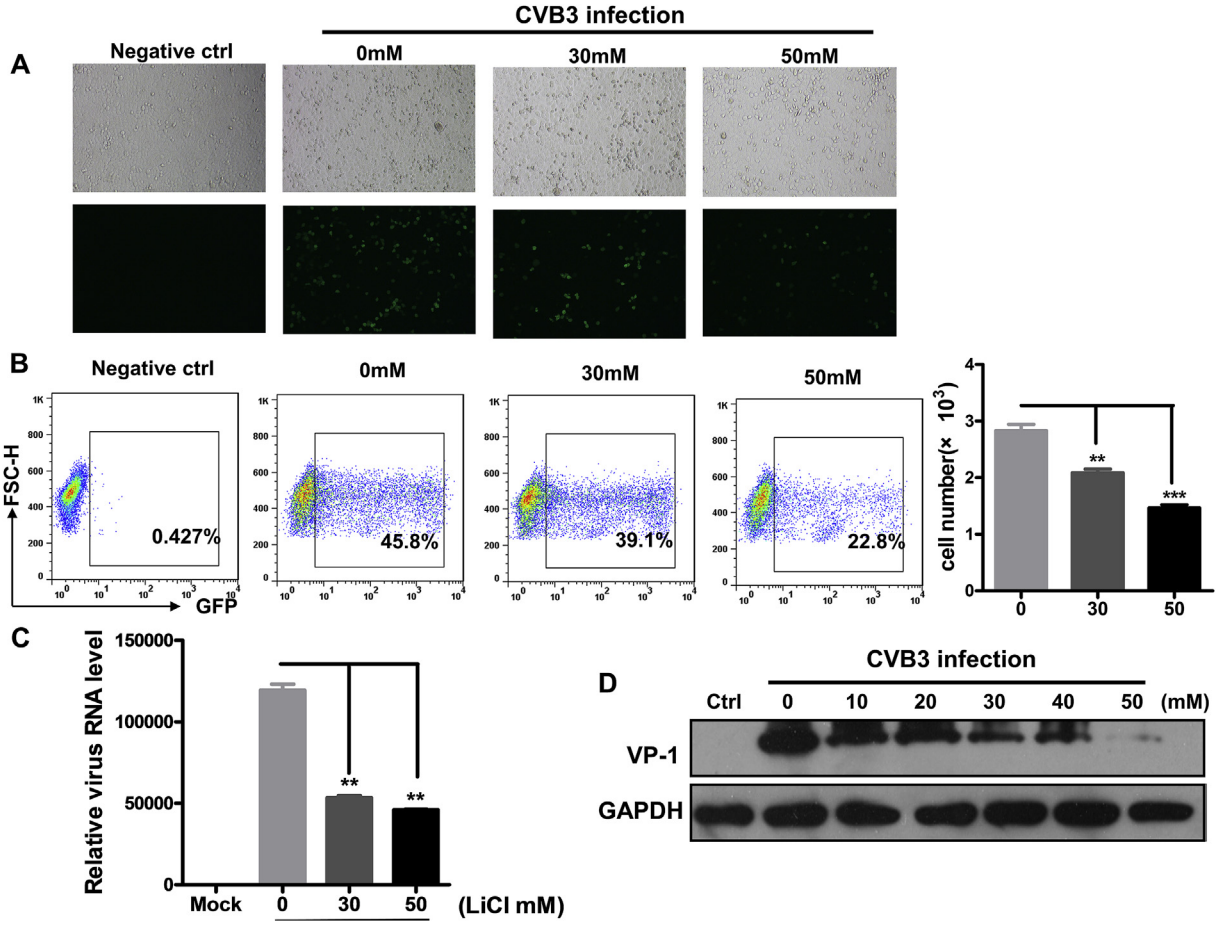

Fig. 2. Effect of LiCl treatment on CVB3 infection.(A–B) HeLa cells were infected with CVB3-eGFP(MOI = 10). After 1 h, cells were washed by PBS and then treated with different concentrations of LiCl for 24 h. (A) The GFP^+ cells were photomicrographs were captured under a fluorescence microscope. (B) TheGFP fluorescence and GFP^+ cell number were analyzed by flow cytometer. (C–D) HeLa cells were infectedwith CVB3 (MOI = 10) for 1 h. After washing with PBS, cells were treated with LiCl for 24 h. (C) TheRNA level of CVB3 was determined by Q-PCR. (D) Protein level of CVB3 VP1 was determined by Western blot.

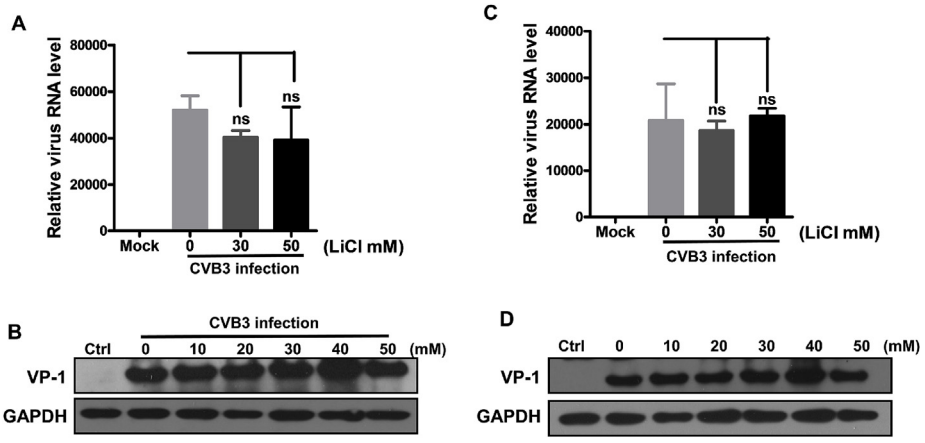

Fig. 3. Effect of LiCl treatment on CVB3 attachment and entry.(A–B) Different concentrations of LiCl were mixed with CVB3 (MOI = 10), and then the mixture was treated into HeLa cells and incubated at 4 °C for 1 h. After washing with PBS, HeLa cells were cultured in the medium at 37 °C for 24 h. The relative RNA level of CVB3 was assessed by Q-PCR (A) and VP1 expression was detected by Western blot (B). (C–D) HeLa cells were co-treated with LiCl and CVB3 at 37 °C for 1 h and washed by PBS. Cells were cultured at 37 °C for 24 h. The level of CVB3 RNA was detected by Q-PCR (C) and cell lysates were collected and analyzed VP-1 expression by Western blot (D).

section is involved; grade 2, 10–30%; grade 3, 30–50%; grade 4, 50–90%; grade 3, more than 90%. To evaluate the effect of LiCl on viral myocarditis, each mouse was infected intraperitoneally with CVB3 after which they were divided into two groups (8–10 mice/group), PBS and LiCl treatments. Considering the effective dose of LiCl in cells (10–50 mM), the fraction of drug absorbance through i.p. approximately 60% and the bodyweight of the mice, LiCl (100 μL, 50 mM/per mouse) were daily applied to mice i.p. upon infection.

2.7. Immunohistochemistry (IHC)

Immunohistochemistry was performed to assess cardiac levels of cleaved caspase-3 according to the manufacturer's instructions (Mouse and Rabbit Specific HRP/DAB Detection IHC Kit, ab64264, Abcam). Briefly, the paraffin sections of hearts were incubated with primary antibodies against cleaved caspase-3 (1:250, Cat. No. 9664, Cell Signaling Technology) at 4 °C overnight. After washing, sections were stained with Biotinylated Goat Anti-Polyvalent for 10 min at room temperature. After washing, the sections were incubated with

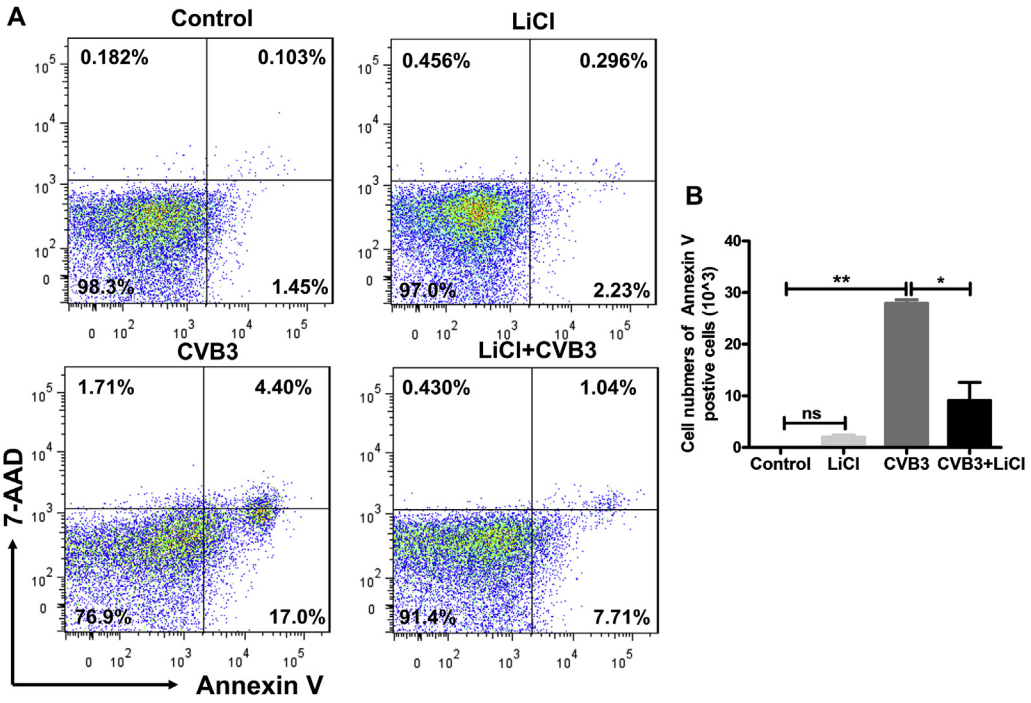

Fig. 4. Effect of LiCl treatment on virus-induced apoptosis. HeLa cells were infected with CVB3 at MOIof 10, and then cells were added with LiCl for 24 h. Cell were stained with Annexin V and 7-AAD and apoptosis was determined by flow cytometry. The apoptosis was quantified and indicated in Annexin V^+ cells (A). The absolute number of Annexin V^+ cells were shown as mean ± SEM (B).

Streptavidin Peroxidase and DAB Substrate. Stained sections were imaged with a Nikon Eclipse TE2000-S microscope and five images were captured under high power fields (100× magnifications) randomly.

2.8. Statistical analysis

GraphPad Prism software was utilized to analyzed data from the tests. All results are shown as mean ± SD or SEM. Experiments are repeated at least three times independently. The survival rate was analyzed using survival curves generated from the Kaplan–Meier tool with the Lonrank test. Student's *t*-test was applied to compare two group. P values, $0.05 \geq P > 0.01$; **, $0.01 \geq P > 0.001$; ***, $P \leq 0.01$ were considered statistically significant.

3. Results

3.1. Cytotoxicity of LiCl in HeLa and 293T cells

As previously reported, 10–60 mM was applied as nontoxic concentration range of LiCl for antiviral effect in F81 cells [14]. So we firstly detected the cytotoxicity of LiCl in CVB3-replicated HeLa or 293T cells. As shown in Fig. 1A and B, cell viability was > 90% in LiCl at 10–50 mM doses for 72 h. It has reported that LiCl could affect cell apoptosis in several tumor cell-lines. So we next analyzed the LiCl's effect on the apoptosis of HeLa or 293T cells by staining Annexin V and 7-AAD. The result of the flow cytometry revealed that compared with control, LiCl at concentrates of 30 mM and 50 mM could not induce cell apoptosis in HeLa or 293T (Fig. 1C). Therefore, we chose the concentration of 50 mM as the maximum concentration of LiCl for antiviral experiments.

3.2. LiCl suppresses CVB3 infection

To explore the antiviral effect of LiCl on CVB3 infection, CVB3-eGFP (MOI = 10) was used to infect HeLa cells followed by treatment with a series of doses of LiCl (30 and 50 mM). As shown in Fig. 2A, a large number of GFP + cells, which reflect virus replication, were observed upon CVB3-eGFP infection, while LiCl treatment could significantly reduce the number of GFP + cells. Flow cytometry analysis further confirmed it and revealed that LiCl treatment decreased the GFP fluorescence after CVB3 infection, implying LiCl could inhibit CVB3 replication (Fig. 2B). Next, we measured the caspid protein level of VP-1 by Western blot and viral RNA expression by Q-PCR. These results showed that both viral RNA and VP-1 protein levels were obviously decreased by LiCl treatment (Fig. 2C and D), suggesting LiCl could potentially restrict CVB3 replication in HeLa cells.

3.3. LiCl does not affect CVB3 attachment and entry

We firstly evaluated the influence of LiCl on CVB3 attachment to HeLa cells. LiCl of different doses (0, 10, 20, 30, 40 and 50 mM) were mixed with CVB3, after which the mixture was added into cells at 4 °C for 1 h. Subsequent to changing the medium and PBS-wash, cells were cultured at 37 °C for 24 h and were analyzed by Q-PCR and Western blot. The results showed that the viral RNA level and VP-1 expression were not altered after treatment with LiCl (Fig. 3A and B). Next to rule out the effect of LiCl on viral entry, HeLa cells were infected with CVB3 together with LiCl treatment followed by incubation at 37 °C for 1 h. Subsequent to changing the media, HeLa cells were cultured for 24 h at 37 °C and then used to carry out Western blot and Q-PCR and. As shown in Fig. 3C and D, we did not observe any significant difference in levels of viral RNA and capsid protein VP-1 observed between LiCl- and mock-treated groups. All these results implied that LiCl treatment has no influence on CVB3 attachment and entry into cells.

3.4. LiCl inhibits CVB3-induced cell apoptosis

CVB3 infection could induce cell apoptosis and LiCl is reported to be involved with several tumor cell apoptosis. So we intended to explore the impact of LiCl on virus-induced cell apoptosis. As shown in Fig. 4A, LiCl treatment had no effect on the apoptosis in normal HeLa cells but CVB3 infection could significantly induce cell apoptosis (21.4% Annexin V^+). Notably, LiCl treatment obviously decreased CVB3-induced cell apoptosis (8.85% Annexin V^+). The result of absolute number of Annexin V positive cells was consistent with above (Fig. 4B), suggesting LiCl could protect cells against virus-induced apoptosis.

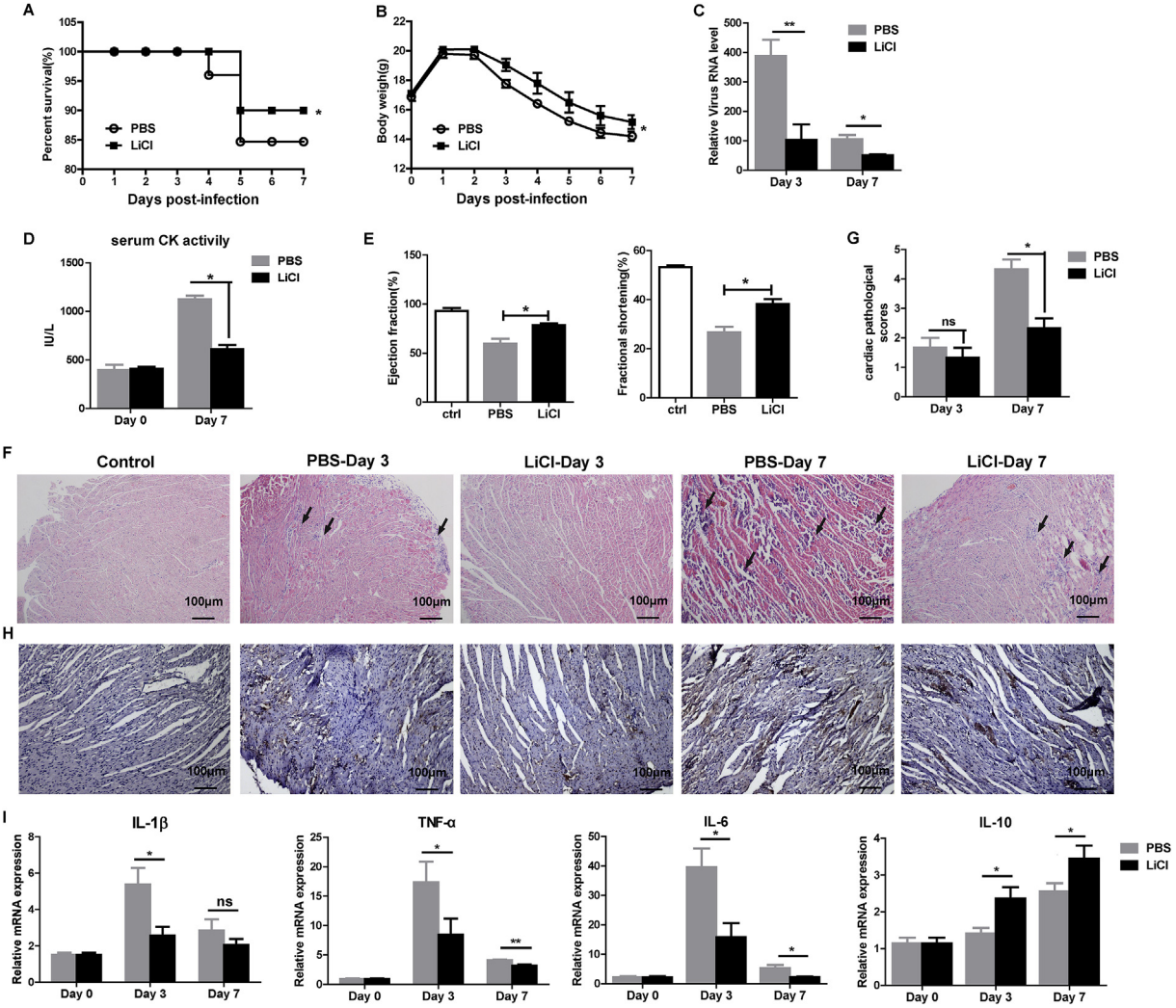

Fig. 5. Effect of LiCl treatment on CVB3-induced myocarditis.
Mice were infected intraperitoneally with 1500 $TCID_{50}$ of CVB3 and then divided into two groups (8–10 mice/group), PBS and LiCl treatments. After CVB3 infection, LiCl were daily applied to mice i.p. The survival rate (A) and body weight (B) of the mice were recorded daily until day 7 post infection (p.i.). (C) At day 3 and 7 p.i., viral RNA level in hearts were analyzed by Q-PCR. (D) Serum CK activity as indicative of cardiac injury was detected at day 0 and 4 post infection. (E) Transthoracic echocardiography was performed to each mouse at day 7 p.i (n = 4). LVEF and LVFS were calculated from measured ventricle dimensions. (F) At day 3 and 7 p.i., paraffin sections of heart tissues were subjected to HE analysis and the arrows indicated inflammatory foci. (G) Pathological scores of the hearts are shown. (H) At day 7 p.i., hearts were sectioned and subjected to IHC assay with primary antibody against cleaved caspase 3. Five images were captured for each section and representative one was shown. (I) At day 3 and 7 p.i., the mRNA levels of inflammatory cytokines in heart tissues were measured by Q-PCR. Data were presented as mean ± SD of three representative independent experiments.

3.5. LiCl treatment confers protection to mice against CVB3-triggered myocarditis

Next we establish CVB3-induced myocarditis in mice to evaluate antiviral effect of LiCl on the viral infections *in vivo*. LiCl was applied daily to the mice after infection, after which we analyzed myocarditis and cardiac viral replication. It was found that the bodyweight loss and survival rate of mice were markedly improved in mice treated with LiCl (Fig. 5A and B). As expected, the viral RNA level was obviously decreased in hearts of mice after LiCl treatment (Fig. 5C). LiCl treatment also significantly decreased the activity of serum creatine kinase (CK) and echocardiographic measurements showed that the left ventricular ejection fraction (LVEF) and left ventricular fractional shortening (LVFS) were markedly increased after treated with LiCl, which suggested that LiCl could obviously improve the cardiac function (Fig. 5D and E). Consistently, HE staining analysis displayed that control mice showed severe myocarditis with necrotic lesions and inflammation, LiCl treatment significantly alleviated myocarditis with less inflammatory foci and necrosis (Fig. 5F and G). IHC analysis showed that LiCl treatment decreased the expression of cleaved caspase 3 within the myocardium, which further supported that LiCl inhibit virus-induced cardiomyocytes apoptosis (Fig. 5H).To further verify the role of LiCl on the inflammation within the myocardium, we analyzed the productions of pro-inflammatory cytokines in hearts by Q-PCR. As shown in Fig. 5E, the levels of IL-6, TNF-α and IL-1β were significantly decreased after LiCl injection while anti-inflammatory cytokine IL-10 was slightly increased with LiCl treatment (Fig. 5I). Thus, these results indicated that LiCl could significantly suppress CVB3 replication and inhibit the virus-induced inflammation *in vivo*.

4. Discussion

The anti-depressant drug lithium is a well-known Food and Drug Administration-approved drug for nearly 60 years. Accumulating evidence suggests that LiCl have diversity of functions on physiological and pathological processes, including cancerogenic or anti-

cancerogenic properties, immune-stimulatory and immunosuppressive functions [6,7]. There are several studies exploring the roles of LiCl on virus infection and suggesting that LiCl inhibit certain virus replication, such as transmissible gastroenteritis coronavirus, HIV, canine parvovirus, PRRSV and so on [8–15]. In this study, we demonstrate that LiCl can also restrict CVB3 infection *in vivo* and *in vitro*.

Cumulative evidence indicates that LiCl acts as an agonist to enhance Wnt/β-catenin pathway and inhibits the glycogen synthase kinase-3β (GSK3β) pathway through competition for Mg^{2+} [18,19]. GSK3β regulates a wide range of cellular functions, including protein translation, cell cycle, development and apoptosis. GSK3β regulates apoptosis in a variety of cell types [20,21]. It is well-known that interferon (IFN) pathway plays a key role on virus replication and GSK3β is also reported to regulate IFN-β production. However, CVB3 has evolved mechanisms to antagonize the IFN response by directly cleaving MDA5 and MAVS. We also explored the effect of LiCl on IFN production upon CVB3 infection and found that the expression of IFN was not altered by CVB3 infection in HeLa cells and LiCl treatment also did not influence the IFN expression. Besides, we also checked type-I interferon responses in mouse peritoneal macrophages after CVB3 stimulation. We found that IFN-β production was induced by CVB3 and inhibited after LiCl treatment, which cannot explain LiCl's antiviral effect on CVB3 infection. Moreover, it seems that IFN-β inhibition induced by LiCl in immune cells also apply to other virus or stimuli. We found similar result in Sendai virus (SeV) infected peritoneal macrophages (data not shown). Wang et al. also reported similar phenotype. They found that, in macrophages, LiCl attenuated IFN-β production and IFN regulatory factor 3 activation induced by LPS-, polyinosinic-polycytidylic acid-, and SeV, but this is in a glycogen synthase kinase-3β-independent manner [22].

GSK3β was reported to induce apoptosis in neuron cells and fibroblasts, but recent studies have suggested that the role of GSK3β on apoptosis is complicated [23,24]. It has been suggested that CVB3 can induce apoptosis in infected cells, which contributes direct cytopathic effect [24]. VMC is caused by the direct virus-infected cardiomyocyte injury via necrosis and apoptosis, and virus-induced inflammatory responses [1]. Apoptosis has been suggested to facilitate viral progeny and spread [25,26]. In 2005, J Yuan et al. reported that CVB3 infection activated GSK3β pathway and inhibition of GSK3β suppressed CVB3-induced CPE and apoptosis via stabilizing β-catenin [27]. Consistently, our results also demonstrated that LiCl treatment could significantly inhibit CVB3-induced apoptosis in HeLa cells.

So far, the antiviral properties of LiCl in viral infected-animal model have not been fully explored. We deeply explore the role of LiCl on viral myocarditis in mouse and revealed that LiCl treatment could markedly restrict virus replication in heart tissues and alleviate the inflammation within the myocardium. Here, we report that LiCl restricts the replication of CVB3 through inhibiting virus-induced cell apoptosis, thus expanding the range of LiCl targets to CVB3. Moreover, we also confirm the antiviral potential of LiCl in hearts of VMC mice and demonstrated that LiCl potently protects mice against CVB3-induced myocarditis, indicating a therapeutic target of LiCl in virus-induced inflammatory diseases.

Ethical approval

The animal experiments were performed in accordance with Soochow University institutional guidelines, and the study was approved by the Ethics Committee of Soochow University in written form. Euthanasia of mice was performed by carbon dioxide inhalation with minimum fear, anxiety and pain.

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Author statement

Li Min: Conceptualization, Methodology, Writing -Original Draft. **Zhao Yinxia and Yan Kepeng:** Investigation, Project administration. **Wang Yanqi and Cai Jiamin:** Validation. **Wei Lin:** Writing - Review & Editing. **Li Shuijun and Xu Wei:** Methodology, Supervision.

Declaration of competing interest

The authors declare no conflict of interest.

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