Antiviral effect of lithium chloride on infection of cells by canine parvovirus

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Abstract Canine parvovirus type 2 causes significant viral disease in dogs, with high morbidity, high infectivity, and high mortality. Lithium chloride is a potential antiviral drug for viruses. We determined the antiviral effect of Lithium Chloride on canine parvovirus type 2 in feline kidney cells. The viral DNA and proteins of canine parvovirus were suppressed in a dose-dependent manner by lithium chloride. Further investigation verified that viral entry into cells was inhibited in a dose-dependent manner by lithium chloride. These results indicated that lithium chloride could be a potential antiviral drug for curing dogs with canine parvovirus infection. The specific steps of canine parvovirus entry into cells that are affected by lithium chloride and its antiviral effect in vivo should be explored in future studies.

Abbreviations
CPV Canine parvovirus
LiCl Lithium chloride
CC50 50 % cytostatic concentration
IFA Indirect immunofluorescence assay
CPE Cytopathic effect
TCID50 50 % tissue culture infectious dose

Introduction
Canine parvovirus type 2 (CPV-2), which was first identified and described in 1978 in both the United States and Australia, is closely related to feline panleukopenia virus (FPV) [1, 2, 15, 28]. This virus was named CPV-2 after an unrelated virus, canine parvovirus type 1 (CPV-1), which causes neonatal death in puppies [4, 5]. CPV-2 causes severe diarrhea and vomiting, and it has a predilection for young puppies, resulting in high mortality due to myocarditis and enteritis [31]. CPV-2 has spread rapidly, becoming globally distributed only two years after it was first identified, and it has been demonstrated to be a contagious pathogen to all populations of canids [12, 24]. Furthermore, CPV-2 has been evolving, and genetic variants continue to be identified. During 1979 and 1980, CPV-2 was completely replaced globally in canids by a new variant, CPV type 2a (CPV-2a) [22–24]. The virus underwent further antigenic drift, and a new variant, CPV type 2b (CPV-2b), was observed [25]. Subsequently, in 2000, another novel CPV variant (CPV-2c) was detected in several countries [10]. The mutations of variants have been mapped for VP2, which is the most abundant structural protein (CPV-2a: Val-555-Ile, Asp-305-Tyr, Ala-300-Gly, Ile-101-Thr and Met-87-Leu; CPV-2b: Ile-555-Val reversion and Asp-426-Asn; and CPV-2c: Asp-426-Glu).
CPV-2 is highly infectious and can lead to high morbidity and mortality in dogs. Currently, vaccination is the best measure for prophylaxis against CPV infection. Nevertheless, regardless of the relatively high costs, there are some concerns about the effectiveness of the existing vaccines [8, 9, 11, 14] and some concerns about the efficacy of existing clinical therapies, which, in addition to symptomatic treatment, include antiserum and interferon treatment. Therefore, drug therapy for CPV-2 infection, as an alternative strategy, warrants more attention.

Lithium salts are significant therapeutic agents that are used to treat several non-infectious diseases [6, 19, 20, 29]. In 1980, the antiviral effect of lithium chloride (LiCl) on DNA and RNA viruses was investigated. LiCl inhibited replication of the DNA virus herpes simplex but did not inhibit the replication of the RNA viruses encephalomyocarditis virus and influenza virus [29]. More recently, several reports have demonstrated the antiviral effect of LiCl on DNA viruses, such as herpes simplex virus [33], pseudorabies herpesvirus [30], and porcine parvovirus [7]. Lately, the antiviral effect of lithium chloride (LiCl) on RNA viruses, such as bronchitis coronavirus [13, 16], transmissible gastroenteritis virus [27], and type II porcine reproductive and respiratory syndrome virus [18], has also been demonstrated. These reports indicate that LiCl might be a potential antiviral drug for other viruses.

In this study, we investigated whether LiCl could inhibit the replication of CPV-2 in vitro and further explored the antiviral mechanism of LiCl.

Materials and methods

Viruses, cells and drug

The newest strain CPV-2c used in this study was maintained in our laboratory. The virus was isolated from a sick dog in Guangdong province, China, in 2014. VP2 was sequenced by the PCR method and found to contain the substitution Asp-426-Glu. Feline kidney cells (F81) were obtained from the American Type Culture Collection (ATCC). They were cultured in Dulbecco’s modified Eagle medium (DMEM) (Gibco, USA) containing 10 % fetal bovine serum (FBS) (Hyclone, Logan, UT) and 1 % penicillin-streptomycin (Gibco, CA) at 37 °C and 5 % CO₂. LiCl (Sigma, St. Louis, MO, USA) was dissolved in DMEM and sterilized by passage through a 0.22-μm filter.

Cytotoxicity assay

Cytotoxicity assays were performed according to the manufacturer’s instructions for CCK8 (Donjindo, Japan). For the assay, 1 × 10² F81 cells were cultured in 96-well plates in serum-free DMEM (to prevent cell replication) at 37 °C and 5 % CO₂ for 1 hour to allow the cells to adhere to the plates. The cells were washed three times with PBS and then incubated with 100 μl of LiCl at a series of concentrations (10, 20, 30, 40, 50, 80, 100, 200 mM) in DMEM with 2 % FBS (five wells/dilution) for 48–72 h. As a control, five wells were mock treated. After washing with PBS, 80 μl of DMEM and 20 μl of CCK8 solution were added to each well, and the plate was incubated at 37 °C for 1–4 h. The optical density (OD) value was measured using a microplate reader (Bio-Rad, USA) at a wavelength of 450 nm. The relative cell viability was calculated as (mean OD₄₅₀ drug)/(mean OD₄₅₀ control) × 100 %. The 50 % cytotoxic concentration (CC₅₀) was calculated using GraphPad Prism (GraphPad Software, San Diego, USA).

Effect of LiCl on viral infection

F81 cells (1 × 10⁴ cells) were cultured in 24-well plates, and nontoxic concentrations (0, 10, 20, 40 and 60 mM) of LiCl mixed with CPV (1 × 10⁻² TCID₅₀/cell) were added to the cells, which were then incubated at 37 °C for 72 h. As a control, cells infected with the same dose of CPV were not treated with LiCl. Subsequently, the antiviral efficacy was evaluated by analysis of viral RNA levels, protein expression level and CPE. In addition, interferon (INF-α and INF-β) expression levels were also determined.

Effect of LiCl on viral attachment

F81 cells (1 × 10⁴ cells) were cultured overnight in 24-well plates with 1 % FBS DMEM, and nontoxic concentrations (0, 10, 20, 40 and 60 mM) of LiCl mixed with CPV (1 × 10⁻² TCID₅₀/cell) was inoculated into cells, which were then incubated for 1 h at 4 °C (maximal binding) [3]. As a control, cells were infected with the same dose of CPV with no LiCl treatment. After removing the drugs and the unbound viruses by washing with cold DMEM, the cell lysates were subjected to three freeze-thaw cycles in preparation for measuring viral loads.

Effect of LiCl on viral entry

F81 cells (1 × 10⁴ cells) were cultured overnight in 24-well plates with 1 % FBS DMEM and infected with CPV (1 × 10⁻² TCID₅₀/cell) at 4 °C for 1 h. After removing the unbound viruses with cold DMEM, the cells were incubated with nontoxic concentrations (0, 5, 10, 15, 20, 25, and 30 mM) of LiCl at 37 °C for 1 h. As a control, cells were infected with the same dose of CPV with no LiCl treatment. After washing with cold DMEM, the cells were cultured in 10 % FBS DMEM for 24 h. The cell
lysates were subjected to three freeze-thaw cycles in preparation for measuring viral loads.

**Effect of LiCl on viral replication**

F81 cells (1 × 10^4 cells) cultured overnight in 24-well plates with 1 % FBS DMEM, and infected with CPV (1 × 10^{-2} TCID_{50}/cell) at 37 °C for 1 h to allow virus entry [3]. After washing with cold DMEM, the cells were treated with nontoxic concentrations (0, 5, 10, 15, 20, 25, and 30 mM) of LiCl and cultured in 10 % FBS DMEM at 37 °C for 24 h. As a control, cells were infected with the same dose of CPV with no LiCl treatment. Subsequently, the cell lysates were subjected to three freeze-thaw cycles in preparation for measurement of viral loads.

**Real-time quantitative PCR**

Total DNA was extracted using a Ra Pure Viral DNA Kit (Magen, China) according to the instructions of the manufacturer. The PCR primers for the VP2 gene of CPV are listed in Table 1. Total mRNA was extracted with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and converted to cDNA using oligo d(T) primers and PCR primers for INF-α and INF-β, which are listed in Table 1. Real-time quantitative PCR was performed using a 7500 Real-Time PCR System (Applied Biosystems, USA) with a SYBR® Green PCR Master Mix Kit (Applied Biosystems, USA), according to the instructions of the manufacturer. The 2^-ΔΔCT method with normalization to GAPDH was used to calculate the relative mRNA expression levels [17].

**Virus titration**

F81 cells cultured in 96-well plates were inoculated with the cell lysates that were serially diluted tenfold in DMEM with five replicates. After cultivating at 37 °C for 72 h, cells were observed for cytopathic effect, and the TCID_{50} was calculated by the method of Reed and Muench [26].

| Table 1 | Primer sequences used for real-time PCR assays to detect gene expression |
|---------|-------------------------------------------------------------------|
| Primer name | Sequence (5'-3') |
| VP2-F | GAAATGAAAGAGCTACAGGATCTGG |
| VP2-R | TTAAAATGCAAGATTCGTGTTGAG |
| IFNα-F | ACCAGTCACCAAGGGCGCAAGGCTTC |
| IFNα-R | GGGTCAGCTGCCGATCAAGTCCGT |
| IFNβ-F | TGACGCTACAGGGCTGGATCCAC |
| IFNβ-R | CATCCTGTCCTTTAGGCGAAATTTAG |
| GAPDH-F | ATGTGAAGGTCGGAGTCAACG |
| GAPDH-R | TCATACTGGAAACATGTACACCATGT |

**Indirect immunofluorescence assay**

Cells were washed with PBS, fixed with 4 % paraformaldehyde for 15 min, and permeabilized with 0.2 % Triton X-100 for 10 min. After washing three times with PBS, the cells were incubated with mouse anti-CPV antibody (1:2000) (Abcam, ab140431, Britain) for 1 h. Subsequently, FITC-conjugated goat anti-mouse IgG (1:500) (Zhongshan, China) was used as the secondary antibody. As a reference protein, nuclear staining was done with 4',6-diamidino-2-phenylindole (DAPI) according to instructions of the manufacturer (Invitrogen, Carlsbad, CA, USA). Finally, fluorescence was observed under a Leica DMI4000 B microscope (Leica, Wetzlar, Germany).

**Statistical analysis**

All experiments were performed in triplicate, and the results are reported as the mean ± standard deviation (SD). The significance of differences between experimental groups was determined using an unpaired t-test and a one-way ANOVA using Prism 5.0 software (GraphPad Software). A p-value < 0.05 was selected to indicate significance.

**Results**

**Cytotoxicity of LiCl in F81 cells**

The 50 % cytotoxic concentration (CC_{50}) of LiCl was 170.73 mM (Fig. 1). LiCl caused serious cellular toxicity at high concentrations (e.g., 200 mM). Concentrations of 10, 20, 30, 40, and 60 mM were above the 90 % cytostatic concentration (Fig. 1) but had no effect on cell morphology.

**Fig. 1** Cytotoxic effect of LiCl treatment on F81 cells. Cells were treated with a series of concentrations (10, 20, 30, 40, 50, 80, 100, 200 mM) of LiCl for 24 h. The relative cell viability was calculated as (mean OD_{450} drug)/(mean OD_{450} control) × 100 %. The dotted line indicates the CC_{50}. 

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when compared with mock-treated cells (data not shown). Therefore, 10-60 mM was used as the nontoxic concentration range of LiCl for antiviral tests.

**LiCl inhibits PCV infection**

To investigate the antiviral activity of LiCl against CPV, LiCl was added in a series of concentrations (10, 20, 40, 60 mM) prior to CPV infection. For real-time qPCR assays, the mean relative viral DNA level of mock-treated cells and cells treated with 10, 20, 40, 60 mM LiCl was 100.00 %, 98.00 %, 64.00 %, 30.33 %, and 22.27 % (with mock-treated cells set at 100 %), respectively (Fig. 2A). For virus titration (50 % tissue culture infected dose, TCID50), the viral titers of mock-treated cells and those treated with 10, 20, 40, and 60 mM LiCl were 5.29, 4.89, 2.93, 1.33, and 1.40 log10TCID50/ml, respectively (Fig. 2B). For indirect immunofluorescence assay (IFA), mock-treated F81 cells produced stronger fluorescent signals at 72 hours after infection with CPV. The fluorescence signals declined after treatment with 20, 40, and 60 mM LiCl (Fig. 2C). Examination by microscopy showed that CPV infection results in the detachment of many cells, while 60 mM treatment prevented most of cells from detaching (Fig. 3). These results indicate that treatment of F81 cells with LiCl inhibits CPV infection and reduces the cytopathic effect in a dose-dependent manner. The relative mRNA levels of INF-α and INF-β were also determined.
but the levels in cells that had CPV, LiCl, and CPV+LiCl were not significantly different when compared to the mock-treated cells (Fig. 3C).

**LiCl inhibits PCV entry**

Viral attachment, entry, and replication assays were performed to determine which step in the viral life cycle is affected by LiCl treatment of F81 cells. No significant differences in the relative levels of viral VP2 gene DNA or viral titers were observed between drug-treated and mock-treated cells, indicating that LiCl had no effect on CPV attachment and replication in F81 cells (Fig. 4A and B).

In viral entry tests, the relative levels of viral VP2 gene DNA in mock-treated cells and those treated with 10, 20, 40, and 60 mM LiCl were 100.00 %, 96.67 %, 72.67 %, 33.00 % and 24.00 %, respectively (Fig. 4A), and the viral titers of mock-treated cells and those treated with 10, 20, 40, and 60 mM LiCl were 5.05, 4.57, 2.73, 1.40, and 1.30 log_{10}TCID_{50}/ml, respectively (Fig. 4B). These results indicate that CPV entry into cells is inhibited in a dose-dependent manner by treatment with LiCl.

**Discussion**

**LiCl has potential as an antiviral agent**

The purpose of this study was to determine whether LiCl could be used as a potential curative agent for CPV-2. First, LiCl concentrations of 0-60 mM were determined to have no significant toxicity in F81 cells and no significant effects on cell morphology. Second, after LiCl treatment, viral DNA and viral protein levels decreased and cell morphology improved. These results indicated that LiCl inhibits CPV infection of F81 cells. However, IFN expression was not affected by LiCl treatment, which indicates that the innate immune system is not impacted by LiCl. Thus, the
potential antiviral effect of LiCl should be explored. The viral life cycle of CPV infection includes attachment to cells, entry into cells, and replication in the nucleus. These stages of the viral life cycle were evaluated after LiCl treatment in this study. CPV attaches to specific receptors on F81 cells in the initial step of the viral life cycle. The attachment step is significant for virus host tropism. We found that viral attachment was not affected by LiCl treatment. CPV replicates in the nucleus and requires that certain cellular factors are expressed during the S phase of the cell cycle. We also found that viral replication was not affected by LiCl treatment. CPV enters cells via an endocytic route that involves microtubule-dependent delivery of CPV to endosomes. This process is mediated by rapid removal of virus via clathrin-coated vesicles [21, 32]. Our results indicate that the CPV entry into F81 cells is inhibited in a dose-dependent manner by LiCl, which indicates that clathrin-coated vesicles might be affected by LiCl treatment.

In conclusion, CPV infection was inhibited in a dose-dependent manner by LiCl treatment of F81 cells. The antiviral effect of LiCl was at the step of CPV entry into cells, and this inhibition might involve clathrin-coated vesicles. Further research is required to determine how CPV entry into cells is affected by LiCl and whether LiCl has an antiviral effect in vivo.

Acknowledgments This work was supported in part by the National Natural Science Foundation of China (31372448) and the Special Fund for Agro-Scientific Research in the Public Interest (201303042).

Compliance with ethical standards
Conflict of interest The authors declare no competing financial interests.

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