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Short Communication

In vitro protective efficacy of Lithium chloride against *Mycoplasma hyopneumoniae* infection

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ABSTRACT

Mycoplasma hyopneumoniae (*M. hyopneumoniae*) infection affects the swine industry. Lithium chloride (LiCl), is a drug used to treat bipolar disorder and has also shown activity against bacterial and viral infections. Herein, we evaluated the antibacterial activity of LiCl on PK-15 cells infected with *M. hyopneumoniae*. Incubation of LiCl (40 mM) with cells for 24 h, did not significantly affect the cell viability. The qRT–PCR showed ~80% reduction in *M. hyopneumoniae* genome when LiCl added post-infection. A direct effect of LiCl on bacteria was also observed. However, treatment of cells with LiCl prior infection, does not protect against the infection. Antibacterial activity of LiCl was further confirmed by IFA, which demonstrated a reduction in the bacterial protein. With 40 mM LiCl, the apoptotic cell death, production of nitric oxide and superoxide anion induced by *M. hyopneumoniae*, were prevented by ~80%, 60% and 58% respectively. Moreover, caspase-3 activity was also reduced (82%) in cells treated with 40 mM LiCl. LiCl showed activity against various strains of *M. hyopneumoniae* examined in our study. Collectively, our data showed that LiCl inhibited the infection of *M. hyopneumoniae* through anti-apoptotic mechanism.

Mycoplasma hyopneumoniae (*M. hyopneumoniae*) causes porcine enzootic pneumonia (PEP) that affects the swine industry (Clark et al., 1991). Treatment of PEP is limited to antibiotics and vaccines that provide partial protection (Haesebrouck et al., 2004). Development of therapeutic agents against the infection is required.

Lithium chloride (LiCl) is a drug used to treat bipolar disorder (Bowden et al., 2005). It modulates several biological functions, including apoptosis, glycogen synthesis and inflammation (Machado-Vieira et al., 2009). LiCl active against viruses including avian coronavirus (Li et al., 2009) and transmissible gastroenteritis virus (TGEV) (Ren et al., 2011) in addition to bacteria such as *Francisella tularensis* (Zhang et al., 2009) and *Pseudomonas aeruginosa* (Chen et al., 2013). However, it un-clear whether LiCl could also inhibit the infection of *M. hyopneumoniae*.

Reactive oxygen species (ROS) (Beere and Green, 2001), nitric oxide (NO) (Brüne, 2003) and caspases, are important apoptosis inducers. LiCl was found to prevent apoptosis by inhibiting production of NO (Wang et al., 2013), ROS (Lumetti and Galli, 2014) and caspases (Ren et al., 2011). *M. hyopneumoniae* infection was reported to induce apoptosis (Bai et al., 2013). Thus, we aimed to investigate the inhibitory properties of LiCl on PK-15 cells infected with *M. hyopneumoniae* and to underline the possible mechanism.

Swine tracheal epithelial cells (STEC) are the ideal model to study Mycoplasma *in vitro*. However, porcine kidney-15 (PK-15) cells model used in our study was previously established in our laboratory with time and titer above 4 h and 1×10^5 CCU/ml (Che et al., 2013; Che et al., 2014; Che et al., 2012; Zhang et al., 2013) and was also described by others (Burnett et al., 2006; Wang et al., 2016).

M. hyopneumoniae isolates Xiaoling Wei-1(XLW-1), Nanjing (NJ) and attenuated strain (168 L) were grown in KM2 medium (Bai et al., 2013). PK-15 cells were cultured in DMEM containing 10% FBS.

Cytotoxicity of LiCl (MW = 42.39, Sigma (U.S.A.) on PK-15 cells was analyzed by MTT. We observed that LiCl at concentrations and time used in our had no toxic effect on PK-15 cells (data not shown).

Cells (1×10^5 cells/well) in 24-well plates were infected with *M. hyopneumoniae* (1×10^6 CFU/ml) at 37 °C for 10 h, washed and inoculated with LiCl (10–40 mM) for 12 h. After which, the cells were washed, total DNA was extracted and the effect of LiCl was evaluated by qRT–PCR following previous methods established in our laboratory (Wu et al., 2012). Briefly, the genome sequence of *M. hyopneumoniae* (GenBank CP002274.1) was used to PCR amplify 460 bp of p97 gene (GenBank: ADQ90328.1). This fragment was cloned in pMD18-T vector (pMD18-T-P97) to generate a log–linear standard curve, from which the DNA copy numbers was determined by qRT–PCR (based on

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Ct value). The qRT–PCR was performed on ABI PRISM 7500 Sequence Detection System (Applied Biosystems, USA) with SYBR® Premix Ex Taq™ Kit (Takara). The qRT–PCR mixture (25 μl in total) consisted of 12.5 μl Premix Ex TaqTM (2×), 0.5 μl ROX Reference Dye (50×), 1 μl Forward primer (10 μM), 1 μl Reverse primer (10 μM), 0.125 μl Probe, 9 μl ddH_2O and 1 μl DNA while the cycling condition was set at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. An about 90 bp of p97 gene was detected using p97 primers Forward- 5′ CCAGAACCAAATTCCTTCGCTG 3′and Reverse- 5′ ACTGGCTGAACTTC ATCTGGGCTA 3′and p97 probe 5′ FAM-AGCAGATCTTAGTCAAAGTGCC CGTG-TAMRA 3′ which labeled at the 5' with the reporter dye 6-carboxyfluorescein (FAM) and at the 3' with a Black-Hole-Quencher.

We found that LiCl induce a dose-dependent decrease in the amount of bacterial DNA. At 40 mM, LiCl caused approximately 80% reduction in the relative amplification of p97 gene of *M. hyopneumoniae* (Fig. 1A). This was consistent with the inhibitory effect of LiCl on DNA synthesis of herpes simplex virus (Ziaie and Kefalides, 1989). Previous studies shown that *M. hyopneumoniae* introduced into PK-15 has infected the cell after 10 h (Che et al., 2014). Herein, we added LiCl to cells 10 hrs post-infection, indicating that, the inhibition of LiCl occurred during bacterial replication. We further investigated the direct effect of LiCl on cells or bacteria. LiCl-treated cells (1×10^5 cells/well) were infected with untreated bacteria (1×10^6 CFU/ml), and untreated cells were infected with LiCl-treated bacteria (1×10^6 CFU/ml). A direct effect of LiCl on bacteria was also observed but not on cells (Fig. 1B and Fig. C) which is consistent with the effect of LiCl on IBV (Harrison et al., 2007).

We further infected cells (1×10^5 cells/well) in 96-well plates with *M. hyopneumoniae* (1×10^6 CFU/ml) at 37 °C for 10 h. Cells washed and inoculated LiCl (10–40 mM) for 12 h. Expression level of *M. hyopneumoniae* surface antigen (p46) was evaluated by IFA using p46 monoclonal antibody (our laboratory) and FITC-conjugated goat anti-mouse secondary antibodies (Boster Biological Technology Company, China). Fluorescence was measured immediately at excitation 485 nm and emission of 535 nm using an infinite F200 Tecan Microplate Reader (Huppert et al., 2010), and the data were acquired using i-control software (Tecan Trading AG, Ma¨nnedorf, Switzerland). We also observed that LiCl inhibited p46 expression (Fig. 1D) which indicated that LiCl treatment led to substantial inhibition of *M. hyopneumoniae* infection.

It has been reported that, *M. hyopneumoniae* infection could induce apoptosis in PBMC and STEC cells (Bai et al., 2015; Ni et al., 2014). As the infection model of *M. hyopneumoniae* with PK-15 cells was already developed in our laboratory, it's un-clear whether PK-15 cells undergoes apoptosis upon infection with this bacterium. In this study, by using AO/EB staining (Normal/Apoptotic/Necrotic Cell Detection kit, Beyotime Biotech., China), we observed that, the infection of PK-15 cells with this bacterium could also induce apoptosis in PK-15 cells (Fig. 2A). We further investigated the ability of LiCl to prevent *M. hyopneumoniae* induced apoptosis. Briefly, cells (1×10^5 cells/well) on 96-wells plate infected with *M. hyopneumoniae* (1×10^6 CFU/ml) for 10 h, washed and treated with or without LiCl (10–40 mM) for 12 h. Cells washed again, added 35 μl of AO/EB solution, examined by fluorescence microscopy and the apoptosis was quantified. Number of positive cells were calculated by selection of five view fields. Apoptotic rate was calculated as follows: apoptotic rate (%) = number of positive staining cells/number of total cells × 100%. Viable cells appeared green with intact nuclei. Nonviable cells had bright orange chromatin. Apoptotic cells appeared shrinked with condensation and fragmentation of nuclei. Apoptotic cells were distinguished from necrotic cells because the latter appeared orange with a normal nucleus (Renvoize et al.,

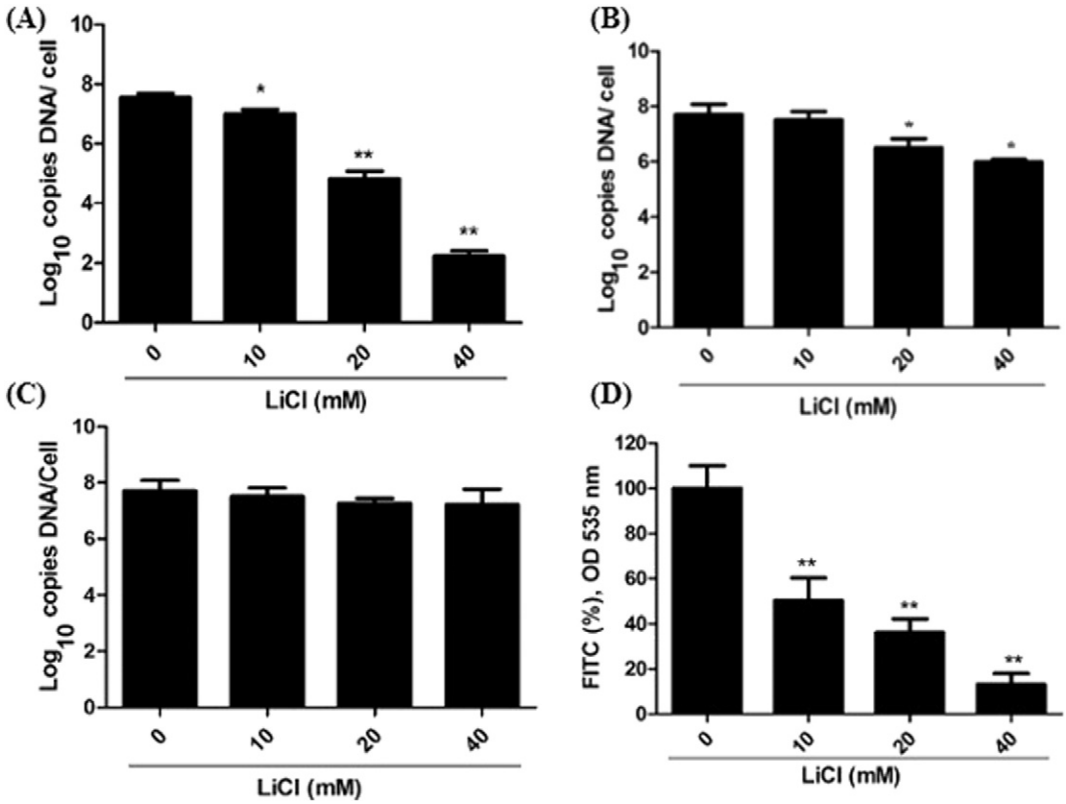

Fig. 1. Antibacterial activity of LiCl against *M. hyopneumoniae* (1×10^6 CCU/ml) infected PK-cells (1×10^5 cells/well). Cells infected with *M. hyopneumoniae*, XLW-1 for 10 h and treated with LiCl for 12 h. (A) DNA extracted and quantified by qRT–PCR (log10 copies of DNA/well). Data presented as Means ± SD (n = 3). $^{*}p < 0.008$ and $^{**}p < 0.001$ *vs*. control. (B) *M. hyopneumoniae* treated with LiCl (direct inactivation) before inoculating into cells. (C) Cells treated with LiCl then infected with *M. hyopneumoniae*, XLW-1 (pre-infection). DNA was extracted and quantified by qRT–PCR as above. A direct effect on bacteria was observed but not in cells. Data presented as Means ± SD (n = 3) $^{*}p < 0.05$ and $p < 0.54$ *vs*. control. (D) Effect of LiCl treatment was evaluated by IFA. Cells washed, fixed and probed with primary (p46 monoclonal antibody) and secondary-FITC conjugated antibodies. FITC-fluorescence was measured at OD 535. $^{**}P < 0.001$ *vs*. control.