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In vitro protective efficacy of Lithium chloride against Mycoplasma hyopneumoniae infection

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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.

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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 ddH2O 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′-CCAGAACAAATTCCTTCGCTG 3′ and Reverse: 5′-ACTGGCTGAACCT 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 showed 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).

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, Maennedorf, 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 shrunk 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 10 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.](image-url)
As shown in Fig. 2A, *M. hyopneumoniae* induced apoptosis were significantly decreased in a dose-dependent manner after treatment with LiCl. Counting apoptotic cells at 24 hpi, confirmed this (Fig. 2B) and indicated ~80% inhibition of apoptosis at 40 mM LiCl. These results imply that LiCl maybe a universal anti-apoptotic agent with limited selectivity to cell types and apoptosis inducers. Although the result is contradictory with studies on *Burkholderia pseudomallei* (Maniam et al., 2013) in which pretreatment of macrophages and A549 epithelial lung cells inhibited the infection, these data on post-infection treatment indicate that whether LiCl executes an anti-apoptotic or a pro-apoptotic effect is largely associated to the cell type and stimulating factor.

Low concentrations of NO inhibits apoptosis, and excessive production of NO triggers apoptosis (Shen et al., 1998). We evaluated whether LiCl inhibits production of NO in PK-15 cells infected with *M. hyopneumoniae* by using NO assay kit (Beyotime Biotech., China). Cells (1 × 10^5 cells/well) infected with *M. hyopneumoniae* (1 × 10^5 CFU/ml) and treated with or without LiCl (10–40 mM). Supernatants were mixed with Griess-I and Griess-II and examined at 570 nm. NO concentrations in the supernatants was reduced by 60% at 40 mM LiCl (Fig. 2C) and this might correlate with the cells protection.

Superoxide anion is involved in the early apoptosis. We treated cells (1 × 10^5 cells/well) with or without LiCl post-infection and the level of ROS was measured by a superoxide assay kit (Beyotime Biotech., China). Compared to controls, addition of LiCl reduced ROS production by 58% (Fig. 2D). This inhibitory activity of LiCl against NO and ROS was previously observed (Lumetti and Galli, 2014; Wang et al., 2013). Inhibitors of GSK3β increase CREB (cAMP Response Element-Binding Protein) DNA binding activity and IL-10 expression, that reduce the production of inducible nitric oxide synthase (iNOS) and NO cells (Bécherel et al., 1995; Seyler et al., 1997). Different results were reported regarding LiCl treatment. LiCl was found to potentiate LPS-induced iNOS expression in astrocytes, but not in mouse macrophages (Feinstein, 1998), and LiCl alone slightly increased iNOS expression in hepatocytes and in vivo (Du et al., 2006). These variable findings may signify stimulus or cell type-selective regulatory effects of GSK3β inhibitors on iNOS expression. However, majority of reports likely indicate attenuation of NO production by GSK inhibitor. Excessive ROS lead to mitochondrial dysfunction and activates caspases and apoptosis (Lu et al., 2011), suggesting that LiCl might act through the mitochondrial pathway.

*M. hyopneumoniae* infection could activate caspase-3 (Bai et al., 2015; 2013). By using caspase-3 activity assay kit (Beyotime Biotech., China), we evaluated whether LiCl blocks *M. hyopneumoniae* activated caspase-3. Cells (1 × 10^5 cells/well) infected for 10 h as indicated above and treated with or without LiCl (10–40 mM) for 12 h. Cells lysed in lysis buffer (~10^8 cells/ml), centrifuged and the supernatant was quantified by Bradford method (Beyotime Biotech., China). An
about 50 µl of cells lysate (100 µg) was mixed with 40 µl reaction buffer and 10 µl caspase-3 substrate (Ac-DEVD-pNA). After incubating at 37 °C for 2 h, caspase-3 activity was measured at 405 nm. As shown in (Fig. 2E), M. hyopneumoniae infection significantly increased caspase-3 activity. When PK-15 cells treated with LiCl, caspase-3 activity was significantly reduced by 82%, suggesting that LiCl may act upstream of caspase-3 activation. LiCl induced cells neuro-protection against glutamate was found to be associated with up-regulation of anti-apoptotic protein Bcl-2, down-regulation of pro-apoptotic proteins glutamate was found to be associated with up-regulation of anti-

M. hyopneumoniae by LiCl may be a general feature of Mollicutes.

In conclusion, our data indicated that LiCl inhibited M. hyopneumoniae infection on PK-15 cells via antiapoptotic mechanism. These results are potentially relevant to the search for antimicrobials against M. hyopneumoniae infections, as well as other Mollicutes.

Competing interests

The authors declare that they have no competing interests.

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