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Antiviral effect of copper chloride on feline calicivirus and Synergy with Ribavirin *in vitro*

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Research article

Keywords: Feline calicivirus, copper chloride, antiviral effect, synergistic protective effect, antagonistic effect

Posted Date: March 13th, 2020

DOI: https://doi.org/10.21203/rs.2.20385/v2

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Version of Record: A version of this preprint was published at BMC Veterinary Research on July 6th, 2020. See the published version at https://doi.org/10.1186/s12917-020-02441-0.

Abstract

Background: Feline calicivirus (FCV) is a common pathogen causing widely prevalent upper respiratory disease for kitten and felines in recent years. Due to the substantial genetic variability of the viral genes, existing vaccines cannot provide complete protection. Therefore, researches on FCV antiviral drugs have received much attention.

Results: In this study, we found that copper chloride had dose-dependent antiviral effects against FCV in F81 cells. We also found that the combination of copper chloride and ribavirin had a synergistic effect against FCV in the F81 cells. In contrast, the combination of and horse anti-FCV immunoglobulin F(ab') 2 showed an antagonistic effect, likely because the copper chloride has an effect on the F(ab') 2 immunoglobulin; however, further research is needed to clarify this supposition.

Conclusions: In summary, we found that copper chloride had low cytotoxicity and significant antiviral effects against FCV in F81 cells, providing a new drug candidate for the prevention and treatment of FCV infection.

Background

Feline calicivirus (FCV) is a small, nonenveloped, single positive-strand RNA virus that belongs to the genus *Vesivirus* of the family *Caliciviridae* [1]. Most felines (e.g., cats, tigers, and cheetahs) are susceptible, and infection in dogs has been reported in recent years [2, 3]. Kittens aged 1 to 12 weeks are mainly infected, with a mortality rate of 67% [4, 5]. Infected animals present with oral ulcers, chronic stomatitis, rhinitis, conjunctivitis, and pneumonia [6-9]. The primary prevention at present remains vaccination. However, due to the high evolutionary rate of the FCV capsid protein, with as many as 1.3×10^{-2} to 2.6×10^{-2} substitutions per nucleotide per year [10], traditional vaccination cannot completely protect the animals from re-infection of mutant or wild-type strains, even if the FCV was considered to have only one serotype [11]. Therefore, it is necessary to develop a safe and effective antiviral drug as a monotherapy or as part of a combination treatment.

In previous studies, various compounds had been found to have anti-FCV effects, such as minomorpho oligophosphate (PMO), lithium chloride, and germacrone [12-14]. The combination of mefloquine and recombinant cat interferon-ω had a synergetic effect against FCV [15]. Copper is an indispensable element used in the production of livestock and poultry. Basic copper chloride is an essential additive in feed [16]. Researchers previously found that copper and copper compounds had antiviral effects against dengue virus, influenza virus, and human immunodeficiency virus (HIV) *in vitro* [17-19]. The copper(II) chloride complex has also been used for anticancer effects *in vitro* in human cell lines of cervical cancer, colon cancer, ovarian cancer, and melanoma [20]. However, there have been no reports on the antiviral effect of copper chloride against FCV.

In this study, we found that copper chloride had low cytotoxicity in F81 cells. We evaluated the antiviral effects of copper chloride *in vitro* in a dose-dependent manner by measuring the $TCID_{50}$ and using RT-qPCR to analyse the effects against FCV. Additionally, the combination of copper chloride and ribavirin showed synergistic antiviral effects against FCV.

Results

Cytotoxicity test of copper chloride on F81 cells

The results of the cytotoxicity assay showed that the relative cell viability was above 85% after treatment with copper chloride at a concentration of 20~80 μM for 24 h or 72 h. When the concentration of the copper chloride was less than or equal to 200 μM (below CC_{50}), the relative cell viability of the F81 cells was higher than 50% after 24 h or 72 h of

treatment, indicating that copper chloride can be regarded as non-toxic in this concentration range. Therefore, a concentration of 200 μM copper chloride was used as the maximum concentration for the antiviral experiments.

Fig. 1 Cytotoxicity assay of copper chloride to F81 cells. A CCK-8 assay was used to measured cytotoxicity to F81 cells exposed to copper chloride at concentrations of 400, 200, 100, 80, 60, 40, and 20 μM during incubation at 37 °C and 5% CO_2 for 24 h or 72 h. The relative activity of 0.4% DMSO-treated F81 cells was considered to be 100%, and the cytotoxicity was shown as the percentage of cell activity with respect to the DMSO mimetic treatment. Each value represents three independent replicate experiments.

Antiviral effect of copper chloride at different doses

To evaluate the antiviral effects of different doses of copper chloride against FCV, we examined the virus titre and RNA levels of FCV in the F81 cells treated with different doses of copper chloride. The results showed that the virus titre was significantly lower than that of the mock group after the cells were exposed to 60, 80, 100, and 200 μM concentrations of copper chloride ($p < 0.001$) (Fig. 2A). The relative RNA levels of the FCV were significantly reduced compared to the mock group when the copper chloride concentration was 80 or 100 μM ($p < 0.005$), and the decrease was highly significant at 200 μM ($p < 0.001$) (Fig. 2B). In addition, all the results indicated that the antiviral effect of copper chloride on the F81 cells was dose-dependent. The IC_{50} of the copper chloride to FCV was determined to be 5.1 μM (Fig. 2C).

Fig. 2 The antiviral effect of different doses of copper chloride (20-200 μM) on the 100 $TCID_{50}$ FCV-infected F81 cells. After incubation for 28 h at 37 °C and 5% CO_2, the virus titre (A) and the relative RNA levels (B) of the FCV were detected. (C) The IC_{50} of the copper chloride to the FCV was determined; * $p < 0.0332$; ** $p < 0.0021$; *** $p < 0.0002$; and **** $p < 0.0001$.

Indirect immunofluorescence assay

To further evaluate whether the antiviral effect of copper chloride against FCV was dose-dependent, we performed an indirect immunofluorescence assay (IFA). The results showed that intense green fluorescence signal was observed in the 0 μM group, and only weak fluorescence signals were observed at the concentrations of 60, 40, and 20 μM, indicating a dose-dependent relationship after greyscale scanning (Fig. 3B). There was almost no fluorescence signal at the concentrations of 80, 100, and 200 μM or for the control group compared to the mock group (Fig. 3A). In addition, the F81 cells treated with 200 μM copper chloride showed slight rounding and polymerization. This finding also validates the range of non-toxic concentrations of copper chloride previously described.

Fig. 3 IFA verified the antiviral effect of copper chloride. (A) F81 cells were infected with different concentrations of copper chloride (20-200 μM) and FCV (100 $TCID_{50}$), and a mock treatment group containing 0.4% DMSO and a negative control group uninfected with FCV were used as controls. After incubation for 1 h at 37°C and 5% CO_2, IFA of the F81 cells was performed. (B) Two experimental wells were selected in the 96-well cell plate, and fluorescence signals were scanned in randomly selected areas in each well using Image J software. The experimental result was expressed as the average of two fluorescence signals. The antiviral effect of the copper chloride was evaluated by scanning the fluorescence signal; **** $p < 0.0001$.

Antiviral effect of copper chloride for different treatment times

To evaluate the antiviral effect of copper chloride to FCV at different treatment times, we examined the virus titer and RNA levels of FCV in F81 cells at different treatment times. The experimental data showed that 80 μM copper chloride had good antiviral effect on FCV and lower cytotoxicity. Therefore, we selected this concentration of copper chloride for subsequent experiments. The results showed that the virus titre and the relative RNA level of the FCV were significantly

lower in the copper chloride treatment group after -1, 0, and 1 h of FCV infection compared with that of the mock group ($p < 0.001$), and there was also a significant difference after 4 and 8 h of infection ($p < 0.002$). After 16 h of virus infection, there was no significant difference between the virus titre or relative RNA level of the FCV and the mock group, indicating that the antiviral effect of copper chloride (80 μM) was not significant after 16 h of FCV infection (Fig. 4A and B). The RT-qPCR data also obtained the similar results. Thus, the antiviral effect of copper chloride on FCV was mainly in the early stages of virus proliferation.

Fig. 4 The antiviral effect of copper chloride on FCV at different time points. The F81 cells were infected with 100 $TCID_{50}$ FCV and then treated with 80 μM copper chloride for -1, 0, 1, 2, 4, 8, and 16 h. After incubation for 24 h at 37 °C and 5% CO_2, the virus titre (A) and relative RNA levels (B) of the FCV were detected. NS, $p > 0.1234$; ** $p < 0.0021$; *** $p < 0.0002$; and **** $p < 0.0001$. ('-1 h' indicated that the cells were treated with CuCl2 1 h before infection)

Antiviral effect of copper chloride on different strains

For other strains of FCV, the antiviral effects of copper chloride were validated. As previously described, we determined the viral titre and relative viral RNA levels of the different strains after copper chloride treatment. The results showed that 80 μM copper chloride used to treat the other strains of FCV (CH-JL1, CH-JL3, CH-JL4, and CH-SH) significantly reduced the viral titre and relative viral RNA levels of the infected F81 cells (Fig. 5A and B). These results indicated that copper chloride had a strong antiviral effect against strains of FCV with differing genotypes.

Fig. 5 The antiviral effect of copper chloride for different FCV strains. The F81 cells were infected with different FCV strains at 100 $TCID_{50}$ and treated with 80 μM copper chloride. After incubation for 28 h at 37 °C and 5% CO_2, the virus titre (A) and the relative RNA levels (B) of the FCV were detected; * $p < 0.1234$ and ** $p < 0.0021$.

Synergistic effect of ribavirin and the antagonistic effect of F(ab')2

The checkerboard method was used to mix different compounds, and the antiviral effect of the compound combination was evaluated using SynergyFinder. The results showed that the combination of copper chloride and ribavirin had a synergistic effect on the protection of FCV-infected F81 cells within a specific concentration range (Fig. 6A). The ZIP model was used to calculate the average score for the synergy of the two compounds, and it was found to be 9.687 (Fig. 6B). Conversely, the combined use of copper chloride and F(ab')2 showed antagonism, and the average score of the two compounds calculated by the ZIP model was -20.798 (Fig. 6C and D).

Fig. 6 The antiviral effect of the compound combination. (A and C) The compounds were diluted to the indicated concentrations and used in combination to treat FCV infection. The results of the RT-qPCR were statistically analyzed by the methods described above, and the effects of the drug combination were evaluated using SynergyFinder. (B and D) Different concentrations of compound interaction scores were calculated using the ZIP model. The synergy score for the ZIP model was expressed as the average of all δ scores in the dose-response landscape, and the red portion of the graph indicates synergy. All experiments were repeated three times.

Discussion

In previous studies, copper or copper ions had an antiviral effect on herpes simplex virus (HSV) [21]. However, they were mainly used to inactivate virions since copper ions can destroy the activity of certain proteins containing hydroxyl radical [22, 23]. However, when the concentration of the copper ions was too high, its toxicity to the organisms was fatal because copper chloride can inactivate specific proteins and induce a large number of reactive oxygen species in cells to induce apoptosis [24]. In our study, we found that low concentrations of copper chloride induced low cytotoxicity in the F81 cells. The F81 cells treated with copper chloride concentrations up to 200 μM for 24 h and 72 h showed cell activity

greater than 50%, which was similar to previously reported soluble copper chloride cytotoxicity results. That was, the cytotoxicity of copper chloride was very slight within the range of cytotoxic concentration that did not destroy the steady state of copper[25]. Previously, copper chloride dihydrate-treated dengue virus exhibited excellent antiviral activity but the antiviral effect of copper chloride on FCV had not been reported [26]. In this study, we found that copper chloride had an antiviral effect against FCV with an IC_{50} of 5.1 μM. The IFA results showed that the inhibition of FCV by copper chloride was dose-dependent and significantly reduced the cytopathic effect (CPE) caused by FCV in the F81 cells. After treatment with different concentrations of copper chloride, the viral titre and RNA expression levels of FCV were significantly different from those of the control.

Then, we demonstrated the inhibitory effect of copper chloride on the viral titre and RNA levels of FCV at different time points. FCV was significantly inhibited when copper chloride was added 8 h after FCV was used to infect the cells, but copper chloride did not significantly inhibit FCV after 16 h. Our findings were similar to those of previous studies using fexaramine, LiCl, ginger extracts,or CSX [13, 14, 27, 28], we speculated that copper chloride could hinder attachment and entry of FCV and inhibit the replication inside the cell, but its mechanism of inhibiting replication needed further verification. Past research had shown that copper ions had the ability to disrupt DNA through cross-linking with single-stranded polynucleotide chains, as well as intra- and intra-strand[27]. Numerous studies had reported that copper ions could inhibit neuraminidase (NA) in cells and tissues [28, 29]. Both influenza virus NA [30, 31] and Sendai virus NA [32] could be inhibited by copper, but it had not been determined whether the antiviral effect was related to NA inhibition. The copper ions caused abnormal morphology of the virus, which might indicate that structural changes could be related to antiviral effects [31]. We found that copper chloride also inhibited other strains of FCV replication in the F81 cells, which was further evidence of the antiviral activity of copper chloride against FCV. The ZIP model in SynergyFinder was used to analyze the combined effects of the drugs. The synergy score for the ZIP model was expressed as the average of all δ scores in the dose-response landscape [26, 41]. In a later experiment, we found that the broad-spectrum antiviral drugs ribavirin and copper chloride had synergistic effects in inhibiting FCV. The most synergistic area scored 15.5, with an average score of 9.7. However, copper chloride and an immunoglobulin $F(ab')_2$ showed a robust antagonistic effect. There has been reported in the literature that copper ions could induce IgG cleavage [33, 34]. Past research had found that when metal ions were present in a specific stereochemistry in the appropriate conformation, metal ions could increase the rate of peptide cleavage and catalyze the reaction [35, 36]. In fact, copper ions could bind and enhance the degradation of proteins such as monoclonal antibodies and hydrolyze small peptides and bovine serum albumin, trace amounts of copper ions might be sufficient to enable site-specific, metal-mediated monoclonal antibodies degradation [37]. When copper chloride and immunoglobulin $F(ab')_2$ were applied to F81 cells infected with FCV at the same time, there might be mutual effects to cause antagonistic effects. But further research would be required to prove.

Conclusions

In summary, we verified that copper chloride was low cytotoxicity in the F81 cells and had an antiviral effect on FCV *in vitro*. Later, we found that copper chloride played a role in the replication stages of FCV-infected cells, and it had a synergistic effect with ribavirin, but the drug resistance should be determined in a future study. This study indicated that copper chloride could be a promising candidate drug for FCV infection in felines.

Methods

Viruses, cells and compounds

The FCV CH-SH strains and F81 cells were provided by the Institute of Military Veterinary Medicine, Academy of Military Medical Science. FCV CH-JL1/CH-JL2/CH-JL3/CH-JL4 were isolated and stored in our laboratory [38]. The horse anti-

FCV immunoglobulin F(ab')$_2$ was produced and stored in our laboratory [39]. The copper chloride chloride dihydrate (Aladdin, China) product number was C111685. The ribavirin (Aladdin, China) product number was R101754.

Cytotoxicity assay for copper chloride

The copper chloride was diluted with DMSO to a concentration of 100 mM as a base solution, and the base solution was diluted to different concentrations gradient (400 μM, 200 μM, 100 μM, 80 μM, 60 μM, 40 μM, 20 μM) with MEM containing 2% FBA. The cells were incubated at 37 ℃ and 5% CO_2 for 24 h or 72 h. After washing the cells twice with PBS solution, 180 μL of FBA-free MEM and 20 μl of CCK-8 (CCK8; Dojindo, Japan) were added to each well. After incubation at 37 ℃ for 1-2 h, the optical density (OD) was read at 450 nm using a Cmax Plus microplate reader (Molecular Devices, USA). The cell viability was calculated by the formula [OD_{450} (compound) - OD_{450} (blank)] / [OD_{450} (control) - OD_{450} (blank)] × 100%. The copper chloride concentrations that were 50% or less than the cytostatic concentration (CC_{50}) were defined as non-toxic [40].

Virus titre and genome detection

The virus solution to be detected was diluted in a 10-fold gradient. Each concentration was added to each column at 100 μL per well, and repeated 8 times for each concentration, followed by the addition of 100 μL of 2% MEM. The control was established in virus-free medium and cultured at 37 °C and 5% CO_2. The virus $TCID_{50}$ was calculated using the Reed and Muench formula. Relative RT-qPCR was used to evaluate FCV gene expression. Briefly, first, the RNA of the virus was extracted from the mixture of cells and medium (freeze and thaw three times) with the Simply P total RNA extraction kit (Bioflux, China), and then, the RNA was reverse transcribed into complementary DNA (cDNA) (Thermo, USA), followed by RT-qPCR using TB Green Premix Ex Taq II (Tli RNaseH Plus) (TaKaRa, China). The RT-qPCR conditions based on Applied Biosystems 7500 Fast Real-Time PCR System of TB Green Premix Ex Taq II (Tli RNaseH Plus) (TaKaRa, China). The upstream and downstream sequences for FCV primers were 5'-GCAGGTTGGGATAAACATGGA-3' and 5'-CACGAGGCGATTGAGTTGAG-3', and for GAPDH, they were 5’-TGGAAAGCCCATCACCATC-3’ and 5’-ACTCCACAACATACTCAGCACCA-3’.

Antiviral effect of copper chloride on FCV

F81 cells at 100% confluence were infected with 100 $TCID_{50}$ of FCV CH-JL2, while 200μL different concentrations of copper chloride (200-20 μM) were added to the medium. The MEM containing 0.4% DMSO was used as a mock treatment group. After incubation for 28 h at 37 °C and 5% CO_2, the $TCID_{50}$ levels of virus and the RT-qPCR results were determined to assess the antiviral effect against FCV. The half-maximal inhibitory concentration (IC_{50}) of the copper chloride to FCV was also determined, and the results were plotted using GraphPad Prism 8.

Indirect immunofluorescence assay (IFA)

To more intuitively observe the antiviral effect of copper chloride on FCV, we performed an IFA [39]. As described above, a monolayer of F81 cells infected with 100 $TCID_{50}$ of FCV was exposed to different concentrations of copper chloride and then fixed in 80% cold acetone for 30 min. Next, the cells were washed 5 times with PBS containing 0.05% Tween-20 (PBST). Then, primary antibody (VMRD, USA) diluted 300-fold with 1% BSA was added and incubated for 1 h at 37 °C. The supernatant was discarded, and the cells washed 5 times with PBST, and a 200-fold diluted FITC-labelled rabbit anti-cat secondary antibody (Bioss Antibody, China) was added in the dark and incubated at 37 °C for 1 h. The fluorescence was observed under an inverted Leica DMi8 fluorescence microscope (Leica, Germany), and the fluorescence results were analyzed by greyscale scanning.

Effect of different treatment times of copper chloride on FCV

To determine whether the antiviral effect of copper chloride in F81 cells was time-dependent, a final concentration of 80 μM copper chloride solution was administered -1 h, 0 h, 2 h, 4 h, 6 h, 8 h, and 16 h after FCV infection. The MEM containing 0.4% DMSO was used as a mock treatment group. After incubation for 24 h at 37 °C and 5% CO_2, the $TCID_{50}$ level was determined and the RT-qPCR results were assessed to determine the amount of virus.

Antiviral effect of copper chloride on different strains

To determine whether copper chloride also has antiviral effects against other FCV strains, we also diluted different strains of FCV (CH-JL1, CH-JL3, CH-JL4, and CH-SH) to 100 $TCID_{50}$ and infected the F81 cells, which were subsequently treated them with a final concentration of 80 μM copper chloride solution. After incubating the cells with virus for 28 h at 37 °C and 5% CO_2, the $TCID_{50}$ level was determined and the RT-qPCR results were assessed to determine the amount of virus.

Combinations of copper chloride and ribavirin or F(ab')2

To evaluate the combined action of the compounds, we diluted the different drugs by the checkerboard method and mixed the two drugs thoroughly to determine the combined effect against FCV of different concentrations of copper chloride (0–40 μM) and ribavirin (0–15 μM)/F(ab')2 (0–300 μg). The results from the RT-qPCR experiments were statistically analyzed in a manner described previously, and the effects of the drug combination were evaluated using SynergyFinder [41]. The zero-interaction-efficiency (ZIP) model [26]was used to calculate the mutual scores of the different concentrations of the drug. For each treatment, at least three triplicate data sets from independent experiments were analyzed. The results were expressed as the mean and mean standard error (SEM).

Statistical methods

All experiments were performed three times independently in triplicate. The data were expressed as the mean ± standard deviation (SD). The significance of the differences between the groups was determined by paired t-test and one-way/two-way analysis of variance.

Abbreviations

PMO: minomorpho oligophosphate; $TCID_{50:}$ 50% tissue culture infective dose; RT-qPCR: Real time quantitative polymerase chain reaction; CC_{50}: 50% cytostatic concentration;PBS: phosphate-buffered saline; IC_{50}: the half-maximal inhibitory concentration; IFA: Indirect immunofluorescence assay; SEM: Standard error of the mean; FITC: fluoresceine isothiocyanate; ZIP: the zero-interaction-efficiency; F81 cells: feline kidney fibroblast (F81) cells.

Declarations

Acknowledgement

The authors would like to thank Xiaoxueying Chen, Yue Teng, Yuxing Tan(Jilin Agricultural University) for invaluable technical assistance.

Author Contributions: Dengliang Li, Zhanding Cui and Guixue Hu conceived and designed the experiments. Dengliang Li and Zhanding Cui performed the experiments. Dengliang Li and Zhanding Cui analyzed the data. Guohua Li, Liangting Zhang, Ying Zhang, Han Zhao, Shuang Zhang, Yanbing Guo, Yanli Zhao, Fanxing Men, Shihui Zhao, Jiang Shao, Dongju Du, Hailong Huang, Kai Wang contributed reagents/materials/analysis tools. Dengliang Li and Zhanding Cui wrote the paper. Guixue Hu, Tiansong Li and Yongkun Zhao requested financial support. All authors read and approved the manuscript.

Funding

The present work was supported by the National Key R&D Program for the 13th Five-Year Plan, the Ministry of Science and Technology of China and the National Key R&D Program (2016YFD0501002).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Figures

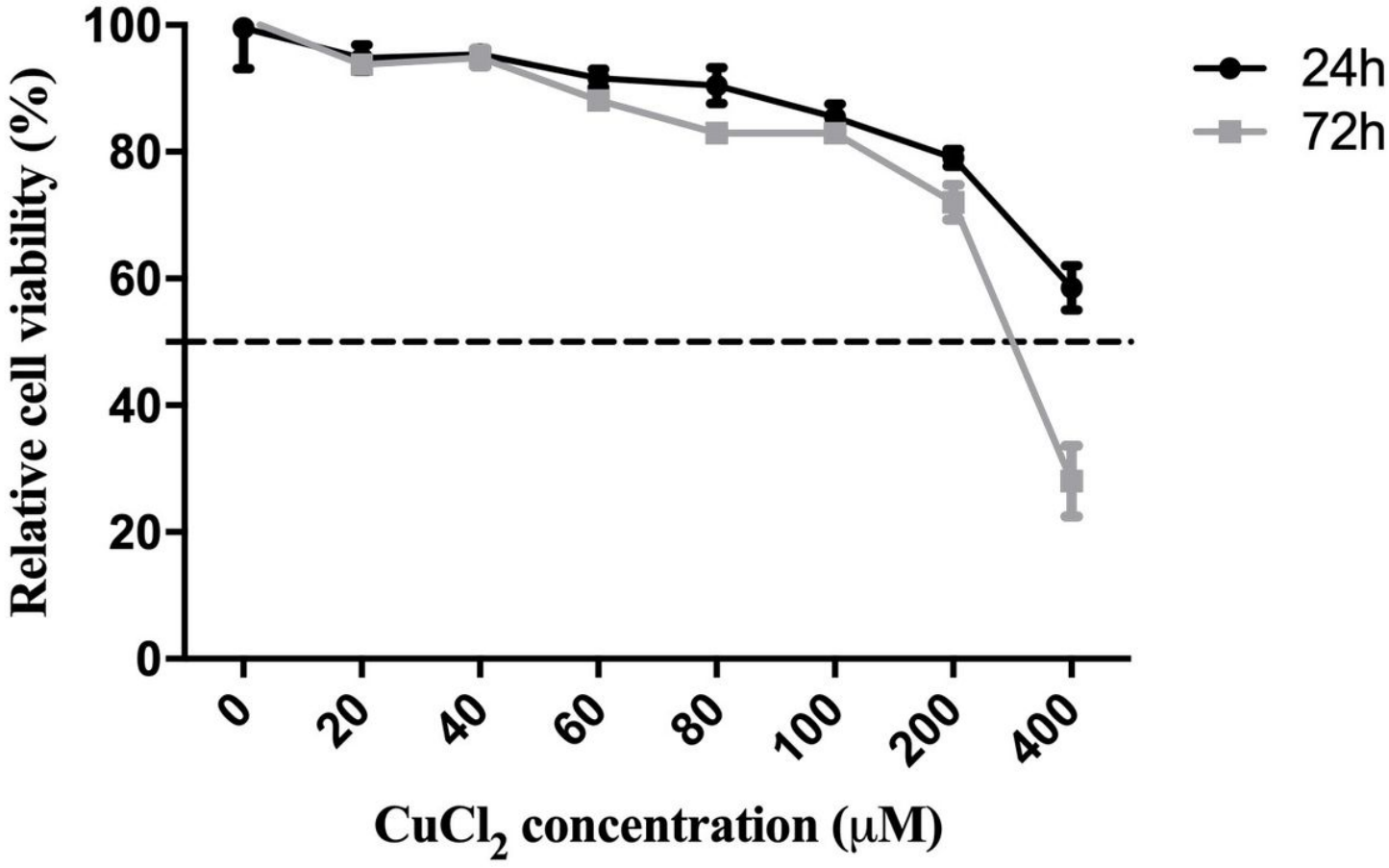

Figure 1

Cytotoxicity assay of copper chloride to F81 cells. A CCK-8 assay was used to measured cytotoxicity to F81 cells exposed to copper chloride at concentrations of 400, 200, 100, 80, 60, 40, and 20 μM during incubation at 37 °C and 5% CO2 for 24 h or 72 h. The relative activity of 0.4% DMSO-treated F81 cells was considered to be 100%, and the cytotoxicity was shown as the percentage of cell activity with respect to the DMSO mimetic treatment. Each value represents three independent replicate experiments.

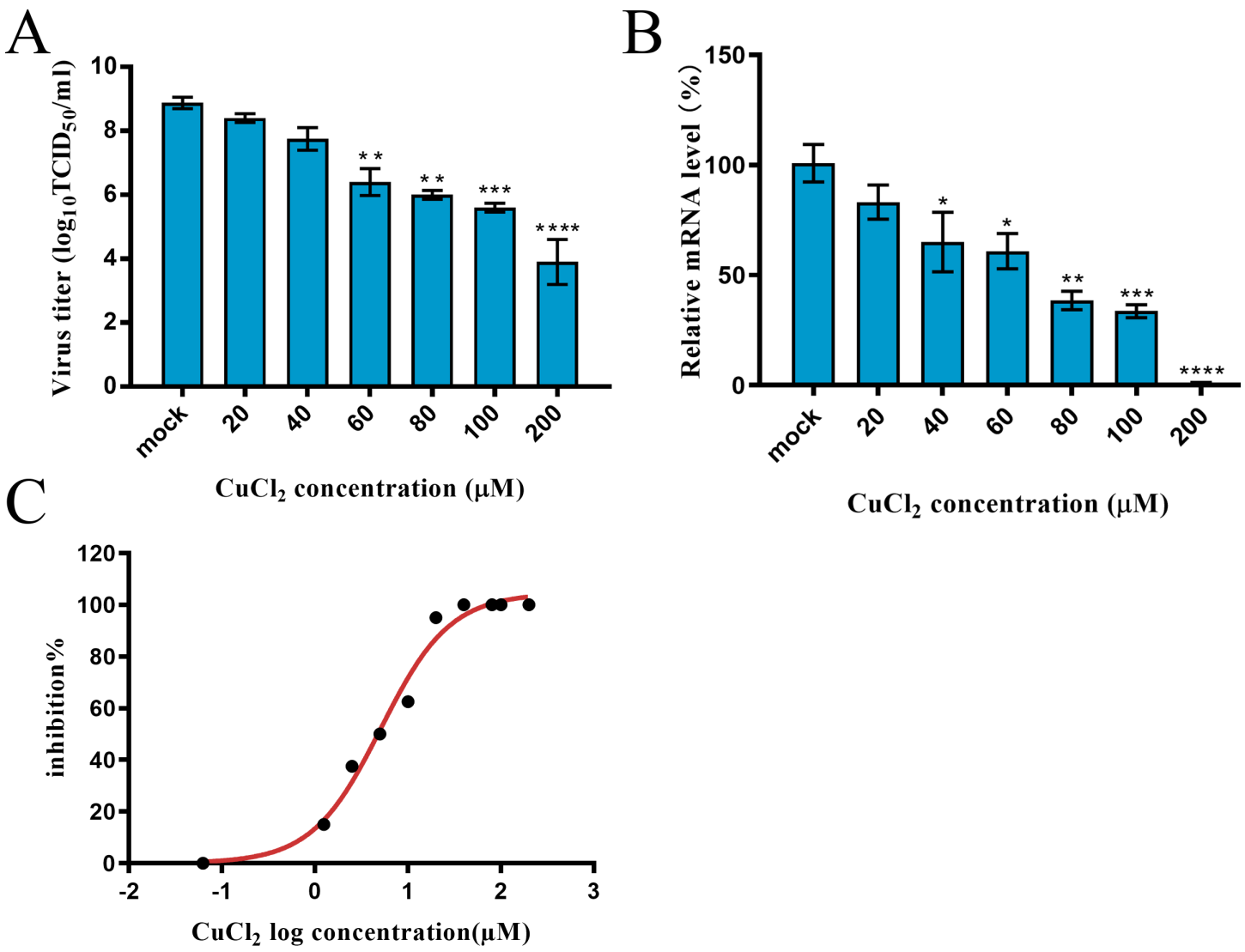

Figure 2

The antiviral effect of different doses of copper chloride (20-200 μM) on the 100 TCID50 FCV-infected F81 cells. After incubation for 28 h at 37 °C and 5% CO2, the virus titre (A) and the relative RNA levels (B) of the FCV were detected. (C) The IC50 of the copper chloride to the FCV was determined; * p <0.0332; ** p <0.0021; *** p <0.0002; and **** p <0.0001.

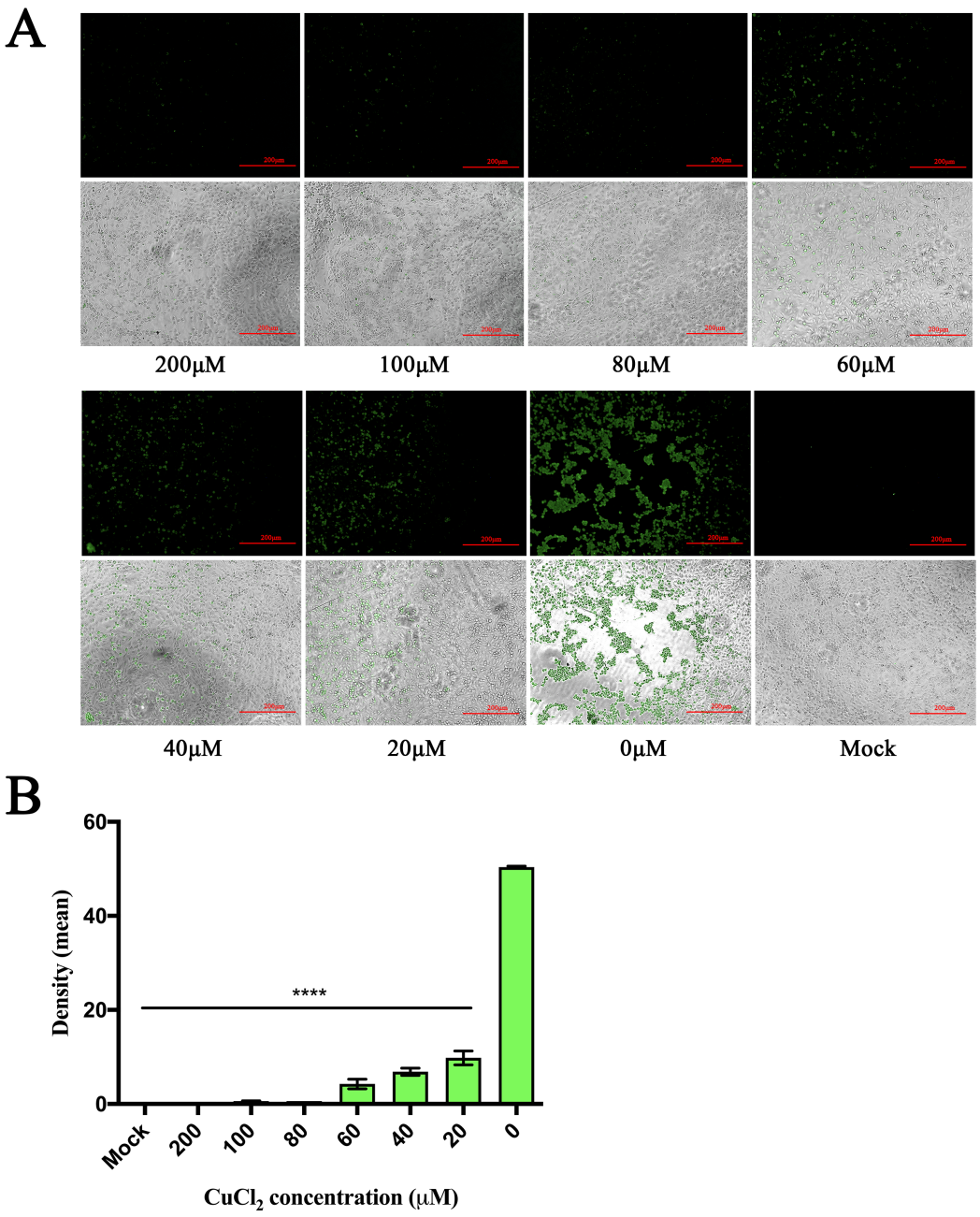

Figure 3

IFA verified the antiviral effect of copper chloride. (A) F81 cells were infected with different concentrations of copper chloride (20-200 μM) and FCV (100 TCID50), and a mock treatment group containing 0.4% DMSO and a negative control group uninfected with FCV were used as controls. After incubation for 1 h at 37°C and 5% CO2, IFA of the F81 cells was performed. (B) Two experimental wells were selected in the 96-well cell plate, and fluorescence signals were scanned in randomly selected areas in each well using Image J software. The experimental result was expressed as the average of two fluorescence signals. The antiviral effect of the copper chloride was evaluated by scanning the fluorescence signal; **** $p < 0.0001$.

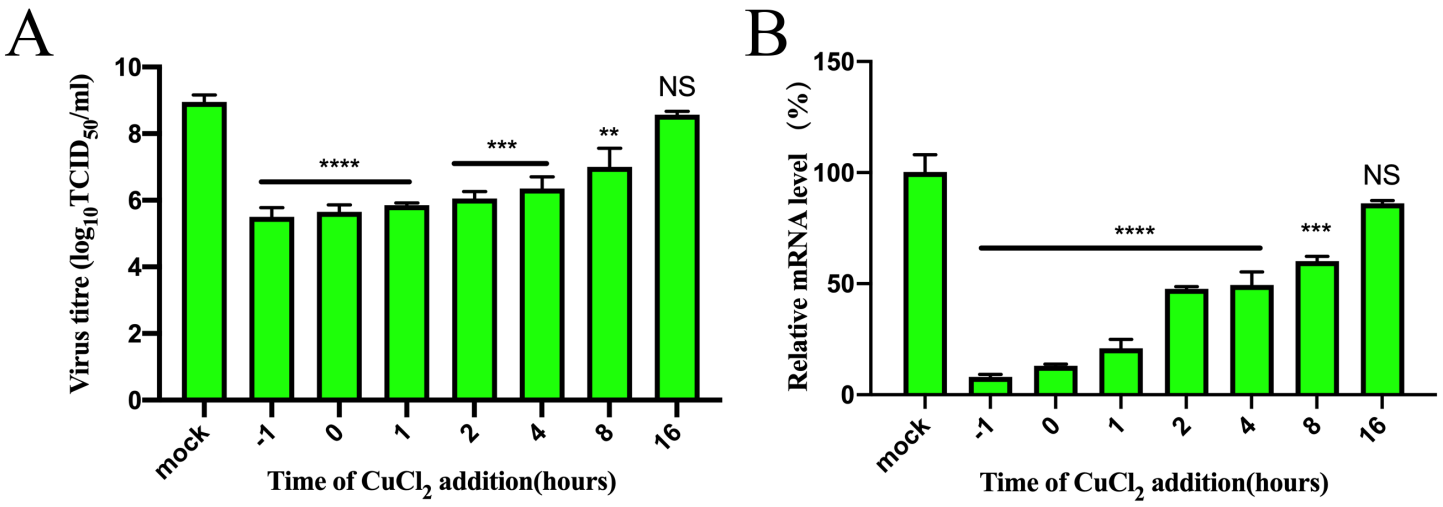

Figure 4

The antiviral effect of copper chloride on FCV at different time points. The F81 cells were infected with 100 TCID50 FCV and then treated with 80 μM copper chloride for -1, 0, 1, 2, 4, 8, and 16 h. After incubation for 24 h at 37 °C and 5% CO2, the virus titre (A) and relative RNA levels (B) of the FCV were detected. NS, p > 0.1234; ** p <0.0021; *** p <0.0002; and **** p <0.0001. ('-1 h' indicated that the cells were treated with CuCl2 1 h before infection)

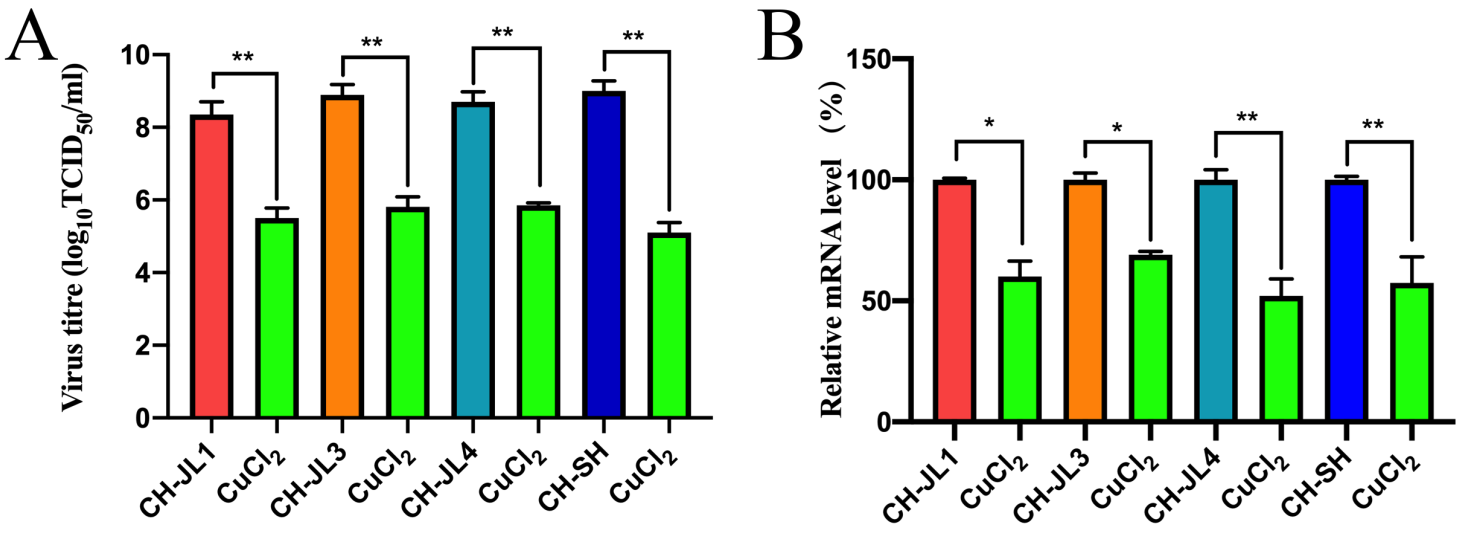

Figure 5

The antiviral effect of copper chloride for different FCV strains. The F81 cells were infected with different FCV strains at 100 TCID50 and treated with 80 μM copper chloride. After incubation for 28 h at 37 °C and 5% CO2, the virus titre (A) and the relative RNA levels (B) of the FCV were detected; * p < 0.1234 and ** p < 0.0021.

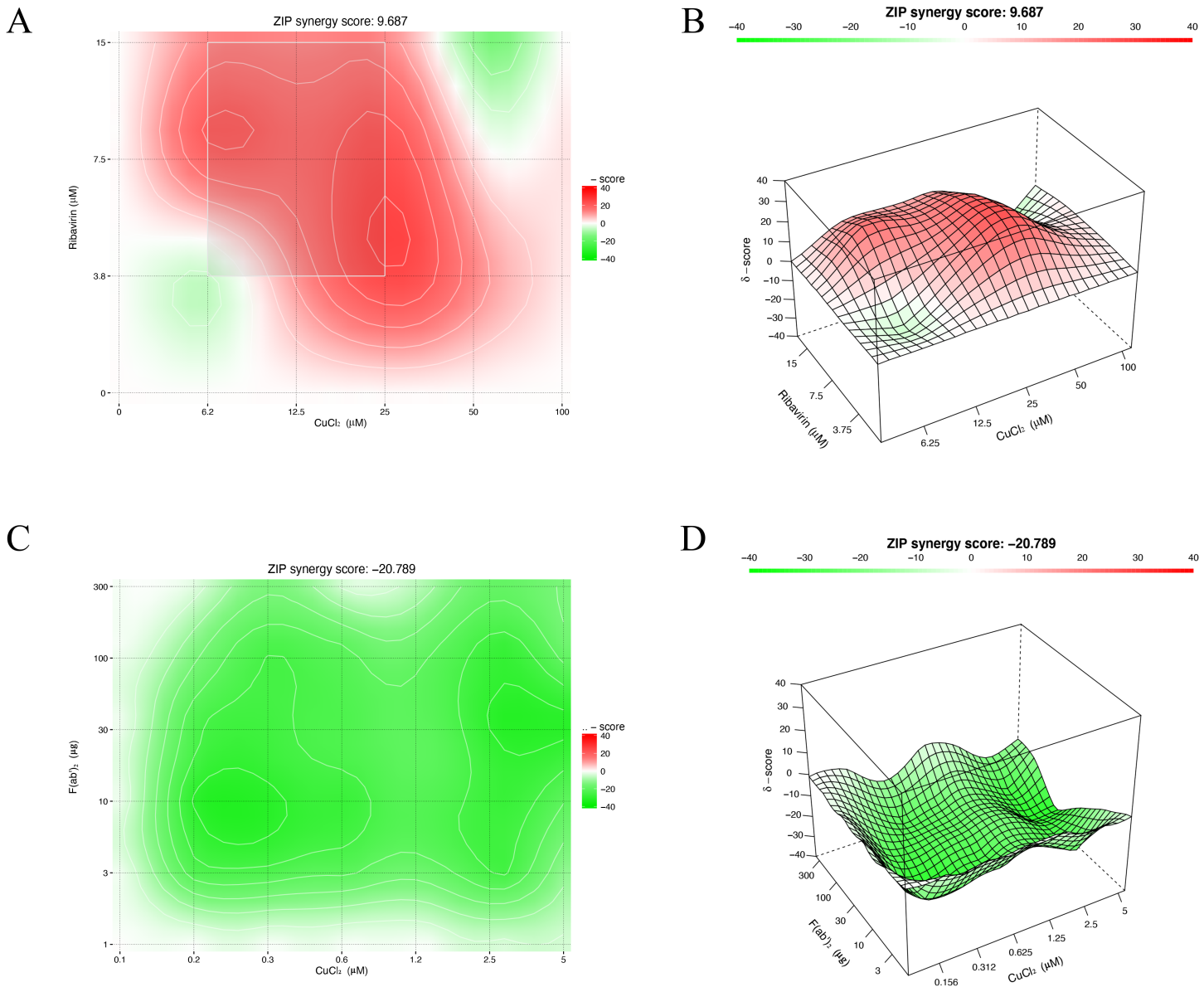

Figure 6

The antiviral effect of the compound combination. (A and C) The compounds were diluted to the indicated concentrations and used in combination to treat FCV infection. The results of the RT-qPCR were statistically analyzed by the methods described above, and the effects of the drug combination were evaluated using SynergyFinder. (B and D) Different concentrations of compound interaction scores were calculated using the ZIP model. The synergy score for the ZIP model was expressed as the average of all δ scores in the dose-response landscape, and the red portion of the graph indicates synergy. All experiments were repeated three times.