QiHong Prevents Death in Coxsackievirus B3–Induced Murine Myocarditis Through Inhibition of Virus Attachment and Penetration

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Viral myocarditis affects about 5% to 20% of the population. So far, there are not many effective antiviral treatments available. QiHong, the combination of the extracts from *Astragali* (Huangqi), *Rhadiola rosea* (Hongjingtian), and *Sophora flavescens* (Kushen), was developed based on laboratory research. The aim of this study was to investigate the effect and mechanism of QiHong on coxsackievirus B3 (CVB3)–induced myocarditis. The antiviral activity of QiHong *in vitro* was evaluated on HeLa and Vero cells infected by CVB3. Ribavirin was chosen as positive control. Our results showed that QiHong possessed potent antiviral effects on CVB3 by sodium 3′- [1-(phenylamino-carbonyl)-3, 4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid and plaque-forming assay (50% inhibitory concentrations [IC_{50}] were 7.16 ± 0.8 μg/ml and 2.63 ± 0.5 μg/ml, respectively). The 50% cytotoxicity concentration (CC_{50}) was 16-fold higher in QiHong-treated cells than in ribavirin-treated cells. Time course studies demonstrated that the antiviral effect of QiHong was mainly found during 0–4 hrs of infection, and it blocked the attachment and penetration of CVB3 into cells. *In vivo* 4-week-old male Balb/C mice were used and inoculated intraperitoneally with CVB3 suspension or normal saline. At 48 hrs after inoculation, the infected mice were gavaged with QiHong or ribavirin. On Day 6, myocardial virus titers were significantly lower in the QiHong-treated group than in the viral-infected groups. On Day 14, QiHong significantly ameliorated CVB3-induced myocardium necrosis; on Day 28, QiHong treatment increased survival rate 4-fold compared with CVB3-infected controls (64% vs. 16%; $P < 0.05$). The results showed that QiHong is a very promising potent antiviral agent with a highly significant favorable effect on survival and pathologic changes in CVB3-induced myocarditis with less toxicity than ribavirin. The antiviral activity of QiHong is at least partially due to an inhibitory effect on virus attachment and penetration. Exp Biol Med 232:1441–1448, 2007

Key words: QiHong; coxsackieviruses; viral myocarditis; herbal medicine

Introduction

Myocarditis is a common cardiac disease that affects an estimated 5% to 20% of the population (1). It may progress to chronic dilated cardiomyopathy and heart failure (2, 3), a major cause of sudden, unexpected death in adults younger than 40 years (4). Coxsackievirus B (CVB) has been proven to be the most common cause of viral myocarditis (5). The therapeutic methods in viral myocarditis still are mainly supportive, such as: bed rest, vitamin C, and coenzyme Q_{10} (5). Until now, few effective drugs are available to treat the disease, and severe cases have a malignant prognosis without heart transplantation (6). As an optional drug in treating viral myocarditis, ribavirin has been found to exhibit potent antiviral properties crucial for the clinical treatment of viral diseases (7), but its use remains controversial due to its questionable efficacy, side effects, and high cost.

According to references of traditional Chinese medicine, about 40 herbs have been reported to treat common colds, other virus-induced disorders, or inflammation-induced heart dysfunction in China. In our preliminary experiments, the antiviral effects of 120 extracts from 39 herbs were tested with sodium 3′-[1-(phenylamino-carbonyl)-3, 4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid (XTT) assay, plaque-forming assay, and cytotoxicity assay, and it was proved that *Astragali* (Huangqi), *Rhadiola rosea* (Hongjingtian), and *Sophora flavescens* (Kushen) not only possessed

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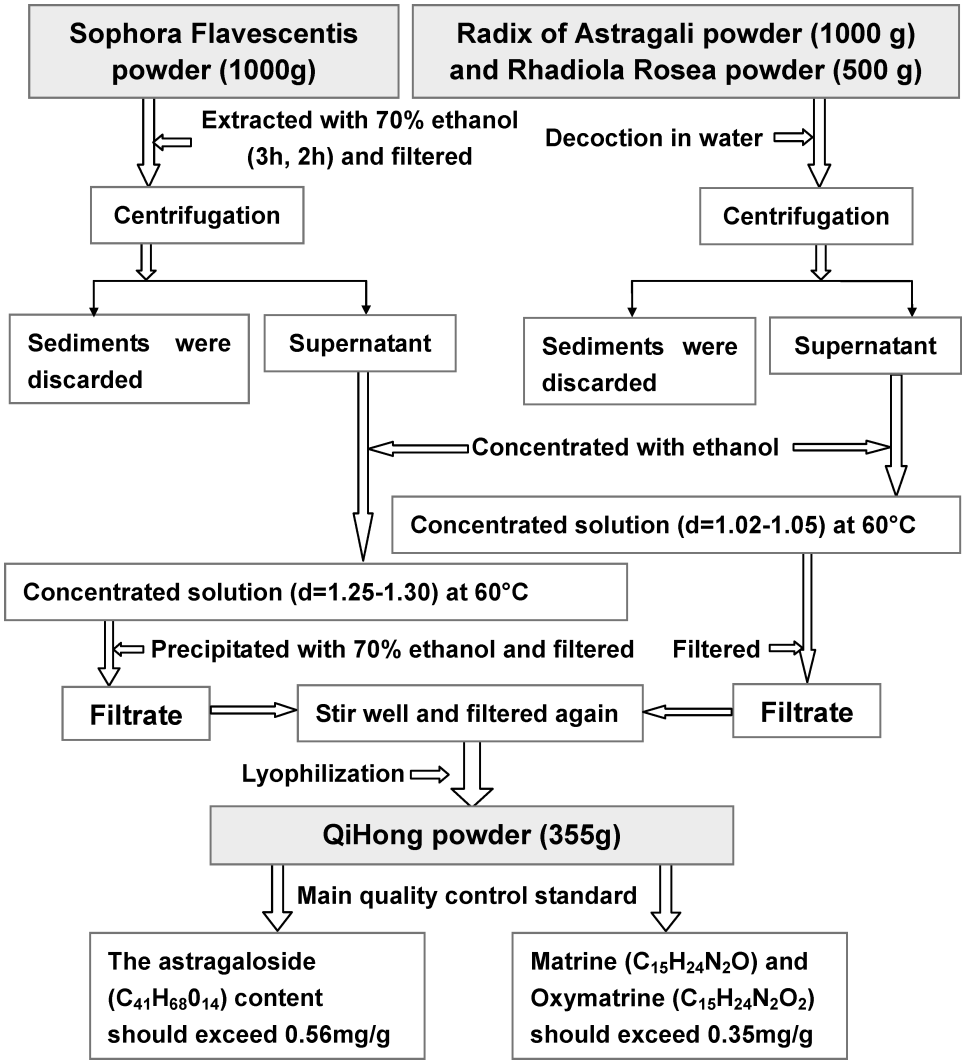

Figure 1. The technical flow and quality control standard of QiHong preparation. Through primary screening by XTT assay, plaque-forming assay, and cytotoxicity assay, the extracts from *Astragali*, *Rhodiola rosea*, and *Sophora flavescens* were identified as having certain antiviral effects. Then the technical flow for preparation of QiHong was conducted using the orthogonal method in the Lanzhou Institute of Physical Chemistry, Chinese Academy of Sciences. The major quality control standard for QiHong stipulates that the content of astragaloside ($C_{41}H_{68}0_{14}$) should exceed 0.56 mg/g (powder of QiHong), and the contents of matrine ($C_{15}H_{24}N_2O$) and oxymatrine ($C_{15}H_{24}N_2O_2$) should exceed 0.35 mg/g (powder of QiHong).

more powerful antiviral effects than other herbs, but also that the combination of the three extracts exhibited synergistic effects on inhibiting virus replication *in vitro*. Therefore, we speculated that the combination of the three herb extracts, named QiHong, might have a more potent antiviral activity than each one of the three extracts alone *in vivo*, and that the combination may become a promising drug for patients suffering from viral myocarditis. In this study, the principal aim was to test whether QiHong has protective effects on CVB3-infected mice and to explore its possible mechanism.

Materials and Methods

Drug Preparation. QiHong was prepared as a combined mixture from extracts of the radix of *Astragali* (Huangqi, *Astragalus membranaceus* [*Fisch.*] *Bge. var. mongholicus* [*Bge.*] *Hsiao*), *Rhodiola rosea* (Hongjingtian, *Rhodiola sachalinesis A. Bor*), and *Sophora flavescens* (Kushen, *Sophora flavescens Ait*). These herbs, authenticated by high-performance liquid chromatography (8), came from a canonical farm in Inner Mongolia of China, and the extracts were prepared at LanZhou Institute of Physical Chemistry, Chinese Academy of Sciences (Lanzhou, China). The technical flow and the quality control standard for QiHong preparation (Fig. 1) have been approved by the State Food and Drug Administration of China (clinical trial approval number 2006L00164). In toxicity tests on rats, neither mutagenicity nor teratogenicity was found, and no noticeable changes were detected in body weight, food intake, urine and feces, blood analysis, plasma biochemical analysis, and pathologic examination of the main organs (data not shown).

To investigate the effect of QiHong on CVB3 virus replication *in vitro*, QiHong powder was first dissolved in

dimethyl sulfoxide at the concentration of 60 mg/ml, and then was diluted to 600 μg/ml in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Gaithersburg, MD). The solution was centrifuged at 1500 *g* for 5 mins at 4°C (Biofuge Stratos, Sorvall, Newtown, CT) to remove insoluble ingredients, and then the supernatant was sequentially sterilized using 0.45-μm and 0.22-μm filters (Carrightwohill, Co. Cork, Ireland). In following experiment *in vitro*, the solution was diluted to six concentrations of 1, 3, 10, 30, 100, and 300 μg/ml with the DMEM. Ribavirin (Sigma, St. Louis, MO) was used as positive control with an identical concentration to QiHong.

Virus and Cells. The experiments were carried out in a biosafety level-3 (BSL-3) laboratory. CVB3 (Nancy strain) was obtained from Dr. Daowen Wang's laboratory, Hua-Zhong University of Science and Technology, China. The virus was prepared in Vero cells (African green monkey kidney), cultured in DMEM, and harvested by CsCl density gradient centrifugation at 416,000 *g* for 2 hrs at 4°C (Biofuge Stratos, Sorvall). Virus stock had a titer of more than 10^9 plaque-forming units (PFU) per 0.1 ml, as determined by plaque assay. The suspension was stored at –70°C until use. Vero and HeLa cells were grown in DMEM supplemented with 5% fetal bovine serum (FBS; Hyclone, Logan, UT).

XTT Assay. The antiviral activity of QiHong was evaluated XTT (Sigma) as described (9). Briefly, 10^4 cells/well were seeded into 96-well culture plates (Costar, Cambridge, MA). After 4 hrs of incubation at 37°C with 5% CO_2, cells were infected with CVB3 at a multiplicity of infection (MOI) of 0.5, and then QiHong was added at the concentration of either 1, 3, 10, 30, 100, or 300 μg/ml. The infected cells were incubated with media containing 2% FBS for another 72 hrs. The media were then aspirated, cells were rinsed with phosphate-buffered saline (PBS), and then XTT reagent was added. The plate was reincubated for an additional 2 hrs to allow the production of formazan. Optical densities were determined with a plate reader (BioRad 550; Bio-Rad, Hercules, CA) at a wavelength of 492 nm and a reference wavelength of 690 nm. The antiviral activity of QiHong was determined according to the following formula (10): antiviral activity (%) = $[(OD_T)_{CVB} - (OD_C)_{CVB}] / [(OD_C)_{mock} - (OD_C)_{CVB}] \times 100\%$. Whereby $(OD_T)_{CVB}$ is the optical density measured with a given concentration of the QiHong in CVB3-infected cells; $(OD_C)_{CVB}$ is the optical density measured for the control untreated CVB3-infected cells; and $(OD_C)_{mock}$ is the optical density measured for control untreated mock-infected cells. The minimal required concentration of QiHong for inhibiting 50% CVB3 growth (IC_{50}) was evaluated according to the method of Weislow *et al*. [9].

Plaque Reduction Assay. The antiviral activity of QiHong was tested in Vero cells. Vero cells were seeded into 24-well culture plates (Costar) at a density of 10^5 cells/well and incubated until reaching at least 95% confluence. Cells (reached to monolayer) were then infected with 100 PFU CVB3 in the absence or presence of QiHong at the concentrations indicated in QiHong preparation, and they were further incubated for 1 hr. After 1 hr of absorption, the cell monolayer was overlaid with 1 ml of 1× DMEM in 0.5% agar (1:1 mixture of 2× DMEM at 37°C and 1% agar at 55°C). The overlay medium was removed 2 days later, and the infected cells were fixed and stained with 10% formalin and 1% crystal violet, respectively. The antiviral activity of QiHong was determined by the following formula: percent of inhibition = $[1 - (\text{number of plaque})_{tested} / (\text{number of plaque})_{control}] \times 100\%$.

The minimal concentration of QiHong required to reduce the 50% plaque formation (IC_{50}) was calculated by regression analysis of the dose-response curves generated from the data (11).

Cytotoxicity Assay. Cell viability was assayed by the XTT method in HeLa cells with no CVB3 treatment (12). Cytotoxic concentration of QiHong toward cells was calculated by the following formula: percent of survival cells = $[OD_T/OD_C] \times 100\%$. Whereby OD_T and OD_C indicated the absorbencies of tested compounds and solvent control, respectively. The concentration of 50% cellular cytotoxicity (CC_{50}) of tested compounds was calculated according to the method of Weislow *et al*. (9).

Time Course of QiHong Effects on CVB3 Infection. The antiviral activity of QiHong or ribavirin was evaluated at various time points up to 24 hrs as described (13). Briefly, Vero cells were seeded into 12-well culture plates (Costar) at a density of 2×10^5 cells/well and were incubated at 37°C under 5% CO_2 for 24 hrs. Cell monolayers were then infected with 1×10^5 PFU CVB3/well. QiHong (10 μg/ml) or ribavirin (10 μg/ml) was added into wells concurrently with CVB3 infection (0 hrs) or at intervals of 2, 4, 8, and 12 hrs after infection. After 24 hrs of infection, infected cells were scraped and viruses were released from cells by freeze thawing three times. Cell debris was removed by centrifugation at 1100 *g* at 4°C for 5 mins (Biofuge Stratos). Virus titers in supernatant were determined by plaque-forming assay. The percent of inhibition was calculated as the reduction in virus titer observed in infected cells containing QiHong or ribavirin relative to that of infected cells containing DMEM as vehicle.

Attachment Assay. Vero cells (reached to monolayer) grown in 24-well culture plates were prechilled at 4°C for 1 hr. The media was aspirated, and the cells were then infected with 200 PFU CVB3 in the absence or presence of serial concentrations of QiHong. After further incubating the infected cells at 4°C for another 3 hrs, the media were aspirated to remove unabsorbed virus. Cell monolayers then were washed with PBS three times and overlaid with 1% methylcellulose medium. The cell monolayer was incubated at 37°C for another 48 hrs before it was fixed and stained. QiHong inhibition of CVB3 attachment to Vero monolayers was calculated by the following formula (11): percent of inhibition = $[1 - (\text{number of plaque})_{tested} / (\text{number of plaque})_{control}] \times 100\%$.

Penetration Assay. Vero cells (reached to mono-

Table 1. Anti-CVB3 Activity, Cytotoxicity, and Selectivity Index of QiHong on Vero Cells[a]

	Antiviral activity, IC_{50} (μg/ml)[b]		Cytotoxicity, CC_{50} (μg/ml)[b]	Selectivity index[c]	
Compound	XTT	PRA	XTT	XTT	PRA
QiHong	7.16 ± 0.8	2.63 ± 0.5	1648 ± 219	230	627
Ribavirin	4.35 ± 0.4	1.92 ± 0.3	103 ± 14	24	54

[a] Antiviral activity was determined by XTT and plaque-reduction assays (PRA). Cytotoxicity was determined by XTT assay.
[b] Values represent the mean ± SD of three independent experiments.
[c] Selectivity index was the ratio of CC_{50} to IC_{50}.

layer) were grown in 24-well culture plates and prechilled at 4°C for 1 hr. The cells were then infected with 200 PFU CVB3 and incubated at 4°C for another 3 hrs to allow the attachment of CVB3 to cells. After 3 hrs of incubation, QiHong (10 μg/ml) or ribavirin (10 μg/ml) was added. Then the cell monolayer was incubated at 37°C to maximize the penetration of virus into the cells. At 10-min intervals, infected cell monolayer was treated with PBS (pH 3) for 1 min to inactivate unpenetrated virus. PBS (pH 11) then was added immediately to neutralize acidic PBS (pH 3). The neutral PBS was removed and cell monolayer overlaid with overlay media. After further 48 hrs of incubation at 37°C, cell monolayer was fixed and stained. Plaques were counted, and the inhibition of penetration was calculated by the following formula (11): percent of inhibition $= [1 - (\text{number of plaque})_{\text{tested}} / (\text{number of plaque})_{\text{control}}] \times 100\%$.

Antiviral Effects Tested in Experimental Animals and Protocols. All animal experiments in our studies were conformed to the guiding principal of China National Law for Animal Use in Medical Research and were approved by the FuWai Hospital Committee for Animal Care and Use.

Survival Rate. Male Balb/C mice, 4 to 6 weeks old, were obtained from the Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences. They were kept under strict hygienic barriers at 23°C ± 1°C in the BSL-3 laboratory. The mouse model of myocarditis was made by inoculating intraperitoneally 0.1 ml CVB3 suspension containing 10^3 PFU, and the drugs (QiHong or ribavirin) were gavaged twice a day (at 0800 and 1800 hrs), beginning 4 hrs after virus inoculation, for 28 days.

QiHong was solubilized at a concentration of 120 mg/ml and was then gavaged using feeding needles. The survival rate was evaluated in the infected mice with either saline ($n = 25$) or QiHong at 200 $mg \cdot kg^{-1} \cdot day^{-1}$ ($n = 25$), 600 $mg \cdot kg^{-1} \cdot day^{-1}$ ($n = 25$), and 1800 $mg \cdot kg^{-1} \cdot day^{-1}$ ($n = 25$); ribavirin (90 $mg \cdot kg^{-1} \cdot day^{-1}$, PO) was used as positive control ($n = 25$). Twenty-five mice were treated with saline only and served as normal control.

The mice were observed daily. Dead mice were necropsied as early as possible during the course of the experiment. Mice surviving until the end of the experiment were killed by bleeding from retroorbital plexus under intraperitoneal administration of sodium pentobarbital (0.04 mg/body wt), and their hearts were removed for pathologic analysis.

Histologic Examination and Virus Titers Analysis of Murine Hearts. Hearts were arrested in diastole with PBS per 20 m*M* KCl solution, weighted and fixed in 10% neutral-buffered formalin, and then cut at the horizontal short-axis plane, dehydrated, embedded in paraffin, and sectioned (5 μm thick). The heart sections were stained with hematoxylin and eosin at the level of the papillary muscle. Pathologic changes were analyzed and quantified by ImagePro Plus 4.5 imaging software (Media Cybernetics, Bethesda, MD).

Another 120 Balb/C mice were divided into six groups (similar to the groups above). For assay of infectivity, half of the mice were randomly chosen from each group for virus titer assay on Day 6, and other mice were sacrificed on Day 14 in heart weight/body weight (HW/BW) assay and histologic examination. Hearts were removed aseptically, weighed, and homogenized in DMEM. After centrifugation at 1500 *g* at 4°C for 10 mins, the supernatant (0.1 ml) was inoculated into Vero cell monolayers for 60 mins at 37°C in 5% CO_2. After absorption, the cells were overlaid with 3 ml media containing 5% FBS and 1% methylcellulose. After two days of inoculation at 37°C in a humidified atmosphere containing 5% CO_2, cells were fixed with glacial acetic acid and methanol (in a 1:3 ratio) and stained with 1% crystal violet, and plaques were counted under an inverted microscope. The myocardial virus titer was expressed as $\log_{10}$ PFU/mg heart tissue.

Statistical Analysis. All experiments *in vitro* were carried out in triplicate. The statistic analysis was performed with SPSS 13.0 (Chicago, IL). Group comparison was performed with one-way ANOVA followed by the Bonferroni test for multiple comparisons. Comparisons between two groups were performed by an unpaired Student *t* test. *P* value < 0.05 was considered statistically significant. Survival rate was analyzed by the Kaplan-Meier method.

Results

QiHong exhibited antiviral effects during 0–4 hrs after CVB3 infection by inhibiting the viral attachment and penetration *in vitro*. Studies *in vivo* showed that QiHong treatment increased survival rate by 4-fold, protected CVB3-induced pathologic changes, and reduced virus titer significantly in myocardium compared with CVB3-infected controls.

Antiviral Activity, Cellular Toxicity, and Selectivity Index of QiHong. Table 1 shows the effect of QiHong on CVB3, demonstrating that QiHong possesses

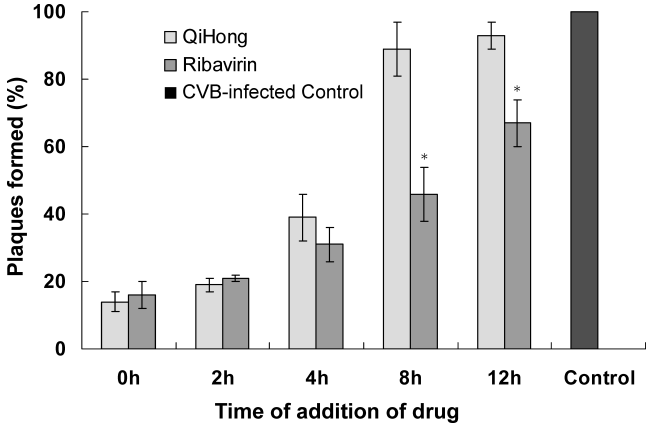

Figure 2. Effects of QiHong and ribavirin on the CVB3 life cycle. Vero cells were seeded into 12-well culture plates and infected with CVB3. QiHong (10 μg/ml) or ribavirin (10 μg/ml) was added into wells either concurrently with CVB3 infection (0 hrs) or at intervals of 2, 4, 8, and 12 hrs after infection. After 24 hrs of infection, virus titer in infected cells was determined by plaque-forming assay. The result suggested that QiHong inhibited virus-specific events within the first 4 hrs of CVB3 infection. The data are expressed as the mean ± SD of three independent experiments. $^*P < 0.05$ QiHong versus ribavirin.

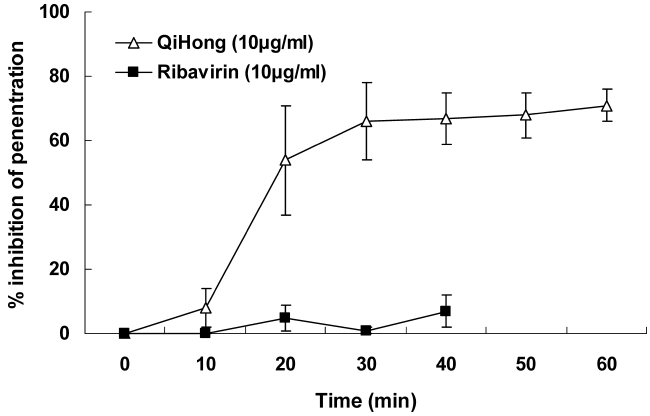

Figure 4. Effects of QiHong (Δ) and ribavirin (■) on CVB3 penetration. Vero cells (reached to monolayer) were prechilled at 4°C for 1 hr and then infected with CVB3 at 4°C for another 3 hrs to complete the virus attachment process. After that, QiHong or ribavirin was added, and the cell monolayer was incubated at 37°C to maximize the penetration of viruses into the cells. The inhibition effect on virus penetration was determined at 10-min intervals. QiHong exerted significantly inhibitory effects on virus penetration into the cells after 20 mins of infection. Each point represents the mean ± SD of three independent experiments.

potent antiviral activity *in vitro*. The IC_{50} in XTT and plaque-reduction assays were 7.16 ± 0.8 μg/ml and 2.63 ± 0.5 μg/ml, respectively. The cytotoxic effect of QiHong on Vero cells was evaluated to ensure that there was no cytotoxic effect on cell viability at concentrations that block CVB3 infection. As determined by XTT assay (Table 1), the CC_{50} of QiHong was 1648 ± 219 μg/ml. The values of selectivity index (ratio CC_{50}/IC_{50}) for XTT and plaque reduction assays were 230 and 627, respectively.

QiHong Inhibited the Viral Attachment and Penetration. Time course results showed an inhibitory effect of QiHong on CVB3 virus at the early stage of CVB3 infection, but not after 4 hrs of infection (Fig. 2), suggesting that QiHong might inhibit virus-specific early events, such as viral attachment, viral penetration, and/or the entry of viral RNA into the cell nucleus.

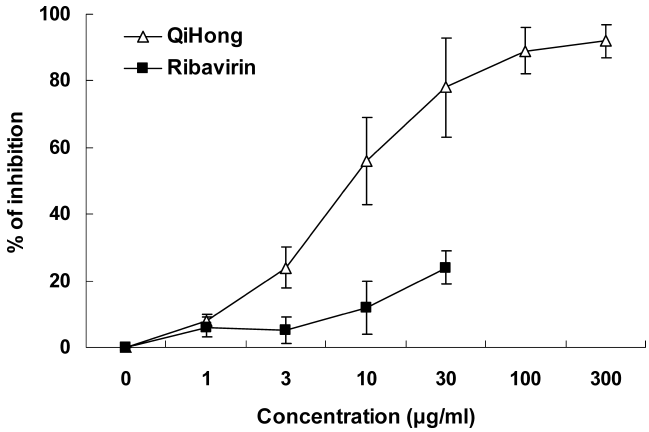

Figure 3. Effects of QiHong (Δ) and ribavirin (■) on CVB3 attachment. Vero cells (reached to monolayer) were prechilled at 4°C for 1 hr to avoid virus penetration. Then, CVB3 and drug were added to the Vero monolayer at 4°C for another 3 hrs. Attachment was inhibited by QiHong in a dose-dependent manner. QiHong inhibited about 80% of the CVB3 attached to the cells at the concentration of 30 μg/ml. Each point represents the mean ± SD of three independent experiments.

We tested whether QiHong inhibited CVB3 attachment to cells, and the results showed that QiHong inhibited CVB3 attachment to cells in a dose-dependent manner (Fig. 3); 80% of the inhibition was found at the concentration of 30 μg/ml. In contrast, ribavirin failed to significantly inhibit virus attachment at concentrations up to 30 μg/ml (when the concentration of ribavirin was greater than 100 μg/ml, a significant cytotoxic effect was noticed). We also tested whether QiHong could prevent the penetration of CVB3 into cells. Results showed that QiHong inhibited viral penetration into cells; 55% of inhibitory effects were identified as early as 20 mins after QiHong administration (Fig. 4).

QiHong Improved Survival Rate in Mice Infected with CVB3. On Day 28, the QiHong group (Group B, 200 $mg \cdot kg^{-1} \cdot day^{-1}$) had a 4-fold higher survival rate than the CVB3-infected control group (64% vs. 16%; $P < 0.05$). The survival rates in Group C (QiHong at 600 $mg \cdot kg^{-1} \cdot day^{-1}$) and Group D (QiHong at 1800 $mg \cdot kg^{-1} \cdot day^{-1}$) were 68% and 64%, respectively, indicating that QiHong might have cardiac protective effects on the mice with CVB3-induced myocarditis, although the survival rate of QiHong-treated mice infected by CVB3 was not QiHong dose dependent. In contrast, the ribavirin group had a 32% survival rate, much lower than that of the QiHong-treated groups (Fig. 5).

QiHong Protected Heart from CVB3 Injury. On Day 0, body weight was comparable between the four groups (data not shown), but on Day 6 it decreased significantly in the CVB3-infected group compared with the QiHong group (14.1 ± 2.8 g in the CVB3 group vs. 19.1 ± 2.6 g in QiHong-

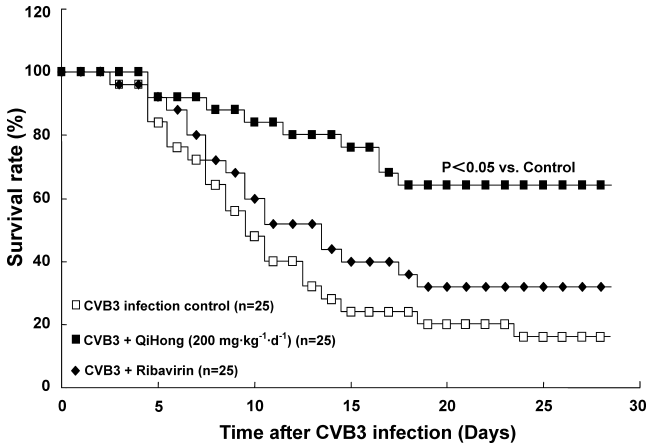

Figure 5. QiHong prevented death in mice with CVB3-induced myocarditis. The mouse model of myocarditis was made by inoculating intraperitoneally 0.1 ml CVB3 suspension containing 10^3 PFU, and the drugs (QiHong or ribavirin) were gavaged twice a day (at 0800 hrs and 1800 hrs) for 28 days. Survival rate was analyzed by the Kaplan-Meier method. The QiHong groups (200 mg·kg^{-1}·day^{-1}) had 4-fold higher survival rates than the control group (64% vs. 16%; $P < 0.05$). In contrast, the ribavirin group had a 32% survival rate, much lower than those of the QiHong-treated groups ($P < 0.05$).

treated group; $P < 0.05$). Heart weight was significantly lower in the QiHong-treated group (92.0 ± 8.2 mg) than in the control group (106.7 ± 9.6 mg; $P < 0.05$). Therefore, the ratio of heart weight to body weight was significantly lower in mice with QiHong ($4.9 \pm 0.7 \times 10^{-4}$) than in the CVB3-infected control group ($8.6 \pm 0.6 \times 10^{-4}$; $P < 0.05$; Table 2).

Myocardial virus titers were significantly higher in CVB3-infected groups than in QiHong-treated groups (3.6 ± 1.1 $\log_{10}$ PFU/mg vs. 1.9 ± 0.6 $\log_{10}$ PFU/mg; $P < 0.05$) on Day 6, indicating that QiHong had an antivirus effect similar to that of ribavirin (1.7 ± 0.6 vs. infection control; $P < 0.05$) in this model (Table 2 and Fig. 6).

In addition, severe inflammation, necrosis, and fibrosis were observed in the heart in the CVB3-infected group; in contrast, no inflammatory reaction was found in noninfected animals *via* hematoxylin and eosin staining. We also found that QiHong protected the heart from CVB3-induced pathologic manifestation in this model (Table 2 and Fig. 7). The ratio of myocardium necrosis over the entire microscope field was lower in the QiHong (200 mg·kg^{-1}·day^{-1})-treated group (2.10% ± 0.13%) than in the ribavirin (200 mg·kg^{-1}·day^{-1})-treated group (7.61% ± 0.94%; $P < 0.05$).

Discussion

The present studies demonstrated that QiHong had protective effects on CVB3-infected mice and possessed a potent antiviral activity against CVB3 *in vitro*. Additionally, ribavirin, the reference drug in this study, showed more powerful antiviral activity than did QiHong *in vitro*. In cytotoxicity experiments, the CC_{50} of ribavirin was 16-fold higher than that of QiHong, suggesting that the toxicity of QiHong was much lower than that of ribavirin. According to the selectivity index values, we also confirmed that the antiviral activity of QiHong was not due to its cytotoxicity. The antiviral activity of QiHong occurred during the 0–4 hrs after CVB3 infection, especially during 0–2 hrs; in contrast, the antiviral activity of ribavirin lasted for 12 hrs. This suggests that QiHong acts at an early stage in the CVB3 replication cycle. Such early stages mainly include viral attachment and penetration into the cell.

CVB3 belongs to the family of nonenveloped picornaviruses that enter into host cells through binding to the coxsackievirus-adenovirus receptor (CAR; Ref. 14). Small depressions surrounding the 5-fold axis, the so-called canyons formed by the viral capsid proteins VP1, VP2, and VP3, bind to CAR (15). Receptor binding induces conformational changes, which facilitate the internalization of viral RNA into host cells (16, 17). Additionally, decay accelerating factor (DAF/CD_{55}) acts as an attachment but not an entry receptor for CVB3 (18, 19). According to our results on viral attachment and penetration assays, QiHong blocked the attachment and penetration of viruses into cells, and the effects might be due to the disturbance of the receptor function of CAR and DAF/CD_{55}.

In addition, this study provided direct evidence that QiHong, when administered to a murine model of viral myocarditis, not only markedly increased the survival rate of the animals, but also protected the heart from CVB3-induced injury.

Of greater interest in the present study is that a concentration of intragastrically administered QiHong as

Table 2. Physical Characteristics and Myocardial Virus Titer in Various Groups[a]

Group	Body weight (g)	Heart weight (mg)	HW/BW ($\times 10^{-4}$)	Myocardial necrosis (%)	Virus titer ($\log_{10}$ PFU/mg)
Normal control	19.3 ± 2.2*	90.1 ± 8.1*	4.7 ± 0.6*	—	—
CVB3 infection control	14.1 ± 2.8	106.7 ± 9.6	8.6 ± 0.6	29.00 ± 3.98	3.6 ± 1.1
CVB3 + QiHong (200 mg·kg^{1}·day^{-1})	17.3 ± 1.9*	95.0 ± 10.3*	5.5 ± 0.7*	2.10 ± 0.13*	2.5 ± 0.7*
CVB3 + QiHong (600 mg·kg^{-1}·day^{-1})	18.2 ± 2.0*	94.1 ± 11.8*	5.2 ± 0.9*	1.70 ± 0.25*	2.3 ± 0.4*
CVB3 + QiHong (1800 mg·kg^{-1}·day^{-1})	19.1 ± 2.6*	92.0 ± 8.2*	4.9 ± 0.7*	0.94 ± 0.07*	1.9 ± 0.6*
CVB3 + ribavirin (90 mg·kg^{-1}·day^{-1})	15.1 ± 3.1	102.8 ± 10.4	6.8 ± 0.7	7.61 ± 0.94*	1.7 ± 0.6*

[a] HW indicates heart weight; BW, body weight. Results are expressed as mean ± SD.
* Versus CVB3 infection control group, $P < 0.05$.

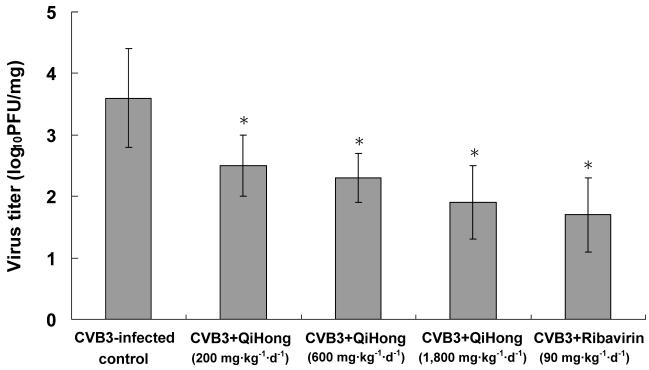

Figure 6. QiHong reduced the virus titer in the myocardium *in vivo*. Sixty Balb/C mice were divided into six groups for virus titer assay on Day 6 of virus CVB3 infection; myocardial virus titers (log_{10} PFU/mg) were significantly lower in the QiHong-treated groups (1800 mg·kg^{-1}·day^{-1}) than in viral-infected groups (1.9 ± 0.6 vs. 3.6 ± 1.1; $P < 0.05$), indicating that QiHong had antiviral effects. Ribavirin also possessed potent antiviral activity (1.7 ± 0.6 vs. 3.6 ± 1.1; $P < 0.05$). *$P < 0.05$ versus CVB3 infection control group.

low as 200 mg·kg^{-1}·day^{-1} significantly reduced the titer of virus in the myocardia and lowered the heart weight. In contrast, although ribavirin reduced myocardial virus titer *in vivo* more markedly than did QiHong, it had no significant beneficial effect on CVB3-induced cardiac histologic changes and death.

Like many other viral illnesses, both direct viral injury and the immune response of the host play important roles in the pathogenesis of viral myocarditis. Results from previous experiments in murine models of viral myocarditis indicated that although the immune response has an important protective role, it may also have deleterious effects on the host. The balance between these protective and deleterious effects may ultimately determine the course of disease after CVB3 infection.

QiHong is a mixture of three herb extracts. It might exert its beneficial effect on CVB3-infected mice *via* multiple pathways. The principal aim of this study was to test whether QiHong has a protective effect on the viral myocarditis model and to explore the possible mechanism of the compound. Our results clearly showed that QiHong-treated mice had much higher survival rates upon CVB3 infection than CVB3-infected controls. Our experiment lasted for 28 days, much longer than the time point (14 days after infection) at which viruses were cleared by the immune system. We speculated that QiHong has some other effects on CVB3-infected myocarditis in addition to blocking the virus attachment and penetration into cells.

In our *in vivo* experiment, the effective antiviral dosage of QiHong was much higher than that of ribavirin. The reason for this phenomenon might be explained by the

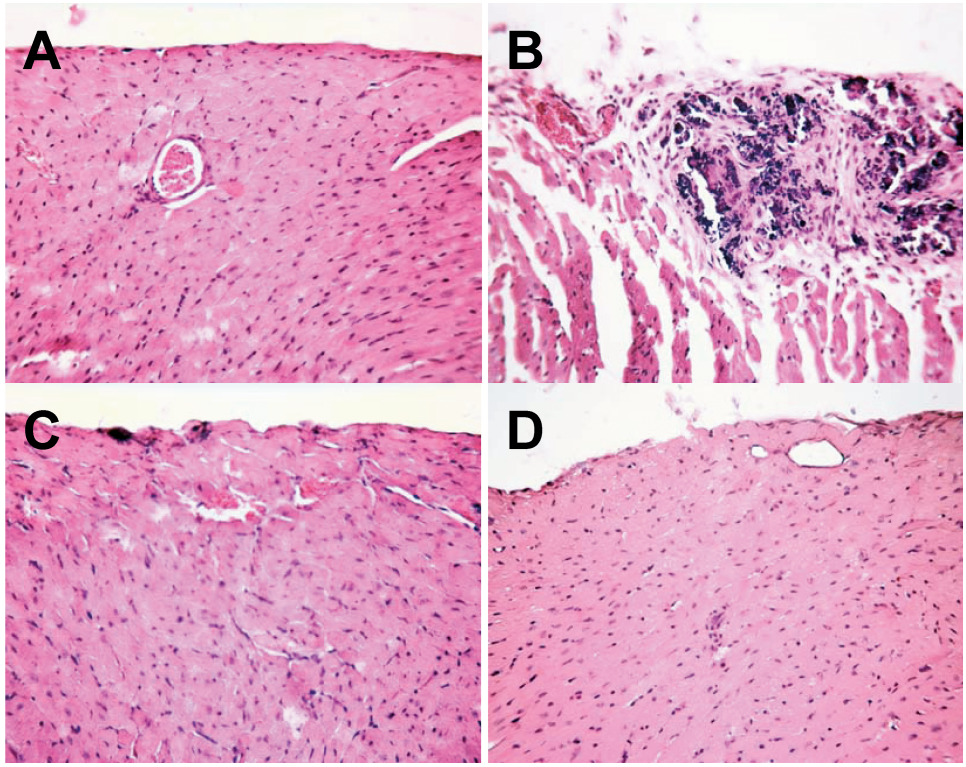

Figure 7. QiHong showed protective effects on the pathologic changes induced by CVB3 infection. (A) Normal control group. (B) CVB3 infection group (0.1 ml CVB3 suspension containing 10 PFU). (C) CVB3 + QiHong (200 mg·kg^{-1}·day^{-1}). (D) CVB3 + ribavirin (90 mg·kg^{-1}·day^{-1}, PO). No myocarditis was observed in mice treated with vehicle, but severe cellular infiltration with myocardial cell necrosis was noted in CVB3-infected mice (ratio of myocardial necrosis: 29.00% ± 3.98%), QiHong (ratio of myocardial necrosis: 2.10% ± 0.13%) exhibited more powerful protective effects on the mice with CVB3 infection than did ribavirin (ratio of myocardial necrosis: 7.61% ± 0.94%) Hematoxylin and eosin staining; original magnification ×380.

difference of clinical dosage between these two drugs. The recommended clinical dosage of ribavirin is 9 $mg \cdot kg^{-1} \cdot day^{-1}$, whereas the clinical recommended dosage of QiHong is 20 $mg \cdot kg^{-1} \cdot day^{-1}$, according to our calculation. Based on body surface area, the dose given in mice is 9 to 12 times the human dose. Thus, the dose of 200 $mg \cdot kg^{-1} \cdot day^{-1}$ in mice is equivalent to a dose of 20 $mg \cdot kg^{-1} \cdot day^{-1}$ in humans, which is within the range of clinical recommended dose. Therefore, this drug is expected to have clinical value in treating viral myocarditis.

In conclusion, our study showed that a moderate dose of QiHong had antiviral effects in a murine model of viral myocarditis induced by CVB3. The antiviral effects might be related to the inhibition of viral attachment and penetration into host cells. These findings suggest that QiHong might be applicable to the treatment of patients suffering from viral myocarditis. Further experiments, however, are required to determine QiHong's mechanism, molecular target of action, and presently undiscovered side effects in humans.

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