The Study of Anti Rabies Virus Effect of Shougong Powder

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ABSTRACT

Rabies is a lethal infectious disease caused by rabies virus (RABV). The mortality rate is very high after the appearance of clinical symptoms, with a survival rate of almost 0%. There is presently no cure for rabies. In the present study, we investigated whether the extract of Shougong powder-a calcium powder prepared from gecko that has demonstrated immunomodulatory properties in mice-is an effective treatment for rabies. The antiviral effects of the extract were evaluated both in vitro and in vivo with the cytotoxicity and antiviral assays and by immunofluorescence analysis, quantitative real-time (qRT)-PCR, and western blotting. The results showed that Shougong powder and its extract increased survival rate in RABV-infected mice is up to 60% and 50% respectively, even in 20 times of LD50. Whereas the control groups treated with Isoprinosine (IPS) or saline are only 20% and 0% survival (P=0.011). qRT-PCR and western blot analyses showed that the extract strongly inhibited viral mRNA expression and protein synthesis in vitro: expression of the N, P, M, G, and L genes of RABV was decreased by 28.8%–45.0% in the IPS group (P<0.05) and by 50.1%–59.0% in the extract group (P<0.05) relative to the control group. These results demonstrate that Shougong powder has antiviral effects against RABV and can potentially be used for the treatment for rabies.

Keywords: Shougong Powder; Extract of Shougong; Rabies; Treatment; Survival rate

List of Abbreviations:

RABV: rabies virus; qRT: quantitative real-time; IPS: Isoprinosine; PBS: phosphate-buffered saline; TCID50: 50% tissue culture infective dose; CC50: half-maximal cytotoxic concentration; IC50: 50% inhibitory concentration; FITC: fluorescein isothiocyanate; HRP: horseradish peroxidase

Introduction

Viral diseases such as Covid-19 [1], rabies [2], and Ebola [3] are a major threat to human health and there are at present no effective drugs for their treatment. Rabies is a highly lethal zoonosis caused by the rabies virus (RABV) that mainly occurs in developing countries, ranking third or fourth among infectious diseases in terms of global prevalence. RABV is usually transmitted from free-range animals and stray dogs and targets the central nervous system, causing encephalitis in humans and other mammals. It is estimated that more than 60,000 people die each year from rabies and over 15 million receive postexposure prophylaxis, with children accounting for 40% of cases [4]. Rabies infection from transplanted organs has also been reported [5]. Once neurologic symptoms appear, the mortality rate is almost 100% [6].

RABV is a single-stranded negative-chain RNA virus. The 12-kb genome encodes 5 structural proteins including nucleoprotein (N), matrix protein (M), phosphoprotein (P), glycoprotein (G), and RNA-dependent RNA polymerase (L). Of these, G protein is the main antigen inducing the innate immune response in the host upon viral infection. There are several reports of cases that have survived RABV infection following treatment with the Milwaukee protocol; however, this was accompanied by severe neurologic sequelae [7]. As such, there is a need for alternative treatment strategies for rabies. It was reported that the combination of ribavirin and interferon (IFN)-α has synergistic effects in the treatment of hepatitis C virus infection, while arabinosine combined with IFN has been effective in the treatment of patients with advanced rabies [8].

Gecko, also known as Shougong in China, is a small lizard found in warm regions [9] that is used in traditional Chinese medicine. The clinical efficacy of Shougong powder for the treatment of a variety of malignant tumors has been reported [10]; moreover, it is less toxic and has fewer side effects than conventional chemotherapeutic drugs [11]. The proprietary Chinese medicine Jinlong Gum capsules prepared from gecko is used for the treatment of colorectal, esophageal, gastric, and lung cancers [12]; the combination of Xiaojie powder and Yiqi Sanjie soup containing Pihu is used for the treatment of esophageal cancer [13]; and the combination of gecko and scorpion is used for the treatment of neck tumors [14]. Thus, Shougong powder has both antitumor and antiviral effects.

In the present study, we investigated whether the extract of Shougong and Shougong powder-a calcium powder prepared from Japanese gecko (Gekko japonicas) that has demonstrated immunomodulatory properties in mice-is effective for rabies treatment using in vitro and in vivo models.

Materials and Methods

Materials

Animals

Female Kunming mice (specific pathogen-free) weighing 18-22g were obtained from Changchun Institute of Biological Products (Changchun, China). The mice were handled in strict accordance with the standards for experimental animals set by the National Deployment Laboratory Animal Management Committee, and were allowed a 3-day acclimatization period in the laboratory before they were used for experiments.

Cells

BHK-21 cells provided by Institute of Military Veterinary Medicine, Academy of Military Medical Sciences were cultured in Minimal Essential Medium containing 5% fetal bovine serum.

Virus

RABV strain CVS-11, which was provided by the Institute of Military Veterinary Medicine, Academy of Military Medical Sciences, was propagated in BHK-21 cells and stored at −80°C. Viral titers were determined as 50% tissue culture infective dose (TCID50)/ml.

Extract of Shougong

Shougong powder was purified by boiling in water for 30 min, treating with ethanol, and dialyzing against water. The dialysate was subjected to ultrafiltration, then freeze drying. freeze drying The purified powder was dissolved in sterile water. Isoprinosine (IPS) was purchased from Toronto Research Chemicals (Toronto, Canada). Both the extract and IPS were dissolved in dimethyl sulfoxide.

Methods

Cytotoxicity Assay

BHK-21 cells were seeded at a density of 2×105/ml in 96-well plates and incubated at 37°C for 24 h. The culture medium was replaced with fresh medium containing various concentrations of the extract (0.69, 1.37, 2.74, 5.47, 10.94, 21.88, and 43.75 mg) or IPS (0.125, 0.25, 0.5, 1, 2, 5, and 10 mM) and incubated for 48 h. The surviving cell fraction was evaluated with the MTS assay. The half-maximal cytotoxic concentration (CC50) was determined by nonlinear regression analysis of the dose–response curves.

Antiviral Assay

BHK-21 cells were seeded at a density of 2×105/ml in 96-well plates and incubated as described above, and then infected with CVS-11 (multiplicity of infection [MOI]=0.1) at 37°C for 1 h. After washing with phosphate-buffered saline (PBS), fresh culture medium containing various concentrations of the extract (0.69, 1.37, 2.74, 5.47, 10.94, 21.88, and 43.75 mg) or IPS (0.125, 0.25, 0.5, 1, 2, 5, and 10 mM) was added to the cells, followed by incubation for 48 h. The antiviral effects of each treatment were assessed by calculating the TCID50 using the following formula: Inhibition rate (%) = [(control group virus titer − treatment group virus titer) / (control group virus titer)] × 100%. The 50% inhibitory concentration (IC50) was calculated by regression analysis of the dose–response curves. The results are expressed as selectivity index (SI = CC50 / IC50).

Immunofluorescence Analysis

BHK-21 cells were seeded at a density of 2×105/ml in 96-well plates. The following day, the cells were infected with CVS-11 at MOI=0.1 and incubated at 37°C for 1 h. After washing with PBS, different concentrations of the extract (0, 2.74, and 5.47 mg) or IPS (0, 0.5, and 1 mM) were added. The cells were cultured at 37°C and 5% CO2 for 48 h and the medium was discarded. The cells were fixed with 50 µl/well of 80% cold acetone at −20°C for 1 h, washed 3 times with PBS, and incubated at 37°C for 1 h with 50 µl fluorescein isothiocyanate (FITC)-conjugated anti-RABV immunoglobulin diluted with PBS. After 3 washes with PBS containing 0.1% Tween-20, 90% glycerin buffer was added to the cells at 50 µl/well. The fluorescent signal was observed and photographed under a fluorescence microscope (IX73; Olympus, Tokyo, Japan).

quantitative real-time (qRT)-PCR

BHK-21 cells were seeded at a density of 2×105/ml in 12-well plates and infected with CVS-11. The culture medium was replaced with fresh medium containing various concentrations of the extract and incubated for 48 h. Total RNA was extracted from cells using TRIzol reagent (Takara Bio, Beijing, China). qRT-PCR was performed with the Mx3000P Q-PCR system (Agilent-Stratagene, La Jolla, CA, USA) according to a previously described protocol [15]. The primers used were listed in Table Ⅰ. The thermal cycling conditions were as follows: denaturation at 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 1 min. Melting curve analysis was performed to verify the specificity of amplification. Relative quantification of target genes was performed with the 2−ΔΔCt method [16]. Each reaction was prepared in triplicate and the experiment was repeated at least 3 times.

Gene	Sequences	Size(bp)
GAPDH-F	GTTCAAAGGCACAGTCAAGG	20
GAPDH-R	ACGCCAGTAGACTCCACAAC	20
CC-qN-F	ATCTTTAGTTGGTCTTCTCT	20
CC-qN-R	TGTCATCAGA GTGTGGTGTT	21
CC-qP-F	CCTCCTTTTGAACCATCCCA	20
CC-qP-R	ACTTGCTTTCTCCTGCCGTG	20
CC-qM-F	GATGATGACTTGTGGCTGCC	20
CC-qM-R	CCGTTTGGACTGCACACCTT	20
CC-qG-F	CCCCATTTA CACGATACCAG	26
CC-qG-R	ACATCCTTCA TCCTCCATAA	21
CC-qL-F	TGAGGAACTCTGACTATAAT	20
CC-qL-R	GTCAGGGTCA TTCGGTAAGG	21

Table 1: Sequences and the sizes of forward (F) and reverse (R) primers of target genes for qRT-PCRWestern

Western Blotting

BHK-21 cells were seeded at a density of 2×105/ml in 6-well plates and infected with CVS-11 for 1 h. The culture medium was replaced with fresh medium containing various concentrations of the extract, followed by incubation for 48 h. Total protein was extracted from the cells using radioimmunoprecipitation assay lysis buffer containing phenylmethylsulfonyl fluoride, and protein concentration was determined with the .BCA Protein Quantification Assay Kit (Beyotime, Shanghai, China). Western blotting was performed as previously described [16]. Protein band intensity was. determined using an imaging system .with Quantity One software (Bio-Rad, Hercules, CA, USA) and was normalized to that of α-tubulin. Polyclonal antibodies against CVS-11 G, N, P, and M proteins were produced in our laboratory. Rabbit polyclonal α-tubulin antibody (AF0001), horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (H+L) (A0216), and HRP-conjugated goat anti-rabbit IgG (H+L) (A0208) were from Beyotime.

Treatment of RABV-infected Mice with Shougong Powder and the Extract

The protocol for animal experiments was reviewed and approved by the Ethics Committee of Animal Experiments at Shanghai Center for Clinical Laboratory. The experimental scheme is shown in Figure 1. Mice were randomly divided into 8 groups (n=10); 4 groups received a 20 times of lethal dose of CVS-11 (20× LD50) and were treated 1 h later with saline (control), Shougong powder, the extract (8.75 mg/day), or IPS (1000 mg/kg) by intramuscular injection into the hind legs once a day. The feeding group was given processed rat food (equivalent to 1 g of purified powder) daily for 11 consecutive days. The other 4 groups were used as healthy controls and treated in an identical manner but without virus infection. All animals were observed daily for 21 days.

After sacrifice, RNA was extracted from half of each mouse brain for qRT-PCR, while the other half of the brain was used for immunohistochemical analysis with FITC-labeled monoclonal anti-RABV nucleoprotein antibody. The fluorescent signal was observed under a fluorescence microscope.

Statistical Analysis

Data are expressed as mean ± SEM. Significant differences between groups were evaluated by one-way analysis of variance followed by the Bonferroni F test, or with the Student's t test or chi-squared test. $P<0.05$ was considered statistically significant.
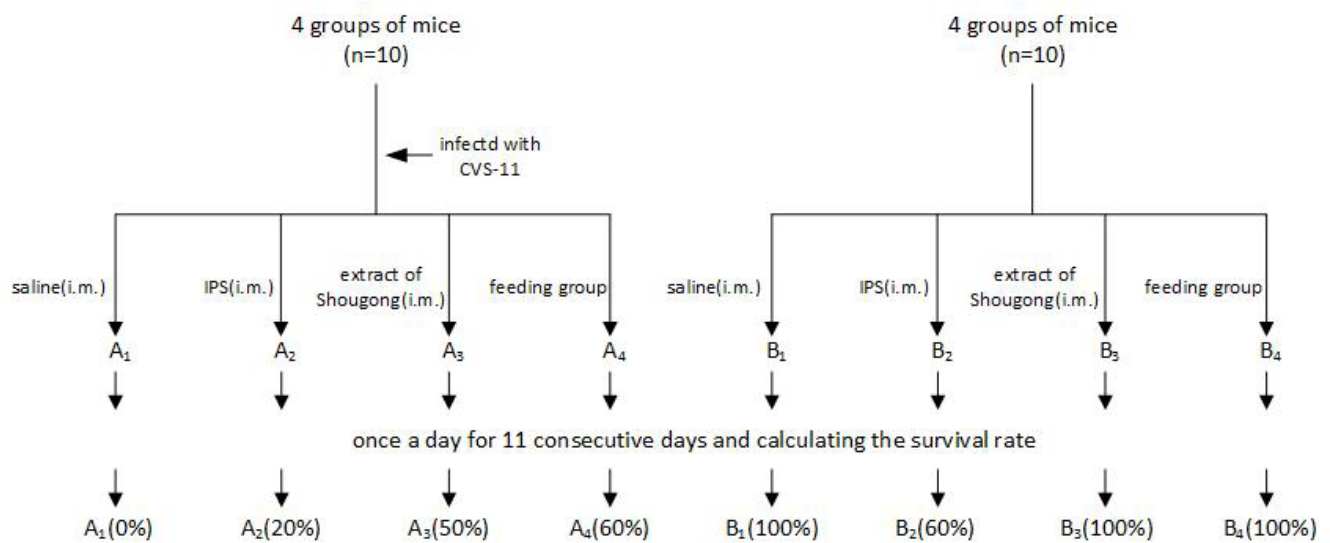

Figure 1: Experimental scheme for evaluating the antirabies effect of Shougong powder and extract of Shougong in RABV-infected mice.

Results

The Extract Inactivates RABV

The toxicity of IPS and the extract in BHK-21 cells was evaluated with the MTS assay. The optical density of each sample was measured and used to calculate survival rate relative to untreated control cells. Cell proliferation was inhibited in a time- and dose-dependent manner by treatment with 10.94 mg the extract and 2 mM IPS (Figure 2A). We selected 5.47 mg the extract and 1 mM IPS as working concentrations for subsequent experiments as they had no significant effects on cell proliferation. In the cytotoxicity assay, the CC50 of the extract and IPS was 28.59 mg and 3.048 mM, respectively. In the antiviral assay, the IC50 of the extract and IPS was 4023.43 mg and 1044.91 mM, respectively. Thus, the SI (CC50/IC50) for each treatment was 0.0071 and 0.0029, respectively. IPS and the extract reduced CVS-11 titer in a concentration-dependent manner by 2.5–60 times and 3.7–70 times, respectively (Figure 2B). These results indicate that both treatments have significant anti-RABV effects in vitro.

**Antiviral Effects of the Extract in Vitro**

The antiviral effect of the extract in BHK-21 cells infected with CVS-11 was evaluated by immunofluorescence analysis, qRT-PCR, and western blotting. Treatment of infected cells with the extract or IPS resulted in a concentration-dependent reduction of the green fluorescent signal (Figure 3A), indicating that viral replication was suppressed. Additionally, the extract and IPS reduced the expression of CVS-11 N, P, M, G, and L genes in a concentration-dependent manner by 28.8%–45.0% ($P<0.05$) and 50.1%–59.0% ($P<0.05$), respectively, relative to the control group, with 12.2%–34.0% ($P<0.05$) lower transcript levels in the the extract group compared to the IPS group (Figure 3B). Similar trends were observed for M, N, G, and P protein levels (Figure 3C). These results indicate that like IPS, the extract inhibits both RABV gene expression and protein synthesis.

Antiviral Effects of Shougong in Vivo

To confirm the above results in vivo, mice were infected with CVS-11 and then treated 1 h later with IPS and the extract for 7 days. Mice in the feeding group were infected with CVS-11 and fed the Shougong powder directly. An identical group was treated
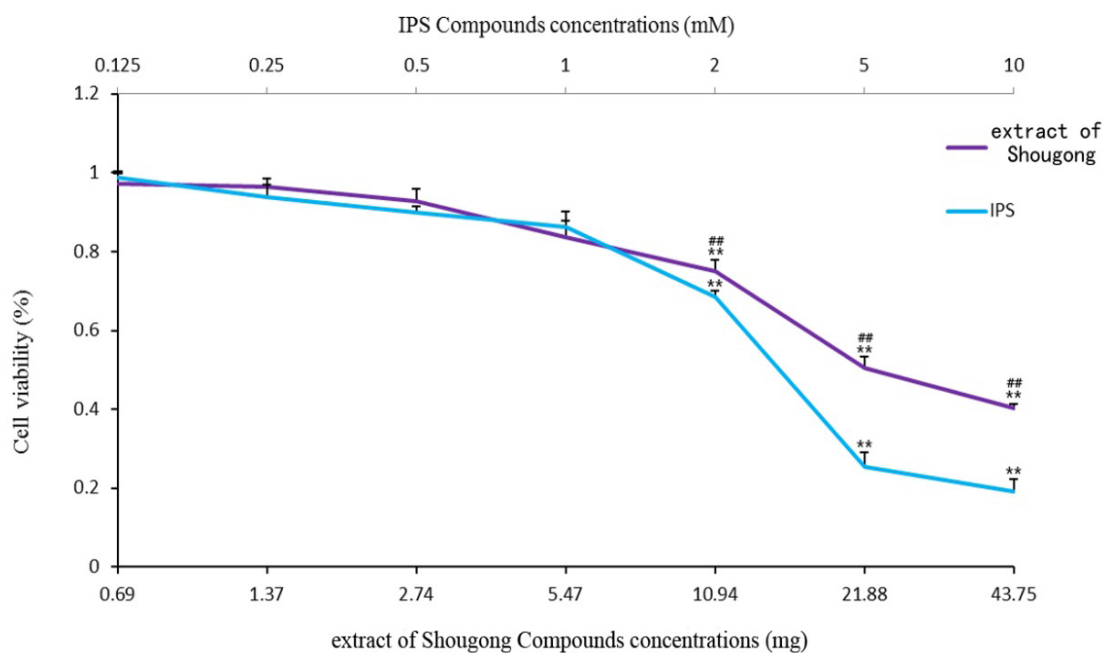
a. The cytotoxicity of IPS and extract of Shougong treated BHK-21 cells with different concentration
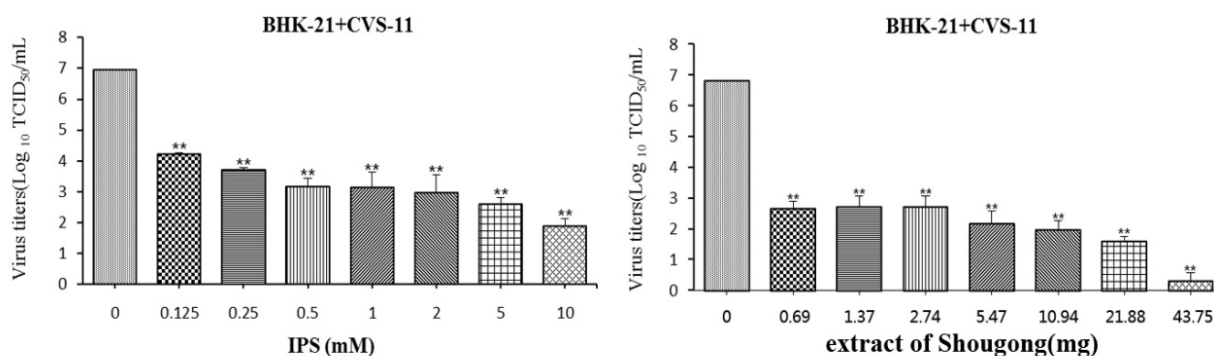

b. Anti-RABV effect of IPS and extract of Shougong

Figure 2: Cytotoxicity of IPS and Shougong extract at different concentrations in BHK-21 cells. **$P<0.01$ vs Control; ##$P<0.01$ extract of Shougong vs IPS.

with IPS (1000 mg/kg) instead of the extract. Body weight, clinical signs, and mortality were monitored for 21 days. Mice in the 4 experimental groups infected with CVS-11 showed significant weight loss from days 5 to 9, which was accompanied by loss of limb coordination, trembling, paralysis, and death (Figure 4). These symptoms were alleviated in mice treated with the extract or IPS and in the feeding groups as compared to untreated control mice. Thus, the extract and IPS alleviate weight loss and clinical symptoms associated with RABV infection.

The 4 groups of mice that were not infected with CVS-11 remained in good condition and gained weight normally, with all but 4 mice in the IPS group surviving until the end of the experiment; this suggested that Shougong powder and the extract were less toxic to mice than IPS. In contrast, among the 4 groups of mice that were infected with CVS-11, all mice from the saline group died on day 8 of the experiment, while the number of surviving mice in the feeding, the extract, and IPS groups was 6, 5, and 2, respectively, at the end of the experiment. The survival rate in mice treated with the extract was 50% as compared to the rates of 20% in the IPS group and 60% in the feeding group. The chi-squared test showed that the survival rates of the feeding and the extract groups were significantly different from that of the control group (P=0.011 and 0.033, respectively), with a higher rate in the former. The results of nucleic acid gel electrophoresis and immunohistochemical analyses showed that mice from the 4 experimental groups infected with CVS-11 all died from rabies, whereas no CVS-11 was detected in uninfected mice from the IPS group (20% mortality).
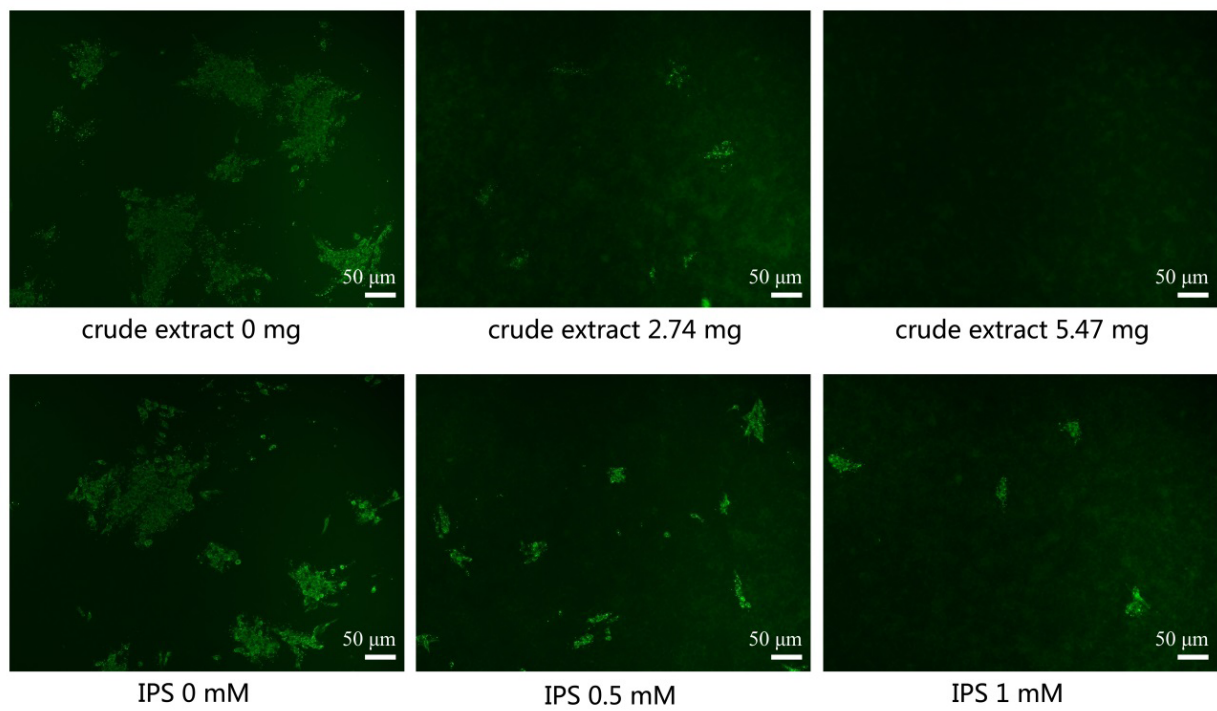
A. Observation green fluorescence signal by fluorescence microscope
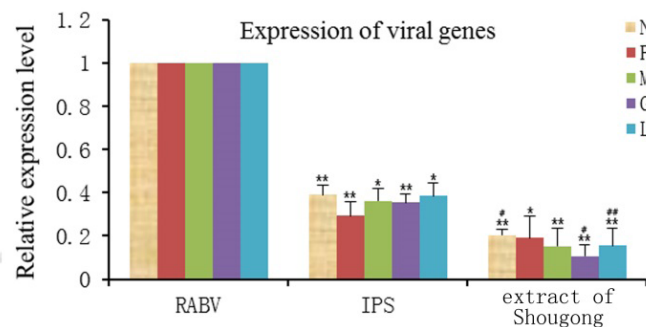
B. Detection the expression of RABV N, P, M, G and L genes by qRT-PCR
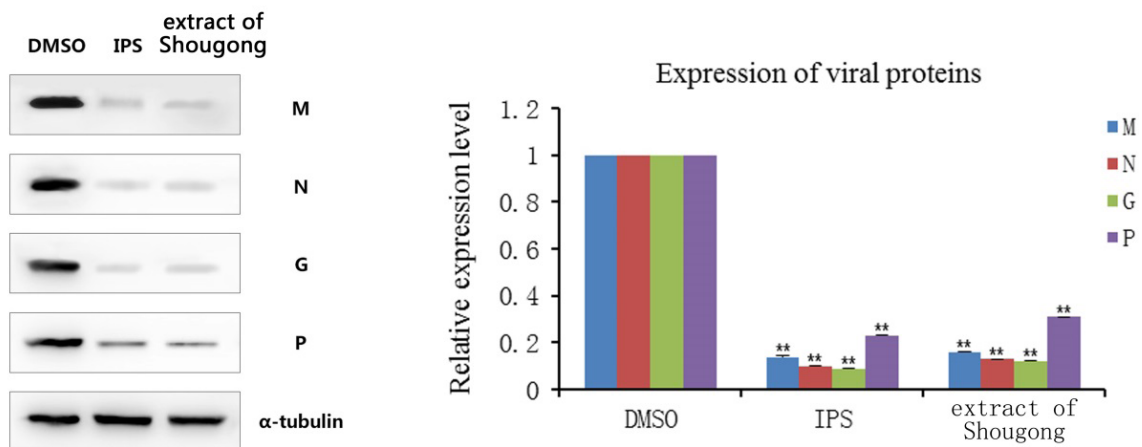
C. Detection the expression of RABV M, N, G and P proteins by western blotting

Figure 3: Shougong extract inhibits RABV replication. BHK-21 cells infected with RABV were treated with extract of Shougong or IPS at indicated concentrations for 48 h, and viral particles were detected by immunofluorescence analysis. (a) FITC-labeled RABV was observed as green fluorescence. Scale bar,100 μm. (b) qRT-PCR detection of RABV N, P, M, G, and L gene expression in cells treated with IPS (1 mM) or extract of Shougong (5.47 mg). (c) Western blot analysis of viral protein expression levels in cells treated with IPS (1 mM) or extract of Shougong (5.47 mg). *P<0.05, **P<0.01 vs Control; #P<0.05, ##P<0.01 vs extract of Shougong.
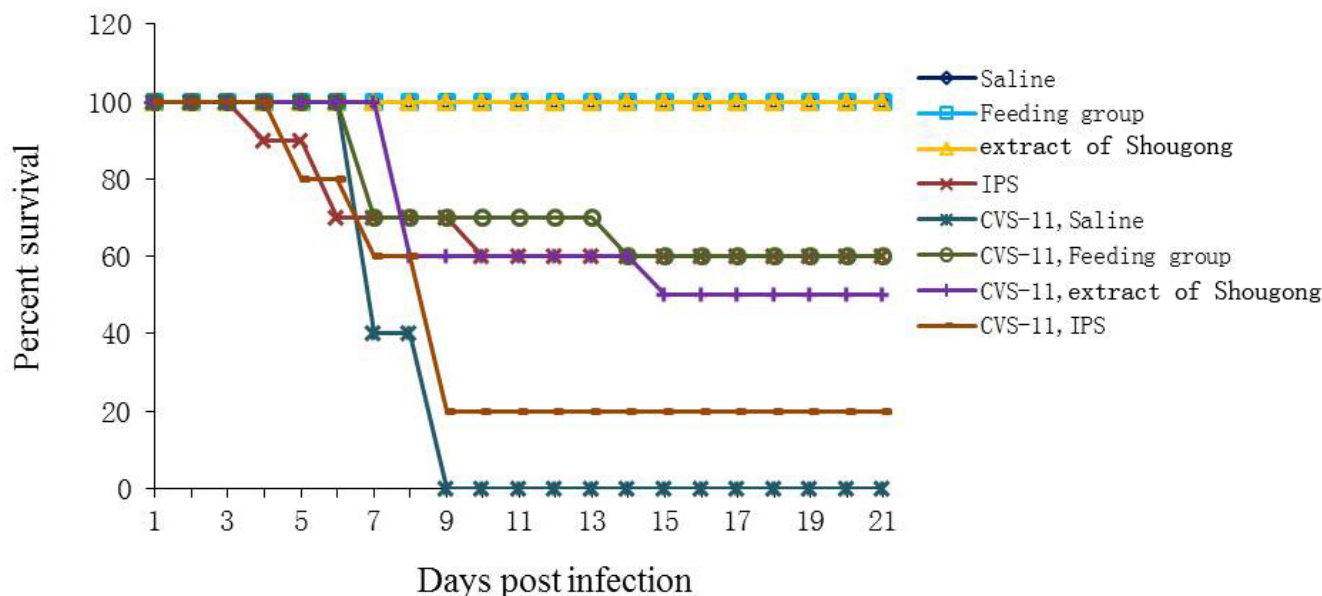
Figure 4: Anti-RABV effect of Shougong in mice. Survival was significantly longer in the feeding and extract of Shougong groups than in the control group (P=0.011 and 0.033, respectively).

Discussion

The earliest record of rabies in China is in the book Zuo Zhuan, which dates back at least 2500 years. Despite the longstanding awareness of this disease, there is no effective cure. Previous studies have identified a variety of compounds that show inhibitory effects on RABV including isoproterenol [17], IFN [18], ketamine [19], ribavirin [20], nucleotide analogs [21], and 1,2,3,4,6-penta-O-galloyl-β-d-glucose [15], but their therapeutic benefits are limited.

Dermaseptins secreted by amphibian skin have been shown to inhibit RABV in vitro and enhance survival in infected mice [22]. IPS inhibits RABV in vitro [17] and is used as a positive control when screening anti-RABV drugs [23]. IPS at a dose of 500–1000 mg/kg increased survival in mice by 20%–30% [15]; we therefore used an IPS dose of 1000 mg/kg in our study. However, the mortality rate of 20% in mice treated with IPS indicates a certain degree of toxicity. Shougong is a type of terrestrial reptile belonging to the Gekkonidae family that has the capacity for tissue regeneration and adhesion to surfaces [24, 25]. In the ancient Chinese books Compendium of Materia Medica and Peaceful Holy Benevolent Prescription, Shougong powder is recommended for the treatment of apoplexy [26]. Our results demonstrate that Shougong powder and it's the extract have inhibitory effects on RABV that are superior to those of IPS in mice, and that it can alleviate clinical symptoms while reducing mortality from rabies. The safety of the powder and the extract as highlighted by our observation that neither was toxic in control mice.

In summary, the results of our study indicate that Shougong powder can improve survival in mice infected with RABV by inhibiting viral replication, while having minimal toxicity. These findings provide evidence for the therapeutic value of Shougong powder for the treatment of rabies, although additional studies are needed to elucidate the mechanistic basis for its effects.

Funding

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Availability of data and material

The datasets supporting the conclusions of this article are included within the article and its additional files and will be freely available to any scientist wishing to use them for non-commercial purposes upon request via e-mail with the corresponding author.

Authors' contributions

HLW conceived and designed the study and revised the manuscript. MZ performed the experiments and statistical analyses and wrote the manuscript text. YF purified the Shougong powder. XW, JW, RC, YX, HYW, and WH provided suggestions and technical support for the study. HP prepared the Shougong powder. All authors contributed to data acquisition and interpretation, and approved the final manuscript for publication.

Ethics approval and consent to participate

This study and the protocol were approved by the Institutional Review Board of Shanghai Center for Clinical Laboratory (Shanghai, China).

Consent for publication

All the authors have approved for publication.

Acknowledgments

Supplementary Materials

There are no Supplementary Materials.

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