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Full Length Research Paper

Study of antivirus, antibacteria and immune functions of Gaoreqing freeze-dried powder

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Gaoreqing freeze-dried powder was effective fraction extracted from *Lonicera japonica* Thunb., *Scutellaria baicalensis* Georgi and Forsythia suspense (Thunb.) Vahl. It was confirmed to have certain anti-virus, anti-bacterial effects and immuno-enhancing activities. Cell culture and influenza viral pneumonia model of mouse were used to study its antiviral effect. Plate culture and pathogenic bacteria-infected model in mouse were established to examine its anti-bacterial effect. Carbon particle clearance test and determination of serum hemolysin were done to observe its immune-enhancing effect. The results showed that Gaoreqing had significant effect on inhibiting cytopathic effect induced by various viruses. Compared with virus control group, high and middle dosage groups could significantly decrease the pulmonary index, pulmonary virus hemagglitination titer in influenza viral pneumonia model of mouse (P < 0.01). It showed obvious inhibitory effects on *Streptococcus pyogenes*, *Staphylococcus aureus*, *pneumococcus* and so on. Gaoreqing also had obvious protective effect on mice infected by *S. aureus* and *pneumococcus*. In addition, phagocytosis and 50% hemolyzing concentration of mice were obviously reinforced by Gaoreqing (P < 0.05 to 0.01). The results obtained here demonstrated that Gaoreqing had obvious anti-virus and anti-bacterial effects and enhanced immune function in mice.

Key words: Gaoreqing freeze-dried powder, anti-virus, anti-bacterial, immune function.

INTRODUCTION

Bacterial and viral infections have been increasing constantly in recent years, so efficient and low toxic drugs of antibacterial and antiviral activity are needed urgently. But certain antibacterial and antiviral drugs have some toxic and adverse effects on central nervous system, blood system and so on. However, Traditional Chinese medicine (TCM) has comprehensive effects on anti-bacterial, anti-virus and improving immune function (Li et al., 2010; Yang et al., 2004). According to WHO reports, 65 to 80% people all over the world use TCM (Liu et al., 2010; Yakubu et al., 2003). Currently, some effective anti-bacterial and anti-viral TCM are found and selected from the herbs and are widely used in clinical treatment in various bacterial and viral diseases (Xu et al., 2010). It was reported that there are hundreds of herbs that have 163.com.

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clear roles and are used widely, such as honeysuckle, forsythia, scutellaria, cloves, garlic, panax, senecio, salvia, licorice, nepeta, bupleurum, berberine, and so on (Liu et al., 2010; Maregesi et al., 2008).Gaoreqing freeze-dried powder (Gaoreqing) is developed by Medical Research and Development Center of China. Gaoreqing is effective fractions extracted from *Lonicera japonica* Thunb. (*Lonicera*), Scutellaria baicalensis Georgi (*Scutellaria*) and Forsythia suspense (Thunb.) Vahl (Forsythia). Active compounds of them were chlorogenic acid, baicalin, forsythol and flavonoids. In this study, they were made into freeze-dried powder injection and this study was undertaken to evaluate its effects on anti-virus, anti-bacterial and improving body immunity.

MATERIALS AND METHODS

Laboratory animals

The study complied with the “Guide for the Care and use of

Laboratory Animals" published by the US National Institutes of Health (NIH publication No. 85-23, revised in 1985) and all animals were approved by the institutional animal experiments committee. ICR mice (20 ± 2 g body weight), half male and half female, were purchased from Vitalriver Experimental Animal Technology Co. Ltd (Beijing, China). Animals were maintained under 12 h light/dark cycle at a temperature approximately 24 ± 1°C with food and water *ad libitum*.

Test substance and reagents

400 mg·ml^{-1} Gaoreqing freeze-dried powder (5 g crude drug, No. 200201) was supplied by Medical Research and Development Center of China. The active compounds were chlorogenic acid, baicalin, forsythol and flavonoids. It was prepared with normal saline. Shuanghuanglian powder injection (Shuanghuanglian, No. 9909008) was purchased from Harbin Pharmaceutical Co. Ltd (Harbin, China).

Virus and strains

Influenza virus FM1, respiratory syncytial virus, adenovirus type 3, measles virus, coxsackie B4 virus, herpes simplex virus, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, *Streptococcus pyogenes*, *Pneumococcus*, *Streptococcus mutans*, *Proteus, Enterococcus*, *Escherichia coli*, *Klebsiella pneumonia* and *Haemophilus influenzae*. All of the aforementioned strains were isolated from clinical specimens and identified and preserved by Pharmacological Laboratory of Medical Research and Development Center of China.

Cells

Hep-2 cells, Madin-Darby canine kidney (MDCK) cells and FL cells were preserved by pharmacological laboratory of Medical Research and Development Center of China.

Effects of Gaoreqing on inhibiting cytopathic effect induced by virus *in vitro*

Determination of virus virulence

Hep-2 cells, FL cells and MDCK cells after 3 days growth of subculture, were diluted to $5 \cdot 10^7$ L^{-1} with medium and inoculated into 96-well plate. After cells grew into monolayer, diluted virus solution with tenfold decrease was added into 96-well plate. Cytopathic effect (CPE) was observed and 50 percent tissue culture infective dose ($TCID_{50}$) of each virus was determined (Yan et al., 2002; Ooi et al., 2006; Li et al., 2009).

Determination of drugs cytotoxicity

After cells grew into monolayer and medium was discarded, 0.2 ml tested drugs of different concentrations and positive drugs diluted by serum-free medium were added into each well. All cells were cultured at 37°C in 5% CO_2 cell incubator and CPE was observed everyday. Drugs cytotoxicity was evaluated and its maximal nontoxic concentration (TC_0) was calculated.

Drugs' inhibition on CPE: 6 virus solutions of different concentrations (10^{-1} to 10^{-6}) was added into Hep-2 cells, FL cells and MDCK cells, then was discarded after 1 h. Non-toxic tested drugs of different concentrations and positive drugs were added into each well and 3 repeated wells, compared with virus and cells controls. All cells were cultured at 37°C in 5% CO_2 cell incubator and CPE was observed under inverted microscope and recorded everyday for continuous 7 days.

Anti-influenza virus experiments of Gaoreqing on mice

LD_{50} determination of influenza virus

Fresh virus solution was diluted with sterilized normal saline by continuous double dilution methods. 0.03 ml different dilution was used to anesthetize mice and they were infected by nasal drip. Percentage of deaths was recorded and LD_{50} was calculated (Yu et al., 2010).

Anti-influenza virus experiments of Gaoreqing on mice

Different dosages of Gaoreqing *in vivo* were 0.76, 0.38 and 0.19 g·kg-1 (equivalent to 10, 5 and 2.5 times of clinical dosages) according to LD_{50} and maximal tolerance dose (MTD). Dosage of Shuanghuanglian, as positive drug, was 0.6 g·kg^{-1} (equivalent to 10 times of clinical dosage). 60 ICR mice were assigned into 6 groups (n = 10 for each group). The same volume of normal saline was injected to mice of virus and normal control groups, respectively. Other groups received different tested drugs by intraperitoneal injection, respectively, on the day before infection. All groups were treated once per day for 5 days. After that, all groups but normal control were vaccinated influenza virus infected mice pneumono-adaption stock FM_1 with administration of nasal drops of 15 LD_{50}. All mice were dissected after infection of 96 h. Lungs of all groups were weighed, lung index and inhibition rate were calculated.

Pathological examination

Lungs of all groups were dealt with formalin fixed, paraffin routinely embedded sections and staining for pathological photomicrograph.

Detection of influenza virus proliferation in mice lungs by hemagglutination test

Animal groups, dosages of tested drugs, injection and successful model methods were the same as that of anti-influenza virus experiments of Gaoreqing on mice. All mice were put to death by cervical vertebra dislocation after virus infection of 72 h, whose lungs were drawn and made into homogenate with 10% normal saline. Supernatants were obtained from it and dealt with doubling dilution. 0.2 ml diluent of different concentrations was dropped into titration plate and 0.2 ml 1% chicken erythrocyte suspension was added. They were treated by well mixing, standing at ambient temperatures for 30 min. After that, hem agglutination titer was determined at the end of erythrocyte agglutination (+ +). The titer was determined by dilution multiple of suspension.

Effects of Gaoreqing on antibacterial functions *in vitro*

Different bacteria was inoculated in their respective plate medium and cultured at 37°C by using plate dilution methods for 12 h. All scraped lawns were diluted with sterile saline and 9×10^6 CFU/ml bacterial concentrations were determined by McFarland Standard.

0.05 ml different bacteria liquids were inoculated in their respective plate with and without medicine and cultured for 24 h. Then, the minimal inhibitory concentration (MIC) was calculated (Belsem et al., 2009; Duraipandiyan et al., 2009; Pavithra et al., 2009).

Protective effects of Gaoreqing on mice infected by staphylococcus aureus and pneumococcus

70 ICR mice were divided into 7 groups (n = 10 for each group), half male and half female. Staphylococcus aureus and pneumococcus were diluted into required concentration with yeast and diluent (equivalent to 100% minimum lethal dose) was given into mice by intraperitoneal injection, respectively. 0.5 ml different drugs were given into different groups mice by intraperitoneal injection immediately and 6 h after infection. The death status of mice were observed and recorded after 7 days 50% effective dose (ED_{50}) and 95% confidence limit of different drugs were calculated by Bless method.

Effect of Gaoreqing on immune function of mice

Effect of Gaoreqing on monocyte-macrophage cells phagocytosis of mice

50 ICR mice were divided into 5 groups (n = 10 for each group), half male and half female. Control group received the same intravenously admitted volume of saline, positive group and Gaoreqing groups received intravenous injection of 0.1 $g \cdot kg^{-1} \cdot d^{-1}$ Shuanghuanglian, 8, 4, 2 $g \cdot kg^{-1} \cdot d^{-1}$ Gaoreqing, respectively. All groups were treated once a day for 8 days. After 1 h of the last set of intravenous injection, mice each group received intravenous injection of $10ml \cdot kg^{-1}$ Indian ink for calculating phagocytosis index and phagocytosis half life after 5, 10, 15 and 20 min.

Effect of Gaoreqing on hemolysin antibody of mice

Animal groups, drugs administration and methods were the same as that of the effect of Gaoreqing on monocyte-macrophage cells phagocytosis of mice. Next day of the last set of intravenous injection, mice each group received intraperitoneal injection of 0.1 ml (each one) sheep red blood cells (SRBC). HC_{50} of each group immunized with SRBC after 6 post-immunization was determined.

Statistical analysis

SPSS13.0 (SPSS, Chicago, IL, USA) was used for statistical analysis. Values were expressed as mean ± S.D. Data were analyzed using one-way ANOVA, followed by Students two-tailed *t*-test for comparison between two groups. $P < 0.05$ was considered to be significant.

RESULTS

Effects of Gaoreqing on inhibiting cytopathic effect induced by virus *in vitro*

Gaoreqing diluted above the concentration of 31.2 $g \cdot L^{-1}$, and Shuanghuanglian diluted above the concentration of 4.687 $g \cdot L^{-1}$ had no toxic effects on FL, Hep-2 and MDCK cells. The concentrations of Gaoreqing and Shuanghuanglian in this experiment are 15.620 $g \cdot L^{-1}$ and 2.343 $g \cdot L^{-1}$, respectively. Results showed that under the situation of 6 virus infection ($TCID_{50}$ = 30 to 100), Gaoreqing within the concentrations of 31.2 to 3.90 $g \cdot L^{-1}$ could significantly inhibited cytopathic effect induced by FM1, RSV, AdV3 and MV virus, while had a certain delaying role on cytopathic effect induced by CVB_3 and HSV-I virus. But the effects were less than that of former (Table 1).

Effects of Gaoreqing on influenzal viral pneumonia in mice

Results showed that high and middle groups of Gaoreqing could decrease lung index obviously and were similar to normal control group. At the same time, hemagglutination titers of high and middle groups were lower than that of virus control group significantly. These two groups had the same effect as positive group (Table 2).

Effects of Gaoreqing on antibacterial functions *in vitro*

Anti-bacterial experiment showed that Gaoreqing could inhibit *S. aureus*, *S. epidermidis*, *S. pyogenes*, *Pneumococcus*, *S. mutans*, *E. coli*, *K. pneumonia* and *H. influenzae* significantly. Meanwhile, Gaoreqing had a certain effect in inhibiting *Enterococcus, Proteus* and *P. aeruginosa*. But the effect was less than that of former (Table 3).

Protective effects of Gaoreqing on mice infected by *S. aureus* and *Pneumococcus*

The result showed that Gaoreqing could decrease mortality of mice infected by *S. aureus* and *Pneumococcus.* ED_{50} and 95% confidence limit were 0.1550, 0.0948 to 0.2535; 0.1354, 0.0653 to 0.2805 $g \cdot kg^{-1}$, respectively (Table 4).

Effect of Gaoreqing on immune function of mice

Result showed that compared with control group, high and middle groups of Gaoreqing could increase phagocytic index of monocyte-macrophage cells ($P < 0.05$) and decrease Carbon half-life ($P < 0.05$) significantly. High, middle and low groups of Gaoreqing could increase HC_{50} ($P < 0.05$ to 0.01), which was similar to positive group. It means that Gaoreqing could improve monocyte-macrophage cells phagocytosis and hemolysin

Table 1. Effects of Gaoreqing on inhibiting cytopathic effect induced by virus *in vitro.*

Virus strain	$TCID_{50}$	Shuanghuanglian (2.343 $g \cdot L^{-1}$)	Gaoreqing (15.620 $g \cdot L^{-1}$)	Cells control	Virus control
FM1	100	-	-	-	++++
RSV	30	-	-	-	++++
AdV-3	100	-	-	-	++++
MV	30	±	-	-	++++
CVB_3	50	±	±	-	++++
HSV-I	100	-	+	-	++++

Note: No cytopathic effect, ± delayed cytopathic effect, + less than 1/4 cytopathic effect, ++ ¼ to 1/2 cytopathic effect, +++ ½ to 3/4 cytopathic effect, ++++ more than 3/4 cytopathic effect.

Table 2. Effects of Gaoreqing on influenzal viral pneumonia in mice ($\bar{X}$ ± s, n = 10).

Group	Dose ($g \cdot kg^{-1} \cdot d^{-1}$)	Lung index	Inhibition rate (%)	Hemagglutination titer
Virus control	-	1.600 ± 0.120	-	32.0 ± 5.5
Normal control	-	1.327 ± 0.070	-	0.0
Shuanghuanglian	0.60	1.337 ± 0.090**	16.43	10.0 ± 6.0
	0.76	1.373 ± 0.090**	14.18	16.0 ± 6.3
Gaoreqing	0.38	1.386 ± 0.740**	13.37	20.0 ± 7.5
	0.19	1.496 ± 0.110	6.50	26.0 ± 10.0

Compared with virus group, **p < 0.01.

Table 3. Effects of Gaoreqing on antibacterial functions *in vitro*.

Virus strain	n	Gaoreqing ($g \cdot L^{-1}$)			Shuanghuanglian ($g \cdot L^{-1}$)		
		MIC	MIC_{50}	MIC_{90}	MIC	MIC_{50}	MIC_{90}
Staphylococcus aureus	49	62.5 - 125	62.5	125	75 - 300	75	300
Staphylococcus epidermidis	47	62.5 - 125	62.5	125	37.5 - 300	150	600
Streptococcus pyogenes	11	62.5 - 125	62.5	125	37.5 - 300	37.5	300
Pneumococcus	10	62.5 - 125	62.5	125	18.75 - 300	62.5	300
Streptococcus mutans	10	62.5 - 125	62.5	125	75 - 300	75	300
Enterococcus	21	250 - 500	250	500	150 - 600	150	600
Escherichia coli	56	62.5 - 125	62.5	125	37.5 - 300	37.5	300
klebsiella pneumonia	43	62.5 - 125	62.5	125	37.5 - 300	37.5	150
Proteus	65	125 - 500	125	500	150 - 600	150	600
Pseudomonas aeruginosa	49	125 - 500	125	500	150 - 300	150	300
Haemophilus influenzae	8	62.5 - 125	62.5	125	75 - 300	75	300

antibody after continuous intravenous administration of 8 days (Table 5).

DISCUSSION

Pathogenicity of the pathogenic bacterium is closely related to its virulence, quantity and pathway of invading the organism and human immune state. Virulence may vary between different strains, even those strains of the same organism. Usually, it was represented by LD_{50} or median infective dose (ID_{50}). The direct infective roles of virus on host cells include cytolytic infection, stable infection, inclusion body, apoptosis and integration of infection. Tissue injury caused by immunopathology is a common problem of virus infection. Antigens inducing

Table 4. Protective effects of Gaoreqing on mice infected by *Staphylococcus aureus* and *pneumococcus.*

Group	Dose ($g \cdot kg^{-1}$)	Logarithmic dose	n	*Staphylococcus aureus* S9811		*Pneumococcus* 3011	
				Death number	Mortality (%)	Death number	Mortality (%)
Control	-	-	10	10	100.0	10	100.0
Shuanghuanglian	0.60	-	10	1	10.0	2	20.0
	0.76	- 0.1192	10	0	0.0	1	10.0
	0.38	- 0.4202	10	2	20.0	2	20.0
Gaoreqing	0.19	- 0.7212	10	4	40.0	4	40.0
	0.095	- 1.0223	10	6	60.0	6	60.0
	0.047	- 1.3279	10	10	100.0	8	80.0

Table 5. Effect of Gaoreqing on monocyte-macrophage cells phagocytosis and hemolysin antibody of mice ($\overline{X} \pm s$, n = 10).

Group	Dose ($g \cdot kg^{-1}$)	Phagocytic index (α)	Carbon half-life ($t_{1/2}$)	HC_{50}
Control	-	3.04 ± 1.73	0.48 ± 0.09	268 ± 112
Shuanghuanglian	0.1	4.67 ± 1.28*	0.36 ± 0.12*	355 ± 137*
	8	4.87 ± 1.11*	0.36 ± 0.10**	436 ± 156**
Gaoreqing	4	4.44 ± 1.08*	0.31 ± 0.13**	418 ± 167**
	2	4.25 ± 1.76	0.32 ± 0.19*	398 ± 138*

Compared with control group, *p < 0.05, **p < 0.01.

immunopathologic reaction include virus and autoantigen induced by virus infection. Furthermore, some virus can invade immune cells directly and destroy their immune functions. These immunopathologic reactions include antibody-mediated, cell-mediated immunoreactions and immunosuppression. Bacteria and virus infections are closely related to body's immune system functions. A major hurdle for western medicine in anti-bacterial and anti-virus therapy is the emergence of drug resistance currently. It also has toxic effect on liver sometimes (Mao et al., 2004). However, theoretical basis of TCM on anti-bacterium and anti-virus is reinforcing the healthy qi and eliminating the pathogenic factors. Meanwhile, TCM has the characteristics of high efficiency, low toxicity, no drug resistance and so on (Bao et al., 1999). Mechanism of TCM, on the one hand, is that it can inhibit some links of adsorption, penetration, replication, mature on virus reproduction. For example, TCM plays an anti-virus role in inhibiting protein and RNA synthesis, adsorption, sialidase activity and membrane fusion of virus. On the other hand, it has anti-bacterial role through interfering with cells wall synthesis, damaging cells membrane, inhibiting cells protein and nucleic acid synthesis and interfering with the genetic code replication.

In our study, Gaoreqing showed obvious antibacterial and antiviral actions. Furthermore, it maintained homeostasis through regulating immune function. Gaoreqing is effective fractions extracted from honeysuckle, scutellaria and forsythia, which included some active ingredients, such as polyphenols, flavonoids and so on and they could inhibit bacterium and virus directly. Anti-virus experiments *in vitro* showed that Gaoreqing could inhibit CPE induced by the most 6 common virus significantly. The degree of lung lesions in viral pneumonia of mice induced by influenza virus was used to be expressed by pulmonary index. The results showed that high and middle groups of Gaoreqing could decreased pulmonary index in mice infected with influenza virus and reduced the pulmonary consolidation degree, which was similar to control group ($P > 0.05$). Influenza virus hemagglutinin was closely related to infectious virus particles (Liu et al., 2003). Hemagglutination (HA) titers could be used in predicting viral loads and virulence to a certain extent. Anti-influenza virus experiments of Gaoreqing in vivo showed that HA titers of high and middle groups were lower than that of virus control group significantly ($P < 0.01$). These two groups had the same effect with positive group. So Gaoreqing had positive antivirus effects by decreasing viral loads of lung lesions induced by influenza virus and inhibiting viral proliferation. The present experiment showed that Gaoreqing had extrasomatic antibiotic functions on Gram-positive bacteria and Gram negative bacteria. Anti-bacterial experiments in vitro showed that Gaoreqing could protect mice infected by *S. aureus* or *S. pneumonia* greatly. So, Gaoreqing had the effects of

broad spectrum bacteriostasis. In conclusion, Gaoreqing showed antibacterial, antiviral functions and enhanced immune function of mice. Its mechanisms need further research.

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