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Final Research Service Report

Date: July 22, 2020

The study of the anti-SARS-CoV activity of RDS

Summary

The study was to test the anti-SARS-CoV activity in a traditional Chinese medicine (TCM), RDS (Respiratory Detox Shot), using Virongy's SARS-CoV S protein pseudotyped GFP or luciferase reporter lentiviruses. The target cells were Vero E6 and ACE2-expressing A549 cell (human lung epithelia cell). Virongy performed experiments to test and quantify the anti-SARS-CoV activity of RDS, and found that RDS possesses anti-SARS-CoV activity. It was discovered that:

1) RDS possesses anti-SARS-CoV activity. The IC50s (50% virus inhibition dosage) were determined to be at 1:160 dilution for the infection Vero E6 cells.
2) In SARS-CoV pseudovirus infection of A549(ACE2) cells, at 1:20 dilution, RDS still inhibited 97.4% viral infection.
3) Mechanistic studies further demonstrated that RDS blocked SARS-CoV S protein-mediated viral entry into ACE2+ target cells.

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Introduction

Respiratory Detox Shot (RDS) is a mixture of traditional Chinese medicine that has been used in China for the management of lung function and SARS-CoV and –CoV-2 infection. We preformed experiments to determine whether RDS has anti-coronavirus activity and quantified its antiviral potency. For the experiments, RDS was serially diluted, and then used to treat Vero E6 or A549(ACE2) target cells to block SARS-CoV pseudovirus infection. Inhibition of viral transduction and viral entry were analyzed for reporter GFP (green fluorescent protein) or Luc (luciferase) expression, and IC50 was determined. The cytotoxicity of RDS was also determined.

Materials and Methods

Viruses: SARS-CoV(GFP) and SARS-CoV(Luc) reporter pseudoviruses were assembled by Virongy LLC using in-house lentiviral assembly system. The infectivity was quantified by infecting in-house A549(ACE2) cells.

Cell culture medium: Cells were maintained in Dulbecco-modified Eagle's medium (DMEM) (Invitrogen) containing 10% heat-inactivated FBS and 1x penicillin-streptomycin (Invitrogen).

luciferase assay: Cells were lysed using Luciferase Assay Lysis Buffer (Promega). Luminescence was measured by using GloMax® Discover Microplate Reader (Promega).

Experimental procedure

A. Testing the anti-SARS-CoV activity of RDS

1) Seeding $1x10^5$ **Vero E6** cells per well in 12-well cell culture plates in 1 ml culture medium. Grow cell overnight at 37°C.
2) The next day, move medium from each well of the 12 well plates. Add 300 ul fresh culture medium,
3) Add 30 ul diluted RDS to treat cells for 30 minutes at 37°C.

   RDS dilution

   A - NO dilution
   B - 1:1 dilution, take 1 ml A + 1ml culture medium
   C - 1:2 dilution, take 1ml B + 1 ml culture medium
   D - 1:4 dilution, take 1 ml C + 1 ml culture medium
   E - 1:8 dilution, take 1 ml D + 1 ml culture medium
   F – 1:16 dilution, take 1 ml E + 1 ml culture medium

4) Add 33 ul Virongy CoV-2-PIE to treat cells for other 30 minutes at 37°C.

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5) Add 100 ul concentrated SARS-CoV(GFP) virus, and then add 10% (V/V) CoV-2-PIE.
6) Incubate for 4 hours.
7) Add 1 ml fresh culture medium with RDS added in concentration as listed above.
8) Analyze cellular GFP expression by flow cytometry at 48 hours post infection.

Results

As shown in **Fig. 1**, RDS inhibited SARS-CoV(GFP) viral infection of Vero E6 cells with an IC50 at around 1:160 dilution. For the experiment, RDS was kept in the cell culture during the whole infection period. For flow cytometry analyses, propidium iodide (PI) was added to stain for dead cells. GFP+ cells were analyzed.

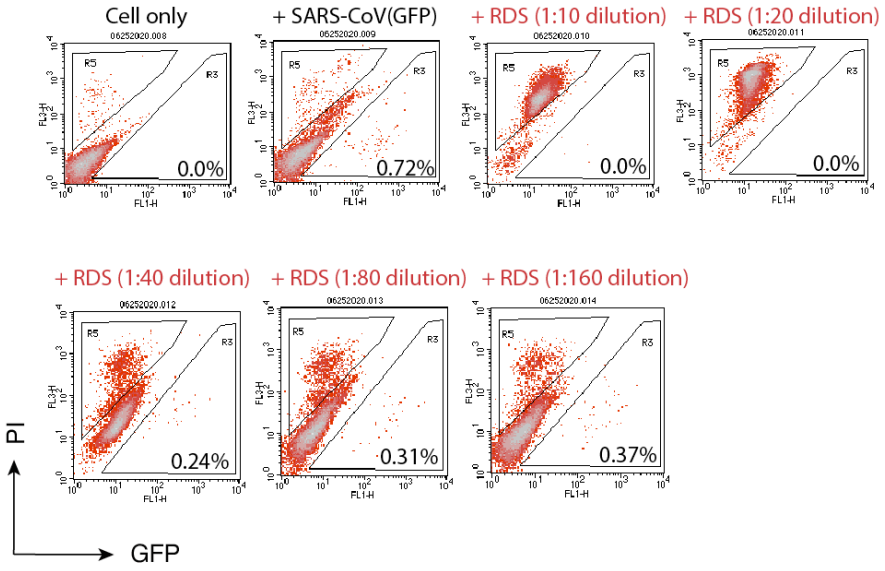

Fig.1. RDS inhibits SARS-CoV(GFP) pseudovirus infection of Vero E6 cells. Vero E6 cells were treated with serially diluted RDS, and infected with SARS-CoV(GFP) pseudovirus, and inhibition of viral infection was quantified at 48 hours post infection by flow cytometry. Uninfected Cell and SARS-CoV(GFP)-infected but RDS-untreated cells were used as controls. The percentages of GFP+ cells are shown. PI, propidium iodide.

B. Testing effects of RDS on the entry of SARS-CoV(GFP) pseudovirus

1) Seeding 1x10^5 **A549(ACE2)** cells per well in 12-well cell culture plates in 1 ml culture medium. Grow cell overnight at 37°C.

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2) The next day, move medium from each well of the 12 well plates. Add 300 ul fresh culture medium.
3) Add 30 ul diluted RDS to treat cells for 30 minutes at 37°C.

RDS dilution
A - NO dilution
B - 1:1 dilution, take 1 ml A + 1ml culture medium
C - 1:2 dilution, take 1ml B + 1 ml culture medium
D - 1:4 dilution, take 1 ml C + 1 ml culture medium
E - 1:8 dilution, take 1 ml D + 1 ml culture medium
F – 1:16 dilution, take 1 ml E + 1 ml culture medium

4) Add 33 ul Virongy CoV-2-PIE to treat cells for other 30 minutes at 37°C.
5) Add 100 ul concentrated SARS-CoV(GFP) virus, add 10% (V/V) CoV-2-PIE
6) Incubate for 4 hours.
7) **<u>After infection, cells were washed twice to remove virus and RDS</u>**
8) Add 1 ml fresh culture medium **without RDS**, culture for 48 hours
9) Analyze cellular GFP expression by flow cytometry at 48 hours post infection.

Results

As shown in **Fig. 2**, RDS inhibited SARS-CoV(GFP) infection of A549(ACE2) cells. For the experiment, RDS was used only during viral infection for 4 hours, and was washed away with the virus after infection. Cells were cultured without RDS following infection. For flow cytometry analyses, propidium iodide (PI) was added to stain for dead cells. GFP+ cells were analyzed only in the live cell population to exclude non-specific effects of cytotoxicity. This experiment demonstrated that RDS blocked viral early infection processes, likely viral entry.

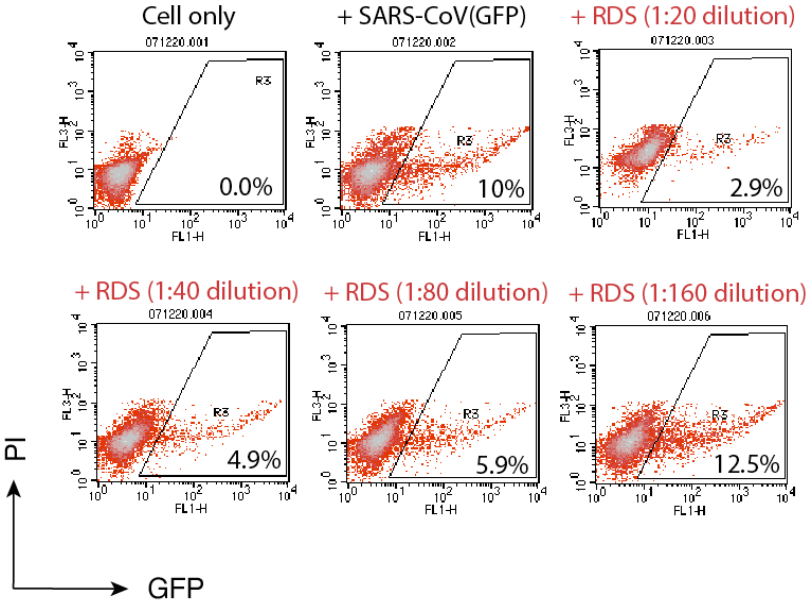

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Fig.2. RDS inhibits SARS-CoV(GFP) pseudovirus entry into A549(ACE2) cells. A549(ACE2) cells were treated with serially diluted RDS, and infected with SARS-CoV(GFP) pseudovirus for 4 hours. Cells were washed to remove the virus and RDS, and cultured in the absence of RDS. Inhibition of viral infection was quantified at 48 hours post infection by flow cytometry. Uninfected Cell and SARS-CoV(GFP)-infected but RDS-untreated cells were used as controls. The percentages of GFP+ cells are shown. PI, propidium iodide.

C. Quantification of the anti-viral activity of RDS using SARS-CoV(Luc) pseudovirus

1) Seeding 1x10^5 **A549(ACE2)** cells per well in 12-well cell culture plates in 1 ml culture medium. Grow cell overnight at 37°C.
2) The next day, move medium from each well of the 12 well plates. Add 300 ul fresh culture medium,
3) Add 30 ul diluted RDS to treat cells for 30 minutes at 37°C.

   RDS dilution

   A - NO dilution
   B - 1:1 dilution, take 1 ml A + 1ml culture medium
   C - 1:2 dilution, take 1ml B + 1 ml culture medium
   D - 1:4 dilution, take 1 ml C + 1 ml culture medium
   E - 1:8 dilution, take 1 ml D + 1 ml culture medium
   F – 1:16 dilution, take 1 ml E + 1 ml culture medium
   G – 1:32 dilution, take 1 ml F + 1 ml culture medium

4) Add 33 ul Virongy CoV-2-PIE to treat cells for other 30 minutes at 37°C.
5) Add 100 ul concentrated SARS-CoV(GFP) virus, add 10% (V/V) CoV-2-PIE
6) Incubate for 4 hours.
7) **<u>After infection, cells were washed twice to remove virus and RDS</u>**
8) Add 1 ml fresh culture medium **without RDS**, culture for 48 hours
9) Analyze cellular GFP expression by flow cytometry at 48 hours post infection.

Results

As shown in **Fig. 3**, RDS inhibited SARS-CoV(Luc) infection of A549(ACE2) cells in a dosage-dependent manner. For the experiment, RDS was used only during viral infection for 4 hours, and was washed away with the virus after infection. Cells were cultured without RDS following infection. The dose-response curve was plotted and the IC50 of RDS was calculated to be at the dose of 1:70.88 dilution.

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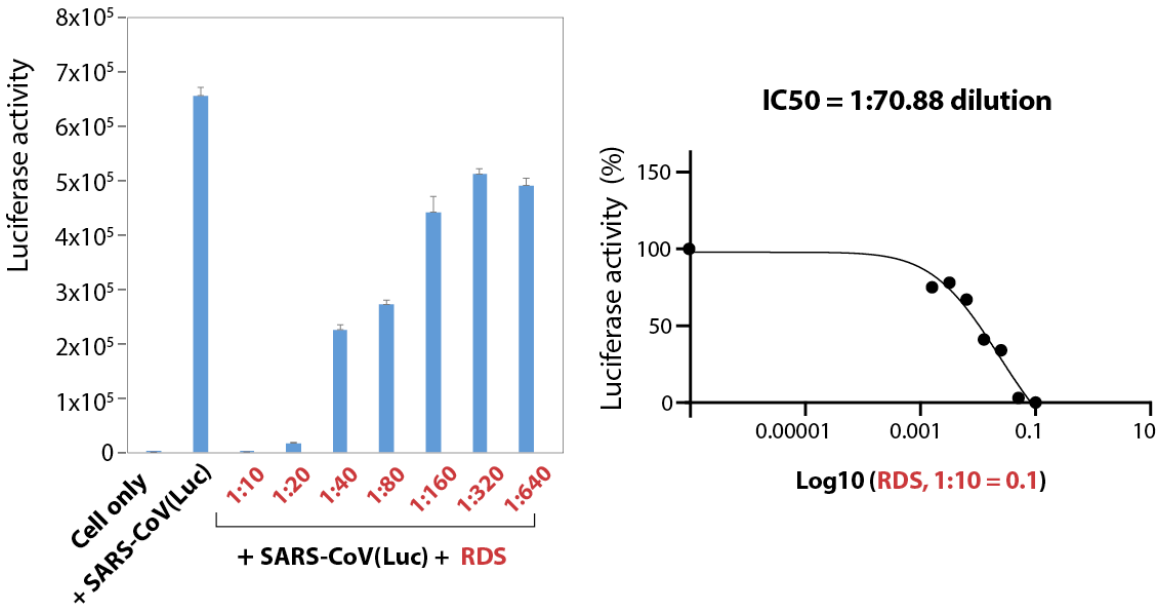

Fig. 3. RDS dosage-dependent inhibition of SARS-CoV(Luc) pseudovirus. A549(ACE2) cells were treated with serially diluted RDS, and infected with SARS-CoV(Luc) pseudovirus for 4 hours. Cells were washed to remove the virus and RDS, and cultured in the absence of RDS. Inhibition of viral infection was quantified at 48 hours post infection by luciferase assay. Uninfected Cell and SARS-CoV-GFP-infected but RDS-untreated cells were used as controls (left panel). The assay was performed in triplicates. The dose-response curve was plotted, and the IC50 of RDS was at 1:70.88 dilution (right panel).

D. Cytotoxicity quantification of RDS on A549(ACE2) cells.

1) Seeding 1x10^5 **A549(ACE2)** cells per well in 12-well cell culture plates in 1 ml culture medium. Grow cell overnight at 37°C.
2) The next day, move medium from each well of the 12 well plates. Add 300 ul fresh culture medium,
3) Add 30 ul diluted RDS to treat cells.

RDS dilution

A - NO dilution
B - 1:1 dilution, take 1 ml A + 1ml culture medium
C - 1:2 dilution, take 1ml B + 1 ml culture medium
D - 1:4 dilution, take 1 ml C + 1 ml culture medium
E - 1:8 dilution, take 1 ml D + 1 ml culture medium
F – 1:16 dilution, take 1 ml E + 1 ml culture medium
G – 1:32 dilution, take 1 ml F + 1 ml culture medium

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4) Incubate for 4 hours.
5) After infection, cells were washed twice to remove RDS
6) Add 1 ml fresh culture medium without RDS, culture for 48 hours
7) Analyze cytotoxicity by staining cells with propidium iodide and flow cytometry.

Results

As shown in **Fig. 4**, for a short treatment of cells with RDS, there was noticeable cytotoxicity at 1:10 and 1:20 dilutions. There was also low-level toxicity at 1:40 dilution, but there was no detectable cytotoxicity at 1:80 dilution. The dose-response toxicity curve was plotted, and the LC50 of RDS was calculated to be at 1:11.89 dilution.

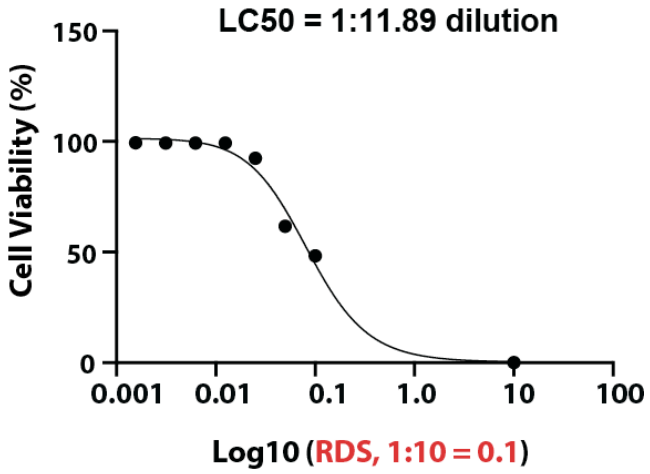

Fig. 4. Quantification of the cytotoxicity of RDS. A549(ACE2) cells were treated with serially diluted RDS for 4 hours. Cells were washed to remove RDS, and cultured in the absence of RDS for 48 hours. The dose-response cytotoxicity curve was plotted, and the LC50 of RDS was calculated to be at 1:11.89 dilution.

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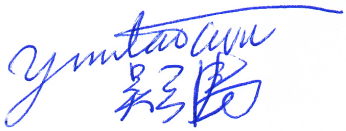
I certify that the experiments and results presented above in this report were conducted in Virongy LLC located at 11100 Endeavor Ct. Lab 119, Manassas, VA 20109, USA.

Signature:



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