The Effect of Sucrose on the Stability of Respiratory Syncytial Virus

By

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An Undergraduate Thesis Submitted to

Oregon State University

In partial fulfillment of the

requirements for the degree of

Baccalaureate of Science in BioResource Research

Biotechnology

5/7/2013

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Abstract

Respiratory Syncytial Virus (RSV) is a major concern in infants, the elderly, and immunocompromised individuals. There is currently no vaccine available. While it causes a significant number of hospitalizations and complications, RSV is a thermo labile virus and not very stable in the environment. This matter complicates the creation of a vaccine, which needs to be a stable live-attenuated virus in order to induce a significant immune response. The purpose of this research is to study the effect of sucrose on RSV. Our hypothesis is that sucrose will have a stabilizing effect on the virus. The two objectives of this research are to determine RSV titers using plaque assays and to study the effects of a variety of sucrose concentrations to determine the optimum concentration that has a significant effect on stabilization of the virus. Our data show that the only concentration that was significant was 0.3% sucrose. These experiments will need to be repeated to ensure reproducibility, but remain as a good first step in determining the optimum sucrose concentration for RSV stability.

Introduction

Respiratory Syncytial Virus strain A-2 (RSV) is an airborne acquired virus that is a major threat to infants, the elderly, and immunocompromised individuals causing significant mortality with a high incidence of disease (1, 2). RSV also causes significant monetary and work-related time losses, with 78,000 hospitalizations per year and an annual hospital cost of ~$650 million (3). In infants, it causes bronchiolitis, a lower respiratory infection of the bronchioles that is characterized by increased airway resistance, wheezing, and breathlessness. Infants can have difficulty feeding.

The virus causes about 199,000 deaths worldwide with a global annual infection of 64 million. There is an estimated 34 million cases of lower respiratory disease (4). The virus can affect severely immunocompromised bone marrow transplant recipients where it can cause pneumonia, there is no cure, and the virus is easily spread (5).

The virus is transmitted either by direct contact or aerosol droplets. In about fifty percent of cases, infants that developed lower respiratory infections due to RSV disease have a persistent wheeze until they are about eleven. Although there is no evidence for causality in this persistent coughing, it does help to note how dangerous this virus can be and how important it is to develop a vaccine (5). Study of RSV disease transmission is important because there is currently no effective treatment or vaccine available.

RSV is a negative strand RNA virus from the Paramyoxviridae family. It is an enveloped virus with a genome-encoded mRNA cap and poly A tail on the mRNA. Its envelope F protein causes syncytia formation in cell culture and in the lungs of infected patients while its G protein is responsible for the attachment of the virus. By forming syncytia, the virus can spread from cell

to cell without alerting the host's immune system. The RSV proteins are depicted in Figure 1. These two proteins are important to the immune response of the patient, activating both the innate and adaptive immune system. These proteins cause an inflammatory response, which leads to a majority of RSV symptoms. RSV buds out of the cell and uses the host membrane with F and G proteins intermingled to spread between cell-to-cell and host-to-host (because of syncytia formation). The nucleocapsid is a group of proteins that are responsible for protecting the RNA genome and antigenome and to shield the virus from the host immune system, by dispersing itself in the host membrane that the virus used when budding out of the cell (5).

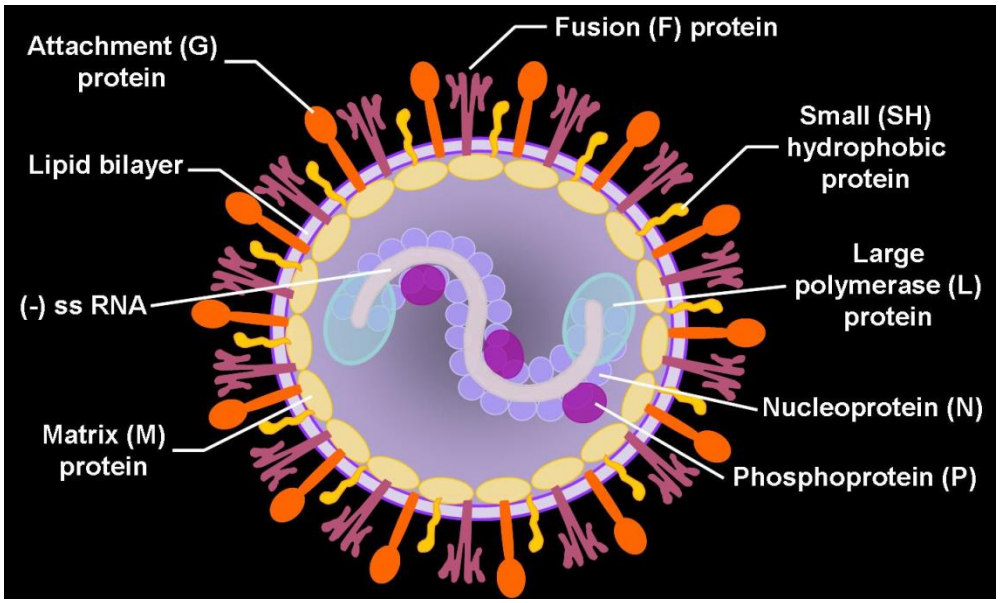

Figure 1: The structure of an RSV virion. From http://www.kuleuven.be/rega/mvr/images/RSV-1.jpg.

Preliminary research (Ausar, et.al) has found that sucrose—and many other sugars—has a stabilizing feature on the RSV coat (3, 6). This feature may protect the virus against denaturing in normal environmental conditions. RSV is a thermolabile virus that only lasts on surfaces for a few hours before denaturing. It is easily destroyed by detergents like soap and ethanol as typical for enveloped viruses. The virus titer is reduced after three months at -70°C. An important part to developing a vaccine is to make the virus more stable so that an attenuated version can warrant a

sufficient protective immune response. Another interesting aspect is how the stabilizing effect of sugar affects the rate of transmission between hosts.

Hypothesis

There are many aspects that could be focused on this topic, but the hypothesis of this research is that sucrose may have stabilizing effects on the RSV envelope and will help shield against environment temperature fluctuations. This increased stability of the virus may help it to not denature as easily and help person-to-person transmission by stabilizing the virus envelope. This also may help in the development of an effective vaccine, which requires the virus to remain stable for a long time. This project will also confirm previous findings in other literature.

Objectives

There are two objectives for this project. The first objective is to optimize RSV cell culture infection protocol and to determine RSV titers using plaque assays. RSV plaques are small and difficult to count, and because there has been difficultly in succeeding in this assay, it would be beneficial to make sure that it is done correctly and good data is obtained. The second objective will coincide with the first. A variety of sucrose concentrations (0.1%, 0.3%, 0.5%,2%, 5%, to 7%) will be assayed to determine the optimal sucrose concentration that will stabilize the virus.

The results of this project could prove to be useful as preliminary data to show any correlations between sucrose and virus stability. For a vaccine to have an effect, the virus must not denature as easily as it currently does. Since RSV can only be stabilized in the environment for a brief amount of time, an RSV vaccine would have to have some type of stabilizing adjunct, a possibility being sucrose, in order for the virus to be stable long enough to replicate in the vaccinated person in order to cause a sufficient immune response.

Materials

Virus and Cell Culture

The type of cell culture used in this research is HeLa cells, a type of immortal cell line from a patient who had cervical cancer. These cells were used because of their convenience as a cell culture model and availability in the lab. Since they come from a human donor and RSV is a human pathogen, this cell type makes a good cell culture model. The type of virus used is RSV A-2. It is grown in cell culture for 5 days, and the released virus in the media is collected and stored in a -80°C freezer after it is purified.

Sugar Media

This is made by dissolving the appropriate amount of sucrose in Gibco® Dulbecco's Modified Eagle Medium (DMEM) to make a 14% concentration and then filter sterilizing it to ensure that there is no contamination. Different lower concentrations of sucrose were made using this stock concentration by diluting in media for the experiments. For this project, 200mls of the sugar media was made, so the calculations are as follows:

14/100=0.14*200=28g of sucrose dissolved in 200mLs of DMEM

DMEM is bought through Invitrogen.

Antibody Step Materials

10xPBS: 80g of NaCl; 2g KCl; 11.5g $Na_2HPO_4*7H_20$; 2g KH_2PO_4; pH≈7.4. Heat and stir the components in 800mLs of deionized water. Adjust the pH to 7.4 and add the remaining 200mLs of water. Autoclave for 25 minutes to sterilize.

1xPBS: This is found using the equation $C_1V_1=C_2V_2$ where C_1 is 10X and C_2 is 1x. V_2 is the total volume and V_1 is the value being determined.

Goat Anti-RSV polyclonal antibody (primary antibody): 1:1500 dilution, 100µL per well; the antibody is diluted using 0.5% Milk Blocking Buffer (MBB) which can be made using the

equation $C_1V_1=C_2V_2$; where C_1 is 5% MBB and C_2 is 0.5%. The amount of 0.5% MBB is determined by multiplying the amount of antibody needed per well (100µL) and the amount of wells used in the experiment. The amount of antibody can then be determined by dividing the 0.5% amount by 1500, because the dilution is a 1:1500 dilution. This antibody is bought at Millipore and aliquoted in 0.5mL tubes. The aliquots are kept in the -80°C freezer.

Donkey Anti-Goat HRP antibody (secondary antibody): This is diluted the same way as the primary antibody, but it is a 1:7000 dilution. 125µL is used per well and the amount of 0.5% MBB calculated is then divided by 7000 to get the amount of secondary needed. This antibody is bought from Rockland Inc. and stored in the fridge. When using it, the lights are kept off at all times. The TMB Small Particle One Component HRP Membrane Substrate is bought through VWR International, LLC and also stored in the fridge.

Methods

The project was split into two parts: testing the titer of the virus and the multiplicity of infection; and testing various concentrations of sucrose on the replication of the virus.

Titer assay

Cell culture:

Day one consisted of seeding a twelve well plate with HeLa cells. The first few steps of all seeding (and passaging of the cells) is to aspirate the media, deposit in the waste, add 5 mLs of PBS to wash the cells, ensuring that they do not dry out and all of the waste media is captured. Then 1 mL of trypsin is added to the flask and the cells are incubated for ten minutes to allow the trypsin to break the cells from the wall of the flask. Once the cells are off of the flask, they are then pipetted vigorously to separate clusters into individual cells. The cell suspension is then

transferred to a sterile beaker and diluted with 5-7 mLs (10 mLs if over 90% confluent) of DMEM to allow single cell counting.

The twelve well plate was seeded at 270000 cells/ml, which means there were 270,000 cells per well. The cells were counted using a hemocytometer, which divides the cells into four grids, and cells/ml is determined by averaging the counts of all the grids and multiplying by 10000. The equation $C_1V_1=C_2V_2$ is used to find the amount of media to dilute the cells. C_1 is the cell concentration found by the hemocytometer. V_1 is the volume that is being determined. C_2 is the cell concentration per milliter that is desired. In this assay it is 270000 cells/mL. V_2 is the total volume that is needed to cover the plate. In this assay, because a twelve-well plate is being seeded and there is 1 mL per well, the final volume is 13 mLs. A little bit extra is needed to ensure that all wells get the right volume.

Once V_1 is found, the cell suspension is added to a sterile beaker and mixed thoroughly, maintaining the single, broken-up cells. 1mL is then transferred to each well and the plate is shaken vigorously to make sure the cells are evenly distributed. This is important so that the virus titer is as accurate as possible because the virus has equal access to all of the cells.

The plate is then incubated for twenty-four hours, allowing the cells to stick and start to grow. The cells need to be 90% or higher confluent so as to allow as much virus as possible to infect the cells. No definite multiplicity of infection is used at this point because this is a titer assay, which will determine the titer of the virus where any amount of virus particles can enter a cell.

Virus infection:

The next day, the virus is diluted using a ten-fold dilution. The dilutions plated are 10^{-6} and $5x10^{-7}$. These dilutions were used because plaques will be visible and countable at this range. Additionally, the same tube was used for all 10^{-6} and $5x10^{-7}$ dilutions.

The plate was infected for an hour, shaking every eight minutes to ensure the infectious material was spread over the whole well.

During this time, the seaplaque agarose overlay was prepared. The overlay is used to keep the virus from spreading to the whole well; instead, confining them to little areas of infected cells, or plaques, that can be counted later. Normally, only 1% of seaplaque is used, but this assay also tested the comparison between using 1% agarose and 2% agarose.

After infection, the agarose was added to the wells and allowed to solidify. The plate was then upturned and incubated for five days to allow the plaques to become big and countable.

Plaque staining and counting:

Day seven is when 4% paraformaldehyde is added to fix the cells and virus, which will allow them to be stained. The procedure for staining is as follows: Aspirate the 4% paraformaldehyde and carefully remove the plugs, taking care not to scratch the wells. Add 500µL of 5% Milk Blocking Buffer (MBB) to the wells and incubate on a rocker for 30 minutes. Aspirate the 5% MBB and add 100µL of the diluted primary antibody. Incubate for 30 minutes. Aspirate the antibody and wash the wells with blot wash buffer three times for five minutes each. Aspirate the wash and add 125µL of diluted secondary antibody. Incubate for 30 minutes. Wash three times with blot wash buffer for five minutes each. Add 125µL of TMB Small Particle One Component HRP Membrane Substrate to the wells and incubate for 30 minutes to allow for the blue stain to appear. The substrate reacts with the peroxide of the secondary antibody, allowing

for the blue color of the plaques to be seen. Once stained, the blue plaques are then counted and the titer is determined by this equation: PFU/mL= average plaque counts of replicates/ (plate dilution X sample dilution)

See **Antibody Materials** above for a full description on how to prepare the materials necessary for all five assays.

Testing replicates of virus dilution using all twelve-well plate for consistency:

The purpose of this assay was to test the consistency between the virus sample dilutions (replicates). The same dilution was used for the four replicates. One twelve well plate was used.

Day one consisted of seeding a twelve well plate. The plate was incubated for over twenty-four hours to ensure 90% confluency. Since this is also a titer plate, the titer will be determined and the average titer will be used throughout the rest of the project.

Day two consisted of using the virus sample dilution that was determined from the first titer plate and gave the most consistent results to infect the twelve well plate.

15mLs of agarose was used: 0.15 grams was heated and dissolved in 3mLs of sterile deionized water. The agarose was then placed in the water bath for the remainder of the infection to ensure that it didn't solidify. 12mLs of DMEM was heated with 15 minutes remaining in the infection. The two were mixed with about two minutes left of infection. After the plugs were allowed to solidify, the plate was incubated for five days.

On the seventh day, 4% paraformaldehyde was added and the plaques were stained according to the procedure mentioned for the first titer assay.

Multiplicity of Infection Assay

The purpose of this assay was to mimic the conditions of the sugar assays while validating expected results. Using the multiplicity of infection (MOIs), 0.01, 0.1, 1, the expected

titers should increase logarithmically. This assay is also testing the consistency between replicates.

Instead of seeding the whole twelve well plate, only 9 wells were needed, bringing the final volume of media containing cells to 10mLs. The plate was seeded as stated above (pg. 5).

Day two consisted of the infection using the following MOIs: 0.01, 0.1, and 1. The stock virus titer used was $4.32x10^8$ PFU/mL. The calculations were as follows:

0.01MOI calculation: the stock virus titer was diluted to 10^{-2} dilution, which gave a new titer of 4,320,000 pfu/ml. This was used to calculate the amount of virus to use in this MOI. MOI= #of virus particles/ # cells. With an MOI of 0.01, the number of virions needed is 2000. The number of virus particles is then divided by the titer and multiplied by 1000 to give a value in microliters. The value is multiplied by 3.5 to account for the number of wells needed. The amount of virus needed from the 10^{-2} dilution is 1.62µL.

0.1MOI calculation: This value was also calculated using the previous 10^{-2} dilution. The amount of virus needed for 0.1 MOI is 16.2µL of the 10^{-2} dilution.

1.0 MOI calculation: This value did not need a dilution and so the resultant amount of pure virus needed was 162µL of the stock virus with a titer of $4.32x10^8$ PFU/mL.

All three of the tubes were vortexed three times before each use. The plate was infected for an hour, shaken every 8 minutes, and incubated with 9 mLs of media for twenty-four hours. Because the plaque assay doesn't actually start until the third day, media needs to be added back to the cells so that they don't die and the infection is allowed to continue. After 48 hours, the cells are then scraped off with the open end of a pipette tip from each of the well and placed into 1.5mL microcentrifuge tubes separately. The infected media from each of the 9 wells will be used for plaque assays to determine the virus titer.

Plaque assay from 0.01, 0.1 and 1.0 MOI infection assay:

Three 24 well plates were seeded with cells on the second or early third day, because they were used in the plaque assay.

Day three: ten-fold dilutions of the virus collected from the infected cells in the twelve-well plate above were made. First, the tubes with appropriate amounts of media are prepared (0.9 mL media and 0.1 mL virus for 10^{-1} dilution).

For the 0.01 MOI, cells were infected with dilutions of 10^{-2} and 10^{-3} in triplicate and 10^{-1} dilution in duplicate. For the 0.1 MOI, cells were infected with dilutions of 10^{-3} and 10^{-4} in triplicate and 10^{-2} dilution in duplicate. The 1 MOI had four dilutions made (10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}) but only three of them were used to infect cells: 10^{-4} and 10^{-5} in triplicate and 10^{-3} in duplicate. 100µL/well of each dilution were used for infection of cells and the plate was infected for an hour with shaking every eight minutes. A total of 40mLs of seaplaque agarose was made as described in the Testing Replicate section and 500µL was added to each well. The plates were then incubated for five days.

Day eight is when 500µLs of 4% paraformaldehyde were added to the 24 well plates and incubated for two hours. The plaques were stained and counted according to the procedure outlined in the Titer assay section.

Sucrose Assay 1

The sucrose media was made according to the protocol outlined in the Materials section. The first day is dedicated to seeding a twelve-well plate according to the procedure outlined above. The plate is then incubated for 24 hours. The sucrose concentrations 0.3, 2, and 7, and a negative control (DMEM) were incubated with virus on ice for one hour. The calculations are as follows:

Desired sucrose concentration is determined using the equation $C_1V_1=C_2V_2$, where C_1 is the sucrose stock concentration (14%), and V_1 is the variable to be found. C_2 is the desired sucrose concentration and V_2 is the total volume (700µL).

The amount of virus is determined by using the MOI calculation: MOI= # virus particles/ amount of cells seeded. The value can then be divided by the titer ($4.32x10^8$ pfu/mL) and multiplied by 1000 and the number of wells needed (3.5) to get the desired virus amount. In this and the next experiment an MOI of 2 will be used. The two values are subtracted by 700 to obtain the amount of media needed. The concentrations are plated in triplicate.

After incubation, the media is aspirated from the plate and 200µL of sucrose treated or mock treated infectious material is added. The plate is incubated for one hour, with shaking every eight minutes. After one hour of infection, the infectious material is then aspirated and 1mL of media is added to each well. The plate is then incubated for 24 hours. Four 24-well plates were seeded and incubated for 24 hours to be used in the plaque assay.

Day three is when the dilutions of sucrose treated virus collected from the above experiment were made to determine the virus titer using a plaque assay. They were prepared the same way as stated in the MOI assay section, with different dilutions prepared as needed. In this experiment the following dilutions were made:

For 7% and 2% sucrose-treated virus: 10^{-2}, 10^{-4}, $5x10^{-5}$ dilutions, with 10^{-4}, $5x10^{-5}$ being plated in triplicate. For 0.3% sucrose and control (DMEM): 10^{-2}, 10^{-4}, $5x10^{-5}$, 10^{-5} dilutions, with $5x10^{-5}$, 10^{-5} being plated in triplicate. These dilutions were chosen because the plaques would be easier to count which would lead to a better confidence that they were counted correctly. $5x10^{-5}$ should have twice as many plaques as 10^{-4}, and half as many plaques as 10^{-5}. The plates were then incubated for one hour with shaking every eight minutes. 40 mLs of agarose was made and

500µL of it was added to each well once the infectious material was aspirated. The plugs were allowed to solidify and the plates were incubated for five days.

Day eight is when 500µL of 4%paraformaldehyde was added to each well and incubated for 2 hours. The 4% paraformaldehyde was then aspirated, the plugs extracted, and the wells were then stained and plaques were counted.

Sucrose Assay 2

The sucrose concentrations used for this assay were 1%, 0.5%, and 0.1%. The concentrations were made using the procedure outlined in the first sucrose assay. The first day consisted of seeding a twelve well plate. The second day consisted of incubating the various sucrose concentrations (in triplicate) along with a control (0.0% sucrose)for one hour and then added to the wells. The rest of the procedure for day two is identical to the first sucrose assay.

On day three, the dilutions were made for each treated virus collected from the above infection. They were prepared the same way as stated in the MOI assay section, with different dilutions prepared as needed. In this experiment, the following dilutions were made: for 1%, 0.5%, 0.1% sucrose concentrations all had 10^{-2}, 10^{-4}, $5x10^{-5}$, and 10^{-5} dilutions with $5x10^{-5}$, and 10^{-5} being plated in triplicate. Control had 10^{-2}, 10^{-4}, and $5x10^{-5}$, with 10^{-4}, and $5x10^{-5}$ being plated in triplicate. The cells were infected for an hour with shaking occurring every eight minutes. The seaplaque agarose was prepared the same way as in the Sucrose assay 1, with 45mLs being used instead of 40mLs. The plugs were applied to the wells after the infectious material was aspirated and allowed to solidify. The plates were incubated for five days.

On day eight, 500µLs of 4% paraformaldehyde was added to the wells and the plates were incubated at room temperature for two hours. The 4% paraformaldehyde was then aspirated and the plugs carefully taken out. The wells were then stained and plaques counted.

Results

Titer Assay:

The purpose of the titer assay was to determine RSV titer in normal conditions without the use of sucrose as a treatment condition. The only condition that was different was that half the plate had a seaplaque agarose concentration of 1% and the other half had a concentration of 2%. The expectation was that the titer would be less than $2.8x10^8$ pfu/mL, which was obtained from a previous experiment conducted in September 2012. The titer obtained in this assay was actually much higher than the anticipated titer. Using the virus dilution of $5x10^{-7}$ for infection that gave plaque counts more consistent results, the titer came out to be $4.16x10^8$ pfu/mL for 1% and $3.93x10^8$ pfu/mL for 2% agarose.

Testing replicates of virus dilution using all twelve-well plate for consistency:

The purpose of this experiment was to confirm the results of the titer assay and to test consistency within replicates and dilutions. The dilution used for initial infection in this experiment was 10^{-6} because it gave higher counts, and since there was a bit of discrepancies in the last experiment, the hope for this assay was to rectify those differences.

The results show that plaque counts were consistent with one another and between replicates as shown in Table 1 with standard deviations of 3.51, 5.77, 2, and 1.73 for replicate 1, replicate 2, replicate 3, and replicate 4, respectively. The average pfu/mL was $4.32x10^8$ and was used throughout the rest of the experiment in the MOI calculations.

Table 1

Replicate 1	Replicate 2	Replicate 3	Replicate 4
86	86	86	86
90	86	84	83
83	96	88	83
$4.32x10^8$ pfu/mL	$4.47x10^8$ pfu/mL	$4.3x10^8$ pfu/mL	$4.2x10^8$ pfu/mL

Table 1: Results from the whole plate dilution assay. The average was $4.32x10^8$ pfu/mL and was used for all subsequent MOI calculations. The replicates together had a standard deviation of 3.68.

MOI assay:

The purpose of this assay was to replicate previous results, to test consistency between replicates, and to imitate the conditions that would be seen with the sucrose assays. For an MOI of 0.01, the most consistent results were from the 10^{-2} dilution with an average pfu/mL being 38444. For an MOI of 0.1, the average pfu/mL was 744444. For an MOI of 1 the average was $2.63x10^6$ pfu/mL. The values were graphed in LOG format to show the expected log increase between the different MOIs (Figure 2).

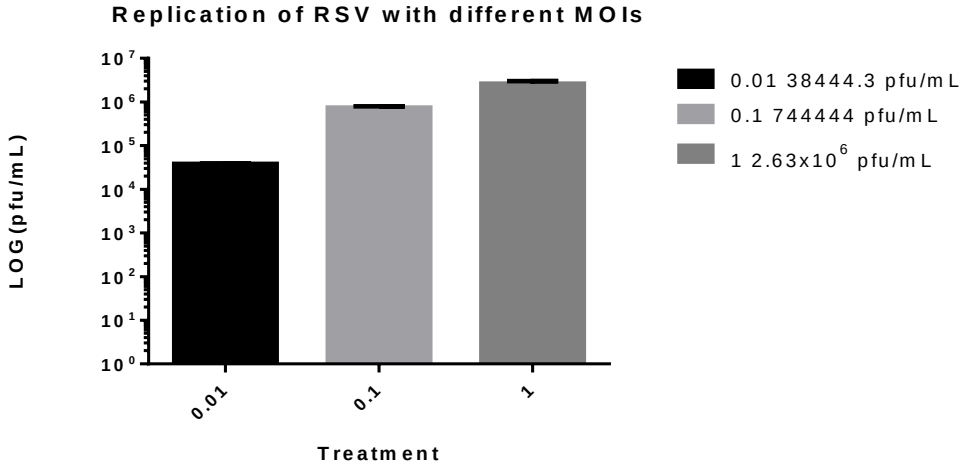

Figure 2: Graph of the replication of RSV using different MOI treatments. The averages are graphed in LOG(pfu/mL) to show differences between the 0.01, 0.1, and 1. Experiments were conducted in duplicates or triplicates and the averages of the MOIs are given in the legend.

Sucrose Assay 1

The purpose of this assay was to test the effects of sucrose concentrations 0.0% (control), 0.3%, 2%, and 7% on the stability of the virus. Equal amounts of the virus stock (3.2µL) were mixed with various concentrations of sucrose and DMEM, incubated on ice for 1 hour, and then plaque-assayed. The results are graphed in Figure 3. The average for the control was $9.622x10^6$ pfu/mL. The averages for 0.3%, 2%, and 7% sucrose treatment were $1.35x10^7$ pfu/mL, $1.09x10^7$ pfu/mL, and 9.24x106 pfu/mL, respectively. The counts were very consistent for the control, 2% and 7% sucrose treatment as shown by the small error bars. 0.3% had a larger error bar and was the only value to be significant against the control (Table 2). This suggests that 0.3% sucrose may increase virus stability leading to higher titer compared to 2%, 7% sucrose concentrations and control.

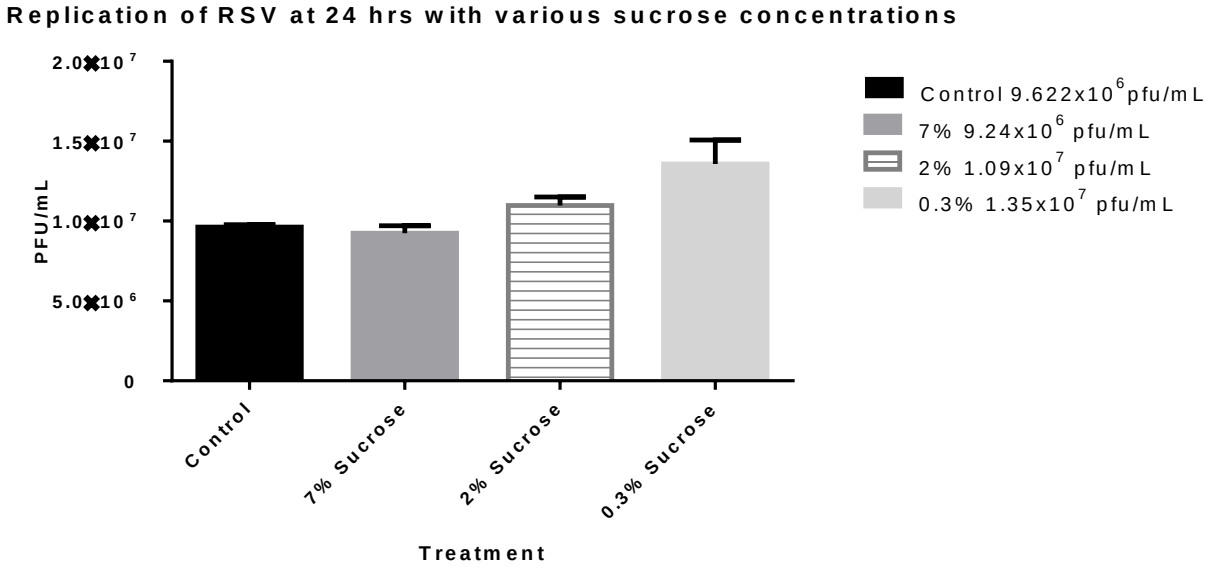

Figure 3: Graph of the replication of RSV at 24 hours after treatment with sucrose concentrations 0.3%, 2%, and 7%. The averages are given in the legend.

Table 2

Tukey's multiple comparisons	Mean Diff.	95% CI of diff.	Significant?	Summary
Control vs. 7% Sucrose	377778	-1.826e+006 to 2.581e+006	No	ns
Control vs. 2% Sucrose	-1.356e+006	-3.559e+006 to 848069	No	ns
Control vs. 0.3% Sucrose	-3.944e+006	-6.148e+006 to -1.741e+006	Yes	**
7% Sucrose vs. 2% Sucrose	-1.733e+006	-3.937e+006 to 470291	No	ns
7% Sucrose vs. 0.3% Sucrose	-4.322e+006	-6.526e+006 to -2.119e+006	Yes	**
2% Sucrose vs. 0.3% Sucrose	-2.589e+006	-4.793e+006 to -385267	Yes	*

Table 2: This shows the comparisons between the effects of different sucrose concentrations on virus titer and the control. It shows the mean difference, the 95% confidence interval of differences, and whether or not the values are significant and how much they are significant. There was significance between the 0.3% and control as well as with 0.3% and 7%, and 0.3% and 2% sucrose concentrations. The p-value was < 0.05 and the R-squared value was 0.8584.

Sucrose Assay 2

The purpose of this assay was to test the effect of sucrose concentrations 0.0% (control), 0.1%, 0.5%, 1% on the stability of the virus. Equal amounts of the virus stock (4µL) were mixed with various concentrations of sucrose and DMEM, incubated for one hour on ice, and then plaque-assayed. A different amount of the virus stock was added then in the previous sucrose assay because the concentration of cells increased as the plate was incubated for longer than 24 hours. The results are graphed in Figure 4. The average for this control was 1.02×10^7 pfu/mL, which is only slightly higher than the first sucrose assay control. Even though these are two different experiments, it was useful to test the significance between the controls, as there have been problems in the past. The controls were not significant. The average virus titer, when treated with 0.1%, 0.5%, and 1% sucrose concentrations were 1.4×10^7 pfu/mL, 1.22×10^7 pfu/mL, and 1.03×10^7 pfu/mL, respectively. The results were consistent, with 0.5%, 1% sucrose

concentrations, and the control having relatively low error bars with none of these values being significant. 0.1% had a larger error bar, but was not significant (Table 3).

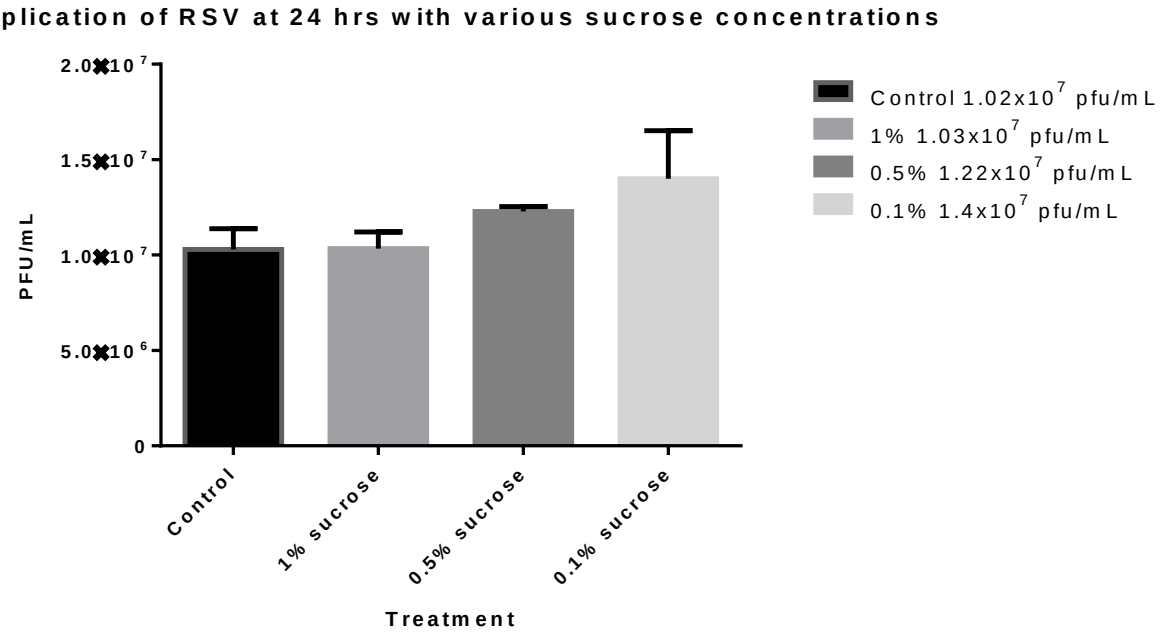

Figure 4: Graph of the replication of RSV at 24 hours after treatment with sucrose concentrations 0.1%, 0.5%, and 1%. The averages are given in the legend.

Table 3

Tukey's multiple comparisons	Mean Diff.	95% CI of diff.	Significant?	Summary
Control vs. 1% sucrose	-44445	-4.340e+006 to 4.251e+006	No	ns
Control vs. 0.5% sucrose	-1.989e+006	-6.284e+006 to 2.307e+006	No	ns
Control vs. 0.1% sucrose	-3.711e+006	-8.007e+006 to 584337	No	ns
1% sucrose vs. 0.5% sucrose	-1.944e+006	-6.240e+006 to 2.351e+006	No	ns
1% sucrose vs. 0.1% sucrose	-3.667e+006	-7.962e+006 to 628782	No	ns
0.5% sucrose vs. 0.1% sucrose	-1.722e+006	-6.018e+006 to 2.573e+006	No	ns

Table 3: This table shows the comparisons between the effects of different sucrose concentrations on virus titer and the control via Tukey's multiple comparisons. The table also shows the mean difference, the confidence intervals, and the significance between each value. There was no significance between the concentrations. The p-value was > 0.05 and the R-squared value was 0.5685.

Discussion

One of the characteristics of an ideal live vaccine is to have stable microbes (which viruses are a type of) that can withstand environmental fluctuations, so that an attenuated version can warrant a sufficient immune response. Preliminary research (Ausar, 2007) has found that sucrose has a stabilizing feature on the RSV coat (3, 6). This feature may protect the virus against denaturing in normal environmental conditions. Our preliminary data show that there is a correlation between sucrose concentration and virus stability.

Because previous experiments didn't yield consistent results and there were many problems with sterile technique and contamination, a set of preliminary experiments was preformed with better sterile technique before the continuation of the sucrose project could commence. The whole project is a conglomeration of assays testing the titer of the virus and using that new titer with a better grasp of the methods to yield consistent results when testing the virus against a variety of sucrose concentrations.

While both of the titer assays gave consistent results, the titer was a lot higher than it was expected. As time progressed, the expected result would have been to see a lower titer than the previous one in September. The titer should also not have changed that much, so to have a bigger titer is an unexpected result. Since the plaque counts were consistent (with a standard deviation of 3.68), the experimental method was probably done right, but because there was a little bit more secondary antibody added than in previous assays the plaques were more visible which could have led to higher counts. It would be beneficial to redo this experiment to confirm the results. There was also a comparison between the seaplaque agarose concentrations, and the data show that there wasn't much of a difference between 1% seaplaque agarose and 2% seaplaque agarose.

Figure 2 shows the expected increase in MOIs ranging from 0.01 to 0.1 to 1. While the results are relatively consistent with each other, the difference between 0.1 and 1 MOI is not as great as expected. When graphed in LOG format, one anticipated seeing a log increase. That is not the case with 1 MOI even though there is a significant increase. There are a few reasons why this could have happened. The plaque counts for 1 MOI are not as consistent between replicates as they are for the 10^{-4} dilution for 0.1 MOI and the 10^{-2} dilution for 0.01 MOI. This could have been a result from not mixing the dilution tubes properly, not doing the dilutions correctly, or not putting enough virus on the cells. Because a repeater pipette was used as it is a quick way to run through the samples, because the plates cannot sit too long, sometimes the pipette would pick up air instead of sample, leading to an incorrect amount of sample in the wells. This would lead to an incorrect plaque count between replicates. To test for consistency, this assay should be repeated.

With the preliminary work done; RSV viability can now be tested under various sucrose concentrations. The hypothesis of this research is that sucrose may have stabilizing effects on the virus, which would keep it from denaturing in the environment and to replicate easier in the host. Through the various sucrose concentrations, it has been determined that 0.3% has the most stabilizing effect by having a higher plaque count than the control and the other concentrations. While there is significance between the control and 0.3%, it is a very small significance. This concentration would have to be tested again to see if it retains its significance. All of the concentrations should be re-tested to make sure that there is no significance between the control and 0.5% or the control and 1%, concentrations that are close in range to 0.3%. 0.1% also was not significant, which means that at some point, the sucrose stops having an effect, if it is having one at all.

The effect that sucrose would be having on the virus is that of stabilizing the surface proteins. Because RSV tends to aggregate when the glycoprotein and the fusion protein change conformations, the sucrose would be acting to stabilize their native conformations by binding and shielding the proteins from the surrounding water so that aggregation does not occur and the virus does not denature (3).

The most interesting findings were treatments with higher sucrose concentrations. Previous literature (Ausar, 2007) states that high concentrations of pure sucrose (20%) has a stabilizing effect on RSV, but 7%, the highest concentration tested here, did not have a significant effect on the virus. The pure sucrose tested in the Ausar study was dissolved in water; whereas, the 14% stock solution was dissolved in a media with a high amount of sugar in the form of glucose (3). After these results were confirmed, future testing could be adding sucrose with virus to media that does not contain the high amount of glucose, but enough nutrients that the cells could still survive without it. This test would show the effects of just sucrose at high concentrations (1%, 2%, and 7%) on the virus. This test would also be good to show the effects of lower concentrations (0.1%, 0.3%, and 0.5%) of sucrose on the virus.

Once all of the results were reconfirmed, it would be good to test the optimum sucrose concentration (in this case 0.3%) at 4 hours, 24 hours, 48 hours, and 3 months incubation with the virus, to see if it still has stabilizing effects. This would be important if one were to use sucrose as a vaccine adjunct, because it would have to stabilize the virus for four to six days in the human host, the common incubation period of RSV (7). It would also be important to test stability after 3 months, because this would ensure that the virus does not denature, and thus the titer is not reduced, after such time in the freezer. Since 0.3% is only slightly significant at one-hour incubation, it probably wouldn't stay as a stabilizing agent at 48 hours incubation.

Conclusion

The results showed that 0.3% sucrose significantly stabilized RSV under the conditions used in these experiments. Other sucrose concentrations tested did not provide a statistically significant stabilization.

Future Work

The stabilizing effect of 0.3% sucrose should be tested at various times after mixing with the virus; for example, at 4 hours, 24 hours, 48 hours, and 3 months. This would provide an additional dimension to the stabilizing effect and would also help in researching the virus, because the virus would not denature during long periods in the freezer.

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