Investigating the Inhibition of Herpes Simplex Virus-1 by Ginsenoside 20(S)-Rg3

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ABSTRACT

Herpes simplex virus, type 1, (HSV-1) is a common viral pathogen. The majority of infections occur during childhood. People who exhibit symptoms from HSV-1 infection can experience genital/oral ulcers (cold sores), latency in sensory neurons, and necrotizing encephalitis, and once HSV has infected the host organism, the individual may be infected for life. This study investigated inhibition of the herpes simplex viruses, type 1 by Ginsenoside 20(S)-Rg3 (Rg3), since this ginsenoside has been reported to inhibit HSV. Mice were dosed with specific amounts of Rg3 and infected with HSV-1. Tissues including brain, spleen, liver, and serum were collected and analyzed for the presence of HSV-1 by the polymerase chain reaction. Although Rg3 treatment appeared to be successful *in vitro*, it was not successful in inhibiting the virus in the living mice.

Introduction

Herpes simplex virus, type 1 (HSV-1), is a widespread pathogen that attaches to a host's cell, where it can enter, spread into other host cells, and replicate in epithelial cells to establish the infection (Amin et al., 2011). Herpes simplex virus type-1 can spread from one host cell to another utilizing viral glycoprotein and host receptors (Amin et al., 2011). The infection may remain in the host organism for life, typically in its latent infectious state (Amin et al., 2011).

Herpes simplex virus type-1 impacts a large portion of the population, having a 67% global prevalence rate (Looker et al., 2015). In the United States, most adults are infected with HSV-1 with the number increasing linearly with age (McQuillan et al., 2018). A larger percentage of females have been reported to be infected than males (McQuillan et al., 2018). The prevalence of HSV was greatest in the Mexican American population and lowest among non-Hispanic white persons (McQuillan et al., 2018).

Most of the population that has contracted HSV-1 will remain asymptomatic throughout their lifetime (McQuillan et al., 2018). People who exhibit symptoms of HSV-1 infection can experience genital/oral ulcers (cold sores), latency in sensory neurons, and necrotizing encephalitis (Ives and Bertke, 2017). Once HSV has infected the host organism, the individual may be infected for life; they will have the potential for severe complications throughout life (Ives and Bertke, 2017). Unfortunately, there is a high infant mortality rate associated with HSV during delivery when the infant contracts the virus at birth from the mother's infection (Ives and Bertke, 2017). The viral infection is particularly dangerous for at-risk populations including immune-compromised individuals, infants, and the elderly (Ives and Bertke, 2017).

Research into HSV-1 is vital to understand the infection and creating more effective treatment options. No vaccine yet exists to prevent the disease (Carter, 2019). Drugs, including acyclovir and penciclovir, are used for the treatment of HSV-1 and HSV-2 symptoms (Piret and Boivin, 2011). Acyclovir and penciclovir are considered the first line of treatment once the symptoms have occurred. As drugs are more widely used and distributed, a large portion of the HSV population has become resistant, creating a need for new drugs or a potential vaccine (Piret and Boivin, 2011).

Time, effort, and money have been dedicated to producing a vaccine for HSV-1, and yet none has been successful (Carter, 2019). A major issue for developers is that the HSV-1 infection can lie dormant in host cells for years before presenting symptoms (Carter, 2019). All these factors make it difficult for developers to create a vaccine that is effective against the infection. Alternative preventive strategies are being investigated.

One way researchers approach finding cures for ailments is by turning to the past. Panax ginseng (P. ginseng) is a root widely used for thousands of years in China for a variety of illnesses, typically consumed as tea (Coleman et al., 2003). Prior to the modern era, it was widely held that if a person had a cold sore (HSV) they could drink the tea and make the cold sore disappear (Coleman et al., 2003). Ginsenosides are bioactive compounds that are used in a variety of ways. Researchers have established a scientific basis back in 2003 for the healing qualities of P. ginseng. The root contains 50 ginsenosides, each with the potential for medical benefits (Coleman et al., 2003). Early results show a correlation between P. ginseng and quality of life (assessment of an individual's overall well-being), although more research needs to be conducted to further understand ginseng's full potential (Coleman et al., 2003).

Ginsenosides are naturally produced glycoside steroids and triterpene saponins that have been shown to have anti-viral properties and immune-boosting abilities (Im et al., 2016). Ginsenoside 20(S)-Rg3, when added to HSV-1 in cell culture significantly inhibited the virus from infection (Wright and Altman, 2020). Additional research and studies in more complex organisms must be completed to better understand the Rg3 virus relationship.

Besides Ginsenoside 20(S)-Rg3, there are several other ginsenosides with promising results including extracts from Korean red ginseng (KRG). Korean red ginseng has various benefits including a link to immune modulation in mice (Cho et al., 2013). Mice were given variable amounts of KRG extract and were then infected vaginally with HSV. It was found that KRG lowered the severity of the HSV infection, increased resistance, and accelerated clearance of the virus (Cho et al., 2013). It also showed an increase in mRNA expression of several mouse genes, which indicates that KRG increases local natural killer cells, stimulating resistance to not just vaginal HSV but other similar infections (Cho et al., 2013).

The work that was conducted builds on previous studies and resources, which have been approved by Middle Tennessee State University's Institutional Animal Care and Use Committee, protocol ID 17-3015. Mice were injected with HSV-1 and specific amounts of Rg3 (10 mg/kg, 3 mg/kg, 1 mg/kg, and 0 mg/kg Rg3). Controls did not receive either HSV-1 or Rg3). Serum, spleen, liver, and brain tissues were collected and evaluated for the presence of infectious HSV-1 by cell culture plaque assay. Plaque assays are a widely utilized tool in virology. They are considered the single best way to ensure recombinant viruses can be isolated as a plaque (Leland and Ginocchino, 2007). While cell culture has long been considered the gold standard for virus detection (Leland and Ginocchino, 2007), others have reported better sensitivity

for the detection of HSV-1 by polymerase chain reaction (PCR) (Wald et al., 2003). The study evaluated samples from mice for the presence of HSV-1 using this more sensitive method. A commonly used method involves the use of a gel electrophoresis, a technique used to separate molecules based on their size and electrical charge (Wald et al., 2003).

Polymerase chain reaction (PCR) has become a staple in biotechnology, forensic science, and research laboratories across the world (Zhu et al., 2020). Polymerase chain reaction amplifies DNA sequences in vitro. Primers are used to amplify desired regions of DNA (Zhu et al., 2020). The success and failure of a PCR test largely depends on the quality of the nucleic acids (Zhu et al., 2020).

A polymerase chain reaction is initiated by denaturing a DNA double helix into two separate single-stranded DNA segments (Zhu et al., 2020). The enzyme Taq polymerase uses the 3′ OH of the primer bound to a SS DNA template to build a complementary strand (Zhu et al., 2020). The result is two identical strands of DNA are repeatedly copied in subsequential rounds of amplification. The process continues exponentially until it is stopped (Zhu et al., 2020).

Polymerase chain reaction drove modern-day forensic science. With PCR scientists can swab areas with suspected bodily fluids and then run the samples through PCR, which then are compared to a DNA database or potential suspects (Gasiorowski-Denis, 2016). In recent years, PCR has become sensitive enough for touch DNA; when individuals touch surfaces, they leave behind trace amounts of DNA which can be swabbed and amplified using PCR (Gasiorowski-Denis, 2016).

A good example of how sensitive touch DNA and PCR can be is evident in the story of the Phantom of Heilbronn. The suspect seemingly had committed over 40 crimes in Austria, France, and Germany (Gasiorowski-Denis, 2016). Following the murder of a police officer where the Phantom's DNA was discovered, researchers took a closer look at the source (Gasiorowski-Denis, 2016). They discovered that the swabs that were being used at the various crime scenes had been contaminated with an employee's DNA (Gasiorowski-Denis, 2016). The employee's contaminated DNA, while seemingly insignificant, when amplified using PCR, created the nonexistent serial killer known as the "Phantom of Heilbronn," (Gasiorowski-Denis, 2016). This illustrates the importance of precautions that need to be implemented to prevent contamination in the lab where the smallest traces can be devastating.

Extreme care was taken to ensure the samples amplified in this experiment were not contaminated by unwanted DNA. Negative controls were run with each sample to ensure no contamination occurred during processing and procedures.

Present Study

The objective of this research was to determine the impact of Ginsenoside 20(S)-Rg3 on the replication of HSV-1 in mice determined by a polymerase chain reaction (and gel electrophoresis) analysis.

Materials and Methods

Samples

Mouse organs were previously collected from mice infected with HSV-1, not infected with HSV-1, or infected with HSV-1 and treated with different amounts of ginsenoside Rg3 (Table 1 & Figure 1) for days 1-3. The tissues collected included the spleen, liver, brain, and serum. A total of 100 samples were evaluated. The experiment was blind to eliminate any bias on part of the researcher. The virus used to infect the mice were HSV-1, the MacIntyre strain, (American Type Culture Collection, Rockville, MD). Each mouse receiving the virus was injected intraperitoneally with 500,000 HSV-1. Two samples were pooled for all HSV and Rg3 samples, while a single sample was used for all others.

Sample Identity	Treatment
No HSV	No treatment
HSV, 6 hour	Infected HSV-1; no Rg3
HSV, 12 hour	Infected HSV-1; no Rg3
10 Rg3: Day 1, 2, 3	Infected HSV-1; 10 mg/kg Rg3
3 Rg3: Day 1, 2, 3	Infected HSV-1; 3 mg/kg Rg3
1 Rg3: Day 1, 2, 3	Infected HSV-1; 1 mg/kg Rg3
0 Rg3: Day 1, 2, 3	Infected HSV-1; 0 mg/kg Rg3

Table 1: 20(S)-Rg3 Treatment Received by Various Groups. The sample's identity and their corresponding treatment are depicted. 0 hours received no treatment, 6-hour infected HSV-1 received no Rg3, 12-hour infected HSV-1 received no Rg3, Day 1, 2, 3: Group 1 infected HSV-1 received 10 mg/kg Rg3, Day 1, 2, 3: Group 2 infected HSV-1 received 3 mg/kg Rg3, Day 1, 2, 3: Group 3 infected HSV-1 received 1 mg/kg Rg3, and Day 1, 2, 3: Group 4 infected HSV-1 received 0 mg/kg Rg3.

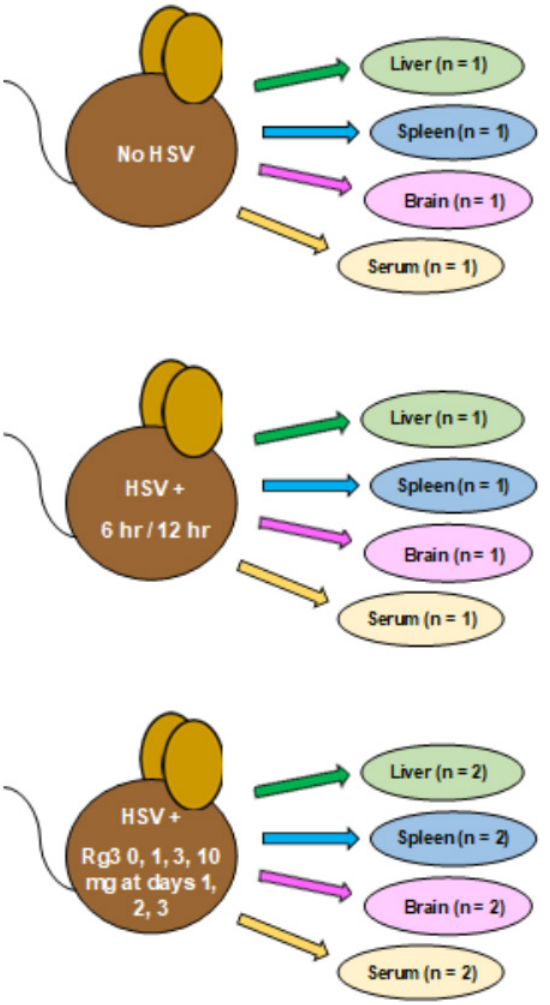

Figure 1: Mouse Treatment: Brain, Serum, Spleen, and Liver. The figure depicts the various treatment each mouse group received. From each mouse, brain (pink), serum (yellow), spleen (blue), and liver (green) were collected from each treatment group. The treatment groups were no HSV, HSV at 6- and 12-hours post-injection, and HSV plus specific Rg3 amounts (0 mg, 1 mg, 3 mg, and 10 mg). "n" represents the number of pooled mice samples.

Sample Extraction

Purified DNA was extracted from previously harvested mice organs stored at -20oC. The extraction process was completed using the IBI Genomic DNA Mini Kit (IBI Scientific, Peosta, IA). All samples of the same type were pooled. From each mouse's sample, 100 µl were pipetted into a 1.5 ml microfuge tube and 30 µl Proteinase K (10mg/ml; Promega, Madison, WI). The microfuge tubes were incubated at 60oC for

15 minutes. Of the GB buffer, 200 µl from the IBI Genomic DNA Mini Kit were added to the microfuge tube and vortexed for 10 seconds. The microfuge tubes were incubated at 70oC for 15 minutes (every couple of minutes the tubes were inverted to ensure good mixing). From ice-cold absolute ethanol (Thermo Fisher, Suwanee, GA) 200 µl were added to the microfuge tube and then vortexed for 10 seconds. The entire volume from the microfuge tube was pipetted into a GD column (IBI Genomic DNA Mini Kit) that had been placed in a 2 ml collection tube and spun at 13,000 rpm for 5 minutes. The flowthrough from the collection tube was discarded. The GD column was placed back into a collection tube. From W1 Buffer 400 µl was added to the GD column and spun for 30 seconds. The flowthrough was discarded, and the GD column was placed into an empty collection tube. From the wash buffer containing ethanol (Thermo Fisher), 600 µl were added to the GD column and spun for 30 seconds. The flowthrough was discarded, and the GD column was placed in the empty collection tube and spun for 3 minutes to dry the column. The GD column was placed into a clean 1.5 ml tube. 50 µl of elution buffer was heated to 70oC. The 50 µl of elution buffer was then added to the GD column and allowed to absorb into the column matrix for 3 minutes. The GD column was spun for 30 seconds, and purified DNA was present in the elution buffer. The samples were stored at -20oC until amplification.

PCR

The DNA was amplified using previously verified PCR primers for HSV-1 (Johnson et al., 2000). Primers were manufactured by Eurofins Genomics, Louisville, KY. Positive control samples were used in a PCR reaction to ensure the reaction produced the expected fragment size for this primer pair, which was 518 base pairs. All reagents were thawed, vortexed, and spun for 5 seconds. The tubes were then kept on ice. In a 0.5 ml tube, 32.5 µl sterile dH2O, 37.5 ul of Promega Master Mix (containing recommended concentrations of buffer, dNTPs, and Taq polymerase), 0.5 µl forward primer, 0.5 µl reverse primer, and 4 µl DNA template were pipetted into the tube. After all the reagents were added, tubes were spun for 5 seconds and kept on ice until tubes were placed in the thermocycler and the cycling began (Table 4).

Tube Number	Tube 1	Tube 2
Sequence Name	HSVfor	HSVrev
Sequence 5' to 3'	GACTTTGCCAGCCTGTACCC	GAGTCCGTGTCCCCGTAGAT
% GC Content	60	60
Tm	64.5	64.5

Table 2: Eurofins Genomics: Oligonucleotide Data Table. The table depicts Oligonucleotide information for the HSV-1 primers including sequence name, sequence 5' to 3', % GC Content, and Tm.

Step	Temperature (°C)	Time
One	93°	2 min
Two	93°	30 sec
Three	50°	45 sec
Four	72°	1 min
Five	Go to step two, ten times	
Six	93°	30 sec
Seven	52°	45 sec
Eight	72°	1 min
Nine	Go to step six, twenty times	
Ten	72°	5 min
Eleven	4°	forever

Table 3: Thermocycler Program. The table depicts the thermocycler program for the PCR samples including the steps, the temperature in Celsius, and the amount of time in each round with a total of eleven steps (thirty-two cycles).

Agarose Gel Electrophoresis

The samples were evaluated by agarose gel electrophoresis to visualize the presence or absence of HSV-1. A positive result for HSV-1 is an amplicon of 518 bp (Johnson et al., 2000). From NuSieve agarose (FMC Bioproducts, Rockville, ME). A 1.25% gel was prepared using 0.5g NuSieve and 40 ml 1 X TAE buffer (40 mM Tris-acetate, 1 mM EDTA) was added to a 250 ml Erlenmeyer flask. The mixture was heated for approximately 30-40 seconds, swirling every 10 seconds to prevent a boil-over. The solution was cooled until it was warm to the touch. The gel tray was taped on both ends and sealed with melted agarose using a Pasteur pipette. The melted agarose was then poured into the tray with an 8-well comb in place. The gel solidified after 20 minutes, the tape and comb were removed, and the gel was placed into the gel box. 1 X TAE containing approximately 80 µl ethidium bromide (10mg/ml; Sigma, St.Louis, MO) was added until it covered the gel. Samples were prepared by combining1.5 µl blue 6 X gel loading dye (BioVentures, Murfreesboro, TN) and 8.5 µl sample PCR product into a new 1.5 ml tube. For the standard ladder, 1.5 µl blue sample buffer and 8.5 µl Biomarker Low (BioVentures) were prepared. All samples were spun down for 10 seconds. The total volume of the ladder, a positive control, a negative control, and samples were all loaded into appropriate lanes of the gel. The gel was run for 1 hour and 15 minutes at 75 volts. Bands were visualized using a UV transilluminator. Fragments located at 518 base pairs were marked as positive for HSV-1.

Results

These studies were undertaken to investigate the ability of various concentrations of Rg3 to inhibit HSV in mice. In addition to evaluating different mouse tissues for HSV following infection, our analysis considered tracking the presence of HSV over 3 days. An example of how these analyses were interpreted is shown in Figure 2.

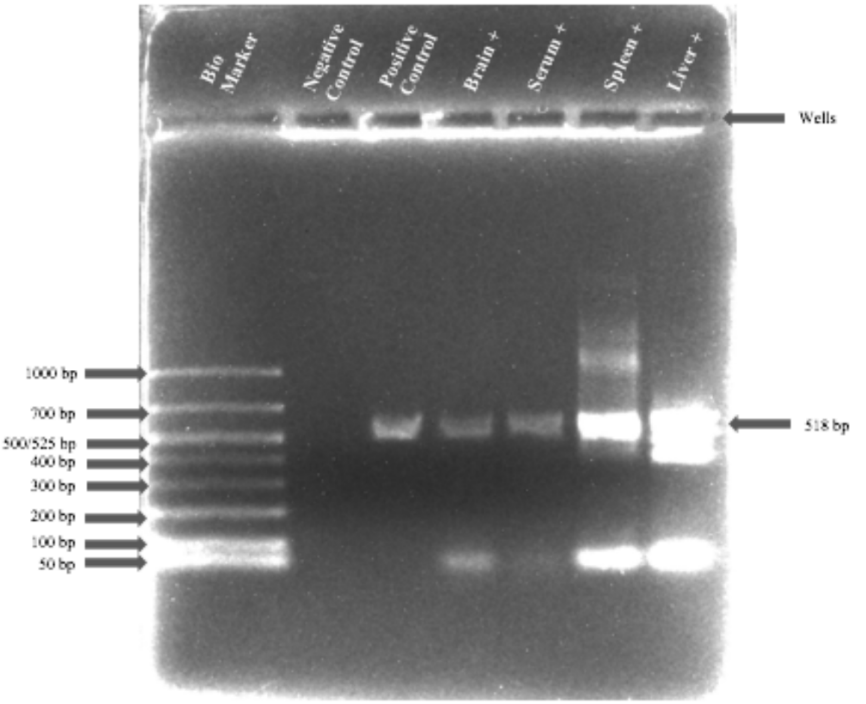

Figure 2: Gel Electrophoresis. Starting on the left, Biomarker Low depicts a ladder with known fragment sizes (fragment sizes: 1000, 700, 500/525, 400, 300, 200, 100, and 50 bp), Negative Control with no bands present, Positive Control with one band at 518 bp, followed by the four tissue types depicting positive results. An arrow points out the wells, the ladder, and where 518 base pairs appear which indicates a positive result.

Table 4 illustrates how the HSV infection takes hold within mice over a specific course of time (6 and 12 hours). This enables a baseline for all treatment groups to be compared. Positive and negative results in both cell culture and PCR were collected for all treatment groups.

Samples	**PCR Results**	**Cell Culture**
6 Hour Serum	+	-
6 Hour Brain	+	-
6 Hour Liver	+	-
6 Hour Spleen	-	-
12 Hour Serum	+	-
12 Hour Brain	+	-
12 Hour Liver	+	+
12 Hour Spleen	+	+

Table 4: HSV Only Post 6- and 12-Hours Post Injection. Samples included all tissue types (serum, brain, liver, and spleen) post 6 hours or post 12 hours from the initial injection of HSV. The results were interpreted using gel electrophoresis, a positive example of each positive tissue type is depicted in figure 2.

In total, 100 samples were collected and processed throughout the course of the experiment. All treatment groups, tissues, and days are depicted in the appendix. The data was compiled into noticeable trend lines depicted in Figures 3, 4, 5, and 6. The goal was to illustrate any trends that were present or absent within the data set as a whole.

Figure 3 looks at any trends between the percent positive of each tissue type in comparison to the treatment day. Over the course of three days, the brain shows a steady increase in percent positive. The brain starts with the lowest percent positive at 25% on day 1 but quickly climbs to 87.5% positive on day 2, where it remains consistent through day 3 at 87.5% positive. The liver shows a decrease in percent positive over the three days. On day 1 liver is 100% positive, falls to 87.5 % on day 2, and finally ends on day 3 at 25% positive. Serum remains consistent over the course of two days at 87.5 % positive; however, no information is available for serum on day 3. Spleen demonstrates a slower decreasing trend over the three days. For the spleen on day 1 and day 2, there is no difference with the percent positive being 100%, and then decreases on day 3 to 87.5 % positive. It is important to note that the liver and spleen both decreased in percentage positive over time, while never increasing. Brain increased in percent positive, but never decreased. The serum remained the same.

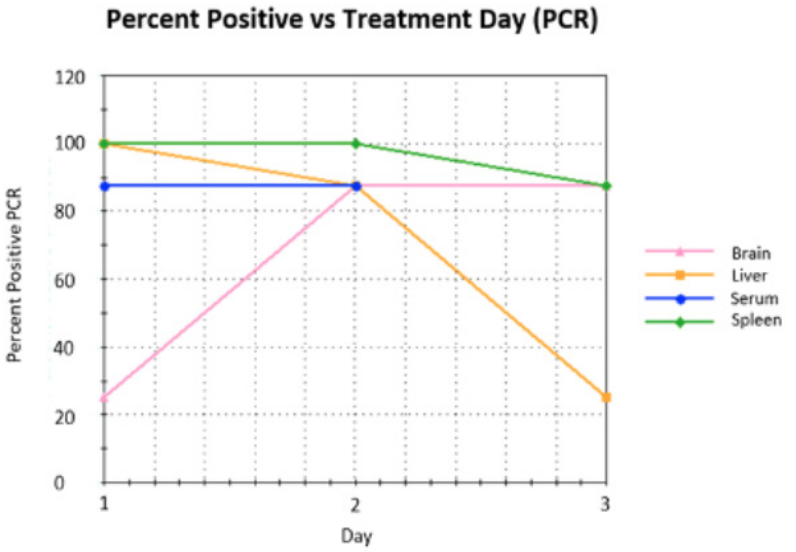

Figure 3: Percent Positive vs Treatment Day (PCR). The figure depicts percent positive results in PCR directly compared to days 1, 2, and 3. A separate line illustrates the four tissue types with the brain being pink, the liver being yellow, the serum being blue, and the spleen being green. No serum samples were available for day 3 of serum.

Figure 4 further demonstrates the trends depicted in figure 3. With all the tissues combined, PCR overall increased from day 1 to day 2, before decreasing overall on day 3. PCR started with a percent positive of 78.13 % on day 1, increasing to 90.63 % positive, and finally decreasing to 66.67 % positive. Cell culture overall decreased from day 1 to day 2, before increasing slightly on day 3. Cell culture started with a percent positive of 43.75%, decreased to 0%, before increasing to 8.33% positive. Not only does the figure illustrate the trends over the course of all three days as a whole, but it also depicts how sensitive PCR is in comparison to Cell Culture.

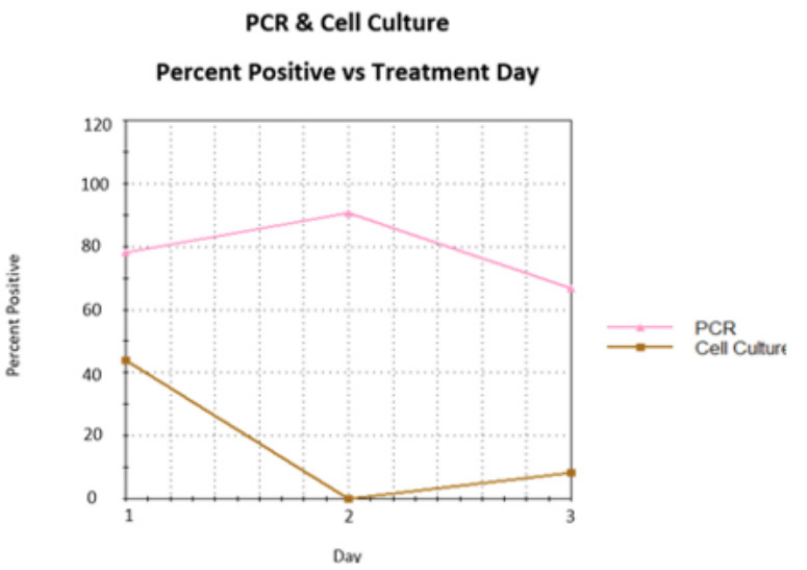

Figure 4: PCR and Cell Culture Percent Positive vs Treatment Day. PCR is represented by a pink line and cell culture is represented by a brown line. Percent positive of all tissue types were directly compared to their corresponding days.

Figure 5 illustrates the relation between Rg3 treatment on various days in comparison to percent positive. As the treatment increased, the percent positive would decrease. Both 0 mg/kg Rg3 and 1 mg/kg Rg3 show an increase from day 1 to day 2, before decreasing on day 3. On day 1 both were 75% positive, increasing to 100% positive, before decreasing to 66.67% positive. Rg3 treatment of 3 mg/kg slowly decreased over the three days. Rg3 of 3 mg/kg started at 87.5 % positive, staying consistent over day 2 at 87.5 % positive, and decreasing to 66.67% on day 3. For Rg3 treatment at 10 mg/kg, it slowly decreased over the three days. On day 1 10 mg/kg of Rg3 was 75 % positive, staying consistent over day 2 at 75% positive, before decreasing to 66.67% positive. The 0 mg/kg and 1 mg/kg acted identically to one another, increasing before degreasing, whereas 3 mg/kg and 10 mg/kg remained consistent between day 1 and day 2, before decreasing slightly. Additionally, all 4 groups ended at 66.67 % positive on day 3.

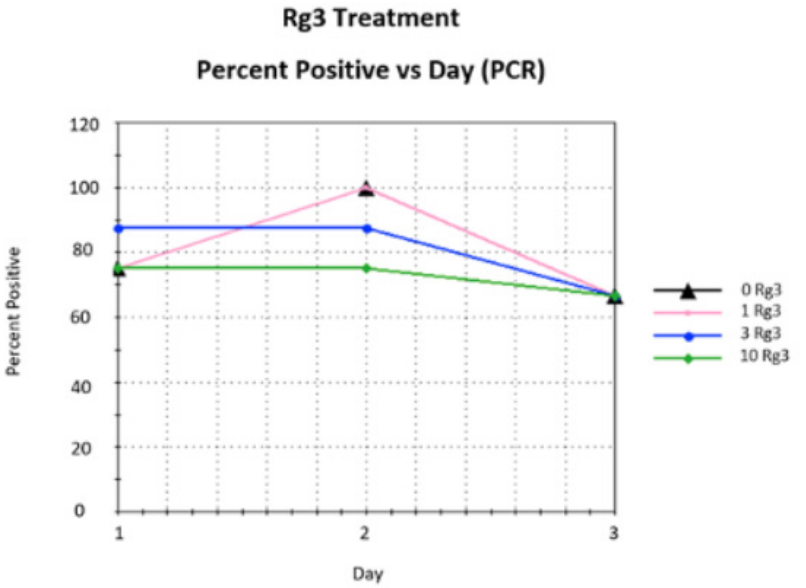

Figure 5: Rg3 Treatment Percent Positive vs Day (PCR). The figure depicts the percent positive on each day concerning specific Rg3 treatments. Rg3 treatment of 0 mg/kg is depicted by a black line, however, it perfectly lines up with 1 mg/kg making it difficult to visualize. Rg3 1 mg/kg is depicted as pink, 3 mg/kg Rg3 is blue, and 10 mg/kg Rg3 is green.

Figure 6 demonstrates the overall trend of all tissue types in comparison to Rg3 treatment and percent positivity. Overall PCR gradually decreased over the specific treatment groups. At Rg3 treatments of 0,1, and 3 mg/kg in PCR had a percent positive of 81.82%, which decreased to 72.73%. Overall cell culture increased before decreasing slightly. At Rg3 treatment of 0 and 1 mg/kg, the percent positive was 13.64%, increasing to 27.27% at 3 mg/kg Rg3, and decreasing to 18.18% positive at 10 mg/kg. PCR, which once again is far more sensitive than cell culture, remained the same over three days, before slightly decreasing. In cell culture, there was a slight jump before decreasing slightly.

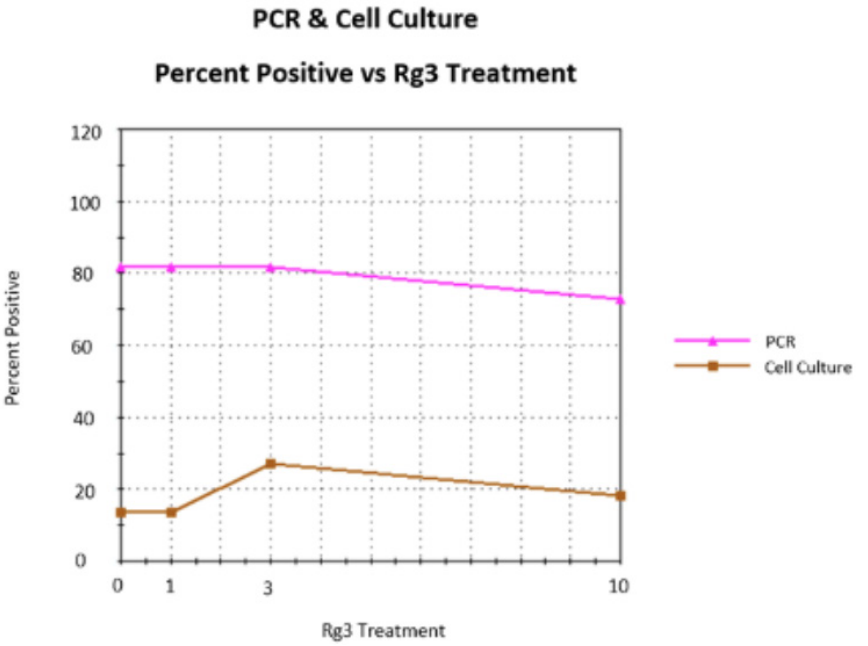

Figure 6: PCR and Cell Culture Percent Positive vs Rg3 Treatment. The figure depicts the trends of all tissue types over the course of increasing amounts of Rg3 in comparison to percent positive. PCR (pink) shows a subtle decline in percent positive as the Rg3 treatment amounts increased. Cell Culture increased in percent positive before decreasing overall.

Discussion

The objective of this study was to evaluate the inhibition of herpes simplex virus type 1 by Ginsenosides 20(S)-Rg3. The four tissue types (spleen, liver, brain, and serum) and three concentrations of Rg3 were accessed using PCR and gel electrophoresis analysis (Wright and Altman, 2020). After all the tissue samples had been analyzed for the presence of HSV, they were compared with the plaque assay

results from the cell cultures. Following the documentation of the specimens, the treatment groups were revealed (blind study). It is vital in understanding that when using PCR, only viral sequences are detected; not necessarily infectious viruses (Wald et al., 2003).

Upon analysis of all the samples, some were unusual. PCR is far more sensitive, able to detect minute details more so than cell cultures (Schrimpf et al., 2020). Therefore, if a cell culture were negative and the PCR were positive, it is not surprising given the sensitivity of PCR (Schrimpf et al., 2020). However, if PCR were negative and cell culture contradicted with a positive result, those samples needed reevaluation. Day 1 liver samples 9-10, 11-12, 13-14, and 15-16 needed retesting due to the contradicting reports. The PCR product in these samples needed re-amplification using the same protocol (except instead of a 4µl DNA template, 1µl of PCR product was used). After re-amplification, the specimens were determined to be positive.

All samples' PCR and cell culture results are shown in the appendix. The groups were then organized in figures 3, 4, 5, and 6. Figure 3 examined the relationship between the tissue types over the course of three days compared to percent positive. Serum remained the same, spleen and liver decreased, with liver decreasing the most, and brain increased in percent positive over the course of three days. These results were not unusual; Eeckout's research showed similar patterns for infected mice's organs over the course of several days (Eeckout et al., 1994). Like this project's research, the spleen and liver cleared out their infection relatively fast (Eeckout et al., 1994). In their project serum cleared the infection similarly to the spleen and liver, while in this project's experiment it was not witnessed, this can be explained by the lack of serum on day 3 (Eeckout et al., 1994). The brain, in both studies, showed the brain takes far longer for the infection to set in, and then to clear out the infection (Eeckout et al., 1994).

Once the specific tissue types were illustrated in figure 3, the overall trend of all the tissue types over the course of the three days is depicted in figure 4 for both PCR and cell culture. In PCR, there was an increase in percent positive from day 1 to day 2, before decreasing on day 3. It illustrates the infection taking hold within the organs between day 1 and day 2. By day 3, the tissues were starting to clear out the infection. PCR in comparison to cell culture was far more sensitive, which is nicely depicted in figure 4. Figures 3 and 4 illustrate the overall trends of the infection over the course of three days, to establish the expected clearance rates for each day. This study's focus was on how specific concentrations of Rg3 impacted the infection.

It was anticipated that as the amount of Rg3 increased, so would the number

of negative results. To demonstrate this, the specific amounts of Rg3 treatment over the course of three days were directly compared to the percent positivity in figure 5. Rg3 at 0 mg/kg and 1 mg/kg both increased from day 1 to day 2, before decreasing on day 3. This is exactly what was illustrated in figure 4, an increase on day 2 before starting to clear the virus on day 3. Rg3 at 3 mg/kg and 10 mg/kg, where it would be anticipated to see the greatest amount of change, showed very little difference. Both remained the same from day 1 to day 2, before decreasing slightly. Interestingly, all treatment groups ended with the same percent positive on day 3. Demonstrating Rg3 did not impact clearance of the virus, rather the variation is due to the days.

The sample groups did not follow any trend that would lead to a correlation between treatment and infection. Other research experiments showed varying amounts of success.

Researcher Cho evaluated the effects of Korean red ginseng (KRG) on mice and found KRG was able to render mice more resistant to HSV vaginal infection, along with other viruses (Cho et al., 2013). Another study found that KRG and its ginsenosides were able to reduce diarrhea in mice who were infected with rotavirus (Yang et al., 2018). Both of these studies did not use cell culture, but they did use live animals (mice). This indicates that KRG is successful in mice for a certain virus, however, there is no direct correlation made between cell culture and the live mice. Although both treatments were successful in mice, these results cannot be translated to cell culture. Conversely, Song conducted research and reported activity against coxsackie virus, enterovirus, and rhinovirus (Song et al., 2014). The researcher only used cell cultures, and never did live animal studies (Song et al., 2014). This illustrates the importance of testing in both arenas. Our study showed promising results in cell culture, much like Song (Song et al., 2014) did, however, the extra step was taken to test in animals. Thus, while Rg3 was successful in cell culture, the same protection was not evident in live animals, which are far more complex with more factors involved.

Appendix

Samples	PCR Results	Cell Culture
Day 1 Serum	+	-
Day 1 Serum	+	-
Day 1 Brain	-	-
Day 1 Brain	-	-
Day 1 Liver	+	+
Day 1 Liver	+	+
Day 1 Spleen	+	-
Day 1 Spleen	+	+
Day 2 Serum	+	-
Day 2 Serum	+	-
Day 2 Brain	+	-
Day 2 Brain	+	-
Day 2 Liver	+	-
Day 2 Liver	+	-
Day 2 Spleen	+	-
Day 2 Spleen	+	-
Day 3 Brain	+	-
Day 3 Brain	+	-
Day 3 Liver	-	-
Day 3 Liver	-	-
Day 3 Spleen	+	-
Day 3 Spleen	+	-

Table 5: 0 mg Rg3 (Virus Only). Table depicts all samples' PCR and cell culture results for tissues that received 0 mg Rg3. Light gray with a "+" represents a positive sample and dark gray with a "-" represents a negative result.

Samples	PCR Results	Cell Culture
Day 1 Serum	+	-
Day 1 Serum	+	-
Day 1 Brain	-	-
Day 1 Brain	-	-
Day 1 Liver	+	+
Day 1 Liver	+	+
Day 1 Spleen	+	-
Day 1 Spleen	+	-
Day 2 Serum	+	-
Day 2 Serum	+	-
Day 2 Brain	+	-
Day 2 Brain	+	-
Day 2 Liver	+	-
Day 2 Liver	+	-
Day 2 Spleen	+	-
Day 2 Spleen	+	-
Day 3 Brain	+	-
Day 3 Brain	-	-
Day 3 Liver	+	-
Day 3 Liver	-	-
Day 3 Spleen	+	-
Day 3 Spleen	+	+

Table 6: Treatment 1 mg Rg3. Table depicts all samples' PCR and cell culture results for tissues that received 1 mg Rg3. Light gray with a "+" represents a positive sample and dark gray with a "-" represents a negative result.

Samples	PCR Results	Cell Culture
Day 1 Serum	-	-
Day 1 Serum	+	+
Day 1 Brain	+	-
Day 1 Brain	+	-
Day 1 Liver	+	+
Day 1 Liver	+	+
Day 1 Spleen	+	+
Day 1 Spleen	+	+
Day 2 Serum	+	-
Day 2 Serum	-	-
Day 2 Brain	+	-
Day 2 Brain	+	-
Day 2 Liver	+	-
Day 2 Liver	+	-
Day 2 Spleen	+	-
Day 2 Spleen	+	-
Day 3 Brain	+	-
Day 3 Brain	+	-
Day 3 Liver	-	-
Day 3 Liver	+	-
Day 3 Spleen	-	-
Day 3 Spleen	+	+

Table 7: Treatment: 3 mg Rg3. Table depicts all samples' PCR and cell culture results for tissues that received 3 mg Rg3. Light gray with a "+" represents a positive sample and dark gray with a "-" represents a negative result.

Samples	PCR Results	Cell Culture
Day 1 Serum	+	-
Day 1 Serum	+	-
Day 1 Brain	-	-
Day 1 Brain	-	-
Day 1 Liver	+	+
Day 1 Liver	+	+
Day 1 Spleen	+	+
Day 1 Spleen	+	+
Day 2 Serum	+	-
Day 2 Serum	+	-
Day 2 Brain	-	-
Day 2 Brain	+	-
Day 2 Liver	-	-
Day 2 Liver	+	-
Day 2 Spleen	+	-
Day 2 Spleen	+	-
Day 3 Brain	+	-
Day 3 Brain	+	-
Day 3 Liver	-	-
Day 3 Liver	-	-
Day 3 Spleen	+	-
Day 3 Spleen	+	-

Table 8: Treatment: 10 mg Rg3. Table depicts all sample's PCR and cell culture results for tissues that received 10 mg Rg3. Light gray with a "+" represents a positive sample and dark gray with a "-" represents a negative result.

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