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Article

Testing the Effects of Salep Derived from the Tubers of *Orchis mascula*, *Aloe vera*, and Alpha-chymotrypsin on Wound Healing in *Drosophila melanogaster* Larvae

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SUMMARY

***Aloe vera* and alpha-chymotrypsin have been used in previous studies to enhance wound healing. Based on this knowledge, we conducted an experiment to determine whether administering these treatments as well as an herb with similar chemical proponents, namely salep flour, would enhance wound healing in *Drosophila melanogaster*. We hypothesized that the three treatments would enhance wound healing by decreasing wound size more effectively. We fed *D. melanogaster* larvae these treatments over a two-week period. We then administered a puncture wound using a steel needle at the larva's dorsal midline. We analyzed the wounds photographically to determine the average percent change of the wound's perimeter over 6 hours. The results of two of the treatment groups, Salep and *Aloe vera*, yielded wound sizes small enough to present a significant percent decrease when compared with the wound sizes of the control group. Therefore, our results show support that both Salep and *Aloe vera* were effective for enhancing wound healing in epithelial cells in *D. melanogaster* larvae.**

INTRODUCTION

The regeneration of epidermal cells is an essential process in most species of the animal kingdom. Epidermal cell regeneration plays a key role in wound closure, and in the prevention of chronic wounds or infections (1). In the field of molecular biology and genetics, the process of wound healing is studied extensively to reduce the time required for healing from surgeries, traumas, and burns (1).

Scientists and researchers extensively use *D. melanogaster,* otherwise known as common fruit flies, as a model organism for the study of wound healing processes due to similarities of their gene pathways to those of humans (2). Researchers have studied the process of wound healing in larvae themselves, and they have observed the activation of multiple gene pathways upon wounding (3). One of these pathways, known as the grainy-head pathway, is a primary contributor to wound closure mechanisms (3). *Grh,* the gene that controls the grainy-head pathway, produces grainy-head transcription factors that regulate epithelial development and barrier formation in a variety of tissues (4). Inhibition of grainy-head signaling impairs the formation of the epidermal barrier and cuticle layers of both fruit fly larvae and mammals (4).

Though research has been conducted on wound healing and its associated intracellular processes, there are a variety of unexplored treatments, such as herbs, that could potentially enhance wound healing mechanisms (2). Currently, the effects of herbal treatments on wound healing remain largely unknown (5). However, past studies have observed the effects of oral administration of alpha-chymotrypsin on the regeneration of epidermal cells in humans (1). Alpha-chymotrypsin is an enzyme that functions to initiate wound healing (1). Alpha-chymotrypsin has been noted for its anti-inflammatory properties and its ability to enhance epidermal growth and tissue repair in acute tissue injuries (1). We used alpha-chymotrypsin as one of the treatment groups in our experiment to compare the effects of a pure protein to those of herbal remedies on wound healing.

In addition to the chemical treatment of alpha-chymotrypsin, we tested two herbal treatments: *Aloe vera* and salep flour. *Aloe vera* is a xerophytic succulent that is known to enhance wound healing upon oral administration (6). The gel of this plant is made up of water and a variety of polysaccharides. The healing properties of *Aloe vera* are directly attributed to a specific polysaccharide: glucomannan (7). Glucomannan is a heteroglycan comprised of glucose and mannose (8). We also chose to investigate the wound healing properties of Salep, a flour derived from the tubers of orchids, because it also contains the polysaccharide glucomannan (9). Though Salep has been used to treat hepatotoxicity in rats, its effects on wound healing have not been tested previously (11).

We hypothesized that when *D. melanogaster* is consistently administered a Salep diet orally over the course of two weeks, the percent decrease in wound size over time will be greater than that of the comparable control group: untreated larvae. Additionally, we hypothesize that *D. melanogaster* kept on an *Aloe vera* diet and those kept on an alpha-chymotrypsin diet will also exhibit a greater percent decrease than the control groups. *D. melanogaster grh* mutants will also be treated to determine whether grainy-head activation is required for wound healing. We hypothesize that the untreated *D.melanogaster* flies and the *D. melanogaster grh* mutants will demonstrate negligent percent decreases in wound size.

RESULTS

The purpose of this experiment was to test whether *Aloe vera*, Salep, or alpha-chymotrypsin would promote a significant decrease in wound size compared to the untreated control group. To measure wound healing, we punctured *D. melanogaster* larvae in the abdominal region and took photographs of the wound with a microscope at 2 and 6 hours after the puncture. We used ImageJ software to analyze these photographs and to measure the percent change in wound perimeter over time. We documented the mortality of each group of larvae at the end of the experiment. The data from each experimental group were then statistically compared to the control group with a t-test. The control group for both parts of the experiment consisted of wild type *D.melanogaster* larvae that were fed untreated fly food medium.

The set-up of our first experiment included 3 treatment groups (wild-type *D. melanogaster* larvae fed fly food medium treated with *Aloe vera*, Salep, or alpha-chymotrypsin) and 1 control group (wild-type *D. melanogaster* larvae fed untreated fly food medium). We measured and compared the average percent change of wound size from 2 to 6 hours after wounding for each of the four groups (**Figure 1**). Salep-treated larvae demonstrated the largest percent decrease in wound size among the groups (-54.47%, compared to *Aloe vera*-treated larvae with -45.92%; alpha-chymotrypsin treated larvae with -31.67%; and untreated larvae with -26.05%). The differences between the average percent change of wound size of Salep-treated larvae compared to the control and *Aloe vera*-treated larvae compared to the control were statistically significant.

After 6 hours of wound healing, 25% of the larvae treated with *Aloe vera* died (**Table 1**). Additionally, 10% of both the control larvae and the *Aloe Vera* treated larvae died. The smallest percent of deaths was seen in the Salep treated larvae, with 0% of the 20 larvae in the sample dying.

The set-up of our second experiment included wild-type *D. melanogaster* larvae and *grh* mutant *D. melanogaster* larvae. As in the first experiment, we wounded these larvae and measured the average percent change in wound size (**Figure 2**). The *grh* mutant had an average percent change of 36.56% (standard error 12.71%) and the wild type control group had an average percent change of -22.06% (standard error 6.20%). The difference in the average percent change of wound size between these two groups was statistically significant.

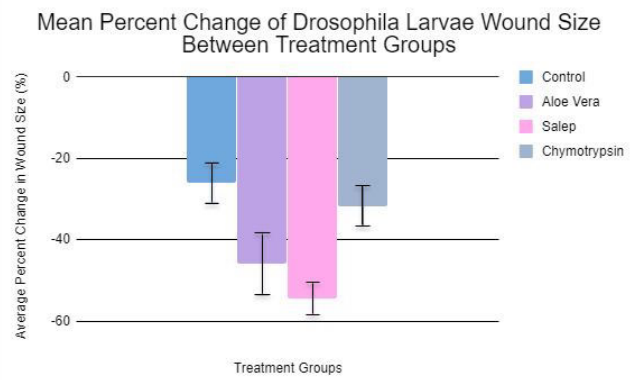

Figure 1. Average percent change in wound size among the four treatment groups: Salep, *Aloe vera*, alpha-chymotrypsin, and the control group. *n*=20 for all groups. Wound size is measured in percent change of wound size from puncture to 6 hours afterwards. The graph shows Salep with percent decrease in wound size being the largest at -54.47% , then *Aloe vera* at -45.92%, alpha-chymotrypsin at -31.67% and lastly the control group at -26.05%. Standard Error Margins have been accounted for as well with *Aloe vera* having the largest SEM. The data were analyzed via Student's t-tests and a Bonferroni correction. Salep and *Aloe vera* showed a significant difference to the control: Aloe vera (p=0.016), Salep (p=0.000031). *p<0.017 and ***p<0.00033, after the Bonferroni correction (0.05 divided by 3 and .001 divided by 3).

After 6 hours of wound healing, 100% of the *grh* mutant larvae had died (**Table 2**). None of the 10 wild type larvae died during the 6 hours.

DISCUSSION

The purpose of our study was to determine whether salep flour, Aloe vera, and alpha-chymotrypsin could enhance wound healing through significantly decreasing wound size over a 6 hour period. The data from the Salep and *Aloe vera* treatment groups demonstrated statistically significant results. Meanwhile, the percent change displayed by the alpha-chymotrypsin groups was not statistically significant. These results support the part of our original hypothesis that states Salep- and *Aloe vera*- treated groups would display a greater percent decrease in wound size from hour 2 to hour 6 in comparison to the control group. Although the alpha-chymotrypsin-treated group also displayed a slight percent decrease in wound size, the difference between this group and the control untreated group was not statistically significant.

Table 1. Mortality from hour 0 to hour 6 amongst the experimental groups. *n*=20. The group with the largest mortality rate is *Aloe Vera* (5 larvae) and the smallest mortality rate is seen in the Salep group (0 larvae). The other two groups, control and alpha-chymotrypsin, each had 2 larvae die during the 6 hour testing period.

Treatment Group Mortality

Groups	Percent Mortality
Control	10%
Aloe vera	25%
Salep	0%
alpha-chymotrypsin	10%

Table 2. Mortality of the grh mutant larvae in comparison to the wild-type larvae (control). *n*=10. Overall, all 10 of the Grh mutant larvae died by the end of the 6 hour testing period, while 0 of the wild type larvae died by the end of the testing period.

Larvae Type Mortality

Larvae Type	Percent Mortality
Grh Mutant	100%
Control	0%

Of the experimental treatment groups, Salep displayed the largest percent decrease in wound size (54.47%) with the smallest standard error (-0.54 +/- 3.98) (**Figure 1**). According to a previous study, salep flour possesses anti-oxidant material that is capable of removing toxins from the liver of rats; this suggests there is a possibility that the root is effective in preventing the entry of potential toxins that could inhibit wound healing (11). These properties may explain the results of our experiment.

Aloe vera displayed the next largest percent decrease in wound size (45.92%) (**Figure 1**). This treatment group demonstrated a statistically significant percent decrease in wound size, yet the standard error for measurements of this group was the largest among all the experimental groups (-0.46 +/- 7.67). A higher standard error suggests higher variance in the data, which may indicate inconsistencies in how *Aloe vera* affected the larvae in this experimental group. The *Aloe vera*-treated group was expected to demonstrate a higher percent decrease in wound size, as the plant is commonly used to heal minor epidermal damage (7). Additionally, potential confounding variables, such as a smaller sample size due to the death of larvae throughout the trial, may have affected the results in this experimental group (**Table 1**). However, calculation of standard error accounted for these deaths, so there should be no effect on the calculated p-value comparing the *Aloe vera*-treated group to the control group.

The large number of deaths in the *Aloe vera* treatment group suggests experimental error, because studies show that *Aloe vera* and Salep share active ingredients; namely, *Aloe vera* and Salep both contain polysaccharides such as glucomannan (6,9). The surviving *Aloe vera* larvae had similar results to those of the Salep treated larvae, narrowing down the cause of experimental error to the wounding of *Aloe vera-treated* larvae. The wounds were likely too large or deep for self-healing to be feasible. This was likely because *Aloe vera* was the last group to be wounded; due to time constraints on the trial period, the procedure may have been rushed, and thus less consistent with the wounding of other groups.

The alpha-chymotrypsin treatment group displayed the smallest percent decrease in wound size (31.67%) (**Figure 1**). The standard of error for this experimental group was relatively low, similar to that of the control group (-0.32 +/- 4.97). Although the average percent decrease in wound size of the alpha-chymotrypsin group was greater than that of control, the percent decrease is not statistically significant at the 0.17 significance level (p=0.21). This may indicate that alpha-chymotrypsin does not assist wound healing via the same mechanisms as Salep or *Aloe vera*. It may also be due to the minute amounts of alpha-chymotrypsin incorporated into the fly medium. We calculated the amount of alpha-chymotrypsin to use based on alpha-chymotrypsin treatments for humans and used a 0.08% concentration of alpha-chymotrypsin for the fly medium, in comparison to the 6% concentrations of the other treatments. When making the food for this treatment group, the measurements of alpha-chymotrypsin were so small that any residue on the weigh boats at the time of transfer could have significantly impacted the concentration in the food. In future studies, it would be more beneficial to test different concentrations of chymotrypsin.

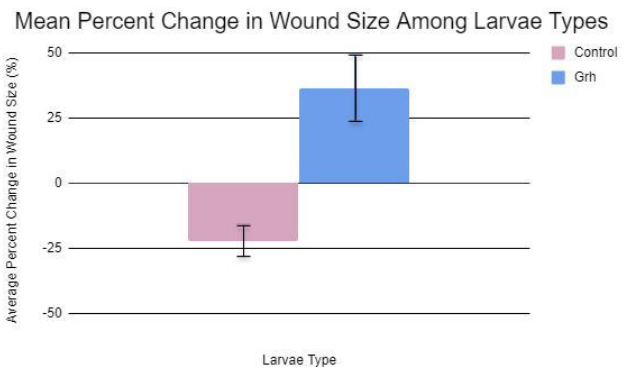

Figure 2. Average percent change in wound sizes among larvae types: wild type and grh mutant. n=10 for all groups. Wound size is measured in percent change of wound size from puncture to 6 hours afterwards. Additionally, the Standard Error margins have been displayed for each group with the grh mutant larvae having a SEM of 12.71. The grh mutant had a positive percent change (36.56%), and the wild type had a negative percent change (-22.06%). The data was analyzed by doing a t- test, comparing the grh mutant to the control. There was a significant difference that was seen between the two groups at the ***p<0.001 level. The p-value of grh was 0.00030.

A separate portion of the experiment determined whether larvae lacking *grh* could effectively heal their wounds the same as wild-type larvae. The results indicated a 36.60% increase in wound size, rather than a decrease (**Figure 2**). The standard error of the *grh* group was (+36.60/12.71). This indicates a large chance that error played a role in the results. The increase in wound size could have been due to the shrinkage of cells after apoptosis (12). Since these larvae lacked an important wound healing gene, *grh*, the cells around the wound site were unable to recover or show signs of inflammation (13). This resulted in the death of the cells surrounding the wound, causing the body to shrivel. This shriveling effect could have interrupted essential metabolic processes involved with wound healing, thus preventing the wound from decreasing in size (12). The death of all the *grh*-lacking larvae is most likely because of the necessity of *grh* in the immune system of flies, as the GRH pathway initiates wound healing in *D. melanogaster* (**Table 2**). The lack of *grh* is typically lethal in *D. melanogaster*, especially in the larval stages; after injury, the larvae are even more susceptible to death (13).

The study conducted allows us to present evidence that treatments of *Aloe vera* and salep flour are significantly effective in decreasing the average wound size in *D. melanogaster*. This research should continue in mammals, and then in humans after further studies on chemical properties and differences between the two treatments. A result we would like to investigate further is the reason *Aloe vera*-treated larvae had the highest mortality. By further

studying the differences between the chemical properties of the two herbal remedies, we could make advances in medical treatments regarding the carbohydrates and molecules that most efficiently enhance wound healing.

METHODS

Making Food and Treatment Groups

We obtained wild-type *D. melanogaster* from Carolina Biological and placed them on four different treatment groups over the course of two weeks: Salep (Salep Sahlep Sahlab Salepi), *Aloe vera* (Bulk Herbs India), alpha-chymotrypsin (Sigma-Aldrich), and control. For the control, we made each vial of food by mixing 5.6 grams of Carolina *D. melanogaster* food medium with 11 mL of water until homogeneous (14). Salep vials were made in the same way as control but with the addition of 0.33 grams of Salep to achieve a concentration of 6% (15). Before we added Salep to the medium, we ground it using a mortar and pestle, filtered using a sieve, and boiled at a temperature of 100 degrees Celsius for 5 mins in 11 mL of water. Alpha-chymotrypsin vials were made in the same way as control vials, but with the addition of 0.05 grams of alpha-chymotrypsin in order to achieve a 0.08% concentration (1). We calculated the amount of alpha-chymotrypsin to use based on alpha-chymotrypsin treatments for humans, proportional to the amount of alpha-chymotrypsin administered to humans in the treatments (1). *Aloe vera* vials were made in the same way as control but with 0.33 grams of *Aloe vera* in order to achieve a 6% concentration (15). Before adding *Aloe vera* to the medium, we first ground it using a mortar and pestle, filtered it using a sieve, and boiled it at a temperature of 100 degrees Celsius for 5 mins in 11 mL of water.

Culturing Flies

We cultured a vial of wild-type flies and a vial of *grh* mutant flies (BDSC stock number: 3720) for three weeks in Carolina *D. melanogaster* food medium. Once the flies in the wild-type vial reproduced to an adequate amount, we transferred 10 flies to each of the four treatment groups. We then gave each vial two weeks to produce larvae that fed only on their respective treatment foods. We kept *grh* mutant flies, obtained from Bloomingdale Drosophila Stock Center and their larvae on control medium for two weeks.

Wounding

After two weeks of feeding, we removed 20 larvae from each treatment group, including the control group, and placed them in a large 24-well array (Carolina Biological). We placed 10 *grh* mutant larvae and 10 control larvae to compare with *grh* in two halves of one array. We then placed each treatment array in the freezer at -20 degrees Celsius for 8 minutes to ensure that the larvae were motionless throughout wounding (16). We then punctured the 20 isolated larvae from each treatment group using a 0.46 mm steel needle (Dritz Inc.). Each larva was punctured at their dorsal midline. (17). We recorded the wounding times of each larva.

Viewing

Two hours after wounding, we viewed the wounds using the 100X magnification lens of an Olympus Bx41 microscope, and we took images using an iPhone 7 camera. This was repeated 6 hours after wounding. We measured the perimeter of the wound in each image in mm using ImageJ. Each perimeter value was the average of three measurements taken by three individuals, in order to ensure greater precision (17).

Data Analysis

We calculated the percent change in the perimeter of the wound size for each larva and performed two-sample t-tests to determine whether the percent change was significantly higher for any of the treatment groups as compared with the control group. There could have been human errors with inconsistencies in the measuring of each wound. For this reason, three individuals scored the data to create a higher precision of the data.

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