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Article

Comparative analysis of vital capacities of athletes, singers and other students of age 13-14 years: a cross-sectional observational study

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SUMMARY

Physical activity when performed regularly has beneficial effects on all systems of the body, including pulmonary functions. This study, conducted at Springdales School in Dhaula Kuan, New Delhi, aimed to determine the effect of sports and singing on the vital capacity (the maximum amount of air a person can expel from the lungs after a maximum inhalation), an important measure of pulmonary health. Vital capacity was assessed in 60 healthy students of 13-14 years of age with an equal number of athletes, singers, and non-athletes non-singers, as well as an equal number of males and females in each group. Vital capacity was measured by Student's spirometer. Anthropometric data was also matched with spirometric parameter. Athletes (3452.5±696.7 cm^3) and singers (3015±346.83 cm^3) had significantly higher vital capacity than the control group (2625±543.74 cm^3). The vital capacities of athletes and singers were also significantly different. Non-athletes non-singers had a significantly higher body mass index (23.87±2.35 kg/m^2) as compared to athletes (20.66±1.52 kg/m^2) and singers (22.6±1.84 kg/m^2). In conclusion, both athletes and singers had better pulmonary function compared to control, with a positive correlation to body mass index. Athletes also exhibited better vital capacity than singers. This implies that encouraging regular exercise and singing in children improves cardiorespiratory functions.

INTRODUCTION

Oxygen is vital for all bodily functions. Maintaining an ideal vital capacity is important because the larger the vital capacity, the more efficiently the body can distribute oxygen to the muscles during exercise (1-3). The Framingham Study (which followed 5,200 individuals for three decades) demonstrated that the greatest predictor of health and longevity was actually lung volume (4). Those with higher vital capacity were healthier and lived longer.

The volume of air occupying the lungs at different phases of the respiratory cycle is subdivided into four volumes and four capacities (5). Air in lungs is measured in terms of lung volumes and lung capacities (**Figure 1**). The volumes that measure the amount of air in and out during breathing include tidal volume (TV), expiratory reserve volume (ERV), inspiratory reserve volume (IRV), and residual volume (RV). The tidal volume measures the amount of air that is inspired and expired during a normal breath. The expiratory reserve volume is the additional amount of air that can be exhaled after a normal exhalation. Conversely, the inspiratory reserve volume is the additional amount of air that can be inhaled after a normal inhalation. Capacities are measurements of two or more volumes. Vital capacity (VC) determines the total amount of air that can be expired after fully inhaling and is the sum of the tidal volume, inspiratory reserve volume, and the expiratory reserve volume (i.e., VC=TV+IRV+ERV). Spirometer determines all the above except the residual volume, which is the amount of air that is left after the expiratory reserve volume is exhaled.

Physiological factors that influence lung volumes/capacities include age, gender, weight, height, ethnicity, and physical activity (6, 7). The lung volumes increase steadily from birth to adulthood and then decrease. Due to larger anthropometric measurements, males are more likely to have increased lung volumes and capacities. Vital capacity increases with height. Formulae to roughly estimate vital capacity are (6, 7):

Vital capacity of female: (21.78-0.101a)*h
Vital capacity of male: (27.63-0.112a)*h

where vital capacity is measured in cubic centimetre (cm^3), a is age in years, and h is height in centimetre (cm).

Ethnic dissimilarities in the lung volumes/capacities are also attributed to anthropometric differences between ethnic groups. Lung functions are negatively affected by pulmonary pathology and air pollution (6, 7).

In a survey conducted by the Heal Foundation, it was observed that more than a third of school children in four big cities of India suffer from reduced lung capacity, with Delhi showing the worst lung capacity among the four cities (8). Delhi is ranked 6 in the top 500 cities by PM2.5 annual mean concentration measurements as documented by the 2018 version of World Health Organization database (9). According to India's National Health Profile in 2015, there were almost 3.5million reported cases of acute respiratory infection (ARI) in 2014, a 140,000 increase from the previous year and a 30% increase since 2010 (10). Exposure to ambient air pollution can lead to clinically important deficits in lung functions. Given the magnitude of its observed effects in India and the importance of lung functions as a determinant of morbidity,

it is important to identify strategies to deal with the effects of air pollution besides reducing levels of air pollutants (11). Exposure to a high level of particulate matter in air pollutants has been associated with radiological evidence of bronchiolar disease and mild bronchial wall thickening on computerized tomography scans in children. Chronic inflammation in the distal airways induced by air pollution can lead to remodeling of the airways (12-14). In a three-year study published in 2010, the Kolkata-based Chittaranjan National Cancer Institute and the WHO found that key indicators of respiratory health, lung function, and blood pressure in children in Delhi between 4 and 17 years of age were far worse than those of children elsewhere (15). The tests were conducted on a total of 11,628 school-age children from 36 schools throughout Delhi and 15 rural schools in West Bengal and Uttaranchal. Forty-three and a half percent of the Delhi school children suffered from "poor or restrictive lungs," as compared to 22% of the kids in the rural schools (15). Alveolar macrophages (AM) are lung-resident immune cells that ingest microorganisms and dust particles, and act as the first line of cellular defense against inhaled pollutants. AM were 2-3 times more frequent in Delhi school children than in rural children, indicating that the Delhi children had greater exposure to particulate pollution (16). The report concluded that about half of the 4.4 million children who reside in Delhi already have irreversible lung damage (15).

Regular exercise improves cardio-respiratory function by improving the VO2 max, which is the maximum oxygen consumption during exercise. Increased oxygen intake and lung usage allow the lungs to grow in strength, and therefore can expand more readily to take in more air (17, 18). Singing involves a fast and strong inspiration followed by prolonged and controlled expiration. This requires diaphragm to contract for inspiration followed by prolonged contraction of the respiratory muscles in order to vibrate the vocal folds. Thus, singing also strengthens chest muscles and promotes deep breathing to further increase the lung capacity (19-20).

We conducted a study on the vital capacities of middle school children. We hypothesize that playing sports and developing hobbies like singing could improve their vital capacity and ability to tolerate air pollution. We compared the vital capacities of athletes, singers, and other students of 13-14 years with the objective of correlating the effect of exercise and singing on the pulmonary functions.

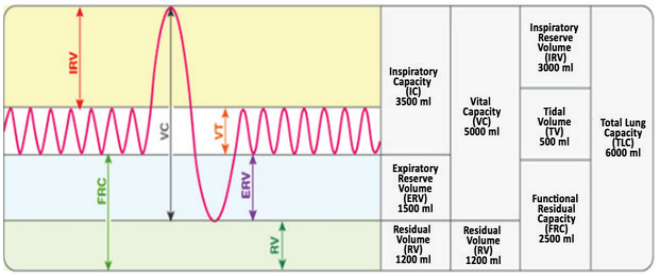

Figure 1: Standard lung volumes and capacities from a spirometer trace. TV= Tidal volume; IRV= Inspiratory reserve volume; ERV= Expiratory reserve volume; RV= Residual volume; IC= Inspiratory capacity; FRC= Forced residual capacity; VT= Vital capacity; TLC= Total lung capacity.

RESULTS

The cross-sectional, observational study was conducted over a period of 3 months in the Innovation Laboratory of our school on a sample size of 60 students who were divided in 3 groups of 20. Since vital capacity depends on age and sex, bias was eliminated by taking equal numbers of boys and girls of the same age group.

Demographic profile

The demographic profile of the males and females of the three groups were compared along with the vital capacity. Each group consisted of 10 males and 10 females, separated as follows: Group A (actively involved in sports over the previous one year), Group S (singers were a part of the school choir over the previous one year) and Group Non-AS (non-athletes, non-singers). There was no statistically significant difference between the mean ages of all the three groups. Hence all groups were comparable in age distribution (**Table 1**).

The Non-AS group had a significantly higher mean weight than Group A (p-value < 0.05). This can be attributed to the increased fitness level of the athletes. However, there was no significant difference in the mean weights of Group S and Group Non-AS (p-value > 0.05) (**Figure 2A**). The three groups were comparable in height distribution (**Figure 2B**). The mean body mass index (BMI) of Group A was significantly lower than that of Group Non-AS (p-value < 0.001), but there was no statistically significant difference between Group S and Group Non-AS (**Figure 2C**).

Vital capacity

Similar to previous studies on vital capacity, the males (3196.67±735.78cm3) in our study had a significantly higher vital capacity than females (2865±478.17cm3, p-value < 0.043) (**Figure 3**).

Both Group A and Group S exhibited a significantly

Age	Grp A	Grp S	Grp Non-AS	Total A & Non-AS	Total S & Non-AS
13	12 (60%)	13 (65%)	15 (75%)	27 (67.5%)	28 (70%)
14	8 (40%)	7 (35%)	5 (25%)	13 (32.5%)	12 (30%)
	20 (100%)	20 (100%)	20 (100%)	40 (100%)	40 (100%)

Table 1: Age distrbution of experimental groups.

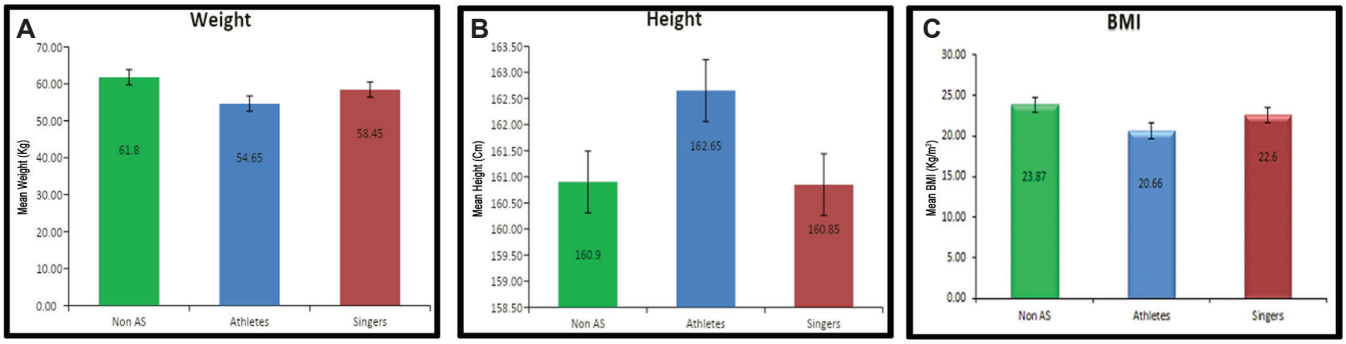

Figure 2: Participant demographics of athletes, singers, and non-athlete non-singer groups. The bar graph depicts mean weight. The bar graph depicts mean height (B). The bar graph depicts the mean BMI Above the 85thpercentile was considered overweight. For males, a value of BMI above 22 for 13 years and above 22.5 for 14 years was considered overweight. For females, value of BMI above 22.5 at 13 years and above 23.5 at 14 years was calculated as overweight (C). The error bars represent the standard deviation.

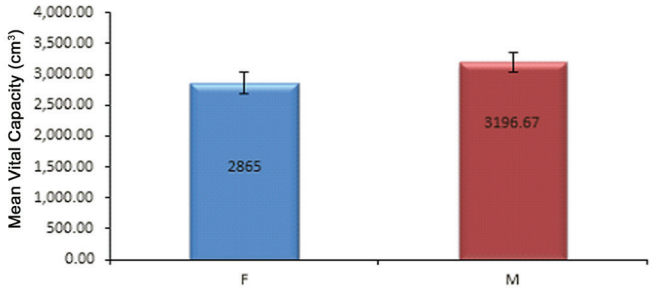

Figure 3: The vital capacity of males (n=30) and females (n=30) of all three groups. Males had significantly higher vital capacity than non- athletes, non-singers. The error bars represent the standard deviation.

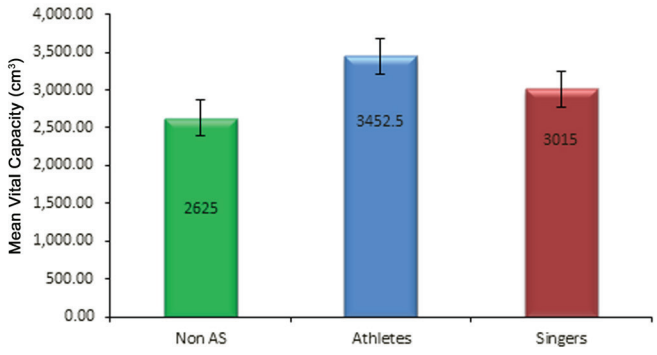

Figure 4: Athletes and singers had significantly higher vital capacity than non- athletes, non-singers. The error bars represent the standard deviation.

higher vital capacity than the control group (p-value 0.0002 and p-value 0.01, respectively) thus reflecting that athletes and singers had a better vital capacity than control group. Athletes had significantly higher vital capacity than singers (Group A 3452±696.7cm3 and Group S 3015±346.83 cm3, p- value 0.016) (**Figure 4**).

Regression analysis was used to determine factors affecting vital capacity. Univariate linear regression demonstrated that male gender and height significantly affected lung capacity. The unstandardized coefficients B in males was 331.667 as compared to females; thus, the vital capacity was significantly higher in males than females (p-value 0.043). Increasing the height by 1 cm increased the vital capacity by 36.450 mL (p-value 0.034).The unstandardized coefficients B in athletes and singers was 827.500 and 195.00 as compared to Non-AS; thus, the vital capacity of athletes and singers was significantly higher than Non-AS (Group A p-value 0.0002, Group S p<0.010). Weight, however, did not affect vital capacity (p-value >0.05). Since there was no statistically significant difference in the ages of participants in the three groups, age did not affect the vital capacity either.

DISCUSSION

The aim of our study was to determine if sports and singing could improve vital capacity. All groups were comparable in age distribution. The vital capacity of males was found to be better than that of females. Similar results have been observed in other studies and sex differences have been attributed to dissimilarities in lung development and physiology (21, 22).

The BMI of group A was significantly lower than that of group Non-AS, but there was no statistically significant difference between groups S and Non-AS. This is in agreement with several studies which have observed the effects of obesity in the adolescent age group (23). Increased weight causes a major change in the respiratory system, resulting in loss of thoracoabdominal synchronism and limitation of diaphragmatic mobility (24, 25). Thus, BMI showed a positive correlation with pulmonary function.

Increased metabolic activity during physical activity improves pulmonary functions through strengthening of the pulmonary muscles. We demonstrated that both athletes and singers had better vital capacity than the control group, similar to other studies (18, 20, 26, 27). Though there are studies which have compared athletes and singers separately with the control group, only one study has compared all three. Athletes exhibited a better vital capacity in our study in comparison to singers; however, Imam et al. found no

Figure 5: Student's spirometer. Apparatus for measuring the volume of air inspired and expired by the lungs by water displacement method.

STUDY PROFORMA

A. Student Particulars: Name: Age: Sex:

B. Brief History: Past History: Of URI or LRTI over last 15 days/ Chronic lung disease/ Any other systemic disease

C. Pre Procedure Formalities: Informed written consent is taken

Subject Information Data Sheet

Height
Weight
Vital Capacity =
Any Comments :

Figure 6: Study proforma. Details recorded for each participant. Three values of vital capacity were observed and the highest was taken.

conclusive difference (28).

Children are most susceptible to the harmful effects of air pollution because they spend more time outdoors than adults and are outdoors when air pollution levels are higher. Their bodily functions demand significantly higher oxygen levels, so their respiration rates are higher. Additionally, because of their small stature, their breathing zone is closer to the ground where the most polluted air is. Furthermore, the diameters of the alveoli in their airways are narrower, which makes them more easily affected by inflammation caused by air pollution (15, 29, 30). A study conducted by Paul Mohai et al. concluded that air pollution around schools was linked to poorer student health and academic performance (31). However, restricting outdoor activities is not a practical solution for children who cannot miss school for a long time. Moreover, school life is all about activities of various forms for the wholesome development of the child. Although exercise promotes positive morpho-physiological adaptations in the cardiorespiratory system, the positive effects of exercise can be suppressed by air pollution. This presents an interesting challenge of balancing the beneficial effects of exercise with the detrimental effects of air pollution upon health. Exercisers can mitigate the adverse health effects of air pollution exposure during exercise by exercising for a period of no more than 30 minutes, avoiding the peak traffic hours of the day, and strictly watching air quality index levels and land use planning (32-33). Opting for a healthy lifestyle and exercises done correctly may be an effective coping strategy for dealing with the menace of air pollution.

Since we planned to evaluate the same age group to avoid any confounding results based on age differences, our sample size was small due to the limited number of students of either sex who actively participated in sports or were in choir. Measuring lung capacity with a simple instrument like the Student's spirometer produced limited results. We will need more sophisticated equipment to measure other parameters and timed vital capacity. Long-term experimental studies need to be conducted to confirm the effect of singing training and outdoor sports by comparing the initial lung vital capacity to vital capacity after the training sessions.

In the future, we would like to conduct a prospective study on the control group by introducing them to regular exercises for a period of six months and observe the change in vital capacity. To understand the association with air pollution, we would conduct a study to observe the daily exposure to air pollution by air quality index and correlate it with the lung functions of students.

We concluded that the vital capacities of both athletes and singers were significantly higher than that of non-athletes non-singers, and that the performance of athletes was even better than singers. The vital capacities of males were also significantly higher than females.The BMI of athletes was significantly lower than singers and Non AS.

METHODS

With the approval of the institutional scientific and ethical committee and written informed consent of the students, a pilot study was initially performed to calculate the power of the study. On the basis of the pilot study, mean values of vital capacity of singers was 3310 ± 394.33cm3, of athletes was 3410 ± 424.85cm3 and of non-singers and nonathletes was 2720 ± 465.83cm3, measured using a Student's Spirometer of capacity 6000 mL (**Figure 5**). Taking these values as a

reference, minimum required sample size with 95% power of study and 5% level of significance was determined to be 14 participants in each study group. To reduce margin of error, the total sample size taken was 60 (20 per group). Informed consent was gained from the participants after explaining the significance of the study and importance of their participation. A brief history was used to rule out exclusion criteria, such as students unwilling to consent, those with a history of allergies, chest infections, severe systemic illness, or drug intake. Participants who fulfilled the inclusion criteria were given more information about the study and invited to participate. Weight and height along with the other details were filled in the study proforma using a height chart and weighing scale (**Figure 6**). Students were instructed how to perform the procedure. Males and females were matched in terms of their height and their weight to remove any confounding factors. The open tube end of the spirometer was cleaned with a spirit swab and gloves. The participant was instructed to pinch the nostrils, take a deep breath, hold it, and then exhale into the open end of the tubing. The lung capacity was measured by the amount of air blown into the tubing. Three such values taken three minutes apart were noted and then the highest of the three was used for calculations. The mouth tubing was washed after each subject.

Statistical Testing

Quantitative variables were compared using unpaired t-test/Mann-Whitney Test (when the data sets were not normally distributed) between the three groups. Qualitative variables were compared using Chi-Square test/Fisher's exact test. Univariate linear regression was used to identify significant factors affecting lung capacity. A p-value of less than 0.05 was considered statistically significant. The data was entered in MS EXCEL spreadsheet and analysis was done using Statistical Package for Social Sciences (SPSS) version 21.0.

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Article

Are Teens Willing to Pay More for Their Preferred Goods?

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SUMMARY

Past research has suggested that brand-loyal adults are willing to pay more for their preferred brand. This study investigated if African American teens are willing to pay more for their preferred brand of honey bun and if, in a real store scenario, a price predetermined by a survey of brand-loyal customers will generate the highest profit. By using a survey of customers who prefer a name-brand honey bun, we found that brand-loyal customers reported they were willing on average to pay $1.26, while non-brand-loyal customers were willing to pay only $1.08. The surveyed price was used in a real store scenario by setting three prices: one lower than the surveyed price, one at the surveyed price, and one higher than the surveyed price. Customers shopped at the store regularly without instruction during the three weeks of price changes. We found the surveyed price produced the most profit. Our findings suggest that not just brand-loyal adults, but also brand-loyal African American teens are willing to pay more for a preferred brand of food product. Additionally, our study shows that surveying customers is an effective way to identify brand-loyal consumers and determine the ideal price to maximize profit for a particular product.

INTRODUCTION

Brand loyalty refers to a person's commitment to a product or company and preference for that brand over others (1,2). Many different factors affect the amount of brand loyalty a person has. For example, one study found that brand trust (when the consumer has feelings of reliability, honesty, and confidence in the brand) and brand affect (the brand's ability to cause positive emotions in the customer) are antecedents of brand loyalty Researchers found a positive effect of both brand trust and brand affect on brand loyalty as well as on price tolerance, with brand-loyal consumers willing to pay more for their preferred brand (3). Further research has investigated the maximum price brand-loyal consumers are willing to pay for their preferred brand compared to non-loyal consumers of the same product (1). In that study, a questionnaire was created to assess participants' brand loyalty to a specific brand of toothpaste, and asked questions regarding how much the participant would pay for the preferred brand. The results of the study revealed that, on average, brand-loyal participants were willing to pay 10.3% more than non-loyal participants for their preferred name-brand toothpaste.

In addition to brand loyalty, advertisements and promotions can also affect a consumer's attention towards a particular brand. A study analyzing the purchasing habits of 14,000 German families concluded that brand-loyal customers are attracted to name-brands over generic brands and are willing to pay more for their favored product (4). Overall, this research demonstrates that the majority of consumers are willing to pay more for a preferred brand.

However, little research has investigated whether these findings about brand-loyal adults can be extended to brand-loyal teenagers. Because teenagers have fewer financial responsibilities than adults, they may make different decisions on how they spend money. The average U.S. teenager is estimated to spend $428 monthly, with African American teens spending 6% more than average on a monthly basis, and one-third of African American teens purchasing food with their own money (5). These spending habits affect advertiser spending choices; in 2004, advertisers spent $1.6 million on cookie and cracker advertisements targeted towards African Americans (5). Therefore, understanding how brand loyalty affects the spending habits of African American teenagers is of significant interest to product manufacturers and retailers. Most of the studies done focused on brand loyalty and its connection to consumers' willingness to pay more for a product using surveys (1, 2, 3). The current study is unique because in addition to a survey, the brand-loyal price premium will be tested in a real store scenario to determine if that price will maximize profit. We hypothesize that people who say they are more brand loyal to specific brand will be willing to pay more for their preferred brand. This agrees with previous research (1, 3, 4). Also, we hypothesize that the average price premium of brand loyal customers from the survey will cause the highest profit in a real store scenario.

RESULTS

The purpose of this study is to see if brand-loyal teenagers are willing to pay more for their preferred name brand product, as has been previously demonstrated for brand-loyal adults. Additionally, the survey data collected to assess brand loyalty was used to calculate an optimal product price, which was then tested in a real store scenario to assess whether the chosen price produces the most profit for a store. We hypothesize that people who say they are more loyal to a specific name brand (Krispy Kreme honey buns) will be willing to pay more for their preferred brand. This agrees with previous research (1, 3, 4). Also, we hypothesize that the average price premium of brand-loyal customers from the survey will generate the highest revenue in a real store scenario.

Comparing Brand Loyalty Price Willing to Pay for Non-Brand Loyal Willingness to Pay

A total of 126 students, in grades 6-12, were surveyed regarding their preferred brand of honey bun and how brand loyal they were to their preferred brand. The 94 students who reported preferring Krispy Kreme honey buns also reported the maximum amount they were willing to pay. The sample was split into brand-loyal and non-brand-loyal customers using the median score on the brand loyalty questions. Seventy-one students were brand-loyal. A t-test for independent samples revealed that there was a significant difference between a brand-loyal customer's willingness to pay for a Krispy Kreme honey bun compared to a non-loyal customers willingness to pay ($t(92) = -2.13$, $p = 0.018$) (**Figure 1**). On average, brand-loyal customers were willing to spend $1.26 for a name-brand honey bun (SD = 0.42), while non-brand-loyal customers were only willing to spend $1.08 (SD=0.34) (**Figure 1**).

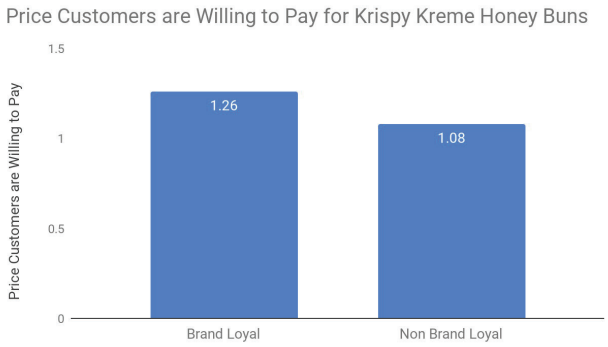

Figure 1. Willingness to Pay for Krispy Kreme Honey Buns. The price customers are willing to pay for a name brand honey bun is higher for brand loyal customers than non-brand loyal customers ($p=0.018$).

Maximizing Profit in a Real Store Scenario

In week one, Krispy Kreme honey buns were sold at $1.00, a lower cost than either brand-loyal or non-brand-loyal customers reported they were willing to spend. In this week, there were 30 Krispy Kreme honey buns sold and 34 Duchess honey buns (an alternative brand) sold, each for $0.75 (Table 1). However, the supply cost for Krispy Kreme honey buns was $0.83 each, compared to $0.37 each for Duchess honey buns. Although more revenue was earned from Krispy Kreme sales, a profit of only $5.07 was generated from these sales, compared to a profit of $12.82 from sales of Duchess honey buns. Under this pricing scheme, consumers who reported being brand-loyal to Krispy Kreme bought their preferred brand 50% of the time; customers who self-identified as brand-loyal to Krispy Kreme purchased 20 Krispy Kreme honey buns and 20 Duchess honey buns during week one.

In week two, the price for Krispy Kreme honey buns was raised to $1.25 to match the average price that brand-loyal consumers reported they were willing to pay when surveyed.

	Week 1 Krispy Kreme $1.00 Duchess $0.75	**Week 2** Krispy Kreme $1.25 Duchess $0.75	**Week 3** Krispy Kreme $1.50 Duchess $0.75
Total Number of Krispy Kreme Sales	30	42	18
Total Number of Duchess Sales	34	35	40
Total Supply Cost of Krispy Kreme	$24.93	$34.90	$14.96
Total Supply Cost of Duchess	$12.68	$13.06	$14.92
Krispy Kreme Revenue	$30	$52.50	$27
Duchess Revenue	$25.50	$26.25	$30
Krispy Kreme Profit	$5.07	$17.60	$12.04
Duchess Profit	$12.82	$12.94	$15.08
Total Profit	$17.89	$30.54	$27.12
Number of Krispy Kreme Purchases by Customers who are Brand Loyal to Krispy Kreme	20	19	9
Number of Duchess Purchases by Customers who are Brand Loyal to Krispy Kreme	20	19	14

Table 1. Weekly Sales Statistics for Krispy Kreme and Duchess Honey Buns. The table displays the sales count, revenue, cost, and profit for both brands of honey buns. Each column represents a different price charged for Krispy Kremes in a given week. The price charged for Krispy Kreme honey buns during the second week was determined by calculating the average price surveyed brand-loyal consumers reported they would be willing to pay.

In week two, 42 Krispy Kreme honey buns were sold, compared to 30 Krispy Kreme honey buns sold in week one (**Table 1**). Similarly, more Duchess honey buns were sold in week two than week one, with 34 sold in week one and 35 sold in week two. Krispy Kreme honey buns generated a higher revenue in week two ($52.50 generated in week two compared to $30.00 generated in week one), but Duchess revenue did not change from week to week ($26.25 generated in week two compared to $25.50 generated in week one). This price scheme produced the largest total honey bun profit for the store ($30.54 in week two compared to $17.89 in week one). The brand-loyal purchasing rate did not change from 50% of purchases.

In week three, the price was raised to $1.50 for a Krispy Kreme honey bun (higher than the average price surveyed brand-loyal consumers reported being willing to pay), and Krispy Kreme sales decreased from 42 sold in week two to 18 sold in week three (**Table 1**). Conversely, Duchess honey bun sales increased from 35 in week two to 40 in week three. Consequently, Krispy Kreme revenue decreased and Duchess revenue increased from week two to week three. This suggests the price change made people abandon their

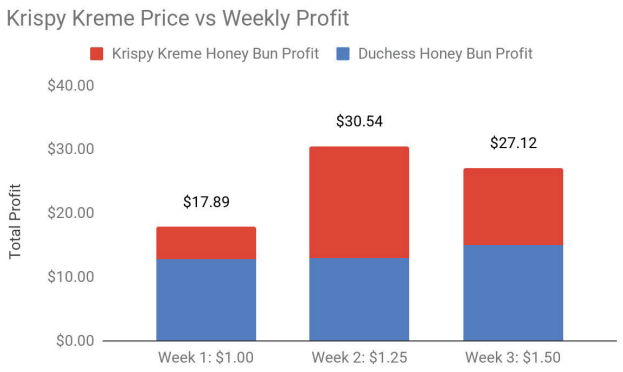

Figure 2. Krispy Kreme Price vs. Weekly Profit. The figure shows how much honey bun profit the store made from Krispy Kreme's price changes, separated by brand of honey bun. The label above each column represents the total honey bun profit.

preferred brand (Krispy Kreme); this conclusion is also supported by our finding that Krispy Kreme honey bun purchase frequency for brand-loyal customers dropped from 50% in weeks one and two to 40% in week three. Interestingly, the overall profit was almost the same in week three as it was in week two ($27.12 earned in week three compared to $30.54 earned in week two), suggesting people might be motivated to buy the more profitable Duchess honey bun when their preferred brand of honey bun (Krispy Kreme) was priced too high.

DISCUSSION

We hypothesized that people who self-report that they are loyal to a name brand (Krispy Kreme) will be willing to pay more for their preferred brand. We also hypothesized that using survey data to calculate the average price brand-loyal customers are willing to pay for a product would allow us to determine the price point that would generate the highest revenue in a real store scenario. Both hypotheses were supported by our data. Brand-loyal participants were willing to pay an average of $1.26 for a Krispy Kreme honey bun, while non-brand-loyal participants were only willing to pay $1.08 for the same product, supporting the claim that brand-loyal customers will pay more for a product than non-brand-loyal customers. During week two, when Krispy Kreme honey buns were priced at the average price brand-loyal customers reported being willing to pay, the store generated higher revenue than was generated when Krispy Kreme honey buns were priced at a lower or higher value (weeks one and three, respectively).

We found that brand-loyal teenagers, similar to brand-loyal adults (1), are willing to pay more for a brand compared to a non-brand-loyal consumers. Our results were generally consistent with those seen in previous studies; for example, while a study surveying consumers about their willingness to pay for toothpaste concluded that brand-loyal customers were willing to pay 10.3% more than a non-brand loyal consumer for their preferred toothpaste (1), our study found that brand-loyal teenagers were willing to pay 16.7% more for their preferred brand on honey bun. However, our study expanded upon these survey results to find that pricing a product at the average price brand-loyal consumers are willing to pay for it generates the maximum profit in a real store scenario; since previous research did not test survey data in a similar scenario, our work contributes novel information to the field. While there are concerns that self-reported survey data may not accurately reflect consumer behavior, our research supports the idea that, at least in this context, survey data is an accurate reflection of the amount consumers are willing to pay for a product.

There are some possible confounding factors that may have affected this study. The first possible source of error was that the store was not allowed to sell to middle-schoolers (6-7th graders) on the last two days of the last week, as administrators claimed that the store was causing them to be disruptive in class. This may have altered our results, but we do not feel that the effect was significant enough to decrease confidence in our overall conclusions. In week two, 26% of Krispy Kreme honey buns were bought by middle schoolers, but during the first two days of week three, only 5.5% of sales were to middle schoolers. Therefore, we believe that the exclusion of middle-school consumers from the last two days of week three should not have affected our results in any meaningful way, as the price change likely caused them to stop buying Krispy Kreme honey buns independently of the administrative ban. Other external events that may have influenced our experimental results were several snow days during week two that prevented sales two days out of the week before school was canceled because of the snowstorm. To obtain a full set of data at these price points, week two data was gathered the following week instead. This might have caused consumers to get used to the new price because they were exposed to that pricing scheme for a longer amount of time; under this hypothesis, when the Krispy Kreme honey bun price was raised to $1.50 in week three, they may have reacted in a more negative way due to additional familiarity with the week two price.

We recommend to future researchers to run the store under each pricing scheme for one month rather than one week to collect more robust data. We believe that this would allow consumers to get used to each price chance and remove any shock to the consumers caused from the price change happening every week. Future studies could also be expanded to investigate different types of products, like a comparison of name-brand tea (Pure Leaf or Gold Peak) to a generic brand (e.g., Tuner's tea). These proposed experiments would allow researchers to determine if our findings can be generalized to all snack foods and drinks, or if particular products are subject to different purchasing behavior.

In conclusion, we recommend that business owners use surveys of potential customers to help determine optimal

pricing for a name-brand product compared to a comparable generic product. Furthermore, entrepreneurs should use a survey to measure the amount of brand loyalty their target consumers have towards particular brands so marketers better understand their market segment and how to optimize product pricing to maximize business revenue.

MATERIALS AND METHODS

The purpose of this study was to investigate if brand-loyal African American teenagers (ages 12-18) were willing to pay more for a name-brand honey bun versus a generic brand honey bun in a real-life scenario. To assess this, we used a self-created survey given to the entire student body of 126 African American students attending a college preparatory school enrolled in grades 6-11. The student body contains 14% 6th graders, 14% 7th graders, 18% 8th graders, 15% 9th graders, 23% 10th graders, and 16% 11th graders. The school is 52% male, and 48% female in the surveyed grades.

Before completing the survey, the participants were given two types of honeys bun to sample. The first one was the name-brand "Krispy Kreme" honey bun, and the other was a "Duchess" honey bun, a common generic brand of bakery items known for their low cost and wide accessibility in local corner stores in the area of the school. On the survey, participants were asked to indicate which of the two honey buns they preferred. Additional questions were designed to assess brand loyalty; the questions were a combination of self-created questions and questions adapted from a similar study (3). Each question required a response from 1-5 to describe how they agreed with the statements, with 1 being "not at all" and 5 being "very much." Finally, surveyed students who initially indicated that they preferred Krispy Kreme honey buns over Duchess honey buns were asked how much they would pay for a Krispy Kreme honey bun by choosing from a list of prices ranging from $0.75 to $2.00. The participants were considered brand-loyal if they had median score of 28 or higher on the brand loyalty questions. This cutoff was set at the median score from all surveyed participants, meaning that customers considered brand-loyal are more brand-loyal than the average consumer, while customers considered non-brand-loyal are less brand-loyal than the average consumer. Our participant pool consisted of 71 brand-loyal consumers and 55 non-brand-loyal consumers. Ninety-four of the participants preferred Krispy Kreme honey buns and 32 of the participants preferred Duchess Honey Buns.

The price name-brand-loyal customers were willing to pay was determined by taking the average of all responses to the last question from the survey. The average was calculated at $1.26; however, the price value used in the real-store scenario was rounded down to $1.25, as the store does not use pennies and the true average price would have therefore brought up too many problems with change. To test if this price would produce the highest profit, we also selected a price lower and a price higher than the average. Therefore, Krispy Kreme honey buns were sold for $1.00 (lower price) during week one, for $1.25 (surveyed price) during week two, and $1.50 (higher price) during week three.

The school store is run by the study authors out of an office during high school and middle school lunch periods, as well as during an afternoon snack period for a total of 90 minutes each day. The store is open four days a week. Participants' purchases were tracked by store receipts, which included customers' names on them and could therefore be matched with their survey responses. During the four weeks prior to the start of the experiment, the store averaged around 38 customers making an average of $412.75 in revenue per week.

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Article

Development of a Novel Treatment Strategy to treat Parkinsonian Neurodegeneration by targeting both Lewy Body Aggregation and Dopaminergic Neuronal Degradation in a *Drosophila Melanogaster* Model

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SUMMARY

Parkinson's Disease (PD) is characterized by the progressive degradation of dopaminergic neurons in the substantia nigra of the brain and is triggered by both environmental and genetic factors. PD is characterized by symptoms that can range from muscle dysfunction to severe mood disturbances. The pathology of PD is two-fold: (1) degradation of dopaminergic neurons due to mitochondrial calcium overload and (2) deposits of α-synuclein that increase cytosolic calcium levels in the brain. In this study, we tested a therapy that targets both pathologic manifestations of PD in fruit flies. Specifically, we used combinations of the drugs Ruthenium red (RuR), a mitochondrial channel uniporter inhibitor, and Ambroxol, a pharmacological chaperone of the lysosomal enzyme glucocerebrosidase that digests α-synuclein, to treat PD in fruit flies. We then measured the climbing ability, ATP content, and glucocerebrosidase activity in the fruit flies. While the drugs showed positive results individually, we found that the drugs had a synergistic effect when used together that resulted in a statistically significant increase in climbing ability, ATP content, and glucocerebrosidase activity. The results of this study indicate that these drugs could be used in treatments for PD.

INTRODUCTION

Parkinson's Disease (PD) is a chronic neurodegenerative syndrome of the central nervous system that affects approximately 1% of all individuals over 60 years of age. PD is primarily associated with symptoms of motor dysfunction, including shaking, rigidity, slowness of movement, and difficulty walking (1). The etiology of PD remains poorly understood, but several environmental and genetic factors are known to contribute to increased risk of PD. Exposure to certain chemicals and mutations of various genes can also produce parkinsonian symptoms (2). However, due to our lack of knowledge of its specific etiology, parkinsonian disorders are characterized primarily by two neuropathologic findings: (1) the degradation of pigmented dopaminergic neurons in the substantia nigra pars compacta and (2) the presence of Lewy body aggregates and Lewy neurites (3).

The presence of Lewy body aggregates is a feature in all postmortem analyses of human parkinsonian degeneration. While PD in fruit flies does not produce the same deposits of protein, inserting the Synuclein Alpha (SNCA) gene into the fruit fly genome leads to the production of human α-synuclein protein and to parkinsonian neurodegenerative effects in flies. Lewy body aggregates, or clumps of protein that often include the protein α-synuclein, have been shown to contribute to both motor and non-motor symptoms of PD (4). Alterations of the SNCA gene which cause misfolding of the α-synuclein protein and those which cause excess production of α-synuclein have been found to cause parkinsonian symptoms and these misfolded or excess α-synuclein proteins cluster together in Lewy bodies (5). While very little is known about these deposits, most research indicates that the deposits have negative effects on the human nervous system. α-synuclein has been implicated in the release of harmful pro-inflammatory cytokines, which exacerbate dopaminergic neurodegeneration by activating microglia and facilitating neuronal death (6). Furthermore, α-synuclein aggregates bind to and activate the SERCA calcium pump in vitro, leading to increased mitochondrial calcium concentrations by passage of the ions through the Mitochondria-Attached Membranes (MAM) and the Mitochondrial Channel Uniporter (MCU) (7). This research suggests that targeting these protein deposits may be critical for effective PD therapy.

Furthermore, additional research indicates that the dissolution of protein aggregates may also be critical for effective PD therapy. The neurotransmitter dopamine is oxidized into neuromelanin when a burdened neuron cannot sequester the dopamine into vesicles before its antioxidant defenses take over and this process increases with age. Due to the accumulation of neuromelanin, the lysosomes cease to function and are unable to digest α-synuclein (8). α-synuclein is normally present in the cell in small concentrations, but this lysosomal enzymatic dysfunction leads to accumulation of the protein. One key lysosomal enzyme that is implicated in this process is the enzyme glucocerebrosidase (GBA) (9). Mutations of the GBA enzyme increase the risk of PD approximately 20-fold, indicating that GBA dysfunction may contribute to these protein deposits and parkinsonian disorders (10). Increasing α-synuclein digestion by improving lysosomal function could be an effective therapy for the reduction of α-synuclein levels. One drug that has potential to reduce protein aggregation is Ambroxol (C13H18Br2N2O).

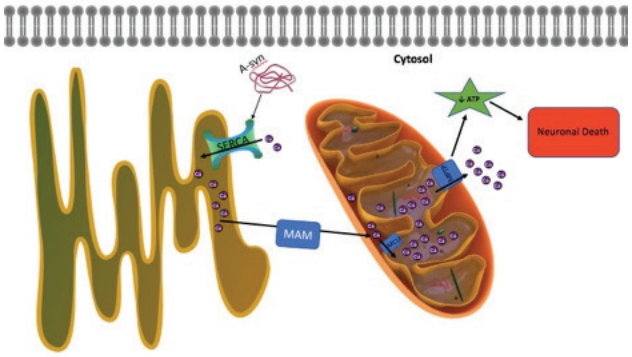

Figure 1. Dysregulation of calcium ions leading to neuronal death. This diagram shows how the α-synuclein protein activates the calcium pump SERCA and causes an influx of calcium ions inside the endoplasmic reticulum (ER). Through the mitochondrial-attached membranes (MAM), the calcium ions enter the mitochondria and through the mitochondrial calcium uniporter (MCU), pass through the inner mitochondrial membrane.

Ambroxol is a drug used in the treatment of respiratory diseases associated with excessive mucus. Ambroxol has recently been shown to increase activity of GBA and has been used as an effective treatment for neuronopathic Gaucher disease, which is a lysosomal storage disease characterized by a deficiency of GBA. Oral Ambroxol reduced symptoms in Gaucher's patients in a pilot study, indicating that this drug crosses the blood-brain barrier (11). Although much research has been done on substances that reduce α-synuclein production and aggregation, few are able to cross the blood-brain barrier.

Recently, oxidative stress has been implicated as one of the environmental causes of parkinsonian neurodegeneration. Oxidative stress is an imbalance between the production of free radicals and the ability of the body to counteract or detoxify their harmful effects. Research on common oxidative stressors, such as MPTP (1-methyl-4-phenyl-1,2,3,6-tetrabydropyridine) and rotenone, demonstrated their ability to cause parkinsonian pathology (12). One study linked PD symptoms in patients to consumption of the illicit drug MPTP, and postmortem analyses revealed destruction of the substantia nigra (13). In addition, many studies have confirmed the presence of oxidative stress in postmortem animal and human models of PD (14). In another study, dosing fruit flies with potent concentrations of the drug rotenone generated parkinsonian phenotypes such as neuromotor dysfunction due to oxidative stress (15). Any process that decreases oxidative stress and lowers the production of reactive oxidative species (ROS) could reduce or prevent Drosophila neurodegeneration.

New research has implicated mitochondrial calcium dysregulation in the pathogenesis of PD (16). The production of oxidative stress through the opening of the mitochondrial permeability transition pore (mPTP) is explored in this study. The mitochondrial calcium uniporter (MCU) is a calcium channel located on the inner mitochondrial membrane that allows the passage of calcium ions from a cell's cytosol to the mitochondria. The MCU is the primary regulator for calcium influx and key to calcium homeostasis, and has been proven to control the rate of energy production and cell death (17). The excessive calcium ion concentration in the mitochondria is what causes the dopaminergic neuronal death in PD (**Figure 1**). Interestingly, the mutation of genes such as PINK1 and Parkin, which control the opening of the MCU, is a common genetic factor for PD (16). Excessive mitochondrial calcium influx triggers the opening of the mPTP on the inner mitochondrial membrane and triggers the release of harmful ROS, leading to neuronal death by excitotoxicity. Blocking the passage of calcium ions through the MCU is one method to prevent excessive calcium influx and the opening of the mPTP. In a cardiac injury model, mice lacking Mcu, a subunit of the MCU, are protected against Calcium overload by preventing activation of the mitochondrial permeability transition pore (18). Ruthenium red (RuR), an MCU inhibitor, could have a similar mechanism and therefore prevent neuronal death.

We used the organism Drosophila melanogaster in this study to model PD by using both transgenic flies and drug-induced wild-type flies. To model familial Parkinson's in humans, we used α-synuclein transgenic flies that produce Lewy body aggregates. To model sporadic PD in humans, we used an induced model in flies through the introduction of rotenone into the fruit fly medium. This method allows for accurate evaluation of both idiopathic and familial PD. Although both have similar pathophysiology, their etiology remains obscure, so this study will improve knowledge of both forms of PD . The model Drosophila melanogaster was selected due to their rapid reproductive rate and relatively low maintenance expenses, allowing for reliable testing of several experimental conditions. Moreover, models of PD in fruit flies demonstrate the same neuromotor dysfunction observed in humans, which allows us to correlate the neurodegeneration between species (19). Although Lewy bodies do not appear in the wild-type Drosophila model of PD, inserting the SNCA gene for α-synuclein into the mutant fly genome generates the same protein deposits and neurodegeneration found in human brains.

The purpose of this study was to develop a drug therapy targeting parkinsonian symptoms in fruit flies exposed to oxidative stressors or genetically induced to model PD. We hypothesized that the fruit flies given both Ambroxol and RuR therapy in the highest concentrations would display better climbing abilities, higher ATP levels, and higher GBA activity compared to the untreated group. To assess the effects of Ambroxol and RuR on parkinsonian degeneration, we utilized three main assays: a negative geotaxis assay, an ATP bioluminescence assay, and a GBA activity assay.

This research will contribute to the development of a sustainable treatment for PD and advance our understanding of oxidative stressors.

Figure 2. A picture of the negative geotaxis assay apparatus. The apparatus was constructed using two vials and masking tape. A mark at a point 8.5 cm above the bottom of the vial was made.

RESULTS

All in all, the goal of this project is to record and analyze the effects of the two drugs tested here on two different forms of PD in Drosophila melanogaster. We hypothesized that the drugs would improve neurological and cellular function and to assess the effects of RuR and Ambroxol, we measured the climbing abilities, ATP levels, and GBA activity of fruit flies exposed to media hydrated with solutions of 500 μM, 1 mM and 5 mM of Ambroxol and 100 μM, 250 μM and 500 μM of RuR, both separately and with combinations of both the solutions. Results from flies exposed to media with drug treatments were compared to results from flies who were fed medium without any drug treatment. Multiple trials of conditions were conducted to ensure preciseness and accuracy of data. For the negative geotaxis assay, three trials of 10 flies per experimental condition were used. For the ATP bioluminescence assay and GBA enzyme activity assay, we generated 3 sample replicates per condition, which consisted of homogenate made from 20 flies each.

For the negative geotaxis assay, we set up vials with 10 fruit flies and measured the percentage of flies that crossed a mark drawn 8.5 cm from the bottom of the vial (**Figure 2**). We conducted 3 trials per vial before, during, and after the 10- day study (Day 0, 5, and 10). The negative geotaxis assay utilizes the natural tendency of flies to fly up against gravity when agitated to measure their locomotive capabilities. Negative geotaxis is an innate escape response in fruit flies. This assay is sensitive to deficits of motor coordination and can therefore be used to test the effects of RuR and Ambroxol on parkinsonian motor degeneration. We placed 10 flies in a vial and tapped the vial to dislodge the fruit flies to the bottom of the vial. We allowed the flies to climb up the side of the vial for 10 seconds. The number of flies above a certain point on the vial is then measured. Fewer flies climbing up the sides of the vial indicates faster disease progression. Ambroxol and RuR had a significant impact on the flies' neurological function based on the results of the negative geotaxis assay (**Figures 3** and **4**). While most of the treated and untreated PD model flies gradually lost motor function over time, treatment with Ambroxol and RuR significantly increased the number of flies that climbed past the mark (p-value < 0.05). Moreover, treatment with these drugs at higher concentrations had a greater impact on locomotive capabilities in treated flies than treatment at a lower concentration, further suggesting that the observed improvement in locomotive capabilities was due to treatment and not due to age-related or other random factors. The treated flies exhibited improved locomotive capabilities compared to their normal (mutant flies with normal diet and wild-type (WD) flies with rotenone) counterparts.

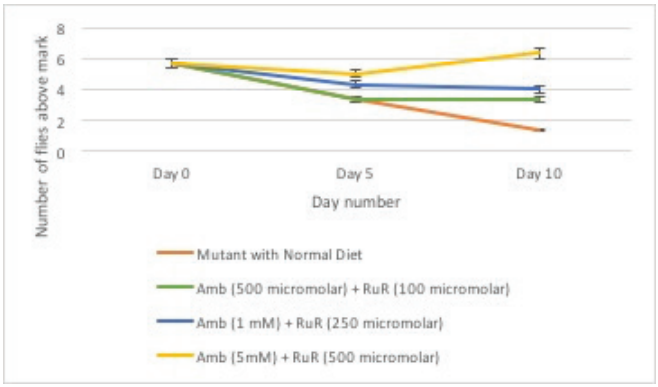

Figure 3. Negative Geotaxis Assay of the Mutant (Mut) Flies cultured with the drugs Ambroxol (Amb) and Ruthenium red (RuR) at different concentrations. The average number of mutant flies above the 8.5 cm mark during the negative geotaxis assay. Three trials of ten flies for each condition (normal diet, 500uM Ambroxol + 100uM RuR, 1mM Ambroxol + 250uM RuR, and 5mM Ambroxol + 500uM RuR) were conducted at Day 0, 5, and 10. Error bars represent a 95 percent confidence interval. Asterisks (*) represent statistical significance (paired sample, one-tailed student's t-test); * = p-value < 0.05, * = p-value < 0.01, and * = p-value < 0.001.

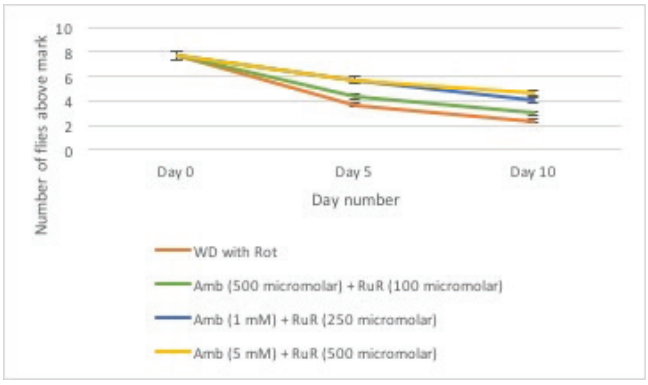

Figure 4. Negative Geotaxis assay of the Wild-Type (WD) Flies with Rotenone (Rot) cultured with the drugs Ambroxol (Amb) and Ruthenium red (RuR) at different concentrations. Flies were tapped down the bottom of the apparatus shown in Figure 2 and were allowed to climb back up for 10 seconds. The number of flies above a mark 8.5 centimeters below the bottom of the apparatus were counted. 3 trials of 10 flies were conducted for each experimental condition. Error bars represent a 95 percent confidence interval. Asterisks (*) represent statistical significance (paired sample, one-tailed student's t-test); * = p-value < 0.05, * = p-value < 0.01, and * = p-value < 0.001.

The ATP bioluminescence assay uses firefly luciferase, a monomeric protein that catalyzes luciferin oxidation using ATP as a co-substrate. Through the conversion of luciferin to oxyluciferin, light is produced in the presence of ATP. The

luminescence can be quantified to yield a value for ATP content in the samples. The ATP assay test was conducted to

on the theory of preservation of mitochondrial integrity by inhibiting the MCU, more ATP should be produced for more

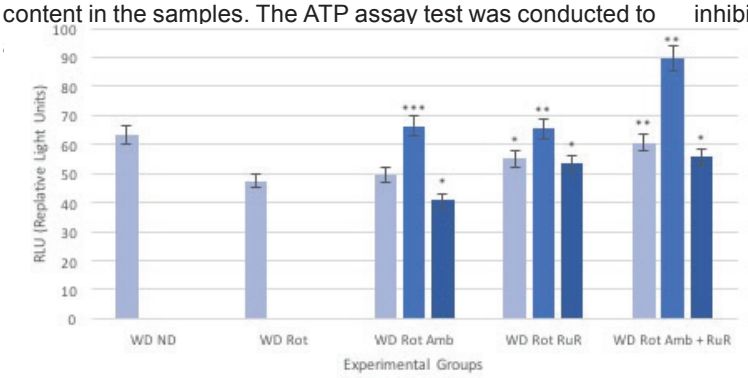

Figure 5. ATP Bioluminescence Assay of Wild-Type (WD) flies with Rotenone (Rot) cultured with the drugs Ambroxol (Amb) and Ruthenium red (RuR) at different concentrations and Normal Diet (ND). Average ATP activity in mutant flies was measured for three concentrations (1 = 500uM Ambroxol and/or 100uM RuR, 2 = 1mM Ambroxol and/or250 uM RuR, and 3 = 5mM Ambroxol and/or 500uM RuR). Averages were from homogenates of three trials of 20 flies, and error bars represent a 95 percent confidence interval. Asterisks (*) represent statistical significance (paired sample, one-tailed student's t-test); * = p-value < 0.05, * = p-value < 0.01, and * = p-value < 0.001.

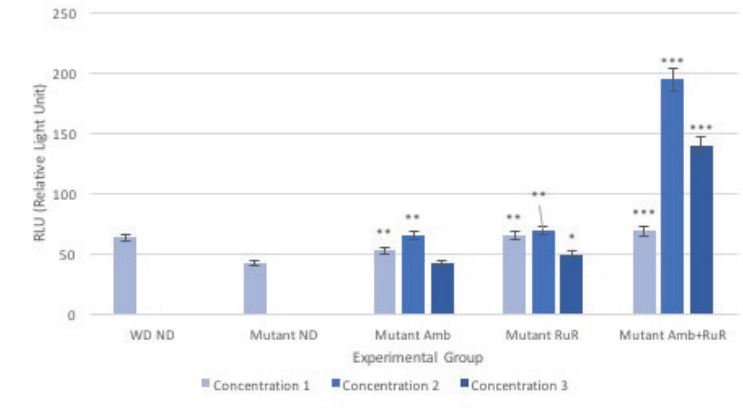

Figure 6. ATP Bioluminescence Assay of Mutant (Mut) flies cultured with the drugs Ambroxol (Amb) and Ruthenium red (RuR) at different concentrations and Normal Diet (ND). Average ATP activity in mutant flies was measured for three concentrations (1 = 500uM Ambroxol and/or 100uM RuR, 2 = 1mM Ambroxol and/or 250uM RuR, and 3 = 5mM Ambroxol and/or 500uM RuR). Averages were from homogenates of three trials of 20 flies, and error bars represent a 95 percent confidence interval. Asterisks (*) represent statistical significance (paired sample, one-tailed student's t-test); * = p-value < 0.05, * = p-value < 0.01, and * = p-value < 0.001.

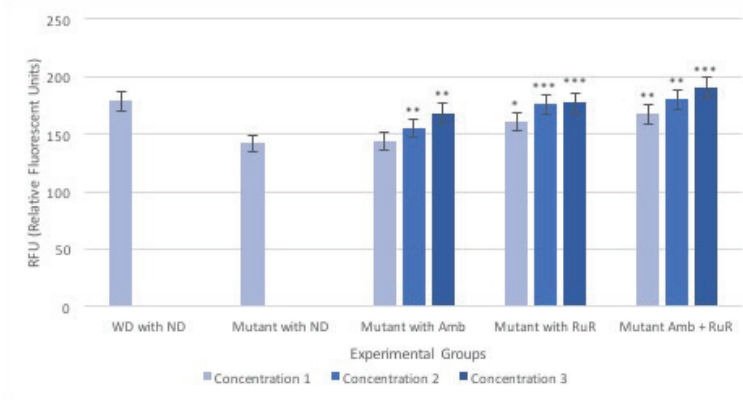

Figure 7. GBA Activity Assay of Mutant (Mut) flies cultured with the drugs Ambroxol (Amb) and Ruthenium red (RuR) at different concentrations and Normal Diet (ND). Average glucocerebrosidase activity in mutant flies was measured for three concentrations (1 = 500uM Ambroxol and/or 100uM RuR, 2 = 1mM Ambroxol and/or 250uM RuR, and 3 = 5mM Ambroxol and/or 500uM RuR). Averages were from homogenates of three trials of 20 flies, and error bars represent a 95 percent confidence interval. Asterisks (*) represent statistical significance (paired sample, one-tailed student's t-test); * = p-value < 0.05, * = p-value < 0.01, and * = p-value < 0.001.

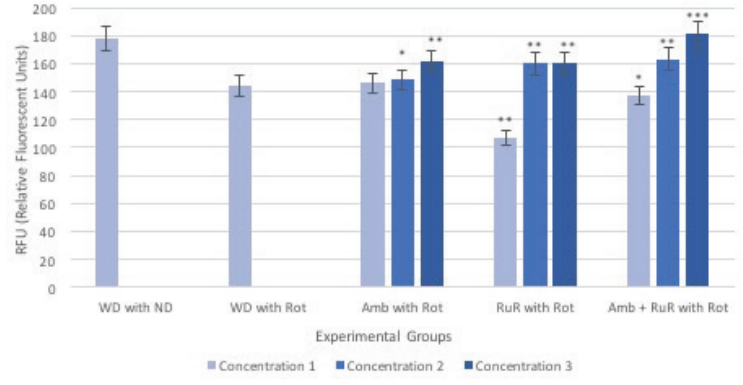

Figure 8. GBA Activity Assay of Wild-Type (WD) flies with Rotenone (Rot) cultured with the drugs Ambroxol (Amb) and Ruthenium red (RuR) at different concentrations and Normal Diet (ND). Average glucocerebrosidase activity in mutant flies was measured for three concentrations (1 = 500uM Ambroxol and/or 100uM RuR, 2 = 1mM Ambroxol and/or 250uM RuR, and 3 = 5mM Ambroxol and/or 500uM RuR). Averages were from homogenates of three trials of 20 flies, and error bars represent a 95 percent confidence interval. Asterisks (*) represent statistical significance (paired sample, one-tailed student's t-test); * = p-value < 0.05, * = p-value < 0.01, and * = p-value < 0.001.

functional mitochondria. Inhibiting the MCU alters calcium homeostasis and prevents mPTP opening, therefore preventing mitochondrial death and allowing the mitochondria to keep producing ATP. The group given RuR and Ambroxol had significantly higher ATP values, and therefore more functional mitochondria than the untreated samples(p-value < 0.05) (**Figures 5** and **6**). Importantly, the middle concentrations of the treatments yielded higher ATP values compared to the low or high doses. This points to a concentration related-toxicity as amounts of the drug increase. More research should be conducted to determine the optimal dosage for this therapy.

In the GBA activity assay, the activity of GBA, a critical lysosomal enzyme that functions in the digestion of the protein α-synuclein, which aggregates in protein deposits in PD is measured. Enhancing lysosomal function provides an important path to dissolving these deposits and lowering the inflammation that speeds neuronal degradation. GBA activity was significantly increased in most groups treated with both Ambroxol and RuR (p-value < 0.05) (**Figures 7** and **8**). While treatment with a single therapy did improve GBA function, combined treatment surpassed the effect of either drug alone. However, low concentrations of RuR and Ambroxol + RuR produced a negative effect on GBA activity in the samples with sporadic PD, but do not show this effect in samples with familial PD. While this could be due to human error, an alternative hypothesis suggests a fundamental difference between the progression of idiopathic and familial PD.

DISCUSSION

The purpose of this experiment was to develop a novel treatment for PD that targets both aggregation of α-synuclein and neuronal degradation. In this study, we tested the synergistic effects of RuR and Ambroxol in a PD model of fruit flies. While the addition of either drug alone demonstrated neuroprotective effects, the combination of these drugs demonstrated greater neuroprotective effects than either of the drugs alone. However, it does have to be said that when given in its highest concentrations, the drugs both alone and together did not have the most optimal ATP production for that experimental group. This indicates that there is a safe medium concentration in which the drugs can be used to their maximum potential. Since the drugs I am testing here have not really been used before for much medical use, my concentrations were estimates. More research should be done into which specific concentrations are the most effective. While as a whole, the data did show the neuroprotective effects of the drugs when given together as proven by significantly higher climbing, ATP production, and GBA activity, concentration related toxicity must be studied further due to the relatively unknown nature of the drugs used in this study.

In this study, we demonstrated that RuR increased ATP production, increased GBA activity, and improved neurological function. While we expected that RuR would influence ATP production by increasing mitochondrial viability, we did not expect RuR to influence GBA activity. It is possible that RuR leads to higher lysosomal activity by activating autophagy pathways that digest damaged mitochondria. The role of PINK1, a gene that functions to ensure lysosomal autophagy of damaged organelles is completed, in PD corroborates this idea. When the PINK1 gene is not present or its protein is mutated, there is a visible parkinsonian phenotype present (20). Lysosomal activity depression has a close correlation with PD progression, as these data show.

There is an urgent need to produce a long-lasting therapy for PD. With an incidence of PD that is still increasing due to continuing harmful pesticide use, more people will come to need a permanent threatment (21). Instead of being forced to rely on expensive cocktails of dopamine precursors that will eventually lose effectiveness, a therapy utilizing these drugs has a much higher chance of preserving neuronal function. The current most common, and only temporary treatment for PD is called the levodopa-carbidopa therapy. This involves the introduction of a dopamine precursor, levodopa, into the body and the brain is able to produce dopamine artificially from this precursor. However, this treatment is not a permanent one, as (1) introducing dopamine into the body does nothing to combat the loss of dopamine production by the brain, (2) temporarily reduces symptoms while actual neuronal degradation is still ongoing, and (3) eventually loses effectiveness as time goes on. Combating the underlying neuronal degradation is important to stop disease progression and this therapy has potential to do this. This project proves that the use of *Drosophila melanogaster* as a model for sporadic PD can help us gain an understanding of its pathophysiology. While the genetic pathway of familial PD is mostly understood, the pathway for sporadic PD remains a mystery. Utilizing a pesticide model provides a viable way of understanding the etiology of sporadic PD. Understanding how it is caused can provide new avenues and methods to treat and potentially cure the disorder.

A major limitation in this project was the time it took for the flies induced to have PD to reproduce. To create the original PD model flies, a driver fly line GAL4 and another fly line UAS were crossed to develop an α-synuclein transgenic fly line. While this fly line is traditionally crossed with itself to grow its population, we found that this PD model flies exhibited a very slow growth rate. To solve this, the GAL4 and UAS crosses were maximized to collect mutants rather than culture the PD model flies in their own vials. Another limitation of this study is the drug delivery method via media. Some of the variations between trials may be explained by the fact that some flies tend to eat more medium than others and the amount of the drug-hydrated medium that an individual fly consumed is impossible to account for and measure. Differences in body weight could have contributed to the differences in the assay values generated from the homogenate. To compensate for this problem, a Bradford assay was conducted and the samples were diluted in TE buffer to normalize signal to

protein content to improve assay precision.

In future experiments, the potential of drug therapies using these two drugs to drastically increase ATP production, even past wild-type values should be explored to create new therapies for disorders like fibromyalgia and chronic fatigue syndrome, or malfunctions of ATP synthase. Fibromyalgia and chronic fatigue syndrome are both characterized by low ATP levels due to mitochondrial dysfunction and increasing ATP levels provides a potential solution to these problems (22). Researchers could also explore this therapy on a long-term basis and observe the effect on total long-term mitochondrial integrity, ATP production, and calcium homeostasis. Brain scans could be analyzed to determine the impact of this therapy on long-term dopaminergic neuron survival. While such scans of fruit flies cannot be taken, specific neuronal areas of interest in the substantia nigra could be observed in murine models, which have a form of PD very similar to the human disease, complete with Lewy body deposit. Currently, Ambroxol is the active ingredient of the drug Mucosolvan and is used as a treatment for respiratory disorders associated with excessive mucus by stimulating the release of surfactant. Since Ambroxol is already used in the treatment of human disease and it can cross the blood-brain barrier, Ambroxol is a strong contender PD treatment (11). While Ruthenium has never been used in a clinical setting or as a therapy in humans, this research shows that it has potential for use in the treatment of PD and further research is warranted.

MATERIALS AND METHODS

Generation of α -synuclein transgenic Drosophila melanogaster

Adult female virgin neural Gal4 promoter transgenic flies and adult male UAS-human α -Syn flies were placed into a vial. A 6:10 female to male ratio was found to maximize egg production. After 5 days, all the adult flies in the vials were removed. All the flies that appear in the vials after will be mutants for the SNCA gene and have a form of familial PD. Flies were transferred out of the vial after anesthetizing them with carbon dioxide gas.

Generation of induced PD models for Drosophila melanogaster

Drosophila culture vials (Carolina Biological) were filled with Formula 4-24® Instant Drosophila Medium (Carolina Biological) to 2/5ths of the volume of the vial. The dry medium was hydrated with 15 mL of 150 μM rotenone (Cayman Chemicals). Approximately five grains of yeast were added and the vial was covered with a vial plug (Carolina Biological).

Control group vials

Drosophila culture vials (Carolina Biological) were filled with Formula 4-24® Instant Drosophila Medium (Carolina Biological) to two-fifths of the volume of the vial. The dry medium was hydrated with 15 mL of distilled water. Approximately 5 grains of yeast were added and the vial was covered with a vial plug (Carolina Biological).

Ambroxol vials

For this experiment, drug concentrations of 500 μM, 1 mM, and 5 mM Ambroxol hydrochloride (Sigma Aldrich) were used. Drosophila culture vials (Carolina Biological) were filled with Formula 4-24® Instant Drosophila Medium (Carolina Biological) to two-fifths of the volume of the vial. For each concentration, the dry medium was hydrated with 15 mL of the drug solution. Around 5 grains of yeast were added and the vial was covered with a vial plug (Carolina Biological). Wildtype flies, fruit flies cultured with rotenone, and α-synuclein transgenic fruit flies were transferred into the drug vials and fed the medium for 10 days.

RuR vials

For this experiment, drug concentrations of 100 μM, 250 μM, and 500 μM RuR (Cayman Chemicals) were used. Drosophila culture vials (Carolina Biological) were filled with Formula 4-24® Instant Drosophila Medium (Carolina Biological) to two-fifths of the volume of the vial. For each concentration, the dry medium was hydrated with 15 mL of the drug solution. Around 5 grains of yeast were added and the vial was covered with a vial plug (Carolina Biological). Wild-type flies, fruit flies cultured with rotenone, and α-synuclein transgenic fruit flies were transferred into the drug vials and fed the medium for 10 days.

Ambroxol and RuR vials

For this experiment, the individual drug solutions were combined into one beaker and gently shaken to mix the solutions. Three groups were used: one using 500 μM Ambroxol HCl and 100 μM RuR, one with 1 mM Ambroxol HCl and 250 μM RuR, and one with 5 mM Ambroxol HCl and 500 μM RuR. Drosophila culture vials (Carolina Biological) were filled with Formula 4-24® Instant Drosophila Medium (Carolina Biological) to two-fifths of the volume of the vial. For each group, the dry medium was hydrated with 15 mL of the combined drug solution. Around 5 grains of yeast were added and the vial was covered with a vial plug (Carolina Biological). Wild-type flies, fruit flies cultured with rotenone, and α-synuclein transgenic fruit flies were transferred into the drug vials and fed the medium for 10 days.

Negative Geotaxis Assay

Using carbon dioxide anesthesia, we sorted the flies into groups of 10. The flies were allowed an hour to recover from anesthesia. To create the apparatus shown in Figure 8 that was used for this assay, we arranged 2 empty polystyrene vials (Carolina Biological) facing each other and joined them with tape. A mark 8.5 cm above the bottom of the vial was placed. For each condition, 10 flies were transferred into the vial and taped in. After an hour, the flies were tapped down and the number of flies that climbed above the 8.5 cm mark

were measured. The assay was repeated for all trials and sample conditions. Three trials of 10 flies were conducted for each experimental condition.

GBA Enzyme Activity Assay

First, the flies were anesthetized with FlyNap (Carolina Biological). We suspended 20 fruit flies in RIPA buffer with a protease inhibitor cocktail and homogenized the mixture with a glass dounce homogenizer. The samples were centrifuged at 15000xg for 20 minutes and the supernatant was aspirated. We conducted a Bradford protein assay and samples were diluted to a protein concentration of 5.5 µg/mL. 40 µL of homogenate was placed in wells of a 96-well microplate and 20 µL of citrate-phosphate buffer was added to each well. The mixture was incubated for 10 minutes and the reaction was terminated with 60 µL of a glycine-NaOH stop solution. The plates were read with a 520/20 filter on a microplate reader (Synergy HTX). Three trials were conducted in this manner for each condition.

ATP Bioluminescence Assay

The flies were first anesthetized with FlyNap (Carolina Biological). We suspended 20 fruit flies in RIPA buffer with a protease inhibitor cocktail and homogenized the mixture with a glass dounce homogenizer. The samples were centrifuged at 15000xg for 20 minutes and the supernatant was aspirated. We conducted a Bradford protein assay and samples were diluted to a protein concentration of 5.5 µg/mL. For this assay, the Promega Cell-titer Glo system was used. 50 µL of homogenate were loaded into wells of a 96-well plate and contents were equilibrated to room temperature for 30 minutes. 50 µL of Promega Cell-Titer Glo reagent (Promega) were then loaded into each well and samples were allowed to incubate for 10 minutes. Luminescence was recorded using a microplate reader (Synergy HTX). Three trials were conducted in this manner for each condition.

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Article

Mapping the electromagnetic field in front of a microwave oven

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SUMMARY

There is limited evidence that EMF may have negative health effects on human beings with extended exposure. This study measured the power density and strength of EMF at different distances and directions in front of a microwave oven. Our results showed that the EMF levels are high (≥ 100 mG) at "one arm's length" distance (61 cm) in all directions, although the power density drops to a range of 0.01 – 0.10 mW/cm2. To achieve a power density of 0.05 mW/cm2 or less and an EMF level of 25 mG or less, the distance should be at least 91 cm and 122 cm, respectively, away from the microwave oven, especially in the left and front directions. Based on the measurement data, we created exposure maps using precautionary thresholds of 0.05 mW/cm2 and 25 mG for power density and EMF, respectively. The measurement results help to understand the EMF distribution in front of the microwave oven. The results suggest that one should avoid staying less than 122 cm away from the microwave oven during operation, and that the "one arm's length" distance (61 cm) may not be sufficient due to the high EMF levels. This study helps to promote awareness of the potential health risks associated with microwave ovens.

INTRODUCTION

A microwave oven is a commonly used electronic appliance that heats food by exposing it to microwave radiation generated by a magnetron (1). Microwaves are a form of non-ionizing electromagnetic energy with frequencies ranging between 0.3–300 GHz, which are used extensively in radar (2). In 1945, while developing magnetrons for radar signals, Dr. Percy Spencer discovered that microwaves could heat up food. Based on this observation, the microwave oven was developed and was first marketed in 1946. Today, most American households have microwave ovens in their kitchens. A microwave oven works by passing microwave radiation, usually at a frequency of 2450 MHz, through food. Water, fat, and sugar molecules in the food absorb energy from the microwave beam and become hot. Microwave beams pass through glass, paper, ceramic, or plastic, but metals reflect microwaves, thus not safe to use.

There have been concerns about the potential adverse health effects upon exposure to microwave energy (3–6). The U. S. Food and Drug Administration (FDA) sets an emission limit from microwave ovens so that "the … power density existing in the proximity of the external oven surface shall not exceed 1 mW/cm^2 at any point 5 cm or more from the external surface of the oven, measured prior to acquisition by a purchaser, and, thereafter, 5 mW/cm^2 at any such point" (7). It is known that the microwave radiation dissipates quickly as the distance increases away from the energy source. At "one arm's length" (approximately 61 cm) away from the microwave oven, the power density would drop to approximately 0.05 mW/cm^2, assuming 5 mW/cm^2 at 5 cm from the microwave (8). A study measured the microwave leakage of 106 microwave ovens and found that only one oven exceeded the 5 mW/cm^2 emission limit (9). Though generally considered as safe when used properly, there are some arguments that microwave exposure at much lower levels could be associated with adverse health effects (5, 10).

In addition to direct microwave radiation, the electromagnetic field (EMF) formed around a microwave oven may also be a concern (11). There is limited evidence that accumulated long-term (hours or more) exposure to EMF may have negative health effects on human beings, which increases the risks for neurological disorders, cataracts, and certain types of cancers (12–14). For example, studies have suggested that extended exposure to power frequency magnetic fields greater than 3 mG may result in increased health risks such as childhood leukemia (14, 15). However, the current knowledge is not sufficient to understand the mechanisms by which EMF exposure leads to cancer development. Currently, there are no universally accepted EMF exposure limits. The Environmental Protection Agency (EPA) recommends avoiding long-term exposure to EMF of up to 2.5 mG (12, 16). The short-term exposure level can be up to 25 mG, 10-fold higher than the EPA recommended safe level for long-term exposure (17).

The purpose of this study was to measure the power density and strength of the EMF at different distances around a microwave oven and to verify if "one arm's length" (61 cm) from the microwave oven is a safe distance. We measured microwave power density and EMF levels at various distances from the microwave oven and in different directions in front of the microwave oven. Since the EMF decreases rapidly with the distance from the microwave oven, we hypothesized that at "one arm's length" distance, the measured power density is no more than 0.05 mW/cm^2 and EMF no more than 25 mG.

RESULTS

Power density measurements

To begin to investigate the relative safety at different points from a microwave oven, we first measured power density using a Tri Field Meter at different distances and directions from a microwave oven. Two independent measurements at different dates were performed with the same microwave oven. The overall mean and standard deviations of the two measurements are summarized in **Table 1**. We calculated the mean and standard deviation of the power density measurements and found the values to range from 0.18–0.33 mW/cm^2 at the front outer surface (distance of 0 cm) of the microwave oven (**Table 1**), which is significantly below the emission limit (5 mW/cm^2) set by the FDA. The power density drops quickly with increasing distance from the microwave oven. At "one arm's length" distance (61 cm), the mean power density readings are in the range of 0.01–0.10 mW/cm^2.

We found that the measured power density levels at 61 cm were 0.05 mW/cm^2 or above in the left-45°, up-45°, down-45°, and front directions; and below 0.05 mW/cm^2 in the right, right-45°, and left directions (**Figure 1**).

The power density dissipated more rapidly at both sides (right, right-45°, and left) than at the front of the microwave oven (down-45°, left-45°, front, and up-45°). In particular, it requires 74–94 cm distance at left-45° and down-45° directions for the power density to dissipate to 0.05 mW/cm^2, compared to 61 cm at front and up-45° directions, and 38–41 cm at right, right-45° and left directions (**Figure 2**).

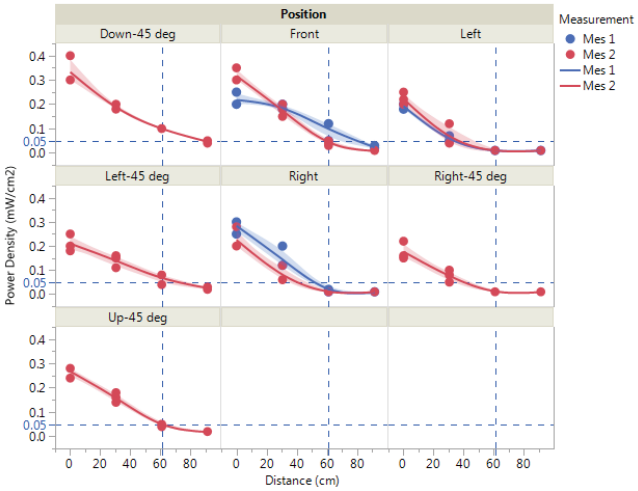

Figure 1: Measured power density at different distances and directions in front of the microwave oven. The graphs illustrate the decreasing trends of the measured power density with the increase of distance from the microwave oven. Two independent measurements, Mes 1 (blue) and Mes 2 (red), were performed with the same microwave oven. The measured power density (dots), regression lines, and 95% confidence areas of regression are illustrated in the graphs, grouped in different directions (horizontal: left, left-45°, front, right-45°, and right; vertical: up-45°, and down-45°). The power density threshold of 0.05 mW/cm^2 and "one arm's length" distance (61 cm) are represented as blue dashed lines.

EMF Measurements

Similar to the measurement of power density, we measured the EMF levels using the Tri Field Meter at different distances and directions in front of a microwave oven. The electric field is 0 V/m at all distances, suggesting that the electric field is shielded completely by the metal pieces of the microwave oven. Thus, the EMF refers to the magnetic field in this study. Two independent measurements of magnetic field levels at different dates were performed with the same microwave oven. The mean magnetic field readings were as high as 100 mG at distance ranges from 61 cm (right) to 104 cm (left-45°) and dropped to approximately 25 mG at distance ranges from 74 cm (right) to 112 cm (left-45°), respectively.

At all directions, at "one arm's length" distance (61 cm), the measured EMF levels were at least 100 mG (**Figure 3**). The EMF levels dissipated more rapidly in the right direction, and more slowly in the left and left-45° directions. To have EMF levels at 25 mG, it requires a distance of 102–112 cm in the left and left-45° directions, respectively, compared to 69 cm in the right direction, and 89–94 cm in other directions.

At the same distance from the surfaces of the microwave oven, the EMF levels were the lowest in the right direction, and were the highest in the left-45° direction. It is clear that at

Distance from microwave oven (cm)	Power density (mW/cm^2) mean ± std dev						
	Front[a]	Left[a]	Left-45°[b]	Right[a]	Right-45°[b]	Up-45°[b]	Down-45°[b]
0	0.27 ± 0.06	0.21 ± 0.02	0.21 ± 0.04	0.26 ± 0.05	0.18 ± 0.04	0.27 ± 0.02	0.33 ± 0.06
30	0.18 ± 0.02	0.06 ± 0.03	0.14 ± 0.03	0.11 ± 0.05	0.08 ± 0.03	0.16 ± 0.02	0.19 ± 0.01
61	0.07 ± 0.04	0.01 ± 0.00	0.07 ± 0.02	0.01 ± 0.01	0.01 ± 0.00	0.05 ± 0.01	0.10 ± 0.00
91	0.02 ± 0.01	0.01 ± 0.00	0.03 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.05 ± 0.01

[a]n = 6. [b]n = 3.

Table 1: Data were obtained from Mes 1 and Mes 2. Values of the mean, standard deviation, and number of readings of power density are grouped based on the distance from the microwave oven in each direction. The measured power density values range from 0.18–0.33 mW/cm2. At "one arm's length" distance (61 cm), the mean power density readings are in the range of 0.01–0.10 mW/cm^2.

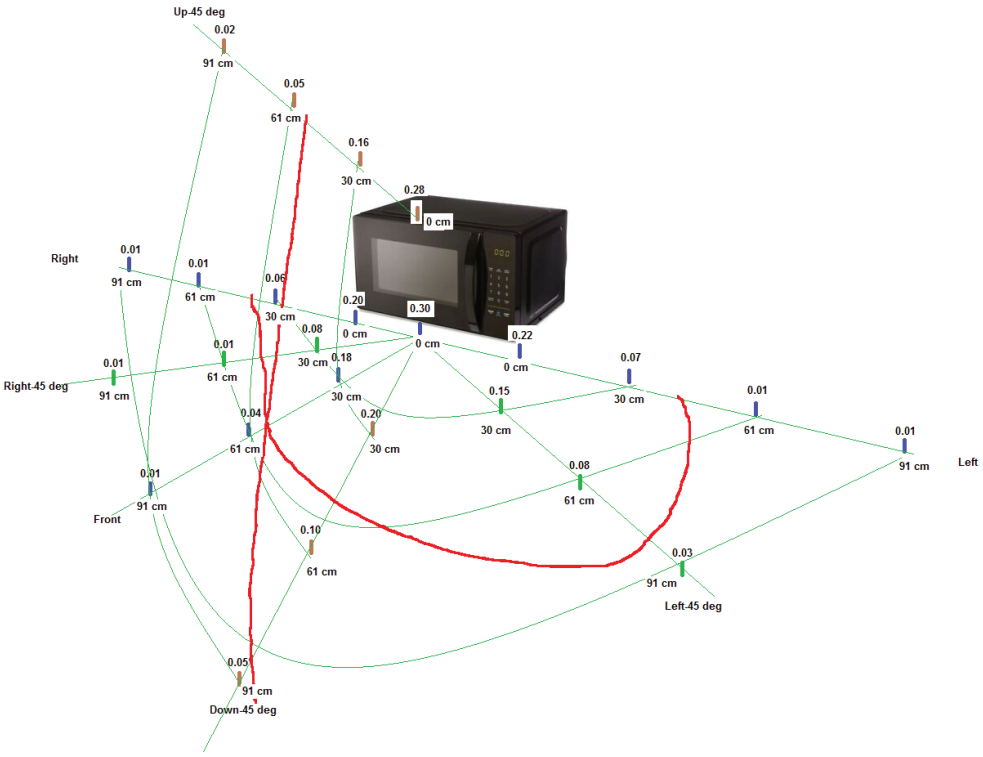

Figure 2: Schematic illustration of the power density distribution in front of the microwave oven. The schematic illustration of the power density distribution on the horizontal and vertical directions in front of a microwave oven. The green lines and curves represent approximate distances (in centimeters) in different directions (horizontal: left, left-45°, front, right-45°, and right; vertical: up-45°, and down-45°); The mean power density (in mW/cm^2, based on Mes 2) and distance are labeled for small bars, which are colored in blue, green, and brown to show different directions; The red lines represent the power density threshold of 0.05 mW/cm^2. It is clear that the power density at "one arm's length" distance (61 cm) in the left-45° and down-45° directions is greater than 0.05 mW/cm^2.

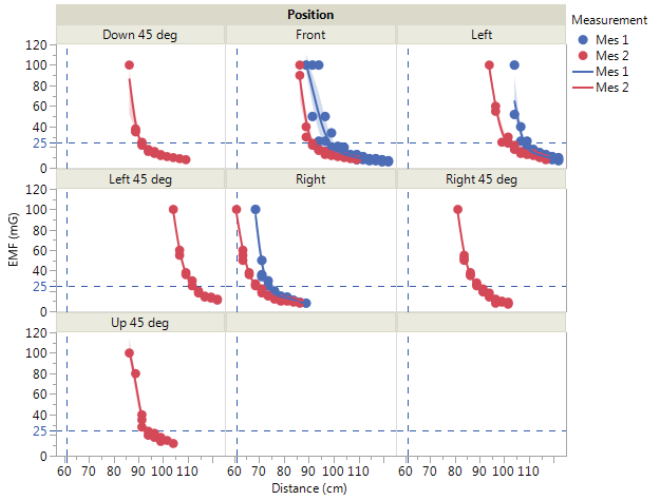

Figure 3: Measured EMF at different distances and directions in front of the microwave oven. The graphs illustrate the decrease of the measured EMF levels with the increase of distance from the microwave oven. Two independent measurements, Mes 1 (blue) and Mes 2 (red), were performed with the same microwave oven. The measured EMF (dots), regression lines, and 95% confidence areas of regression are illustrated in the graphs, grouped in different directions (horizontal: left, left-45°, front, right-45°, and right; vertical: up-45°, and down-45°). The EMF threshold of 25 mG and "one arm's length" distance (61 cm) are represented as blue dashed lines.

the distance of 61 cm, the measured EMF level is 100 mG or greater at all directions (**Figure 4**). To have an EMF exposure of 25 mG or lower, it requires a distance ranging from 69 cm (right) to 112 cm (left-45°) away from the microwave oven.

DISCUSSION

There have been concerns about the potential health risks that a microwave oven could generate by leakage of microwave radiation (3–6) and exposure to the EMF that forms around the oven (12–14). The microwave radiation decreases quickly as the distance increases away from the energy source. A measurement suggested that the power density dropped to approximately 0.05 mW/cm^2 at the distance of 61 cm from the microwave oven (8), which is 100-fold lower than the emission limit set by the FDA (7). Based on a data from EPA, the EMF drops to approximately 25 mG at the distance of 91 cm away from a microwave oven (3). This is approximately 10-fold higher than the long-term exposure limit set by the EPA.

This study aimed to evaluate the level of power density and EMF at "one arm's length" distance (61 cm) from a microwave oven. We measured the power density and EMF levels at different distances and different directions, namely front, left, left-45°, right, right-45°, up-45°, and down-45°, from

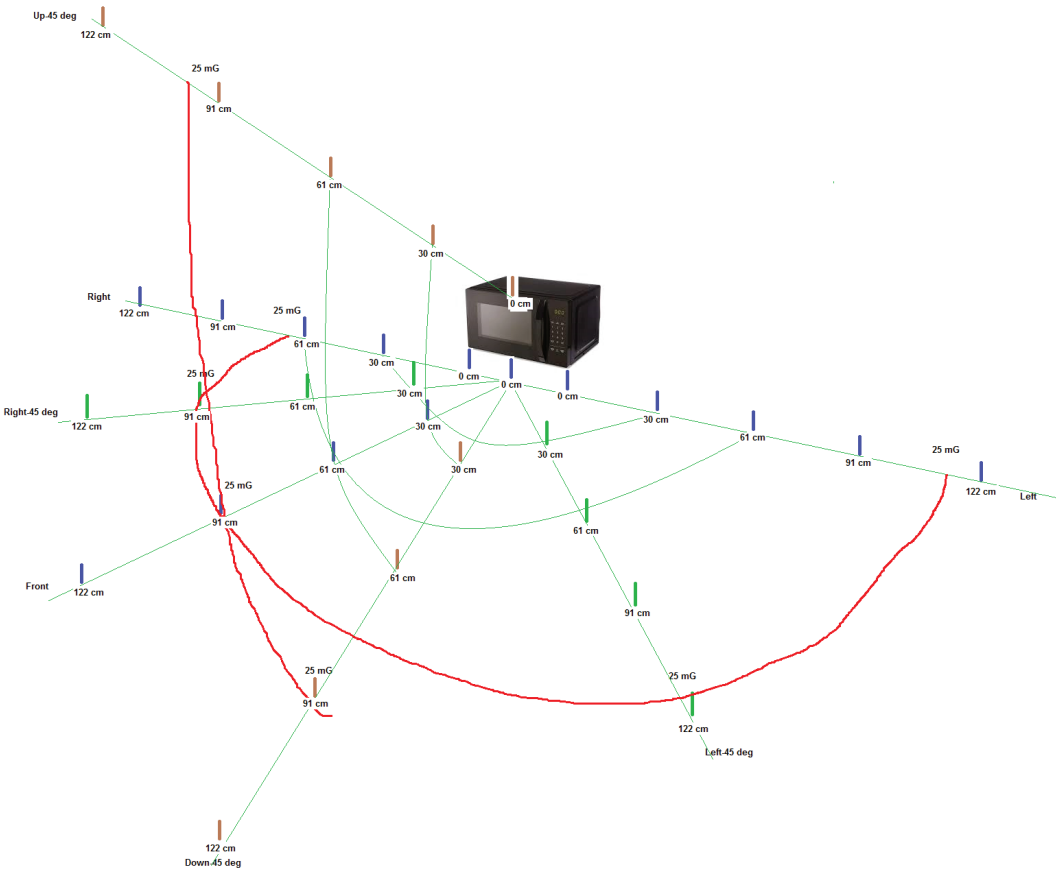

Figure 4: Schematic illustration of the emf distribution in front of the microwave oven. The schematic illustration of the EMF distribution on the horizontal and vertical directions in front of a microwave oven, based on Mes 2. The green lines and curves represent approximate distances (in centimeters) in different directions (horizontal: left, left-45°, front, right-45°, and right; vertical: up-45°, and down-45°); The distance (cm) are labeled for small bars, which are colored in blue, green, and brown to represent different directions; The red lines represent the EMF threshold of 25 mG. It is clear that the magnetic field intensity at "one arm's length" distance (61 cm) in all directions is greater than 25 mG.

a microwave oven. For the purpose of this study, we used the expected levels of 0.05 mW/cm^2 for the power density (8) and 25 mG for the EMF (3), respectively, as precautionary thresholds.

To ensure the accuracy of the measurements, the contribution of the environment power density and EMF near the microwave need to be assessed. We performed background measurements on both power density and EMF levels at different locations around the microwave oven while the microwave oven was disconnected from power. The readings were quite low, 0 mW/cm^2 for power density and ~1 mG for EMF strength, which was negligible and could be disregarded.

Originally, we planned to measure the microwave oven using different power settings, such as 25%, 50%, and 75% of power, but we found that these low power settings were achieved by alternating between high power and low power, which caused a lot of variations in measurements. Thus, we only used the 100% power setting to run the microwave oven to measure the maximum microwave power density and EMF strength formed around the appliance.

The EMF values were not stable, making it difficult to take readings. Some measured values showed high variability. To reduce the risk of estimation error, we performed multiple measurements and analyzed the measured data by log transformed regressions. Two independent measurements, each with replicate readings, were made at two different dates, with the same microwave oven. The microwave oven is placed in a cabinet that leaves free space only in front of the microwave oven. The EMF field behind the microwave oven was not measured due to the lack of free space.

The results of measured power density and EMF both suggest that at least a distance of 91 cm is required for the power density to decrease to the level of 0.05 mW/cm^2 or below, and a distance of 122 cm is required for the EMF to decrease to the level of 25 mG or below. The measured EMF levels are also higher in left and left-45° directions than the data reported from other studies (11, 12). Based on the measurement data, the power density and EMF exposure maps were created. The results suggest that at the down-45° and left-45° directions it requires longer distance than other directions for power density to dissipate to 0.05 mW/cm^2, so

do at the left-45° and left directions for the EMF to dissipate to 25 mG. This is possibly because the magnetron is located on the left side of the microwave oven.

This study helps to understand the distribution of EMF around a typical home appliance and the potential health risks with exposure to elevated EMF. The results suggest that one should avoid staying less than 122 cm away from the microwave oven during operation, and that the "one arm's length" distance (61 cm) may not be sufficient due to the high EMF levels. Further studies could investigate how the factors such as the age of a microwave oven as well as operating power capacity and voltage affect the surrounding power density and EMF strength. The ultimate question is to understand the mechanism how microwave radiation and EMF adversely affect cell functions and metabolism.

In summary, we measured the power density and EMF in front of a microwave oven at different distances. Based on the measurement data, we created exposure maps using precautionary thresholds of 0.05 mW/cm^2 and 25 mG for power density and EMF, respectively. To obtain an exposure level below the thresholds, it requires a distance of 91–122 cm away from the microwave oven, especially in the left and front directions. The measurement results help to understand the EMF distribution in front of the microwave oven. The results suggest that one should avoid staying less than 122 cm away from the microwave oven during operation , and that the "one arm's length" distance (61 cm) may not be sufficient due to the high EMF levels.

MATERIALS AND METHODS

An LG microwave oven Model LCRT1513ST (Power: 120 V AC, 1500 W; microwave output: 1100 W; frequency: 2450 mHz; oven capacity: 1.5 cu ft; purchased in 2015) was used in this study. A measuring tape was used to measure the distance to the microwave oven at different directions, i.e., front, left, right, left-45°, right-45°, down-45°, and up-45°. A big bowl of water was placed inside the microwave oven, and the microwave was run at 100% power for up to 5 minutes before changing water in the bowl. The values of radio/microwave power density and magnetic field were measured using a Tri Field Meter (model 100 XE) standard version (frequency weighted to 60 Hz; EMF measurement range 0–100 mG, resolution 0.2 mG, with accuracy of ± 20% at 60 Hz; radio/microwave measurement range 0–1 mW/cm^2, resolution 0.01 mW/cm^2, and frequency range 50–3000 mHz) (18). Multiple readings were taken at different distances along the measuring tape at each direction. Two independent measurements, namely Mes 1 and Mes 2, on the same microwave oven were performed on two different dates.

The measured data were entered into JMP data tables and analyzed using JMP software (Version 14) (SAS, Cary, NC). The statistical analysis include mean, standard deviation, 95% confidence interval, regression analysis. The graphs were made using JMP Graph Builder to illustrate the measured microwave power density and magnetic field intensity along the different distances. Schematic exposure graphs were made to illustrate the distances with the precautionary thresholds of power density at 0.05 mW/cm^2 and EMF at 25 mG, respectively.

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Article

Antibacterial activity and absorption of paper towels made from fruit peel extracts

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SUMMARY

Barriers to adequate hygiene are important problems throughout the world, as billions of people do not have access to sanitary conditions. In particular, unsatisfactory hand hygiene leads to the spread of bacterial infections from person to person. To address this problem, we developed the PeelTowel, an antibacterial and water-absorbing towel made of a combination of fruit peels and recycled paper waste, which has the potential to make hand-hygiene accessible, sustainable, and environmentally friendly. Kiwi, orange, and lime peels were chosen for this purpose because they contain antibacterial factors such as vitamin C and citric acid as well as water-absorbing cellulose. PeelTowels were produced by creating a paste of crushed fruit peels and paper and then drying thin films of this paste on screens. PeelTowels were tested for their ability to inhibit the growth of bacteria and absorb water. They were incubated with *Escherichia coli*, and bacterial survival was measured by counting colonies on agar plates. Similarly, absorption was quantified by exposing PeelTowels to varying amounts of water. The Lime PeelTowel had the highest antimicrobial activity. It eradicated 50-91% of *E. coli* after exposure for 1 hour and 95-98% after exposure for 18 hours. It also absorbed three times the amount of water as a commercially available paper towel. Our results suggest that Lime PeelTowels have the potential to be an environmentally friendly option for antibacterial and absorptive hand towels.

INTRODUCTION

According to the World Health Organization, around 2.5 billion people (35% of the world's population) do not have access to adequate hygiene, including clean water, sanitizers, and other sanitation products (1). Educational campaigns have been undertaken to improve hygiene in the home, but people in developing nations frequently cannot afford soap or other basic products to maintain good health (2). The resulting lack of hygiene may lead to the spread of infections. Our goal was to develop a clean, eco-friendly, and easy to use paper towel product that has antibacterial activity.

The use of currently available paper towels creates several environmental challenges. Every year in the U.S. alone, 2 billion trees are harvested to make 85,000,000 tons of paper, and the average American uses 680 pounds of paper annually (3). Coincidentally, this usage is mirrored by consumption of fruit. The American Institute of Physics found that 15.6 million tons of citrus peel waste are created annually around the world (4). This waste makes its way to landfills and adds to the growing accumulation of discarded materials in the environment. These statistics highlight the need for new methods to improve hygiene while reducing and reusing waste. In particular, in areas where effective hygiene options are limited or inaccessible, we asked how an antibacterial hygiene product could be created that reduces paper and peel waste.

The peels of orange, kiwi, and lime contain antibacterial factors such as vitamin C, citric acid, flavonoids, and phenolic compounds (**Table 1**; 14-15). These factors work in different ways to kill bacteria. For example, vitamin C facilitates the killing of bacteria by the Fenton Reaction, in which reactive oxygen species are produced that are lethal to bacteria. In the Fenton reaction, ferrous iron reacts with hydrogen peroxide to generate ferric iron and antibacterial reactive oxygen species. Vitamin C aids in this process by converting ferric iron back to ferrous iron, thus allowing the Fenton Reaction to continuously produce reactive oxygen species. As an example of the importance of this reaction, the bacterium *Mycobacterium tuberculosis* is killed by vitamin C due to the reactive oxygen species that are produced (5). In addition to vitamin C, citric acid is also effective in killing bacteria. Citric acid may acidify the bacterium's environment, leading to inhibition of bacterial replication (6). Oranges, kiwis, and limes all contain high amounts of vitamin C, citric acid, flavonoids, and phenolic compounds, suggesting that they have antibacterial properties (7-14).

	Vitamin C (mg per 100 g)	Citric acid in juice (mg per 100 g)	pH	Cellulose content (%)
Orange	58.30	452	3.1 - 3.96	15
Lime	27.78	4124	4.35	14
Kiwi	92.72	1402	2.4	6

Table 1: Properties of orange, lime, and kiwi peels. The vitamin C and citric acid concentrations, pH, and cellulose contents of orange, lime, and kiwi peels (7, 10-14, 16, 17).

Many fruits also contain cellulose, which is highly effective in attracting and absorbing water (15). For this reason, cellulose from wood products is a major component of paper towels. Orange peels, lime peels, and kiwi contain 15%, 14%, and 6% cellulose, respectively (**Table 1**; 16, 17). These values suggest that extracts of orange, kiwi, and lime peels are capable of absorbing water under appropriate conditions.

Many of the infections that result from poor hand-hygiene are gastrointestinal in nature and manifest as diarrhea. For example, *Escherichia coli* is a Gram-negative bacterium normally found as a commensal in the intestines. However, while most strains of *E. coli* are harmless and even beneficial to the gut, pathogenic strains can cause infectious diarrhea. Infectious diarrhea occurs following ingestion of food or water contaminated by fecal material from an infected person or animal. For these reasons, good hand hygiene (e.g. the thorough washing of hands with soap or another disinfectant) can prevent many cases of illness caused by *E. coli* (18). Several other bacterial pathogens, such as *Salmonella enterica* and *Shigella* species are transmitted in a similar way.

The bactericidal and water-absorbing properties of orange, kiwi, and lime peels led us to hypothesize that peels from these fruits could be used with discarded paper waste to produce antibacterial paper towels. In the current study, we performed proof-of-concept tests to demonstrate that certain formulations of paper towels made of fruit peels and recycled paper (designated "PeelTowels") were water absorbent and killed *E. coli* bacteria.

RESULTS

Fruit peels and paper are used to generate PeelTowels

Our goal was to develop a simple, natural, and environmentally-friendly product for improved hand-hygiene, which we called the PeelTowel. Our prototype PeelTowel reused discarded fruit peels and paper and took advantage of the natural antimicrobial properties of fruit peels. Briefly, Orange PeelTowels, Lime PeelTowels, and Kiwi PeelTowels were constructed as follows: The three fruits were washed and peeled, and each peel was emulsified in a blender along with shredded paper and water. The contents of the blender were added to a handmade mold constructed from photo frames and window screens, which drained excess water from the PeelTowel. The PeelTowel was then dried with a cloth, sponge, and dryer. In this way, PeelTowels made from fruit peels and paper were produced for subsequent experiments (**Figure 1**).

PeelTowels demonstrate antibacterial activity following one hour of exposure

To test the antibacterial properties of PeelTowels and fruit peels compared to commercial paper towels, we generated a standard bacterial inoculum of known size. The bacterial concentration of *E. coli* was adjusted to reach approximately 10^8 colony forming units (CFU) per mL – the initial inoculum.

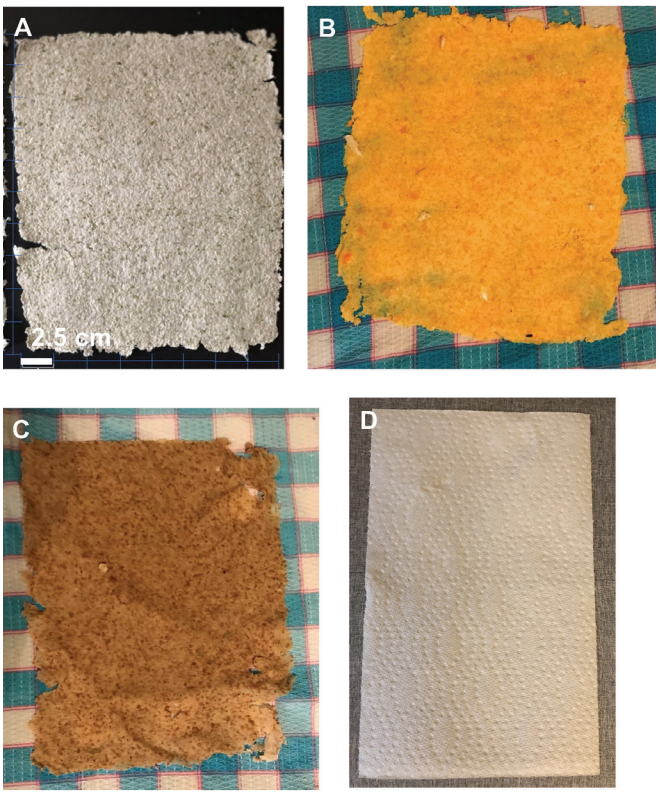

Figure 1: PeelTowels. Photos of **(A)** Lime PeelTowel with scale, **(B)** Orange PeelTowel, **(C)** Kiwi PeelTowel, and **(D)** paper towel.

The actual number of bacteria in the inoculum was then determined by streaking the inoculum on agar plates and counting colonies that had grown by following day. Based on these results it was estimated that a 10-µL inoculum contained 773,000 CFU of *E. coli*. This value was used in subsequent calculations.

To determine whether PeelTowels, fruit peels, and the commercial paper towel have antibacterial activity following one hour of exposure to the bacterial inoculum, we co-incubated these substrates with *E. coli*. We compared the antibacterial activities of Orange PeelTowels, Lime PeelTowels, Kiwi PeelTowels, orange peels, lime peels, kiwi peels, and a commercial paper towel. We added a fixed inoculum of bacteria (10 µL; 773,000 CFU) to a small amount of each substrate in a microfuge tube. We incubated the tubes for one hour at room temperature and then applied a portion of the contents to agar plates. We incubated the plates overnight and counted colonies the next day. In nearly all cases, colonies of a single morphology were observed, suggesting that contamination had not occurred (**Figure 2**). Differing numbers of bacteria were recovered from each substrate (**Table 2**, **Figure 3A**). Of the PeelTowels, the Lime PeelTowel had the highest antimicrobial activity and reduced the bacterial inoculum by around 91%. The lime peels also killed almost all of the bacterial inoculum. Furthermore, the Orange PeelTowel and Kiwi PeelTowel both reduced the bacterial inoculums by 69% and 81%, respectively. All three

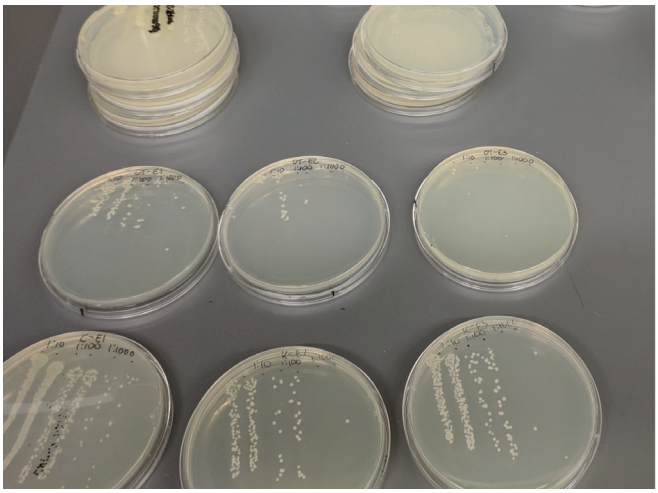

Figure 2: *E. coli* colonies growing on Lysogeny Broth (LB) agar plates for enumeration. Aliquots of 10 µL of diluted samples were spotted onto plates, which were then tilted to allow the suspension to flow across the surface of the agar. The plates were then incubated overnight at 37°C, and colonies of *E. coli* were counted.

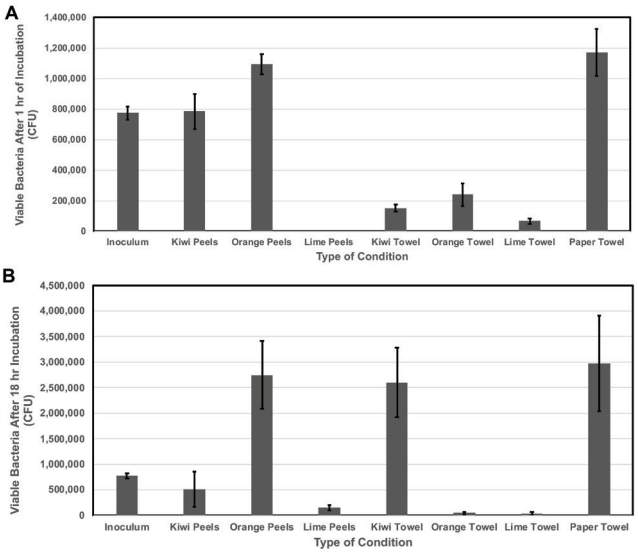

Figure 3: Viable *E. coli* following exposure to fruit peels, PeelTowels, and paper towels. *E. coli* bacteria were incubated with the indicated fruit peel, PeelTowel, or paper towel for **(A)** 1 hr or **(B)** 18 hr, and surviving bacteria were enumerated by plating. Each value represents the mean of three experiments, and each experiment represents the average CFU from two 10-µL samples. Error bars represent standard errors of the mean. When corrected for multiple comparisons, differences between the groups were not statistically significant (pairwise Mann-Whitney U tests adjusted for multiple comparisons using the Holm method, p-value > 0.05).

PeelTowels and the lime peels killed a greater number of bacteria than the paper towel. In contrast, the paper towel, orange peels, and kiwi peels allowed the number of bacteria to increase above that present in the inoculum. While incubation with the paper towel for one hour resulted in a 51% increase in bacteria, incubation with orange peels and kiwi peels yielded 41% and 2% more bacteria, respectively. Of note, none of these differences were statistically significant due to the large number of comparisons made. These results suggest that the PeelTowels have antimicrobial activity after one hour of incubation.

Some PeelTowels suppress bacterial numbers following 18 hours of exposure

We next examined whether PeelTowels, fruit peels, or the paper towel had antibacterial activity following longer incubation times with bacteria. *E. coli* bacteria (10 µL inoculum containing 773,000 CFU) were incubated with the peels and towels for 18 hours, after which viable CFU were measured by plating (**Table 2**, **Figure 3B**). The Lime PeelTowel had the greatest antimicrobial activity, killing 95% of the inoculum. The lime peels killed around 81% of the inoculum. Furthermore, both the Lime PeelTowel and Orange PeelTowel caused a decrease in the numbers of viable bacteria at 18 hours compared to the numbers in the inoculums and the paper towel. In contrast, the orange peels, paper towel, and Kiwi PeelTowel all contained more bacteria than the initial

	Number of viable bacteria after 1 hour of exposure to peels and towels		Number of viable bacteria after 18 hours of exposure to peels and towels	
PeelTowel	Viable bacteria relative to inoculum (%)	Viable bacteria relative to paper towel (%)	Viable bacteria relative to inoculum (%)	Viable bacteria relative to Paper Towel (%)
Inoculum	N/A	-34%	N/A	-74%
Kiwi peels	+2%	-33%	-34%	-83%
Orange peels	+41%	-7%	+255%	-8%
Lime peels	-100%	-100%	-81%	-95%
Kiwi towel	-81%	-87%	+236%	-13%
Orange towel	-69%	-79%	-94%	-98%
Lime towel	-91%	-94%	-95%	-99%
Paper towel	+51%	N/A	+286%	N/A

Table 2: Viable bacteria after 1 hour and 18 hours of exposure to peels and towels. The CFU of *E. coli* recovered by plating following 1 hr or 18 hr of incubation with peels or PeelTowels were compared to the starting inoculum and to the CFU recovered from paper towels incubated with bacteria for the same amount of time. N/A = not applicable. Differences between each pair of tested conditions was not statistically significant (pairwise Mann-Whitney U tests adjusted for multiple comparisons using the Holm method, p-value > 0.05).

inoculum. The orange peels contained 255% more bacteria than were present in the inoculum. Likewise, the paper towel contained 286% more bacteria than were present in the inoculum -- a greater increase than was observed after one hour of exposure. While the Kiwi PeelTowel demonstrated a reduction in the number of bacteria after one hour of exposure, after 18 hours these towels contained 236% more bacteria than were present in the inoculum. While differences in antimicrobial activity between some of the PeelTowels and fruit peels were evident, they were not statistically significant when corrected for multiple comparisons due to the large number of comparisons made.

Lime PeelTowels demonstrate significant antibacterial activity in repeat experiments

As mentioned, the large number of comparisons made prevented us from detecting statistically significant differences between the PeelTowels, fruit peels, and the paper towel. We therefore repeated the antibacterial assays using only the Lime PeelTowel, which had the highest antibacterial activity, and the paper towel. An additional change was that the Lime PeelTowel was exposed to UV irradiation prior to the assay to minimize the number of microbes on its surface prior to commencing the experiment. In this repeat assay, a new *E. coli* inoculum was generated as described above. Plating and enumeration of colonies indicated that a 10 µL volume of this inoculum contained 357,500 CFU of *E. coli*. The bacterial inoculum was incubated with the Lime PeelTowel or a paper towel for 1 hour and 18 hours. The Lime PeelTowel reduced the number of viable *E. coli* by 50% (from 357,500 CFU to 179,944 CFU) after one hour of incubation and contained 61% fewer viable bacteria than the paper towel (**Figure 4A**). The Lime PeelTowel reduced the number of viable *E. coli* from 357,500 CFU to 8,500 CFU (98%) after 18 hours of incubation (**Figure 4B**). In contrast, the paper towel contained 8,000,000 CFU of viable *E. coli*. All of these differences were statistically significant (pairwise Mann-Whitney U tests adjusted for multiple comparisons using the Holm method, p-value < 0.05), demonstrating the antibacterial activity of the Lime PeelTowel after 1 and 18 hours of incubation.

PeelTowels are highly absorbent

We next compared the absorption of PeelTowels and fruit peels to a paper towel. The volume of water absorbed by a 2 cm x 1 cm portion of each PeelTowel and the paper towel following immersion in water for 10 seconds was measured (**Figure 5**). The Lime PeelTowel absorbed almost three times the amount of water (0.93 mL) as the standard paper towel (0.33 mL). The Orange PeelTowel absorbed about the same amount of water as the paper towel (0.30 mL), as did the Kiwi PeelTowel (0.23 mL). In summary, the absorbency of the Lime PeelTowel was greater than that of the paper towel, Kiwi PeelTowel and Orange PeelTowel.

DISCUSSION

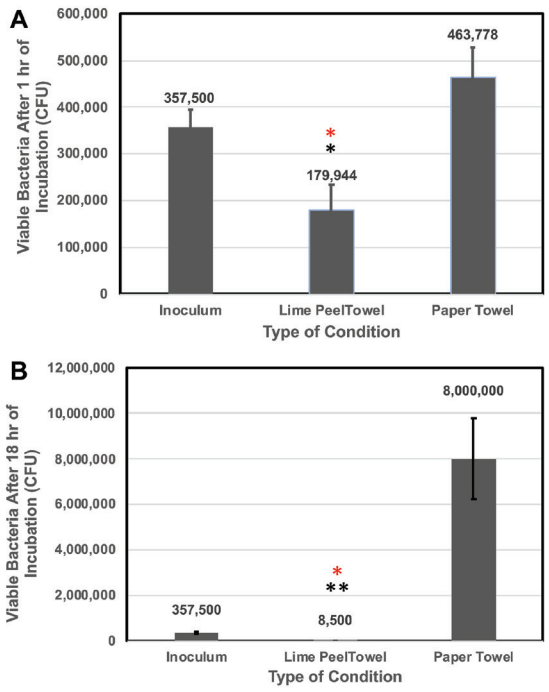

Figure 4: Viable *E. coli* following exposure to UV-irradiated Lime PeelTowels and paper towels. *E. coli* bacteria were incubated with UV-irradiated Lime PeelTowels or paper towels for **(A)** 1 hour or **(B)** 18 hours, and surviving bacteria were enumerated by plating. Each value represents the mean of three experiments, and each experiment represents the average CFU from two 10-µL samples. Error bars represent standard errors of the mean. * $p \leq 0.05$, ** $p \leq 0.01$, red asterisk is comparison to paper towel, black asterisk is comparison to inoculum (two sample independent one-tailed t-test, $p < 0.05$).

Today, lack of hygiene is a significant problem worldwide. Reports suggest that 35% of the world's population (2.5 billion people) in 2012 were without proper hygiene (1). Thus, there is a dire need for innovative and sustainable approaches that facilitate hand hygiene. Of note, commonly eaten fruits contain antibacterial factors that create an inhospitable environment

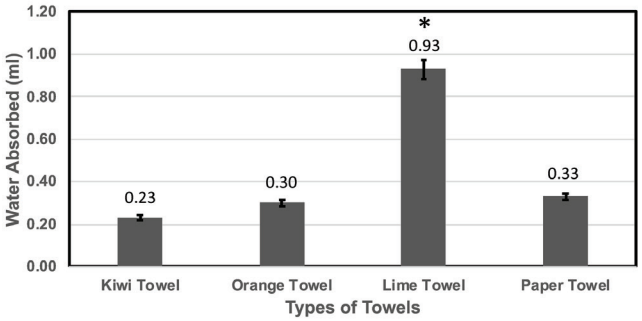

Figure 5: Water absorption by PeelTowels, fruit peels, and paper towels. The PeelTowels, fruit peels, and paper towels were immersed in water to measure their absorption. Each value represents the mean of three experiments. Error bars represent standard errors of the mean. * $p \leq 0.0005$ compared to each of the other conditions (one-way ANOVA, followed by pairwise comparisons using Tukey's multiple comparisons test).

for bacteria (6). Therefore, we designed PeelTowels made from orange, kiwi, and lime peels. Since PeelTowels may remove bacteria from hands either through antibacterial activity or by absorbing bacteria-laden water, we tested their capacity to effectively kill bacteria and to absorb water. A potential advantage of PeelTowels is that they are eco-friendly in that they are both biodegradable and generated from waste products.

Two of the three PeelTowels were antibacterial and performed as well as or better than the paper towel. The Lime PeelTowel reduced the growth of the *E. coli* bacteria by 50-91% after 1 hour and 95-98% after 18 hours of incubation. The lime peels by themselves reduced bacterial CFU by 100% at 1 hour and 81% at 18 hours, explaining the superior performance of the Lime PeelTowels. This is consistent with reports that limes contain more citric acid than oranges or kiwi (**Table 1**). The Lime PeelTowel also absorbed about three times more water than paper towels and the Orange and Kiwi PeelTowels. This may reflect differences in the conformations and degree of drying of cellulose in lime peels compared to other peels, as both these factors may have dramatic effects on water retention by cellulose (19). The Orange PeelTowel reduced growth of the *E. coli* bacteria by 68% and 94% after 1 and 18 hours, respectively, although these differences were not statistically significant. Additionally, the Orange PeelTowel absorbed water to the same degree as the paper towel. In contrast, Kiwi PeelTowels performed well at 1 hour but poorly after 18 hours. This may be due to the presence of antimicrobial factors that are unstable and lose activity over 18 hours. For example, kiwi peels are rich in polyphenols, which have antimicrobial activity but degrade in the presence of oxygen (20-21). The short-lived activity of the Kiwi PeelTowels against *E. coli* suggests that they may not be as effective in facilitating hand hygiene as the other types of PeelTowels. Somewhat surprisingly, kiwi and orange peels exhibited no antibacterial activity after one hour of exposure, whereas Kiwi PeelTowels and Orange PeelTowels demonstrated high activity at this time point. We speculate that the additional processing (i.e. time in blender) of these peels during generation of the PeelTowels may have released more of their antibacterial compounds. These findings demonstrate that peels and PeelTowels differ substantially in their ability to kill or inhibit the growth of *E. coli*.

Our study has several limitations. Ideally, the PeelTowels would be sterile at the start of each experiment. Because our PeelTowels were homemade, contaminating bacteria or fungi were likely present at the start of our initial experiments, and these microbes may have been counted as *E. coli* CFU. However, the colonies we observed on our growth plates were of uniform color and morphology (**Figure 2**), making this possibility less likely. In addition, we repeated antibacterial experiments with UV-irradiated Lime PeelTowels and obtained similar results. A second source of error is that some bacterial colonies were not distinct from one another on the agar plates used for counting, forcing us to use our best judgement in determining whether one colony or two were present. To minimize this error, we plated several dilutions. We only tested *E. coli* bacteria, so we do not know how PeelTowels will perform against other bacteria important for hand hygiene, such as *Salmonella* and *Shigella* species. We normalized measurements of absorption to towel area rather than weight. Since the PeelTowels were thicker than the paper towels, this method of normalization favors the PeelTowels. We chose to normalize based on area because we felt individuals would use a single towel sheet (regardless of its thickness) for hand hygiene. Finally, we measured the performance of PeelTowels under laboratory conditions, which may not accurately reflect their antibacterial activities and absorption under real-use conditions.

In future studies, several additional aspects of PeelTowels need to be examined. The antibacterial effectiveness of the PeelTowels over a shorter period of time (e.g. one minute) that more realistically simulates the usage of the PeelTowel for hand hygiene should be tested. Although the PeelTowels would likely kill a smaller number of bacteria over this short time frame, it is likely that human hands would also carry fewer bacteria than the high numbers used in the current experiments. The shelf life of PeelTowels will need to be tested, since organic material is prone to degrade over time. The tensile strength of PeelTowels will also need to be examined to ensure they are capable of withstanding their intended uses. Other fruit peels with high concentrations of vitamin C, citric acid, or additional antibacterial compounds such as acetic acid, acetone, and alkaloids should also be tested (22).

In summary, we have developed a prototype of an eco-friendly paper towel substitute, which we have designated the PeelTowel, from fruit peels and paper. Lime PeelTowels effectively killed *E. coli* bacteria and successfully absorbed water. With further optimization, these PeelTowels have the potential to reduce waste while improving hand hygiene.

MATERIALS AND METHODS

Production of PeelTowels

Orange, Lime, and Kiwi PeelTowels were produced using a 5-step process. First, a molding was made from two picture frames. We cut out a piece of window screen to match the frame size and hot-glued the window screen to the back of the first frame. The other frame was lightly placed on top of the window screen to complete the molding. Second, we laid a dry cloth on a table in well-lit area and shredded 2 sheets of 8.5-inch x 11-inch paper per towel onto the cloth. Third, we washed and peeled four limes, oranges, and kiwis. For the limes, we used a lime squeezer to squeeze out the juice and retain the peel of the lime. Then, all the peels were separately ground (60-90 seconds) along with the shredded paper and 600 mL of water using a blender and made into a smooth paste. Fourth, we placed the constructed molding inside a large, square-shaped basin that was ¼ filled with water. Using the water as an aid, we spread the paste evenly on

top of the window screen of the molding. Fifth, after removing the molding from the bucket, the PeelTowel was allowed to dry. After five minutes, we carefully lifted the PeelTowel from the window screen and used a sponge and hairdryer to remove any remaining water. Dried PeelTowels were stored at room temperature. For some experiments, each side of the PeelTowel was exposed to UV irradiation for 2.5 hours in a laminar flow hood immediately prior to use. Commercially available Bounty brand paper towels (The Proctor and Gamble Company) were purchased for use as a control.

Estimation of the bacterial inoculum

Antibacterial testing was performed using aseptic technique. *E. coli* strain S17.1 (23) was grown overnight on LB agar plates at a temperature of 37ºC. An individual colony was removed from the plate technique and added to 1 mL of LB medium, which was vortexed. The OD_{600} of the suspension was measured using a spectrophotometer (BioPhotometer D30, Eppendorf, Hamburg, Germany). The OD_{600} of the bacterial suspension was then adjusted to obtain approximately 10^8 CFU/mL using published OD_{600} vs. CFU/mL curves (24). This final suspension was then used in experiments. The actual bacterial inoculum was measured by plating a portion of the suspension onto LB agar plates and counting colonies the following day, as described below.

Measurement of antibacterial activity

Approximately 1 cm x 1 cm sections of PeelTowels or paper towels were placed in microfuge tubes. Ten µL of the *E. coli* inoculum was added to the substrate in each tube. The tubes were then capped and incubated at 37°C for 1 or 18 hours. After the incubation, approximately five small sterile glass beads and 1 mL of LB medium were added to each tube, which was vortexed for approximately one minute. The number of viable bacteria in each tube was then measured by plating and counting colonies, as described below. A similar approach was used to measure the antibacterial activity of fruit peels except that peels from fruit were ground in a blender with approximately 50-150 mL of water for 30-45 seconds, and 100 µL of the resulting fruit peel purée were added to the microfuge tubes in place of the PeelTowels. Following incubation at 37°C for 1 or 18 hours, 900 µL of LB medium was added to each tube. The tube was then vortexed, serially diluted, and plated.

Estimation of bacterial numbers by plating

The number of viable bacteria in a test suspension was determined by serial dilution and plating. Briefly, we removed 100 µL from the test sample and added it to 900 µL of LB medium (1:10 dilution). The mixture was vortexed, and 100 µL was removed from it and added to 900 µL of LB (1:10^2 dilution). This process was repeated up to five times. At this point, 10 µL aliquots of the dilutions were spotted onto an agar plate, the plate was held vertically to allow the bacterial suspensions to form a streak across the plate surface. The plates were then incubated at 37°C for approximately 24 hours, after which the colonies were counted. Each measurement was the average of counts from two different 10 µL samples. The number of dilutions was taken into account to estimate the CFU in the initial test suspension. Most measurements were performed in triplicate, although a few were done in duplicate because technical difficulties caused one of the samples to be discarded.

Measurement of water absorption

The PeelTowels and paper towel were each cut into 2 cm x 1 cm rectangle pieces, and each piece was placed into a 5 mL tube filled with 3 mL of water. After waiting for ten seconds, each piece was removed using forceps. The volume of water remaining in each tube was then measured to estimate the volume of water absorbed by the PeelTowel.

Statistical analysis

Each experiment was performed in duplicate or triplicate, and the means and standard errors were calculated. For bacterial counts, differences between multiple groups were assessed using pairwise Mann-Whitney U tests, and *p*-values were adjusted for multiple comparisons using the Holm method. Differences between two samples were assessed using two sample one-tailed independent t-tests. For water absorption, differences between groups were determined by performing one-way ANOVA, followed by pairwise comparisons using Tukey's multiple comparisons test. Statistical significance was defined as an adjusted *p*-value of ≤ 0.05.

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Article

The parent-child relationship during the college planning process

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SUMMARY

With the dramatic increase in competitiveness in college admissions evident by falling acceptance rates over time, the college planning process has become of great importance to high school students and their parents. As a result, high schools are redesigning their college counseling programs to better serve their students in this increasingly competitive environment. However, college counseling programs are just one piece of the puzzle. The parent-child relationship during the college planning process also plays an important role in achieving a successful outcome. This study presents results from a survey in which high school students answered questions in the form of messages to their parents during the college planning process. We surveyed 138 high school juniors from two private schools that differ in contextual factors (e.g., a private boarding school versus a private non-boarding parochial school). We applied the Grounded Theory coding method to code open-ended survey responses into common themes that were used to test three hypotheses regarding school-specific contextual factors and the frequency of common themes in student messages to their parents. One key result from our analysis is that students at private boarding schools are likely to express greater fear of parental control and disappointment than students at private non-boarding parochial schools. We also found that students at schools with less extensive college planning programs express greater need for parental help in the college planning process. These findings provide evidence that contextual factors are important in the college planning process and could be useful to the design of college planning programs at high schools.

INTRODUCTION

Planning for and applying to college are stress-inducing tasks for high school students, because college choices will affect future outcomes such as potential earnings, career choices, and social capital (1). Parent involvement during the college planning process can have both positive and negative effects on students' stress levels. Contextual factors, such as the type of school a student attends, may affect parent involvement and thus may affect student stress (2). In the current study, we solicited messages from students to their parents regarding the college planning process and examined how these messages differ among students based on contextual factors of the schools they attend. This research can be useful to high school college counseling programs by identifying common issues affecting the parent-child relationship during the college planning process.

Parents play an important role in a student's college planning process. Many parents have high expectations for their student's educational and occupational achievements. Parent involvement in academics can be helpful, as parent involvement is shown to improve a student's academic performance (3). However, due to the growing competitiveness in college admissions, parents increasingly manage students' academic activities to maximize likelihood of college admission (4). Many negative effects, however, can result from this aspect of parental involvement. Parents who consistently intrude on the physical, emotional, and intellectual space of students may interfere with students' abilities to develop independent thinking skills (5). For example, Nelson, Padilla-Walker, and Nielson show that "helicopter parenting," generally defined as an overly-involved parenting style, is associated with student instability or stress (6). The authors also provide evidence that the absence of parental warmth can cause physiological problems in emerging adulthood.

Previous studies have shown that parental educational background can also be a factor in their level of involvement in the college planning process. Kalenkoski finds that less-educated parents tend to be less involved in students' college planning process (7). Students whose parents did not go to college tend to report lower academic expectations than their peers. This may be due to the fact that parents, who did not go through the college planning process themselves may feel ill-equipped to help students during this process (8). Parental educational level is not the only factor that influences involvement in the college planning process. Rowan-Kenyon et al. found that the selectivity of the colleges their student is applying to, socioeconomic factors, and state academic requirements also contribute to differences in parental involvement (4).

Parents who choose private high schools for their children do so for a variety of reasons which may have implications for the college planning process and students' development. There are several specific factors that can also influence the development of high school students. Bryk and Driscoll found that parochial schools shape their students in many aspects including their academic capabilities and personal character

Contextual Factor	School A (n = 108)	School B (n = 26)
Boarding	Yes	No
Urbanicity	Suburban	Rural
Geography	East coast	East coast
Counseling Programs (description)	Extensive, All students college bound, Focus on admission to Ivy League / competitive institutions	Less extensive, All students college bound, Focus on community engagement
% accepted to Ivy League colleges	~20%	< 1%
Student-counselor ratio	90:1	160:1
Admissions rate	20%	86%
Private vs. Public	Private	Private
Parochial	No	Yes

Table 1: Contextual factors of schools included in study.

(9). They found that parochial high schools, for instance, tend to create organizational structures for their students that encourage strong commitments to social cooperation and meaningful community interaction. Therefore, the authors concluded that it is possible that students attending these institutions exhibit a more caring and connected relationship to family and peers at their school.

When comparing across different educational levels of schools, there are several additional factors that may influence student development during the college planning process. For example, private boarding schools in the United States, are expected to offer a wide array of extracurricular activities (9). This extensive involvement of the school in extracurricular activities may account for a student's feeling of being controlled. It can also add to a student's anxiety level as it creates pressure on students to excel both in and outside of the classroom. Families often place their children in private boarding schools, because they feel these schools prepare students well for admittance to highly competitive colleges and universities (9).

In this study, we were interested in answering the following research question: How do student messages to their parents during the college planning process (e.g., messages conveying fear of parental disappointment, anxiety, and need for independence) differ depending on school-specific contextual factors? The goal of this study was to provide an initial exploration that could generate useful insights on the development of more effective college counseling programs at high schools and identify areas for future research. A deeper understanding of the relationship between contextual factors and the parent-student relationship in the college planning process will allow schools to design their college planning programs to better accommodate the needs of their students and families. For example, private boarding schools may find that implementing counseling methods that foster a better parent-student relationship to be the most effective, since this bond may be lost when a student is not living at home.

Based on previous studies, we generated three hypotheses related to the effects of these contextual factors on the response of the students that we tested in our study. First, based on the findings of the Bryk and Discoll study, we expected students attending the private boarding school to express fewer messages of parental warmth and support than students attending the non-boarding parochial school. Second, also based on the findings of Bryk and Discoll, we expected students at the private boarding school to express more feelings of anxiety, as well as fear of parental control and disappointment, than students at the less competitive non-boarding parochial school. Lastly, we expected students at the private boarding school, which features more extensive college planning programs, to express less need for parental help in the college planning process than students attending the non-boarding parochial school.

We found that students at private boarding schools are likely to express greater fear of parental control and disappointment than students at private non-boarding parochial schools. We also found that students at schools with less extensive college planning programs express greater need for parental help in the college planning process. These findings provide evidence that contextual factors are important in the college planning process and could be useful to the design of college planning programs at high schools.

RESULTS

To test our hypotheses related to the influence of school-specific contextual factors, e.g., private boarding versus non-private boarding, we conducted statistical analyses on data we collected from a survey of high school students that asks them to provide messages they would like to convey to their parents with respect to the college-planning process. In general, we did not find significant differences between the two schools in the frequency of themes like "child asking for independence", "parent controlling", "child anxiety", "gratitude", "difficulty" and "warmth" (**Table 2**, two sample t-test, $p<0.60$).

Differences in several themes ("parent expectations," "confidence," "uncertainty," "excitement," "potential/current child disappointment," "parent supporting independence," and "comparing to others") were weakly significant (**Table 2**, two sample t-test, $0.1<p<0.4$), indicating little, if any, meaningful differences in the responses of the students regarding these themes.

However, for five themes, there was a significant difference in response frequency between the two schools: "potential/current parent disappointment"(two sample t-test, $p<0.05$), "child reassuring parent" (t-test, $p<0.05$), "child asking for help"(two sample t-test, $p<0.01$), "child desires" (two sample t-test, $p<0.01$), and "instrumental/financial support" (two sample t-test, $p<0.1$). School A (a private boarding school located in a suburban area in the eastern United States) had

Themes	Examples	All	School A	School B	*p*-value
Potential / current parent disappointment	"You're going to be disappointed. I'm sorry. I'll probably go somewhere though."	43.3%	51.9%	7.7%	.035**
Child asking for independence	"Let me do me. Let me make a decision that will help me feel more comfortable with my future."	15.7%	15.7%	15.4%	.965
Parent expectations	"It's not going to be as good as you expected."	14.2%	15.7%	7.7%	.294
Parent controlling	"I don't want to be a doctor. If you could stop trying to make me be one, that'll be great."	14.2%	13.9%	15.4%	.846
Child reassuring parent	"Don't worry, I got it."	11.9%	14.8%	0%	.037**
Child anxiety	"I'm stressed."	10.4%	11.1%	7.7%	.612
Confidence	"I got this!"	9.7%	11.1%	3.8%	.265
Child asking for help	"Help me, please."	8.2%	4.6%	23.1%	.002**
Uncertainty	"I am not sure where I want to go to college."	8.2%	9.3%	3.8%	.371
Parent anxiety	"Stop worrying about my grades, where I'm going, or what my test scores are."	7.5%	9.3%	0%	.10*
Child desires	"I want to play college hockey."	10.4%	5.6%	30.8%	.0001***
Gratitude	"I hope all goes well and I'm grateful for their help."	8.2%	8.3%	7.7%	.916
Instrumental / financial support	"Sign me up for ACT registration."	7.5%	5.6%	15.4%	.088*
Difficulty	"It is hard to go to a good school."	5.2%	5.6%	3.8%	.728
Excitement	"I'm ready for an exciting but turbulent process."	3.0%	3.7%	0%	.323
Warmth	"I love you and support everything you do for me!"	4.5%	4.6%	3.8%	.864
Potential / current child disappointment	"I don't think I'm gonna get into that one..."	12.7%	15.7%	0%	.183
Parent supporting independence	"Thank you for supporting me and letting me lead my own path."	1.5%	0.9%	3.8%	.274
Comparing with others	"Mom, this will be easier than dealing with [name] and [name]."	2.2%	2.8%	0%	.394
	Total Responses	134	108	26	

Table 2: Summary statistics of survey results. Two sample t-test was used for analysis, * $p < 0.10$; * $p < 0.05$; ***$p < 0.001$.

a higher percentage of responses than School B (a private non-boarding Catholic school located in a rural area in the northeastern United States) that contained statements of "potential/current parent disappointment" and "child reassuring parent." School B, on the other hand, had a higher percentage of responses than School A for the other three significant themes: "child asking for help," "child desires," and "instrumental/financial support."

DISCUSSION

A few key messages emerge from these results. First, we found no difference in the frequency of messages of warmth, as conveyed by students expressing feelings of love and support toward their parents, between the two schools. Thus, our results suggest that students attending private non-boarding parochial schools will not express more messages of warmth than students attending private boarding schools. Second, although we found no difference between the two schools with respect to direct messages of warmth from students to parents, there was a strongly significant (p=0.0001) difference between the two schools related to the "child desires" theme, with students at School B answering the survey with messages of what they want to do in college (e.g. "I want to play college hockey"). The largest percentage of student responses at School B (30.8%) fell under the "child desires" theme while this was one of the lowest percentages at School A (5.6%).

Third, although we found no significant difference between the schools in students expressing greater anxiety, and fear of parental control, we did find a significant difference between the schools in students expressing "potential/current parent disappointment." The largest percentage of responses from students at School A (51.9%) fell under the theme, "potential/current parent disappointment," while a small percentage (7.7%) of responses from students at School B fell under this theme.

Fourth, although weakly significant (p=0.294), we found that the second highest percentage of responses from students at School A (15.7%) fell under the theme of "parent expectations," while this was one of the lowest percentage of responses from students at School B (7.7%). However, there was no significant difference (p=0.965) in the percentage of responses between the two schools that fell under the theme of "child asking for independence" (15.7% for School A and 15.4% for School B).

Lastly, our results found a strongly significant (p=0.002) and very large difference in the responses from students asking for help between School A (4.6%) and School B (23.1%).

The results of this study have important implications for our expectations based on the prior literature. Based on findings from the literature, we expected students at the private boarding school (School A) to express fewer messages of warmth. From the findings of Bryk and Driscoll, we expected that School A, as a boarding school, would foster less warmth between students and parents in comparison to School B, where students lived at home. However, there was no significant difference found in student responses between the two institutions related to this theme.

However, we did find a significant difference in terms of "child desires." This may be the result of a more community-focused atmosphere at School B relative to School A. School A is a boarding school that expects many of their students to attend elite colleges and universities. However, School B encourages the exploration of other options outside of the traditional college track, such as art schools, technical schools, gap years, community colleges, or collegiate level athletics. This seems consistent with the findings of Bryk and Discoll, who found that parochial schools encourage strong commitments to social cooperation and community interaction among their students.

Our results also found that students from School A had a significantly higher percentage of responses related to "potential/current parent disappointment" when compared to School B. We hypothesize that many students at School A fear disappointing their parents and families when it comes to the college planning process. This may be related to the greater pressure put on students at private boarding schools to get into the most competitive colleges and universities. Parents are paying higher tuition for their students to attend a private boarding school, and students may fear that they will not be able to deliver the outcome parents desire, therefore fearing parental disappointment. Acceptance rates at colleges and universities have fallen dramatically over time, as the process has become more and more competitive (10). Although college admittance rates continue to fall, the expectations that parents place on their students are unchanged. This can lead to students fearing disappointment if they are unsuccessful achieving the outcome they were hoping for during their college planning process.

Our results also suggest that students have a good sense of parental expectations as the college planning process nears. Students tend to have two different responses to the pressure of meeting these expectations. Some students may ask for independence to make their own decision related to their college choice. This was the case for both School A and B, as we found no significant difference between the schools in responses related to "child asking for independence." At the same time, students may also express a fear of disappointing their parents if they do not meet the standards that their families expect of them. We found a weakly significant difference between the schools in student responses related to "parent expectations," with a higher percentage of responses from students at School A.

Lastly, we found strong support for the conclusion that students at schools with more extensive college planning programs will express less need for parental help in the college planning process. We conjecture that this may have to do with the difference in counseling services offered at each school. School A has an extensive counseling program

in place for their students, while School B had less college planning services offered to their students. This difference seems to explain the large response rate from students at School B asking for help in the college planning process in comparison to School A.

Although the results of this study are compelling, there are important limitations to our study that could be addressed with a fuller exploration of this research topic. The use of Grounded Theory coding could raise the possibility of bias in our analysis, as the categories of themes are often changed as researchers notice patterns (11). This may create experimenter bias, caused by a subconscious influence on results to benefit the hypotheses. Though we do not believe that this method altered our data analysis substantially, it may have created bias within the initial design of the study.

Another limitation of our study is the lack of public school participation in our survey. Including survey results from a public high school would have enhanced our analysis by allowing us to compare how student responses differ between public and private high schools. This would have also allowed us to explore the importance of resources/support, which tend to be much higher at private schools. With the majority of children in the United States attending public schools, the insights from our study therefore have limited scope. We surveyed only two schools, both on the East Coast, which may also present limitations to the applicability of our results to schools in other regions. Additionally, administrators at the schools that participated in our survey required oversight on the types of questions we could ask students. Therefore, we were unable to ask students about their parental background in order to gain a better understanding of each student's family life. Though we added a question regarding parental education for students in School B's survey, the administrators required that the question be optional, limiting the number of responses and preventing us from drawing any clear conclusions. An additional question asking how often students communicate with their parents would have allowed us to make stronger claims about the lack of family communication with students who attend boarding schools. Another interesting addition to the study would be to evaluate the messages given from parents to their children. This would allow us to assess how the parents' messages differ depending on contextual factors, as well as how they matched up or did not match up with the students' perceptions of parental expectations.

Lastly, differences in the methods used for survey distribution at each school could lead to bias in our study. In the case of School A, our study used previously collected data that was distributed for the school's own purpose. Because of this, the survey distribution for School A had already taken place before the study was conducted. The survey was distributed at a school-wide meeting for all juniors. Each student was asked to take out a sheet of paper and write their answers to a single question that was displayed on the screen. The papers were then collected and recorded manually. School B, on the other hand, was asked to participate in the study after School A's survey was collected. The administration at School B suggested that we use Google Survey to administer the survey, because they felt it would be the most efficient method for their students to access the questionnaire and would also take up less class time. The difference in these methods may have introduced some bias into our study. Students may have felt inclined to respond more thoroughly to an online questionnaire than one which required pencil and paper.

Regardless of these limitations, we feel that our study offers an important and compelling initial exploration of how students convey their feelings about the college planning process to their parents. Depending on the type of school, these findings could be useful in the design of counseling programs at high schools to better serve their students during the college planning process. For example, based on our results that students at boarding schools expressed feeling greater pressure from their parents than students who attend a non-boarding school, counselors at a boarding school could work toward creating programs that form better and more constructive communication between parents and their students. At highly competitive schools, counselors could take the results found in our study and develop programs that emotionally and mentally support students during this stressful time, as many students felt as if their acceptances into elite colleges influenced what their parents thought of them.

METHODS

To address our research question, we collected data from two private schools primarily serving college-bound students. We coded these data to collect themes related to the parent-child relationship and compared the frequency of these themes to the contextual factors of each student. We were thus able to identify important themes that are related to the type of influence parents have on their children during the college planning process.

Participants from School A (n=108) attended a private boarding school with a competitive admissions process and an enrollment size of approximately 210 students per class (**Table 1**). A large number of students who graduate from School A are accepted to highly competitive universities, including Ivy League institutions. School A is located in a suburban area in the eastern United States. The college counseling programs implemented at the school are extensive and are available to students as early as their freshman year. For instance, School A arranges meetings between students and college admission counselors from their students' desired schools to help students refine their college applications. The counseling programs at School A go to great lengths to ensure that their students have the highest chances of acceptance to elite universities. The student/counselor ratio is relatively low, allowing for more one-on-one interactions. Since School A is a boarding school, the majority of students live on campus. As a result, it can be inferred that most students at School A

do not have daily face-to-face interactions with their parents.

Participants from School B (n=24) attended a small private Catholic school with an enrollment of approximately 32 students per class (**Table 1**). Students, who graduate from School B are college-bound, but not necessarily targeting acceptance to highly competitive universities. School B is located in a rural area in the northeastern United States. The student-counselor ratio at School B is larger than School A, as there is only one counselor to serve all of the students in all four grade levels. The college counseling programs implemented at the school are less extensive than that of School A; however, similar to School A, college planning is also a high priority at School B for students starting as early as their freshman year[1]. The academy is non-boarding, and students generally live at home with their parents. Students at the two schools were asked to respond to the following question: "If you could tell your parents/guardians one thing as you begin the college planning process, what would it be?" Students were asked to write one or two sentences in response to this open-ended question. The study collected 108 student responses from School A and 26 student responses from School B.

At School A, the survey was distributed on paper during a school assembly with all juniors. In addition to questions related to the educational background of their parents, students were asked by their assembly advisors to write a response to the open-ended question on the survey. It was explicitly stated to each student that the survey was completely optional and that they may provide as much information in their responses as they pleased. The responses were recorded anonymously, as students were instructed not to write their names on the paper. The students were given 5-10 minutes to complete the open-ended question on the survey.

At School B, the survey was distributed via Google Survey. The survey was created and distributed to the head directors of the schools, who then emailed the survey to the junior class. Students were asked by their teachers to write a response to the open-ended question on the survey. Along with the open-ended question, there were several additional questions regarding the students' parents' educational backgrounds[2]. It was explicitly stated to each student that the survey questions were completely optional and they may answer as many or as few questions as they pleased. The responses were recorded anonymously. The students were given 5-10 minutes to complete the open-ended question on the survey.

We used open-ended Grounded Theory coding methodology to analyze the survey responses. Grounded Theory coding methodology, developed by Glaser and Strauss (12), involves inductive data analysis and is used when assessing qualitative data (13). As data is reviewed, repeated ideas found within the data are assigned to codes and eventually categorized into concepts. Based upon this analysis, each of the 134 student responses was coded for the following themes: parent disappointment, child asking for independence, parent expectations, parent controlling, child reassuring parent, child anxiety, confidence, child asking for help, uncertainty, parent anxiety, child desires, gratitude, instrumental/financial support, difficulty, excitement, warmth, child disappointment, parent supporting independence, and comparing with others. Student responses were coded for as many themes as were applicable. As there were three researchers involved in the coding process, it was important to ensure consistency of theme assignment. If two or more coders chose the same theme, then the theme assignment was made; else, the assignment was not made (14).

We present summary statistics from our survey results in **Table 2**. Column 1 contains the themes identified by the study authors; Column 2 provides an example of the type of response that would fall under each of the themes in Column 1; Column 3 presents the percentage of responses from students at both schools (134 students total) that fell under each theme; Column 4 presents the percentage of responses from students at School A (108 students total) that fell under each theme; and Column 5 presents the percentage of responses from students at School B (26 students total) that fell under each theme. We used the Stata statistical software package (15), to test whether the differences seen in the percentage of the given responses are statistically significant using a two-sample t-test of difference in means (ttest) where the null hypothesis is defined as: H0: mean(A) – mean(B) = 0 and the alternative hypothesis is defined as: HA: mean(A) – mean(B) ≠ 0. The values shown in the last column of **Table 2** are the *p*-values from the statistical test of the null hypothesis.

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Article

Nitric Oxide Synthesis/Pathway Inhibitors in *Daphnia magna* Reverse Alcohol-Induced Heart Rate Decrease

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SUMMARY

Studies show that alcohol causes oxidative stress and heart rate (HR) depression in humans. Chronic alcohol consumption also causes cardiac myopathy, which afflicts about 500,000 Americans annually. The present study aims to understand how alcohol reduces HR and examine if select chemical agents with inhibitory effects on nitric oxide levels can reverse the effects of alcohol on HR. In this study, we used *Daphnia magna,* a water crustacean with a large and a transparent heart, as an in vitro model to study the effects of agents such as methylene blue (a guanylyl cyclase inhibitor), melatonin (a free radical scavenger and Phosphodiesterase E5 (PDE5) inhibitor), L-glutamate (N-methyl-D-Aspartate (NMDA)-ion channel stimulator), and L-N(gamma)-nitroarginine methyl ester (L-NAME, nitric oxide synthase inhibitor) on HR by alcohol. We also examined the ability of these agents to prevent a decrease in the HR by alcohol by changing the order in which we exposed the *Daphnia* to the substances. Alcohol exposure decreased HR by 27% after 30 minutes of exposure. The experiment measured the ability of various drugs to reverse this decrease in HR. Methylene blue, after 10 minutes of exposure, increased the HR by 15%. Melatonin, after 10 minutes exposure, increased the HR by 27%. Similarly, L-NAME and L-glutamate, after 10 minutes of exposure, increased the HR by 20% and 36%, respectively. These results showed that alcohol's depressive effects on HR could be mediated through nitric oxide generation and confirmed the hypothesis that nitric oxide synthesis/pathway inhibitors can reverse alcohol-induced HR decrease in *Daphnia magna.*

INTRODUCTION

Alcohol is the most frequently used drug in our society. Alcohol consumption leads to both short-term and long-term cardiac health effects in humans, which are thought to be mediated primarily through oxidative damage caused by reactive oxygen species (ROS), promoting cell injury and death (1). Short-term effects of ethanol decrease HR and P-R interval (onset of P wave to the start of the QRS complex) in rats, whereas in humans, ethanol was reported to decrease myocardial contraction, possibly due to compensatory mechanisms involved (2, 3). The described long-term effects in humans include decrease in HR, eventually leading to cardiac myopathy and heart failure. How alcohol mediates these effects is not well understood.

Alcohol mediates its central nervous system (CNS) effects through altering cell membrane structure, decreasing Ca^{2+} ion currents, and binding to ligand-gated ion channels (4-6). Other reported effects of alcohol include direct effects on membrane receptors and ROS generation (7, 8). Researchers have shown that alcohol depresses several antioxidant enzymes such as catalase and superoxide dismutase, and that it increases free radical content in a rat model (9). The above effects contribute to oxidative stress and possible auto-oxidation of catecholamines involved in HR regulation. Alcohol also exerts inhibitory effects on the CNS through gamma-aminobutyric acid (GABA) receptors and increases in nitric oxide (NO), which have been shown to have depressive effects on both contractile force and HR (10, 11).

Among the above mechanisms of alcohol discussed, the role of free radicals, ligand channels, and nitric oxide are less well understood in *Daphnia*'s HR regulation. The present study explored if alcohol's depressive effects on the HR in *Daphnia* are mediated via the NO pathway, using NO inhibitors. We investigated the ability of select chemicals to prevent or reverse alcohol-induced depressive effects on HR. Specifically, we tested methylene blue (guanylyl cyclase inhibitor), melatonin (increases PDE5 phosphorylation), L-Glutamine (NMDA ion channel stimulator) and L-NAME (NO synthase inhibitor), which are reported in the literature to interfere with free radicals, ion channels (NMDA, Ca^{2+}), or the NO pathway.

In the present study, *Daphnia magna* were an in vitro model to study the effects of some chemicals on heart rate. *Daphnia magna* is a water crustacean, and it is used frequently because of its transparent exterior and its visible internal organs, which allowed us to easily monitor the HR. Additionally, the HR of *Daphnia* is inhibited by acetylcholine and potassium, and is stimulated by adrenaline, indicating that the cholinergic and sympathomimetic actions seen in *Daphnia* are similar to those in vertebrates (12). The cardiac effects observed in *Daphnia* were sometimes different from mammals in that adrenaline accelerated cardiac rates only at high concentrations, whereas atropine had a positive chronotropic effect (13). Acetylcholine has similar effects on the heart in *Daphnia* as in mammals (14). These differences do not impact the present study, which aims to investigate agents impacting the NO pathway. The main advantage of

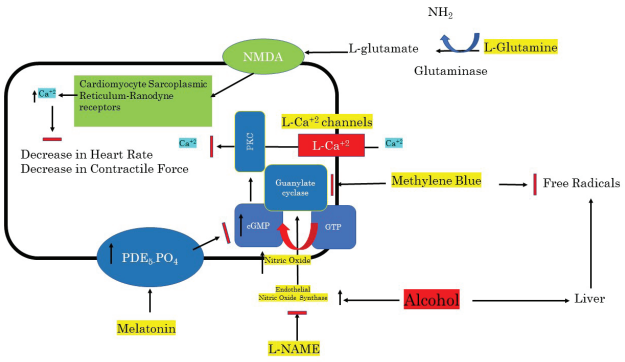

Figure 1: Nitric Oxide-based mechanisms in HR regulation of *Daphnia magna*. The study results suggest a possible therapeutic role for agents that inhibit NO synthesis or signaling pathway in alcohol-induced HR depression. Alcohol increases NO synthesis and cGMP via NO synthase stimulation, which in turn causes a protein kinase cascade to inhibit L-Ca^{2+} channels and Ca^{2+} influx leading to decreased HR and contractile force. Agents which inhibit NO levels directly or indirectly, such as L-NAME (NO synthase inhibitor), melatonin (PDE5 phosphorylation and decreased cGMP), methylene blue (Guanylate cyclase inhibitor) and L-Glutamate (NMDA receptor activator) have resulted in an increased HR in *Daphnia* in this study. All agents reversed the depressive effect of alcohol on the HR and offered protection in post-alcohol treatment. Further investigations are needed to confirm the above proposed mechanisms.

Daphnia magna as a bioassay is its transparency, which allows easy measurement under a microscope, making them a useful model to study the effects of various agents on the heart (15).

Changes in HR and contractile force are modulated by the extent of calcium flux through L-type calcium channels in cardiac myocytes, which are regulated by a cascade of enzymes and their end products (16). The calcium influx through L-type calcium channels is inhibited by protein kinase G (PKG) activation, which is in turn controlled by cyclic guanosine monophosphate (cGMP), leading to a decrease in HR and contractile force (**Figure 1**). Endothelial nitric oxide synthase (eNOS)-derived NO increases the levels of cGMP via soluble guanylate cyclase (sGC) via synthesis from guanosine triphosphate (GTP). In cardiomyocytes, this stimulates the sGC-cAMP-PKG pathway, resulting in decreased HR (17). Agents L-NAME and methylene blue interfere with this pathway. NO synthesized by neuronal nitric

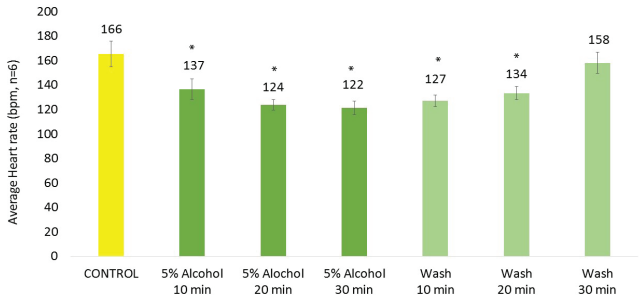

Figure 2: Effect of 5% Alcohol on HR of *Daphnia magna*. *Daphnia* were exposed (n = 6) to 100 µL of 5% alcohol solution in a Petri dish for up to 30 minutes. HR of Untreated (yellow), 5% alcohol-treated *Daphnia*, alcohol-treated *Daphnia* after water washout, were measured in 10-minute intervals for 30 minutes (n = 6 for each condition, excluding control). Error bars represent standard deviation, and asterisks (*) represent statistical significance compared against control HR (student's t-test, *p*-value < 0.05). Control bars, alcohol bars are shown in yellow and green colors respectively.

oxide synthase (nNOS) is coupled to ryanodine receptors in the myocardial sarcoplasmic reticulum and elicits increased calcium influx and increased HR and contractile force, which are opposite to the effects of eNOS-derived NO (17). The NMDA receptor agonist L-Glutamine promotes this activity. Finally, melatonin promotes PDE5, leading to decreased cGMP levels, increased calcium influx, and increased HR (19). Collective use of these agents would allow us to confirm involvement of the NO pathway in *Daphnia magna* HR regulation.

RESULTS

Alcohol causes a dose-dependent (1%-5% v/v) decrease in HR in *Daphnia*. In order to determine the alcohol concentration to be used in the experiments, the effect of 5% (v/v) alcohol was measured after 10, 20, and 30 minutes of exposure to alcohol (**Figure 2**). Treatment with 5% v/v alcohol reduced the HR from 166 ± 10 bpm in the control group to 122 ± 5 bpm (27% of control, *p*-value 0.001)) by 30 minutes (**Figure 2**). There was no further decrease in the HR between 20 and 30 minutes (124 ± 4 bpm and 122 ± 5 bpm, respectively). Therefore, 30 minutes was chosen as an end time to measure the effect of 5% (v/v) alcohol on HR, when the HR has reached a plateau. We chose the 5% v/v alcohol

Table 1: Heart rate comparison of water wash and post-alcohol treatment groups at 10 min and 20 mins (bpm ± std.dev)

Control	Pre-Treatment	10 min	20 min	30 min	Post-Treatment	10 min	20 min	30 min
Treatment					Post-Alcohol Treatment			
166 ± 10	Alcohol (5%)	137 ± 8	124 ± 4	122 ± 5	Water Wash	127 ± 5*	134 ± 5*	158 ± 9
170 ± 6	Alcohol (5%)	146 ± 7	140 ± 7	125 ± 9	Melatonin	159 ± 5**	161 ± 13***	150 ± 12
158 ± 3	Alcohol (5%)	132 ± 7	128 ± 4	123 ± 4	L-Glutamine	166 ± 9**	160 ± 10***	158 ± 3
150 ± 5	Alcohol (5%)	143 ± 5	133 ± 6	122 ± 5	L-NAME	146 ± 9**	148 ± 6***	149 ± 5
166 ± 6	Alcohol (5%)	156 ± 4	141 ± 6	128 ± 4	Methylene Blue	147 ± 3**	152 ± 3***	161 ± 3

* Significance of $p < 0.05$ when compared to Control group (166 ± 10 bpm)
** Significance of $p < 0.05$ when compared to water wash group (127 ± 5 bpm)
*** Significance of $p < 0.05$ when compared to water wash group (134 ± 5 bpm)

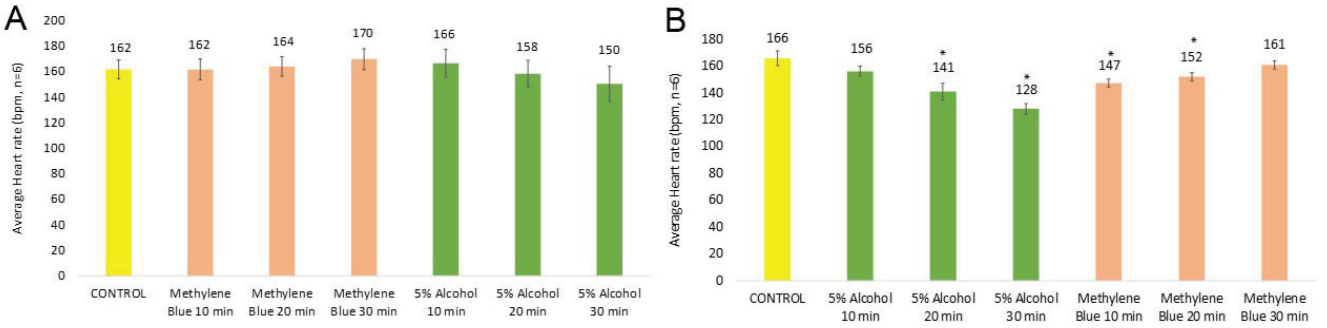

Figure 3: Effect of Methylene Blue Treatment on HR of *Daphnia magna*. A) *Daphnia* were exposed (n = 6) to 100 µL of methylene blue (10 µg/mL) solution in a Petri dish for up to 30 minutes. HR of Untreated (yellow), methylene blue-treated *Daphnia* before alcohol exposure, alcohol-treated *Daphnia* after water washout, were measured in 10-minute intervals for 30 minutes (n = 6 for each condition, excluding control). **B)** *Daphnia* were exposed (n = 6) to 100 µL of 5% alcohol solution in a petri dish for up to 30 minutes. HR of Untreated (yellow), alcohol-treated *Daphnia*, methylene blue-treated *Daphnia* after water washout, were measured in 10-minute intervals for 30 minutes (n = 6 for each condition, excluding control). Error bars represent standard deviation, and asterisks (*) represent statistical significance compared against control HR (student's t-test, *p*-value < 0.05). Control bars, alcohol bars are shown in yellow and green colors respectively.

concentration because it reduced the HR substantially enough (27% of controls) to allow the visual measurement of reversal effects of chemical agents on HR without causing excessive depression or death.

In order to determine the reversibility of the alcohol effect on HR, the *Daphnia* were washed with 1 mL of water and the effect of wash time was observed every 10 minutes for 30 minutes. The depressive effect of alcohol on HR was maintained until 20 minutes (134 ± 5 bpm, *p*-value 0.001 from the control value of 166 ± 10 bpm). By the end of 30 minutes the HR was 158 ± 9 bpm, or 95% of control (**Figure 2**). The HR values were analyzed at 10 and 20 minutes after the water wash and compared to the HR before the wash (i.e., 30 minutes of alcohol treatment) with a significance threshold of $\alpha = 0.05$. This analysis indicated that the HR values at 10 minutes and 20 minutes post-wash were significantly different compared to the control group (166 ± 9 bpm, **Table 1**, *p*-value 0.001). Additionally, we compared the HR values at 10 and 20 minutes of treatment with each of the agents, which were added after 30 minutes of alcohol exposure, to the corresponding HR values with water wash treatment alone and were found to be significantly different ($p<0.05$, **Table 1**). These data indicated that the HR reversal effects of these agents were more pronounced with a quick onset than the water wash effects and could be measured up to 20 minutes. As a conservative measure we measured all reversal effects of chemical agents by 10 minutes.

Select chemical agents which interfere with NO synthetic pathway were used to examine if NO pathway was involved in alcohol induced depressive effects on HR and whether these effects can be prevented or reversed. All chemical agents were screened in a pre-alcohol exposure experiment for selecting a final concentration based on their toxicity levels and concentration where effects were measurable. Methylene blue (MB) by itself at 10 µg/mL did not have any significant effect ($\alpha = 0.05$) on HR over 30 minutes of exposure, when added before alcohol (**Figure 3A**). Pre-alcohol addition of MB for 30 minutes protected from alcohol-induced HR reduction up to 30 minutes (150 ± 14 bpm, 93% of control), as no significant decrease from control values (162

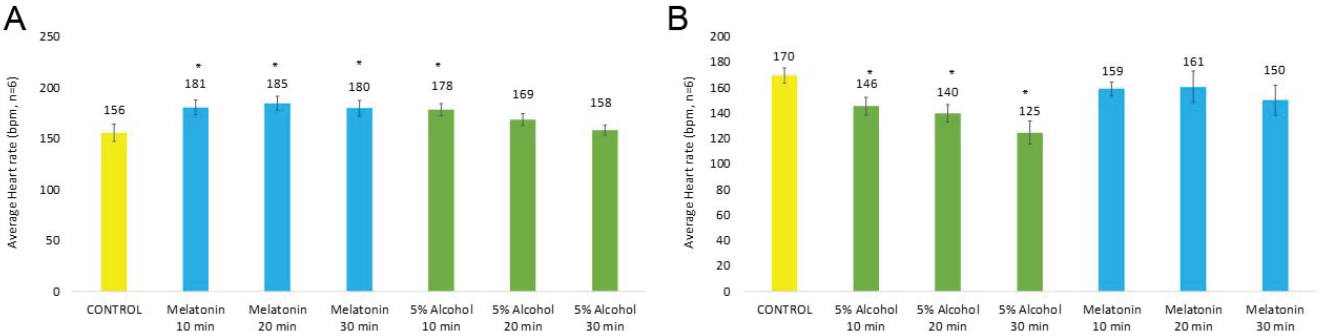

Figure 4: Effect of Melatonin Treatment on HR of *Daphnia magna*. A) *Daphnia* were exposed (n = 6) to 100 µL of melatonin (5 mg/mL) solution in a petri dish for up to 30 minutes. HR of Untreated (yellow), melatonin-treated *Daphnia* before alcohol exposure, alcohol-treated *Daphnia* after water washout, were measured in 10-minute intervals for 30 minutes (n = 6 for each condition, excluding control). **B)** *Daphnia* were exposed (n = 6) to 100 µL of 5% alcohol solution in a petri dish for up to 30 minutes. HR of untreated (yellow), alcohol-treated *Daphnia*, melatonin-treated *Daphnia* after water washout, were measured in 10-minute intervals for 30 minutes (n = 6 for each condition, excluding control). Error bars represent standard deviation, and asterisks (*) represent statistical significance compared against control HR (student's t-test, *p*-value < 0.05). Control bars, alcohol bars are shown in yellow and green colors respectively.

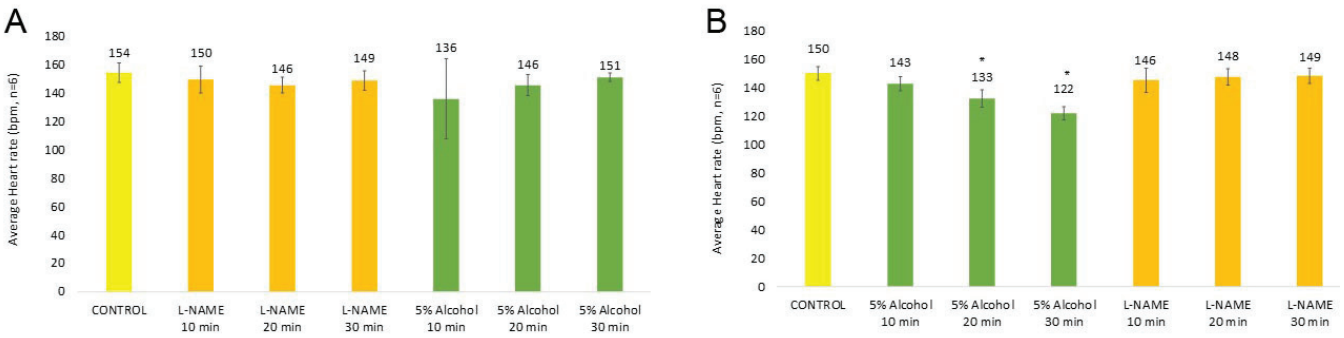

Figure 5: Effect of L-NAME Treatment on HR of *Daphnia magna*. A) *Daphnia* were exposed (n = 6) to 100 µL of L-NAME (3 mg/mL) solution in a petri dish for up to 30 minutes. HR of Untreated (yellow), L-NAME-treated *Daphnia* before alcohol exposure, alcohol-treated *Daphnia* after water washout, were measured in 10-minute intervals for 30 minutes (n = 6 for each condition, excluding control). **B)** *Daphnia* were exposed (n = 6) to 100 µL of 5% alcohol solution in a petri dish for up to 30 minutes. HR of untreated (yellow), alcohol-treated *Daphnia*, L-NAME-treated *Daphnia* after water washout, were measured in 10-minute intervals for 30 minutes (n = 6 for each condition, excluding control). Error bars represent standard deviation, and asterisks (*) represent statistical significance compared against control HR (student's t-test, *p*-value < 0.05). Control bars, alcohol bars are shown in yellow and green colors respectively.

± 7 bpm) were observed. We next added MB after 30 minutes of alcohol exposure, and by 10 minutes, it had HR increased the HR to 147 ± 3 bpm (90% of control HR of 166 ± 6 bpm, *p*-value 0.001) from the previous depressed rate of 128 ± 4 bpm (**Figure 3B**). These results suggest that MB was able to both prevent and reverse alcohol-induced depressive effects on HR.

Treatment with melatonin alone at 5 mg/mL increased the HR from the control value (156 ± 9 bpm) to 180 ± 8 bpm (115% of control) by 30 minutes of treatment (**Figure 4A**). After 30 minutes of alcohol exposure, the HR decreased to 158 ± 5 bpm (101% of control). Melatonin thus protected from the alcohol-induced decrease in the HR. We next added melatonin after 30 minutes of alcohol treatment, and by 10 minutes, the melatonin had increased the HR to 159 ± 5 bpm (94% control HR of 170 ± 6 bpm, *p*-value 0.009) from the previous depressed value of 125 ± 9 bpm (**Figure 4B**). These results suggest that melatonin was able to counter the alcohol-induced decrease in the HR.

L-NAME treatment at 3 mg/mL resulted in a HR of 149 ± 7 bpm by 30 minutes, which is not statistically different from the control HR of 154 ± 7 bpm (**Figure 5A**). With subsequent alcohol treatment, the HR remained unchanged at 151 ± 3 bpm (98% of control HR) by 30 minutes, indicating that L-NAME was able to protect against the alcohol-induced decrease in HR. After 30 minutes of alcohol pre-treatment, we added L-NAME, and the treatment increased the HR within 10 minutes to 146 ± 9 bpm (97% of control HR, *p*-value 0.266) from a previous value of 122 ± 5 bpm (**Figure 5B**). These results suggest that L-NAME was able to both prevent and reverse the alcohol-induced depressive effects on HR.

Thirty minutes of L-Glutamine treatment alone resulted in a HR increase to 177 ± 6 bpm, a 110% increase from a control HR of 160 ± 3 bpm (**Figure 6A**). When alcohol was subsequently added, the HR decreased to 134 ± 26 bpm (83% of control HR) by 30 minutes, suggesting that L-glutamine could not prevent alcohol's effects. However, treating with L-Glutamine for 10 minutes following 30 minutes of alcohol exposure, increased the HR to 166 ± 9 bpm (105% of control 158 ± 3 bpm, *p*-value 0.092) from a previous value of 123 ± 4 bpm (**Figure 6B**). These results suggest that L-Glutamine was only able to reverse alcohol-induced depressive effects on HR, but it could not protect against the alcohol effects.

DISCUSSION

In the present study, exposure to 5% (v/v) alcohol decreased *Daphnia* HR, approximately by 27%, from 166 ± 10

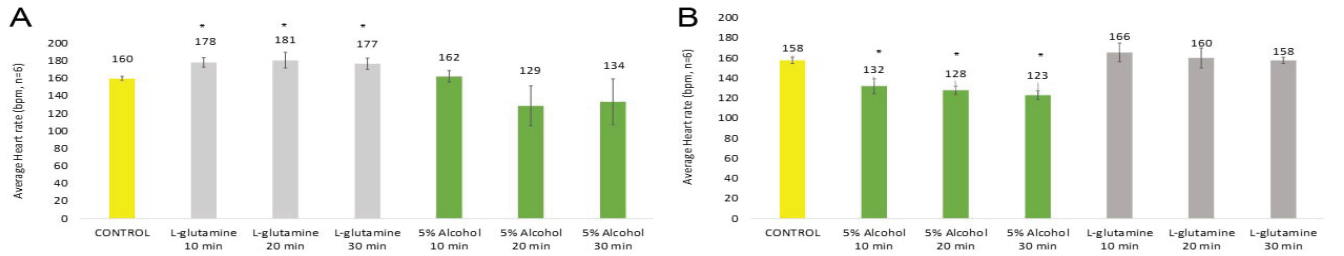

Figure 6: Effect of L-Glutamine Treatment on HR of *Daphnia magna*. A) *Daphnia* were exposed (n = 6) to 100 µL of L-Glutamine (10 mg/mL) solution in a petri dish for up to 30 minutes. HR of Untreated (yellow), L-NAME-treated *Daphnia* before alcohol exposure, alcohol-treated *Daphnia* after water washout, were measured in 10-minute intervals for 30 minutes (n = 6 for each condition, excluding control). **B)** *Daphnia* were exposed (n = 6) to 100 µL of 5% alcohol solution in a petri dish for up to 30 minutes. HR of untreated (yellow), alcohol-treated *Daphnia*, L-NAME-treated *Daphnia* after water washout, were measured in 10-minute intervals for 30 minutes (n = 6 for each condition, excluding control). Error bars represent standard deviation, and asterisks (*) represent statistical significance compared against control HR (student's t-test, *p*-value < 0.05). Control bars, alcohol bars are shown in yellow and green colors respectively.

bpm to 122 ± 5 bpm in controls by 30 minutes (**Figure 2**). The reversal of this decrease was monitored as a parameter in this study to understand if alcohol mediates its effects through the NO pathway with the use of select inhibitors of enzymes — nitric oxide synthase (L-NAME) and sGC (methylene blue). Additionally, the ability of two other agents, one acting through the NMDA receptors (L-Glutamine) and the other via phosphodiesterase 5 (melatonin), on cGMP levels were studied for their ability to reverse alcohol-induced depressive effects on the HR.

Methylene blue by itself did not have any significant effect ($\alpha = 0.05$) on *Daphnia* HR over 30 minutes of exposure (**Figure 3**). Pre-treatment with MB for 30 minutes protected from alcohol-induced HR reduction for up to 30 minutes (150 ± 14 bpm), and no significant decrease from control values (162 ± 7 bpm) was observed. Adding MB for 10 minutes after 30 minutes of alcohol exposure increased the alcohol-depressed HR back up to 147 ± 3 bpm (90% of control HR of 166 ± 6 bpm, p-value < 0.05) from a previous value of 128 ± 4 bpm (**Figure 3A,B**). These effects could be due to MB's inhibitory properties on sGC and the consequent PKG activation (17). L-type calcium channels have been shown to facilitate the action potentials in the atrioventricular node (AV) and contribute to regular HR, and their inhibition by alcohol leads to reduced HR (18). The reversal of alcohol-induced HR depression by MB treatment, as seen in this study, suggests a role for the sGC-cGMP-PKG pathway in alcohol-induced depressive effects on HR.

Melatonin is a free radical scavenger, an antioxidant, and can antagonize nitric oxide activity (19). Melatonin is a hormone produced by the pineal gland and is known to increase the phosphorylation of the enzyme PDE5 four-fold, which in turn decreases the cGMP levels produced by nitric oxide and antagonizes its relaxation effects (20). Exposure to melatonin alone in the pre-alcohol treatment group significantly (p-value < 0.05) increased the HR within 30 minutes to 180 ± 8 bpm from a control HR of 156 ± 9 bpm (**Figure 4A**). Pre-treatment with melatonin for 30 minutes also protected against the alcohol-induced HR reduction up to 30 minutes (158 ± 5 bpm) as no significant decrease from control values (156 ± 9 bpm) was observed. After 30 minutes of alcohol exposure, the subsequent treatment of 5 mg/mL melatonin for 10 minutes increased the alcohol-depressed HR to 159 ± 5 bpm or 94% of control levels (170 ± 6 bpm) in the present study (**Figure 4B**). These protective effects may be the result of the decreased cGMP levels caused by melatonin, which could potentially decrease the PKC and consequent L-type calcium channel activation leading to increased HR (20).

The third agent that was used is L-NAME, which is a well-known non-specific inhibitor of nitric oxide synthase, and it decreases the levels of nitric oxide (21). Sole exposure to L-NAME had no significant ($\alpha = 0.05$) effect on the HR over a 30-minute period (**Figure 5A**). Pre-treatment with L-NAME for 30 minutes protected from alcohol-induced HR reduction up to 30 minutes. After 30 minutes of alcohol exposure, we added 3 mg/mL L-NAME for 10 minutes, which increased the lowered HR from 122 ± 5 bpm to control levels 146 ± 9 bpm (**Figure 5B**). It is possible that alcohol exposure for 30 minutes causes an increase in nitric oxide levels, leading to the activation of the sGC-cGMP-PKC pathway and consequent ligand binding channel (L-channel)/Ca^{2+} influx inhibition (11, 16). This could cause a reduction in the HR. L-NAME, due to its non-selective inhibitory action on nitric oxide synthase could decrease NO production and as a result reverse the inhibitory effects on L-channels/Ca^{2+} influx (21).

The fourth agent used was L-Glutamine, a precursor amino acid for L-glutamate, and L-channel agonist. L-glutamate is formed by deamidation of L-Glutamine by the enzyme glutaminase. L-glutamate is a known agonist for the NMDA ion channels which are a part of the glutamine receptors, which mediate most fast excitatory transmission in the CNS (22). Exposure to L-Glutamine for 30 minutes in the pre-alcohol treatment group increased the HR from 160 ± 3 bpm by 10% to 177 ± 6 bpm (**Figure 6A**). However, pre-treatment with L-Glutamine for 30 minutes did not protect from the effects of alcohol-induced HR reduction up to 30 minutes. After 30 minutes of alcohol exposure, the addition of 10 mg/mL L-Glutamine for 10 minutes increased the lowered HR from 123 ± 4 bpm to 166 ± 9 bpm (105% of control level of 158 ± 3 bpm, **Figure 6B**). L-Glutamine binds to the NMDA glutamine receptor, and its activation increases the synthesis of nitric oxide in the CNS by stimulating the nNOS (22). It is possible that NMDA receptor activation and subsequent NO release in cardiomyocytes results in the ryanodine receptor stimulation in the sarcoplasmic reticulum leading to calcium release, muscle contraction, and AV node activation (23). L-Glutamine also has been shown to inhibit nitric oxide formation by inhibiting nitrogen oxide synthase in bovine endothelial cells (24). This effect was proposed via the metabolism of glutamine into glucosamine, which reduces the cellular availability of the NADPH cofactor for nitric oxide synthase, thereby inhibiting its activity. These mechanisms could possibly explain the reversal of alcohol's depressive effects on HR by L-Glutamine.

Overall these results indicate that indirect and direct inhibition of nitric oxide synthesis/pathway by various agents results in the reversal of the alcohol-induced depressive effects on the *Daphnia* HR (**Figure 1**). These data support the hypothesis that alcohol could mediate its depressive effects on the HR via the nitric oxide pathway. Further studies are necessary to confirm the validity of this hypothesis by the measurement of the actual abundance of nitric oxide synthase, soluble guanylate cyclase, phosphodiesterase-5 as well as nitric oxide, and the verification of the involvement of other potential mechanisms which this study did not aim to do. The verification of the validity of these mechanisms in other mammalian organisms is also desirable. The agents which have direct or indirect nitric oxide inhibitory action would have a beneficial therapeutic effect against alcohol-induced

depressive effects on HR.

MATERIALS AND METHODS

Daphnia magna were ordered from Carolina Biological Supply (North Carolina, NC). They were stored in loosely lidded glass jars filled with spring water at room temperature. *Daphnia magna* were fed yeast culture every 2-3 days. Drug solutions were freshly prepared at various indicated concentrations in the results section by diluting the stock solutions of methylene blue (1% solution), melatonin (10 mg/mL), L-Glutamine (50 mg/mL), and L-NAME (9 mg/mL) with water in polypropylene test tubes (Fisher scientific). Alcohol (ethanol) solution was prepared at 5 % v/v concentration by dilution with water from 95% v/v.

There were six organisms in each group and their HR (bpm) was measured for 15 seconds and calculated for 1 minute, every 10 minutes up to 30 minutes with a hand counter. The mean and standard deviation were then calculated. There were two types of controls. One control was the HR obtained without any treatment (1st column labelled as control in graphs 2-6. The second control was the effect of the chemical agent by itself on the HR measured in the pre-alcohol cohorts (columns 2-4 in each graph), prior to alcohol administration. Finally, the same organism was used for the control, pre-alcohol or post-alcohol effects measurements, and the results from the replicate experiments (n = 6) were averaged.

All chemical agents were screened in a pre-alcohol exposure experiment for selecting a final concentration based on their toxicity levels and concentration where effects were measurable (data not shown). Methylene blue (half maximal effective concentration (EC50) in *Daphnia* ~ 2.28 µg/mL) was tested at 1.1, 3.3, and 10 µg/mL concentration levels (25). At 1.1 µg/mL MB was not effective in preventing alcohol-induced depression, but was from 3.3 µg/mL and higher. The 10 µg/mL concentration was chosen as the effects were more pronounced. Melatonin (median lethal dose for 50% of the animals (LD50) in rats > 800 mg/kg) was tested at 5.0, 1.0, and 0.33 mg/mL concentration levels (26). Melatonin protected from alcohol-induced depressive effects at 1 mg/mL concentration and higher, and a 5 mg/mL concentration was finally chosen. L-NAME (LD50 not available) was tested at 3, 1, and 0.33 mg/mL concentration levels. L-NAME did not protect from alcohol-induced depressive effects at 0.33 and 1.0 mg/mL concentrations, and a 3 mg/mL final concentration was thus chosen. L-glutamine (LD50 in rats ~ 7.5 gm/kg) was tested at 10, 3.3, and 1.1 mg/mL concentrations (27). L-glutamine was not effective at 3.3 mg/mL and below in protecting from alcohol-induced depressive effects, and so a 10 mg/mL final concentration was chosen.

Daphnia was placed in the wells of concave slides using a Nalgene dropper with a wide mouth (provided by Carolina Biologicals) and were given 10 minutes to acclimate (**Figure 7**). The surrounding water was slowly aspirated with the dropper just enough to restrict the mobility of the organism,

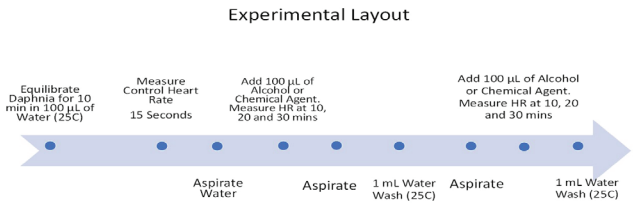

Figure 7: The experimental layout depicts the procedures of equilibration, wash and addition of agents to *Daphnia*. After equilibration at room temperature, control *Daphnia* HR (n=6) is measured and either alcohol or a chemical agent is added. HR is monitored at 10-minute intervals for 30 minutes. After washout with water, the treatment in the previous step is reversed, and the HR is monitored at 10-minute interval for 30 minutes.

and the slide was placed under a digital microscope connected to a personal computer. The HRs were measured for 15 seconds using a timer (Fischer Scientific), and the total beats were calculated per minute, and this was considered the control HR. The surrounding water was then completely aspirated, and 0.1 mL of alcohol solution (5% v/v) was then added. The HRs were again measured for 15 seconds for every 10 minutes, up to 30 minutes. The alcohol solution was then aspirated with a dropper, prior to the HR measurement for 15 seconds and added back to resume the experiment until 30 minutes. Then the alcohol solution was completely aspirated, and the *Daphnia* were washed with 1 mL of water. The wash liquid was completely aspirated, and 0.1 mL of chemical agent solution prepared in water was then added. After 10 minutes equilibration, the solution was aspirated, and the HRs were measured for 15 seconds (for every 10 minutes), and the solution was added back to resume the exposure for 30 minutes. This process was repeated for each drug, using a total of six *Daphnia* for each trial of pre- or post-alcohol exposure. Pre- and post-alcohol exposure sets used different sets of *Daphnia*, and each *Daphnia* once used was never used for any other experimentation.

Chemical agents were added, either before or after the alcohol exposure for 30 minutes for all treatment groups. If chemical agent treatment was after the alcohol treatment (post-alcohol treatment), then the ability of the chemical agent to reverse the decreased HR is observed. If chemical agent treatment was before the treatment with alcohol (pre-alcohol treatment), then the ability of chemical agent to prevent the drop in the HR by alcohol is looked for. All alcohol treatments were performed up to 30 minutes, and the reversal effects of the agents were measured for 30 minutes, but only results at 10 minutes were considered for comparison.

Statistical Analysis

HRs were obtained at 15 second intervals and were multiplied with 4 to calculate the total beats per minute (bpm). The average HR (n = 6) for a group and the standard deviation values were calculated using Microsoft Excel. A student's t-test was also conducted to find the differences between

the control group and drug-treated groups. This was done by determining the *p*-values (at a significance level of 0.05), which showed that the data for drug-treated groups was significantly different from that of the controls.

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Article

The Bioactive Ingredients in *Niuli Lactucis Agrestibus* Possess Anticancer Effects

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SUMMARY

In the field of medicine, natural treatments are becoming increasingly vital towards the cure of cancer. We investigated the effect of various concentrations of *Niuli Lactucis Agrestibus* crude extract on the survival percentage and proliferation rate of COLO320DM adenocarcinoma cells. Our experimental process consisted of the following methods: a Trypan Blue Cell exclusion assay for viability, a cancer cell migration assay, and a protein expression test. These tests were performed using varying concentrations of crude extract to test the impact of the chemicals in the extract on colorectal cancer cells. Our results for the cell viability test showed that the crude extract significantly increased the death rate of colon cancer cells, but not of healthy cells. Furthermore, the results suggested that increasing the concentration of crude extract inhibited colon cancer cell migration. In addition, we observed that the *Niuli* extract significantly upregulated the expression of p21 and p27, two key proteins in the cell cycle. These results indicate that the presence of bioactive ingredients in *Niuli* lettuce can exhibit anti-colon cancer properties. Further experimentation will be carried out to purify and identify the chemicals found within the crude extract that contain the potential for the treatment of colorectal cancer.

INTRODUCTION

The American Cancer Society reported that colon cancer is the third-most diagnosed cancer in the US with over 100,000 cases expected in 2019 (1). Colorectal cancer affects the lower area of the large intestine around the rectum and may only show symptoms after several years (2). Different colon cancer treatments are available for people in different disease stages, with many treatments involving surgery or other costly procedures. Additionally, if patients are not healthy enough, they may be unable to have the surgery, forcing them to opt for chemotherapy (3). Research has proven that chemotherapy, while killing cancer cells, also has a negative effect on healthy cells, resulting in numerous side effects (4). Some of the side effects include mood changes, fertility issues, hair loss (alopecia), infection, nausea and vomiting, sleep problems, urinary and bladder problems, etc. (5). As another option to the current cancer treatments, which are both dangerous and costly, plant extracts have gained increased interest as a source of bioactive molecules for cancer treatment.

For instance, certain substances found in the bark of *Lafoensia* can be composed into a mixture capable of killing proliferating cancer cells (6). Another study stated that incorporating spinach, broccoli, lettuce, carrots, celery, greens, etc, and fruits rich in lutein, reduces the risk of developing colon cancer. In addition, frequent consumption of lettuce, decreases the risk of breast cancer (7). Similarly, one study found not eating salad and other leafy greens significantly increased the risk of cancer (8).

After further research, two cyclin-dependent kinase inhibitor proteins exhibited great potential to aid in the battle against cancer: p21 and p27. Firstly, the tumor suppressor protein, p21, is known for its ability to mediate the cell cycle by inhibiting several cyclin and cyclin-dependent kinase (CDK) complexes (9, 10). p21 also has a role in DNA repair through its interaction with the proliferating cell nuclear antigen (PCNA) (11). Furthermore, p21 and p27 have a crucial role in determining the fate of cancer progression. Although they are known to be tumor suppressor proteins, in some cases they also display oncogenic behavior. Recent research has found the subcellular localization of p21 and p27 to be the predominant factor influencing the dual properties of p21 and p27 (12). Nuclear accumulation of p21 and p27, through various mechanisms, leads to increased effectiveness of cancer treatments. When localized in the nucleus, p21 and p27 inhibit CDK complexes at the G1 or G2/M checkpoints, and therefore promote cell cycle arrest (13).

As hypothesized for centuries, recent research has shown that plants may be a much-needed substitute for our current cancer treatments (14, 15). Natural products shows great promise for the future of medicine and cancer treatment. We tested the extract of *Nuili Lactucis agrestibus*, a type of lettuce, for its ability to inhibit colon cancer cell proliferation. We aimed to investigate whether an increased dosage of the *Nuili* lettuce crude extract inhibits proliferation, increases the distance traveled during cell migration, and enhances the activity of tumor suppressors. We hypothesized that the chemical extract of *Nuili* lettuce would hinder the proliferation of colon cancer cells and that it would stimulate increased p21 and p27 protein expression. This increase in protein expression is hypothesized to be a deterrent to the colon cancer cell growth.

Our results demonstrate that *Nuili* lettuce extract

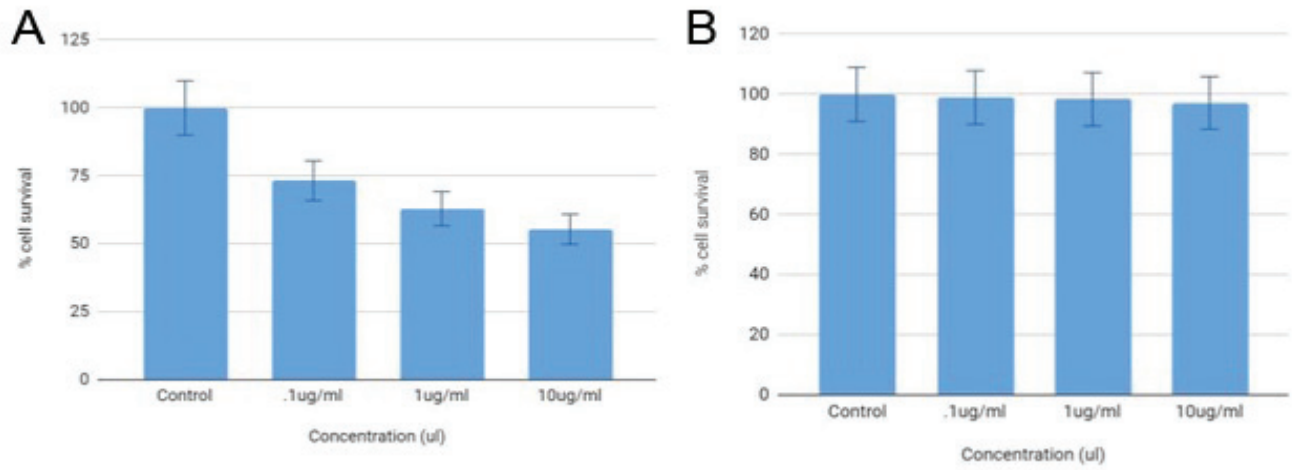

Figure 1: Assessing cell viability of normal and cancerous colon cells treated with *Niuli* crude extract. A) Average cell viability of untreated, 0,1 µg/mL, 1 µg/mL, and 10 µg/mL-Niuli extract treated Colo320 cells (n = 10). **B)** Average cell viability of untreated, 0.1 µg/mL, 1 µg/mL, and 10 µg/mL-Niuli extract treated normal colon cells (n = 10). Error bars depict standard deviation and asterisks indicate statistical significance (student's t-test, *p*-value < 0.05).

inhibited cancer cell migration and proliferation. Additionally, the crude extracts upregulated p27 and p21 expression. These results emphasize the potential of natural products on the treatment of cancer. With further testing, this extract could be a legitimate and natural way to prevent the formation and growth of colon cancer cells in the body.

RESULTS

The Trypan blue exclusion assay can be used to test for cell viability among cancerous and non-cancerous colon cells after treatment of the extract. This experiment will allow us to determine if the extract is able to decrease the viability of colon cancer cells without harming the non cancerous ones. After incubating the colon cells with different concentrations of the *Nuili* crude extract, we observed that crude lettuce extract significantly (student's t-test, *p*-value < 0.05) decreased the percentage of colon cancer cell survival in a dose-dependent manner, relative to the control (**Figure 1A**). The control cell groups were grown without the addition of extract to assess the effect of the extract treatments. At the highest dosage 10 µg/mL, the colon cancer cell survival was reduced by over 50% compared to the control. However, non-cancerous cells also needed to be tested to determine if the extract impacted non-cancerous cell viability. The extract did not significantly affect non-cancerous cell viability even at the highest concentrations tested (**Figure 1B**). The results indicate that an increase in the concentration of crude *Nuili* lettuce extract reduced the survival of colon cancer cells, without significantly affecting the healthy colon cells.

We also wanted to perform the cell migration test to see if the extract was able to prevent the migration of the cancerous colon cells. This would be very beneficial towards stopping the spread of colon cancer cells within a patient's body. Increasing the concentration of the lettuce extract inhibited colon cancer cell migration after 24 hours of incubation. The results show a directly proportional relationship between the dosage and inhibition of migration (**Figure 2A**). This trend indicates that as the dosage of crude extract increased, the distance between the colon cancer cells also increased. We observed the gap between the two main bodies of cells widened after each consecutive concentration (**Figure 2B**). The largest distance was observed when the concentration was at 6 µg/mL (**Figure 2A**). Therefore, the lettuce extract reduced colon cancer cell migration in a dose-dependent manner (student's t-test, *p*-value < 0.05), in respect to the control group.

p21 and p27 are important proteins involved in cell cycle regulation (16). Therefore, we wanted to determine if the lettuce extract influenced the abundance of p21 and p27 in cancer cells. To do this, we performed an ELISA assay to measure p21 and p27 in cell lysates from treated and non-treated cells. The results suggest that the effect that the crude extract had on two key proteins in the cancer cell cycle, p21 and p27. In the graphs, there is a clear direct relationship, indicating that an increase in the dosage of crude extract increased the protein expression of both these cyclin-dependent kinase inhibitors (**Figure 3A and B**). In both cases, the cells were incubated for 24 and 48 hours. Significance was determined by taking the levels of p21 and p27 expressed by the cells and comparing them to those in the control. The levels of p27 were slightly higher compared to the levels of p21 at the same concentrations; however, in both cases, significance was exhibited (student's t-test, *p*-value < 0.05). The subcellular location of p21 and p27 are exceptionally significant in determining their role in cancer (17). Nuclear p21 and p27 have several anticancer properties, while other forms of the proteins can exhibit oncogenic behavior (18). Targeting nuclear accumulation of p21 and p27 could be important in increasing the efficacy of current cancer treatments.

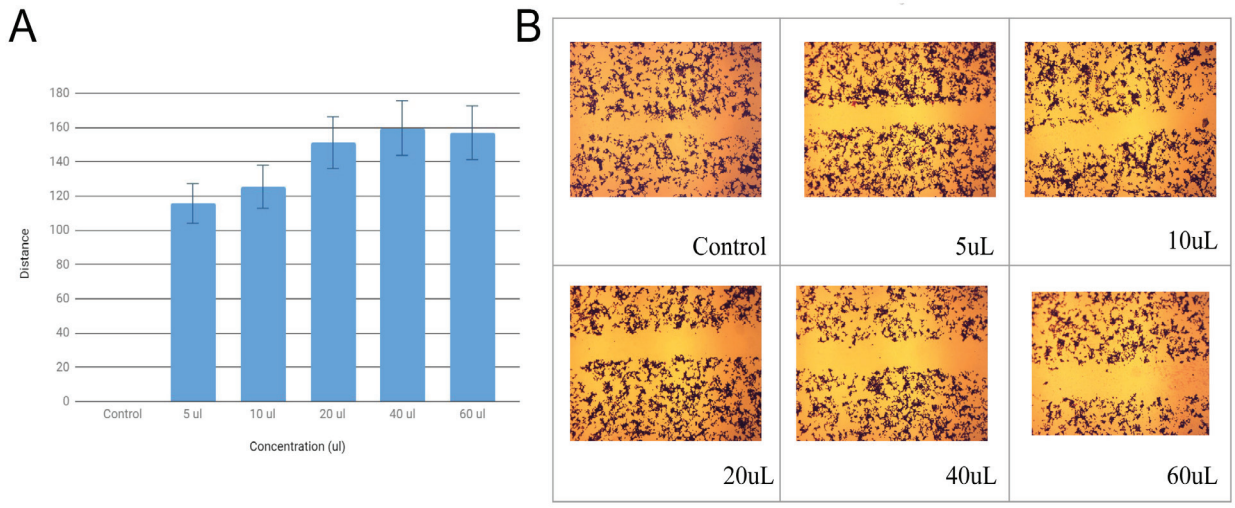

Figure 2: Increasing concentration of *Niuli* crude extraction decreased cancer cell migration. A) Average cell migration of untreated, 5 µL, 10 µL, 20 µL, 40 µL, 60 µL-Niuli extract treated Colo320 cells (n = 9). Error bars depict standard deviation and asterisks indicate statistical significance (student's t-test, *p*-value < 0.05). **B)** The photos visually show the average cell migration of untreated, 5 µL, 10 µL, 20 µL, 40 µL, 60 µL-Niuli extract treated Colo320 cells (n = 9).

DISCUSSION

Our results demonstrate that the *Nuili* lettuce extracts exhibited significant inhibitory effects on the proliferation of colon cancer cells. Increasing the concentration of the lettuce extract significantly increased cancer cell death without affecting healthy cells. *Nuili* lettuce extract also significantly reduced colon cancer cell migration in a dose-dependent manner. Preliminary experimentation indicated bioactive components within this extract are able to regulate key protein (p21 and p27) expression, two important proteins involved in tumor suppression. All the results supported our hypothesis that the chemical extract from *Nuili* lettuce would have anti-cancer effects. Our results demonstrate that there may be many natural options to combat colon cancer. Additionally, the crude extract may be able to prevent the proliferation of colon cancer without the use of painful and expensive treatments. We would also like to implement a wider array of tests, such as an MTT assay which measures metabolic activity of cells, strengthening our results on the cell viability test. Our results provide preliminary evidence that *Nuili* lettuce extract could potentially be tested in other model systems These experiments have also prompted many questions and future studies, such as elucidating the specific chemical or chemicals found in the extract, and performing tests on the specific chemical that has the anti-cancer properties. In the future, more experimentation should also be done to identify the specific pathway at which the extract is able to regulate p21 and p27 expression. Nuclear accumulation of p21 and p27, through various mechanisms, leads to increased effectiveness of cancer treatments (19). Future research can be conducted to discover additional methods through which nuclear accumulation of p21 and p27 can assist cancer treatments.

In summary, we conducted three major experiments to test the effects of the crude extract on colon cancer cell proliferation prevention. At a cellular level, our results demonstrate that *Nuili* lettuce extract exhibited notable inhibitory effects on cancer cell proliferation and migration. Additionally, the crude extracts upregulated p27 and p21 expression, two important factors involved in tumor suppression. Our study provides significant evidence that the extracts from *Nuili Lactucis Agrestibus* could be an effective and natural way to combat colon cancer already in the body and prevent its initial formation.

METHODS

Cell culture

The COLO320DM (COLO) colorectal cancer cell line (ATCC, New York, NY) was grown in RPMI 1640 media supplemented with 10% fetal bovine serum (Invitrogen, USA). The CCD 841 CoN (CCD) human normal colon epithelial cell line (ATCC, New York, NY) was grown in RPMI 1640 media supplemented with 10% fetal bovine serum (Invitrogen, USA). Cells were incubated at 37 °C in 95% O_2/5% CO_2.

Preparation of crude extract

To separate the supernatant from the *Nuili* leaf, 10 g of fresh leaves were placed in a50% ethanol solution, ground using a Kinematica PCU Polytron, and centrifuged for 15 minutes at 4000 rpm. Once a density gradient was established, the supernatant was obtained and further purified using Whatman filter paper. The supernatant was stored in Eppendorf tubes and ethanol and water were dried by Labconco centrivap console (Cryostar industries Inc USA). The dry weight of the extract was measured by subtraction of the empty tube. The dried tube was then suspended with 1 mL of distilled water for further experimentation.

Trypan blue cell exclusion assay

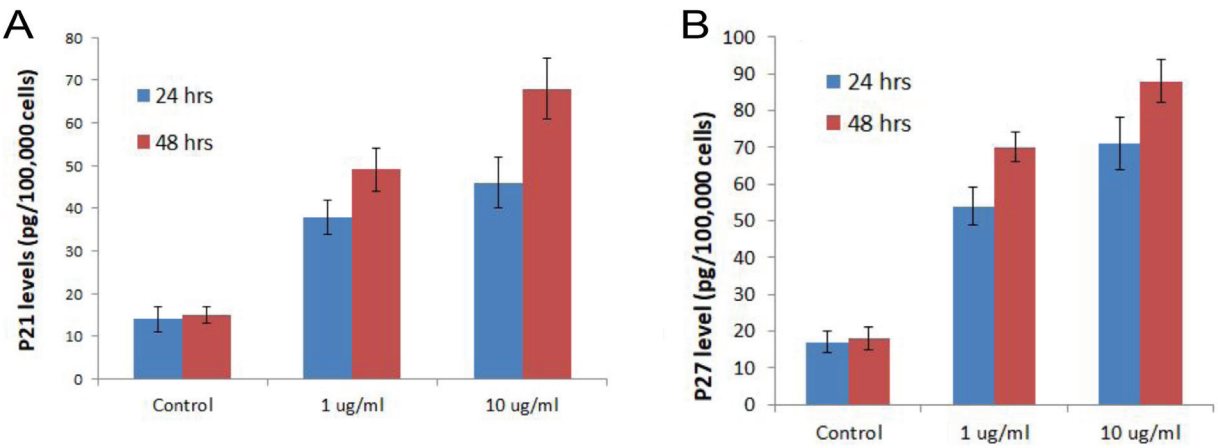

Figure 3: Crude extract treatment increases p21 and p27 expression. **A)** Average levels of p21 expression of untreated, 1 µg/mL, 10 µg/mL-*Niuli* extract treated Colo320 cells for both 24 and 48 hours (n = 2). **B)** Average levels of p27 expression of untreated, 1 µg/mL, 10 µg/mL-*Niuli* extract treated Colo320 cells for both 24 and 48 hours (n = 2). Error bars depict standard deviation and asterisks indicate statistical significance (student's t-test, *p*-value < 0.05).

First, 100 µL of medium containing suspended colon cancer cells for testing were placed in a microcentrifuge tube and 10 µL of 0.4% trypan blue dye was added to the suspended colon cancer cells. The suspension was mixed by pipetting up and down and incubated for two minutes. Next, a coverslip was placed on top of a hemocytometer and 10 µL of the cell suspension was used to gently fill the indent. The hemocytometer was placed on the stage of a light microscope and the cells were counted, both clear (alive) and blue (dead), in the hemocytometer and the results were documented.

Cell migration assay

COLO320DM cells were detached using 0.25% Trypsin-EDTA, and these cells were centrifuged in a 15 mL conical tube at 1,000rpm for 4 minutes at 20 °C. Then the supernatant was aspirated, and the cells were resuspended in the culture media. Approximately 6,000 cells were plated into each well of the 24-well plate. After 24 hours, crude extract was added to the wells in the following volumes: 0 µL, 5 µL, 10 µL, 20 µL, 40 µL, and 60 µL, with the back-side of each well in a 24-well plate was labeled accordingly. Manually, a small wound or scratch was made over the body of cells using a sterile pipette tip. The culture media was inserted against the well wall to cover the bottom of the well and the plate was placed in an incubator. After 24 hours, the plate was observed from under the compound light microscope and snapshot pictures were taken on the computer. To analyze the results of snapshot pictures, the distance of one side of the wound to the other was measured using a scale bar.

ELISA Assay

p21 (CDKN1A) and p27 (CDKN1B) in vitro SimpleStep ELISA® (Enzyme-Linked Immunosorbent Assay) kit (abcam USA) was used for quantitative measurement of target proteins in COLO320DM cells. Before lysing, the cell incubation media is removed and rinsed with distilled water. Later, 1mL of water was added and kept at -80 °C. The cells were lysed by undergoing the freeze-thaw cycle (-80 °C to room temperature) 4 times. The SimpleStep ELISA® employs an affinity tag-labeled capture antibody and a reporter-conjugated detector antibody, which immunocapture the sample analyte in solution. This entire complex (capture antibody/analyte/detector antibody) is in turn immobilized via immunoaffinity of an anti-tag antibody coating the well. To perform the assay, samples or standards were added to the wells, followed by the antibody mix. According to the manufacturer's instructions, the wells were washed to remove unbound material. 3,3',5,5'-Tetramethylbenzidine (TMB) substrate is added and during incubation it is catalyzed by horseradish peroxidase (HRP), generating blue coloration. This reaction is then stopped by addition of Stop Solution completing any color change from blue to yellow. Signal is generated proportionally to the amount of bound analyte and the absorbance is measured at 450 nm.

Statistical analysis

A variety of one-way ANOVAS and t-tests were run to test for statistical significance. One- way ANOVAS were used to compare multiple treatments with one another, while t-tests were run for experiments with only two variables. All data were analyzed on SPSS where significance was defined as *p*-value < 0.05. **p*-value < 0.05, ***p*-value < 0.01, ****p*-value <0.001. Error bars show standard deviation.

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Article

Expression of anti-neurodegeneration genes in mutant *Caenorhabditis elegans* using CRISPR-Cas9 improves behavior associated with Alzheimer's Disease

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American Heritage School, Plantation, FL

SUMMARY

Alzheimer's is ranked as the 6th leading cause of death in the United States, and mainly presented as neurodegeneration. In order to begin to understand its physiology, the specific role of proteins in neurodegeneration, LRP1 and AQP4, need to be studied. This study tested the effect of using the CRISPR-Cas9 system to overexpress the LRP1 and AQP4 proteins, associated with transport of materials and waste across the cell membrane, in *Caenorhabditis elegans* to assess effects on neurodegeneration, such as chemosensation, size, and average speed phenotypes. I hypothesized that combinatorial overexpression of AQP4 and LRP1 would have the greatest effect on reducing neurodegeneration. I tested chemosensory behavior using a chemotaxis test, revealing a decrease in neurodegeneration when both LRP1 and AQP4 were overexpressed. The size of the *C. elegans* did not change, but the speed increased in the strain expressing amyloid beta in the muscle, suggesting that a decrease in amyloid beta allowed muscles more room to contract. These results support our hypothesis and show that the overexpression of LRP1 and AQP4 proteins decrease neurodegeneration and allow *C. elegans* to preserve their olfactory retention. This study will help demonstrate the role of LRP1 and AQP4 in Alzheimer's and determine whether they benefit the system once they are overexpressed.

INTRODUCTION

Approximately 5.7 million people worldwide have Alzheimer's Disease, which eventually causes loss of mental function and memory in those affected. There are a few main hypotheses that try to explain Alzheimer's disease, including the amyloid hypothesis and the Tau hypothesis. The amyloid hypothesis proposes that a protein in the cell membranes of neurons called the amyloid precursor protein (APP) helps transmit signals from the inside of the cell to its environment. APP is eventually broken down by alpha, beta, and gamma secretases. Alpha and gamma secretases break down the protein into a soluble state, but beta secretase activity leads to insoluble APP. Buildup of insoluble APP can generate an amyloid beta plaque that inhibits the connections between neurons (1). Alternatively, the Tau hypothesis suggests that, in Alzheimer's Disease, the Tau protein, which normally stabilizes microtubules, instead separates from these microtubules, causing them to fall apart. The strands of Tau cause tangles, which disable the transport system and destroy the cell (2).

The LDL receptor family functions to bind ligands for internalization and degradation, as well as for cholesterol metabolism. Previous research suggested that LRP1-APP interactions favor APP processing through the amyloidogenic pathway due to LRP1 binding with sAPP770, an isoform of APP. Recent studies have shown that areas of the brain that have decreased amounts of low-density lipoprotein receptor-related protein 1 (LRP1) have an increased amount of amyloid beta plaques. When the activation of LRP1 is inhibited, there is lethality due to molting defects during the L3-L4 transitions. LRP1 has been suggested to aid in the uptake of cholesterol from the environment in *Caenorhabditis elegans* (*C. elegans*) (3).

Aquaporin 4 (AQP4) is a water channel in the central nervous system and plays a vital role in the balance of water and ions in the brain. AQP4 deficiency in the brain leads to deficits in memory and ability to learn (4). Scientists agree that expression of AQP4 protects the brain from amyloid beta plaques. An association of AQP4 and GLT1, a glutamate transporter, is present in plasma membranes and may function as a dynamic signaling platform. When this signal is disrupted, it can cause neural impairment (5).

LRP1 plays critical roles in amyloid beta metabolism and clearance in neurons. LRP1 knockdown results in decreased uptake and degradation of amyloid beta. Similarly, the deletion of AQP4 exacerbates cognitive defects and induces an increase in amyloid beta accumulation (6). Furthermore, a deficiency in AQP4 results in the decreased upregulation of LRP1 and consequently the decreased uptake of amyloid beta. This suggests that AQP4 is important in the upregulation of LRP1 and clearance of amyloid beta (7).

C. elegans are often used as a model organism for studying Alzheimer's Disease. These animals reproduce quickly, as the period from fertilization to hatching only lasts 12 hours. They mature into adults over the course of 3 days, and then lay about 300 eggs over the course of the next 3 days. Furthermore, amyloid beta plaques in Alzheimer's Disease are caused by the APP gene in humans, which is homologous to the apl-1 gene in *C. elegans*.

The current treatments of Alzheimer's Disease interfere

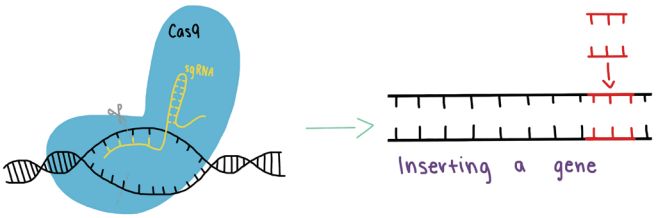

Figure 1: Simplified diagram of CRISPR-Cas9. A double-stranded break is made in the DNA by Cas9, and then the cDNA is inserted.

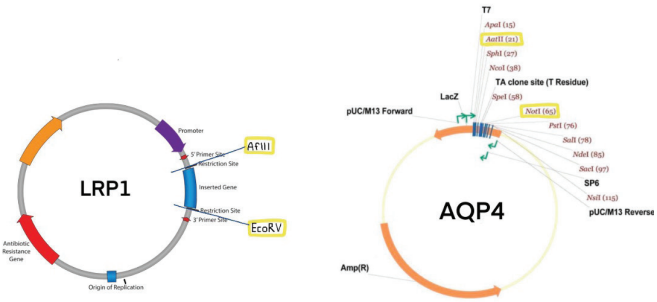

Figure 2: LRP1 and AQP4 plasmid diagrams. Plasmids were commercially obtained. Plasmids were then digested with enzymes depicted to remove the cDNA insert of LRP1(AflII and EcoRV) or AQP4 (NotI and AatI).

with other associated diseases and do not improve cognitive impairment. For example, neprilysin is utilized to break down amyloid beta plaques, but patients with cardiac conditions take a drug called LCZ696 which inhibits neprilysin. Therefore, new therapeutic options are necessary for those that cannot use neprilysin (9).+

A method called CRISPR-Cas9 has been recently implemented to use for gene editing (**Figure 1**). CRISPR-Cas9 requires a single guide RNA (sgRNA) which can be generated from a CRISPR design program. Additionally, a single-stranded oligonucleotide (ssODN) with 5' and 3' homology arms are designed to contain the gene of interest and restriction sites (10). In addition, the Cas9 recombinant protein is used. In this study, the CRISPR Cas9 system was used for gene knock in of LRP1 and/or AQP4.

Plasmids containing genes of interest were microinjected into the *C. elegans* (**Figure 2**).This is the most widely used form of gene editing in *C. elegans* to create transgenic worms. The DNA is inserted into the distal gonad syncytium or directly into the embryos. Transformation markers are inserted with the gene of interest in order to identify the transgenic worms. In order to see which worms show the phenotype, a GFP vector is also inserted which makes the worm or egg glow green to show the presence of the trait (12).

In the study, primers were added to LRP1 and AQP4 genes matching the insertion site in the *C. elegans* genome. Once this construct was developed, the genes were microinjected into the *C. elegans* with Cas9 and a site-specific guide RNA. The effects of the addition of the genes were then tested by running chemosensory, size, and speed assays. The negative control hypothesis was that adding no gene to the *C. elegans* would have no effect on the chemotaxis abilities, brood size, or locomotion. The alternative hypothesis was that given only LRP1 or AQP4 was inserted downstream of the apl-1 promoter, there would be a reduced effect on the chemotaxis abilities, brood size, and locomotion compared to inserting both LRP1 and AQP4 downstream of the apl-1 promoter in conjunction. When both LRP1 and AQP4 are overexpressed, I predict a significant increase in the uptake of beta amyloid, as evidenced by increases in chemotaxis, brood size, and locomotion. This study could be a major step in the research to develop a better method of curing Alzheimer's at the source, which is a DNA mutation. Once the proteins are overexpressed, the primary presentation of Alzheimer's, neurodegeneration, will be limited.

RESULTS

A GFP plasmid was microinjected in conjunction with the LRP1 and AQP4 genes. The fluorescence of the injected *C. elegans* confirmed that the LRP1 and AQP4 genes were being expressed. To assay the predicted changes in the *C. elegans* after the addition of the APQ4 and LRP1 genes, I measured chemotaxis abilities, size, and locomotion. The chemotaxis assay was run using known attractant of *C. elegans* to measure sensory ability. The chemotaxis index shows an increase in chemosensory ability by moving away or towards certain smells. Because neuronal amyloid beta expression is known to induce defects in chemotaxis, I expected that a chemotaxis assay would be an appropriate measure of the effects of various proteins on amyloid beta levels (13). Additionally, apl-1 mutations cause defects in movement and brood size, which correlates with the effects of LRP1 and AQP4 on the apl-1 mutation in Alzheimer's.

To measure the neurological changes after overexpression of the candidate genes, I assayed chemotaxis, brood size, and locomotion. I tested these phenotypes in three different strains of *C. elegans*, including N2 (wildtype), CL2006, a strain that expresses amyloid beta plaques, and VC1246, a strain that expresses mutant apl-1. The chemosensory assay showed that when the *C. elegans* were microinjected with LRP1 or AQP4, there was no significant effect on the chemotaxis index. But, when they were microinjected in conjunction, there was a significant increase in the chemotaxis index in all strains. The VC1246 strain showed an increase in the chemotaxis index by 0.5 when both LRP1 and AQP4 were microinjected (($p= 0.006785$), p-value < 0.05, student's t-test). Whereas, the CL2006 strain showed an increase of 0.12 in the chemotaxis index with both LRP1 and AQP4 overexpressed (**Figure 3**; $p=0.007554$, p-value < 0.05, student's t-test). This was expected and supports the hypothesis that together, LRP1 and AQP4 improve chemotaxis abilities. The simultaneous overexpression of LRP1 and AQP4 improved the neurodegenerative phenotype, but did not completely rescue the phenotype to wildtype levels.

The brood size assay showed that there was no significant increase in brood size for any strains, microinjected with LRP1 or AQP4, or both (**Figure 4**). This was unexpected since the expression of beta amyloid is known to decrease the brood size, and therefore with the addition of LRP1 and AQP4, the brood size should have increased (14). Brood size may have increased growth after a few days and could be tested in the future. In the speed assay, the speed of the *C. elegans* did not increase with the microinjection of LRP1 or AQP4.But when they were microinjected in conjunction, the speed in the VC1246 strain increased significantly by about 20 µm/s overexpressed (**Figure 5**; p=0.0269, p-value < 0.05, student's t-test).This was expected for this strain and suggests that the uptake of the beta amyloid by LRP1 and AQP4 allowed the muscle to contract more efficiently, thereby enabling the animals to move faster. The N2 control did not show a significant increase when microinjected with either of the two genes. Thus, the results support the hypothesis that combined overexpression of LRP1 and AQP4 would increase chemotaxis ability and locomotion more significantly than either protein overexpressed alone.

Overall, the study revealed an increase in the neurological ability of *C. elegans* microinjected with both LRP1 and AQP4 as assessed by an increase in chemosensory ability and speed. These results show that individually, the LRP1 and AQP4 proteins had no significant effect on chemosensation, but when overexpressed in conjunction, they increased the chemosensory capacity of the VC1246, the strain with the apl-1 mutation, and CL2006, the strain with beta amyloid plaques present.

DISCUSSION

Alzheimer's Disease, a disease with an unclear physiology, is a global health issue of increasing importance. Current treatments focus on mitigating side effects, but do not address the underlying mechanism of disease, which is hypothesized to be caused by accumulation of beta amyloid plaques. The results showed that when LRP1 and AQP4 were given in conjunction, this treatment significantly reduces amyloid beta plaques, as seen through the increase in chemotaxis ability and increase in speed in the strain of *C. elegans* that expresses plaques in their muscle. I conclude that LRP1 and AQP4 function in conjunction to reduce the formation of additional amyloid beta plaques and break down the buildup of these plaques that were previously formed. Since the APP mutation in humans results in the depletion of AQP4 and LRP1, replacing these proteins could contribute to a better sense of environment as seen in the VC1246 worms (15). Our analysis suggests that the *C. elegans* overexpressing both LRP1 and AQP4 had the greatest reduction of neurodegeneration, while overexpression of LRP1 or AQP4 alone only improved it to a lesser degree.

Limits on time, budget, and experience restricted the extent of the experiment. The last steps of a gene editing study are to typically sequence the genome and check mRNA and protein expression. These methods were not available due to time and monetary constraints. The expression of the GFP protein, however, suggests a successful edit. Testing of the CRISPR-Cas9 system demonstrated up to an 86% precise genome editing efficiency; while this is high for the current methods of genome editing that exist, it is not perfect (16). Furthermore, genome editing has potential side effects.

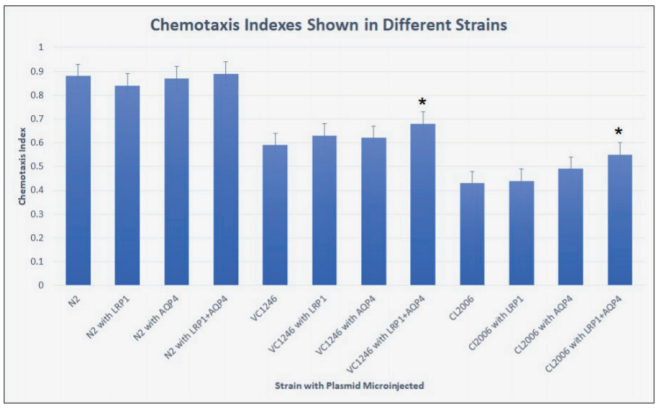

Figure 3: Chemotaxis indexes of different strains after injection. The graph depicts chemotaxis index scores for each strain tested (n=500 for each strain). Error bars represent standard error, and asterisks (*) denote statistical significance (p-value < 0.05, student's t-test).

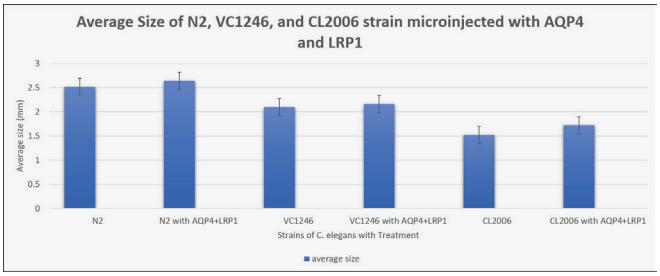

Figure 4: Average size of N2, VC1246, and CL2006 strains microinjected with LRP1 and AQP4. The graph depicts average brood sizes for each strain tests (n=100 for each strain), Error bars represent standard error, and asterisks (*) denote statistical significance (p-value < 0.05, student's t-test).

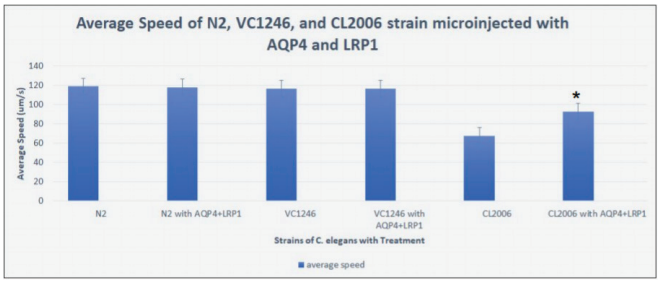

Figure 5: Average speed of N2, VC1246, and CL2006 strains microinjected with LRP1 and AQP4. The graph depicts average speed for each strain tests (n=100 for each strain). Error bars represent standard error, and asterisks (*) denote statistical significance (p-value < 0.05, student's t-test).

The additional copies of LRP1 and AQP4 could potentially interact with other proteins in unknown manners.

Although the data suggests an increase in sensory ability and movement under our experimental manipulations, future research can assess the long-term effects as the worms age, as well as effects on their progeny. The CRISPR-Cas9 system can also be applied to a plethora of other diseases by allowing the correction of many missense mutations. The effect of correcting these mutations can then be studied on a larger scale in other model organisms, such as mice, to assess the potential for reversal of the disease (17). In addition, other genes such as CR1 that control the flow of proteins and waste across cellular membranes can be microinjected to test other possible proteins that could decrease neurodegeneration (18). RNAi feeding strains can also be used to knock out genes to show if the removal of the apl-1 gene influences neurodegeneration. Other strains of *C. elegans* expressing different phenotypes associated with Alzheimer's Disease can be further tested to account for the many different hypotheses currently being debated. Additionally, other properties can be assayed in the future, such as serotonin sensitivity, which can further establish the phenotypic effects of neurodegeneration.

METHODS

C. elegans strains

All strains of *C. elegans* were obtained from the Caenorhabditis Genetics Center at the University of Minnesota. The N2 strain of *C. elegans* is the wildtype strain. The VC1246 strain has the apl-1 gene mutated, which is homologous to an APP gene mutation in humans. The CL2006 strain is categorized with Alzheimer's Disease with the addition of the UNC-54 gene, which codes for amyloid beta in humans. This strain presents amyloid beta in their muscles. The CL2006 strain is temperature-sensitive, meaning that paralysis and egg-laying deficiencies arise when organisms are raised at 20°C.

Transformation of AQP4 and LRP1 plasmids into E. coli with ampicillin selection

Highly Competent DH5alpha E. coli (New England Biolabs, NEB) was thawed on ice and 50 µl were added to LRP1/AQP4 DNA (genomics-online). The reaction was mixed gently by pipetting and flicking the tube 4-5 times. The mixture was placed on ice for 30 min, then heat shocked at 42°C for 30 sec. 950 µl of LB media (NEB) was added, and the tube was placed in a shaking incubator at 37°C for 1 hr. From each culture, 50-100 µl of bacteria were spread onto plates and incubated overnight. The transformed cells were grown on ampicillin plates to select for successfully transformed, ampicillin-resistant bacteria.

DNA extraction

Plasmid DNA was extracted from the transformed bacteria using the Monarch plasmid miniprep kit according to the manufacturer's protocol (NEB).

AQP4 and LRP1 double digests

Using the restriction enzymes around the insertion site, an overnight double digest was run to cut out the cDNA from the cloning vector. For the AQP4 plasmid, the restriction enzymes NotI and AatII (NEB) were used. For the LRP1 plasmid, the restriction enzymes AflII and EcoRV (NEB) were used. Digests were composed of 4µl Multi Core Buffer, 0.2 µl BSA, 10 µL DNA, 1 µl of each enzyme, and 4.8 µl of sterile water and kept in the fridge overnight. To confirm digestion, ethidium bromide (Biolabs) gels were made with 1X TAE Buffer (Biolabs) and then placed into 1X TAE Buffer to run. 1 µl of loading dye and 5 µl of DNA were added per lane. Gels were run at 150 V for an hour. UV light was then used to visualize bands.

PCR and Gibson Assembly to attach primers and DNA fragments

A 25 µl mixture was made with 2.5 µl 10X standard TaqReaction Buffer (NEB), 0.5 µl 10nM dNTPs (NEB), 0.5 µl 10 µM Forward Primers, 0.5 µl 10 µM Reverse Primer, brought up to 25 µl with Template DNA. The reaction was gently mixed and put in the PCR machine. The cycle was 95°C for 30 seconds, 30 cycles of 95°C for 20 seconds, then 60°C for 30 seconds, then 68°C for 1 minute, then 68°C for 5 minutes and finally held at 4°C.

For the Gibson Assembly mix, 1 pmol of DNA fragment, 10 µl of Gibson Assembly Master Mix (NEB), and 10 µl of deionized water were added to a tube and incubated in the thermocycler at 50°C for 60 minutes.

Microinjection

Microinjection was done with the nanoliter (World Precision Instruments) and paraffin oil was used to ensure worms stayed still. A single guide target RNA sequence was created by SYNTHEGO to place the DNA inserts downstream from the apl-1 promoter. The Cas9 protein was obtained from Sigma Aldrich. The final injection mixture was: 5 µmol of single guide RNA, 5 µmol of Cas9 nuclease, 50 ng/µl of DNA insert and GFP plasmid. These components were homogeneously mixed by gentle pipetting. The mixture was loaded into needles and *C. elegans* were injected at the gonad syncytium and then transferred to new NGM plates after recovery.

Chemotaxis Assay

Chemotaxis plates were first labeled with the center in the middle, control on one side, and attractant on the other side. Approximately 100 worms were washed using M9 Buffer three times and then placed in the center, with one drop of sterile water on one end of the plate and a known attractant on the other side of the plate at the marked areas. A disposable plastic pipette was used as the dropper. 2 µl of 0.5M sodium azide (Biolabs) were put on both sides to paralyze the worms. After 1 hour, the plates were chilled at 4ºC for 15 minutes to stop worms from moving and then worms on both sides

of the plate were counted and recorded. The data was then analyzed, and the chemotaxis index was determined. The chemotaxis index is calculated by (number of worms at attractant – number of worms at the control)/ (total number of worms).

Locomotion Assay

Thirty second videos of worms crawling on NGM plates were taken using a microscope. The speed of the worms was quantified by the worm tracker extension in ImageJ.

Measuring Brood Size

Brood size was measured using an application called ImageJ. Images taken of the *C. elegans* were traced using a tool on ImageJ, and the brood size was measured. The sizes were then downscaled based on the amount of zoom in the pictures.

Statistical Testing

T-tests were performed comparing the data to the wildtype and the p values were analyzed in Excel to determine which values were under 5% and therefore statistically significant.

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Article

Repulsion of Ants Using Non-Toxic Household Products

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SUMMARY

Ant invasion causes damage exceeding $5 billion annually in North America. Commercial repellents often contain chemical compounds that are potential carcinogens and maybe harmful to human health. In this study, we aimed to identify natural products with ant-repelling properties. We tested chalk, cinnamon, ginger powder, lemon juice, rosemary powder, table salt, and turmeric powder using a custom ring apparatus designed to quantify ant-repellence. We discovered that cinnamon and lemon were the most effective ant repellents of the tested products. Specifically, we found that cinnamon oil and lemon juice increased the median time required for ants to exit the ring as compared to their vehicle controls; that the lipid-soluble component of cinnamon, i.e., oil, and the lipid-insoluble component of lemon, i.e., juice, were more efficacious at repelling ants than their counterpart fractions; that cinnamon oil and lemon juice repelled ants in a dose-dependent manner; that cinnamon oil was more efficacious than lemon juice; that lemon juice was more potent than cinnamon oil; and that cinnamon oil and lemon juice are synergistic in their ant-repellent properties. These data suggest that compounds found in non-toxic household products, such as cinnamon oil and lemon juice, could be used in low-dose combinations as potent, effective, eco-friendly, and safe ant repellents.

INTRODUCTION

Ant invasions of residential homes are seasonally commonplace. Use of commercial insecticides containing compounds such as bifenthrin, which is classified as a possible human carcinogen and is highly toxic to aquatic life and bees, are often the first response of homeowners and pest control agencies to ant invasions (1). In this study, we aimed to identify non-toxic household products that repel ants in a safe and effective manner as alternatives to commercial insecticides. Ants cause billions of dollars of damage to residential homes and to commercial crops every year, making this area of research broadly beneficial to homeowners, house residents, and the agricultural industry (2).

Previous research suggests that essential oils of plants such as basil, lemon grass, and peppermint repel a variety of arthropods (3). In addition, previous research suggests that various acids secreted defensively by animals act as ant deterrents (4, 5). As a result, we chose to test 7 non-toxic household items for ant-deterrent properties: chalk, fresh ground cinnamon, ginger powder, lemon juice, rosemary powder, table salt, and turmeric powder. We used water as a control.

Preliminary results suggested that cinnamon and lemon were the most effective ant repellents among the tested items. We then hypothesized that the lipid-soluble and lipid-insoluble fractions of cinnamon and lemon would differ in their effectiveness, because cinnamon and lemon have different parts, such as bark, oils, rind, and juice, that may vary in their chemical composition. Subsequently, we hypothesized that cinnamon oil and lemon juice would repel ants in a dose-dependent manner. Specifically, we expected that chemicals in cinnamon oil and lemon juice are responsible for the repellent properties of cinnamon and lemon, and that as the concentration of these chemicals increased, so too would their repellent activity. Next, we hypothesized that cinnamon oil and lemon juice would be synergistic, rather than merely additive, in their repellent activity. We formulated this hypothesis based on our expectation that cinnamon oil and lemon juice might trigger different senses of the ant due to the different physical and chemical properties of these substances.

RESULTS

The experiment was set up in a stainless steel, 12-inch pan. For each trial, we deposited one of the non-toxic household products in a 6-inch diameter ring within the 12-inch pan and then placed a single ant at the center of the pan (**Figure 1**). Ants explore new territories instinctively and attempt to exit the arena when placed at the center of the pan (6). However, if the ant is repelled by the substance in the 6-inch diameter ring, the ant would avoid the perimeter and spend a greater amount of time inside the ring. We used the time taken by the ant to exit the ring as a measure of the repellence of the substance. Water and grapeseed oil were chosen as negative controls for their inability to repel the ants. Specifically, water was used as a negative control for water-soluble substances and grapeseed oil was used as a negative control for lipid-soluble substances.

The 7 household substances exhibited varying degrees of ant repellence (**Figure 2**). Of these substances, cinnamon

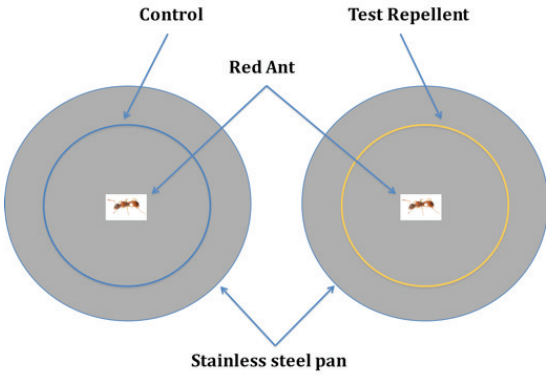

Figure 1. Schematic of apparatus. A stainless steel, 12-inch pan was used as the arena. For each trial, one of the non-toxic household products was deposited in a 6-inch diameter ring within the 12-inch pan. A single ant was placed at the center of the pan to commence each trial.

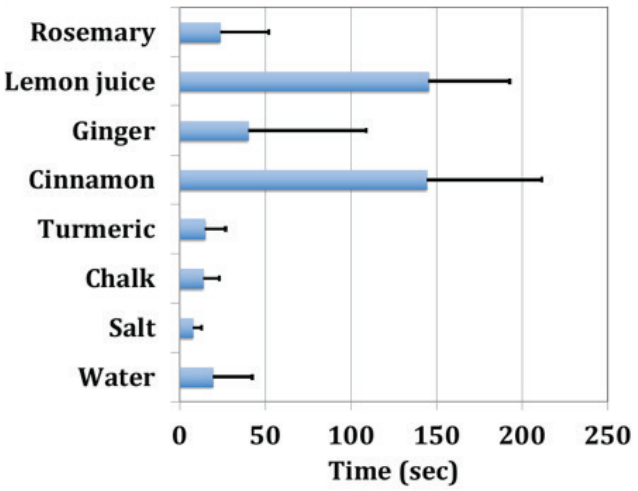

Figure 2. This bar graph shows the times taken by the ants to leave the rings of the various substances. The blue bar represents the mean time taken to leave the ring and the black bar represents the standard error of the mean.

powder (P = 0.005 compared to negative control) and lemon juice (P = 0.002 compared to negative control) were the most effective; thus, we further investigated these 2 substances in subsequent experiments.

We tested whether the lipid-soluble or lipid-insoluble components of cinnamon were responsible for its ant repellence. To determine this, we extracted cinnamon oil from cinnamon by heating it in grapeseed oil. We ran the mixture through a sieve and separated the oil from the insoluble cake. Then we exposed the ants to rings containing grapeseed oil, cinnamon oil, or the insoluble cinnamon cake. Cinnamon oil extract displayed significantly better ant repellence compared with cinnamon cake or grapeseed oil (P < 0.05) (**Figure 3A**).

We then tested whether the lipid-soluble or the lipid-insoluble components of lemon were responsible for its ant repellence. To determine this, we extracted the lemon oil from the peel of the lemon and we heated it in grapeseed oil. We also squeezed out the juice of the lemon. We exposed the ants to rings containing grapeseed oil, lemon oil, water, or lemon juice. Lemon juice displayed significantly better ant repellence than lemon oil (P = 0.013) (**Figure 3B**). Overall, these data indicate that the lipid-soluble fraction of cinnamon and the aqueous (lipid-insoluble) fraction of lemon are superior repellents compared to their aqueous (lipid-insoluble) and lipid-soluble fractions, respectively.

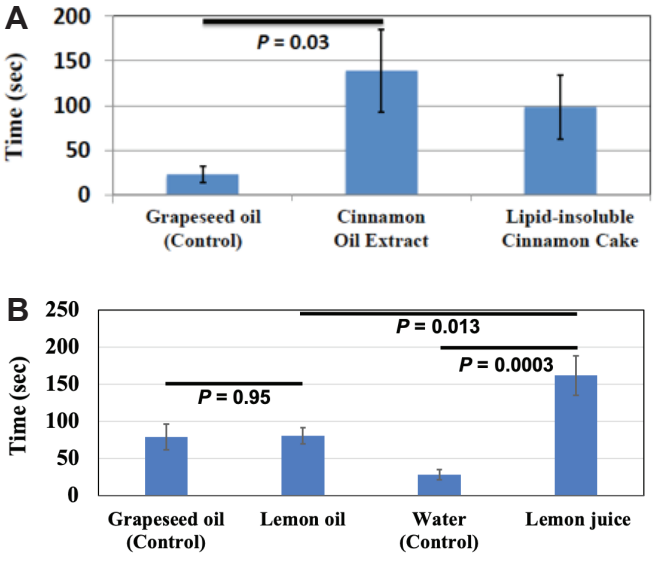

Figure 3. Cinnamon oil extract was more effective than its control, grapeseed oil (A). Lemon juice was more effective than its control, water, and more effective than lemon oil (B). P values by Student's t test.

Kaplan-Meier survival curve analyses showed that the median time to exit the ring was significantly greater with lemon juice compared with water (P = 0.0001), and significantly greater with cinnamon oil compared with grapeseed oil (P = 0.003) (**Figure 4A-B**).

Next, we performed dose-ranging studies of cinnamon oil and lemon juice by testing concentrations from 0.5% to 100% in grapeseed oil and water, respectively. The dose-response curves were fit using an exponential curve model. The mean time to exit the ring increased with increasing

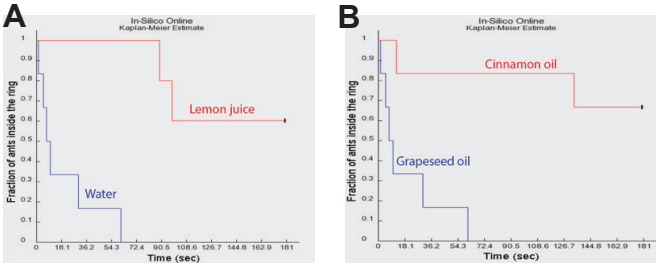

Figure 4. Kaplan Meier survival curves showing the fraction of ants inside the lemon juice (A) or cinnamon oil (B) rings over time. In the water control group, all the ants left the ring before 60 sec. However, in the lemon juice group, half the ants remained inside the ring by the time the experiment ceased (log-rank P = 0.0001). In the grapeseed oil control group, all the ants left the ring before 60 sec. In the cinnamon oil group, nearly 70% of the ants remained inside the ring by the time the experiment ceased (log-rank P = 0.003).

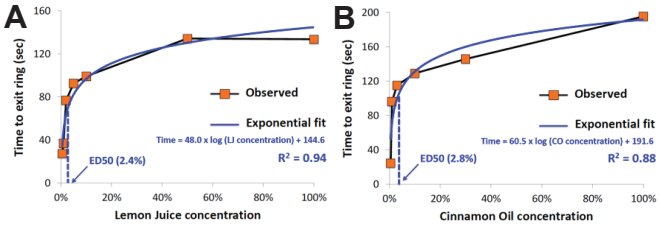

Figure 5. Scatter plots of the observed mean times to exit the ring (orange squares) for various concentrations of lemon juice (A) and cinnamon oil (B). The response was dose dependent: as the concentrations increased the time increased as well. These data followed an exponential trend (blue lines are the exponential curves of best fit). The coefficients of determination were: R^2 = 0.94 for lemon juice and 0.88 for cinnamon oil.

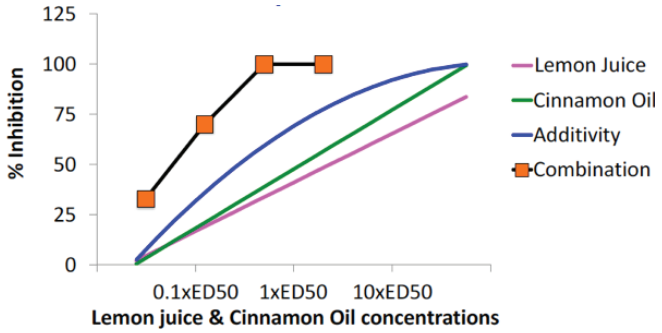

Figure 6. The combination of cinnamon oil and lemon juice is represented by the orange squares and the black line. The line of best fit for lemon juice is represented by the pink line while the line of best fit for cinnamon oil is represented by the green line. This graph shows the % inhibition for each concentration of the combined lemon juice and cinnamon oil. The inhibition was synergistic.

concentrations of cinnamon oil and lemon juice (**Figure 5A-B**). The coefficients of determination (R^2) were 0.88 and 0.94 for cinnamon oil and lemon juice, respectively. This excellent fit indicated a dose-dependent effect of both substances. In addition, most of the variation (88% and 94%) in repellence could be attributed to variation in cinnamon oil or lemon juice concentration.

The efficacy values, defined as the ratio of observed inhibition to theoretical maximal inhibition were 95.4% for cinnamon oil versus 60.9% for lemon juice (**Table 1**). Therefore, cinnamon oil was more effective than lemon juice at repelling ants. Efficacy refers to the maximal effect of a substance regardless of the dose used. Potency is a measure of the activity of a substance in terms of the concentration required to produce a defined effect, quantified in this study as the concentration required to repel at 50% of the maximal effect. The median effective dose, or ED50, values were calculated by plotting a log-linear curve (7). The half-maximal value of inhibition was identified on the y-axis, and the ED50 for this point on the exponential fit line was identified on the x-axis. The ED50 was 2.41% for lemon juice, whereas the ED50 was 2.79% for cinnamon oil (**Table 1**). Therefore, lemon juice was more potent than cinnamon oil at repelling ants.

Next, we sought to determine whether the combination of cinnamon oil and lemon juice would be additive, synergistic, or subtractive in their ant-repellence. We analyzed the interaction between cinnamon oil and lemon juice using the Bliss Independence Model (8). The observed data for the combination of these two substances showed inhibition greater than additivity and the combination indexes at all dose combinations were < 1.0 (**Table 2**).

These data, when plotted on a log-linear graph, demonstrated that the combination of cinnamon oil and lemon juice outperformed both substances individually, and exceeded the expected ant-repellence if their combination was merely additive (**Figure 6**). Therefore, the combination of cinnamon oil and lemon juice was synergistic in repelling ants.

Table 1. Potency and efficacy of lemon juice and cinnamon oil in repelling ants.

Repellant	Potency (ED50)	Efficacy (Maximal inhibition)
Lemon Juice	2.41%	60.9%
Cinnamon Oil	2.79%	95.4%

Potency values, measured as median effective dose (ED50), reflect percentages of lemon juice diluted in water or of cinnamon oil diluted in grapeseed oil. Efficacy values, which reflect ratio of observed inhibition to theoretical maximal inhibition, are provided as percentages.

DISCUSSION

In the first phase of this project, we observed that, of 7 household substances, cinnamon oil and lemon juice repelled ants more effectively than their negative controls. In the second phase, we hypothesized that the lipid-soluble and lipid-insoluble components of cinnamon and lemon would differ in their effectiveness. We hypothesized that cinnamon oil and lemon juice would repel ants in a dose-dependent manner, i.e., as the concentration of their respective repellent chemicals increased, their effectiveness would as well. We determined that the principal components of cinnamon that repelled ants were lipid-soluble and that the principal components of lemon that repelled ants were aqueous. In the third phase, we hypothesized that cinnamon oil and lemon juice might be synergistic in their repellent activity, based on our expectation that each of these compounds might trigger different senses of the ant because cinnamon oil and lemon juice differ in their physical and chemical properties. We concluded that both cinnamon oil and lemon juice repelled

Table 2. Efficacy of lemon juice and cinnamon oil, individually and in combination, in repelling ants

	Lemon Juice + Cinnamon Oil (% Inhibition)			
	1/32 x ED50	1/8 x ED50	1/2 x ED50	2 x ED50
Lemon Juice (Best Fit)	4.3	19.0	33.7	48.3
Cinnamon Oil (Best Fit)	3.2	21.0	38.8	56.6
Lemon Juice + Cinnamon Oil *Expected Additivity*	7.4	36.0	59.4	77.6
Lemon Juice + Cinnamon Oil *Observed*	32.8	70.0	100.0	100.0
Combination Index	**0.226**	**0.514**	**0.594**	**0.776**

Efficacy values, which reflect ratio of observed inhibition to theoretical maximal inhibition, are provided as percentages. Expected additivity values were calculated per the Bliss Independence Model (8) using the formula described in the Methods.

ants in a dose-dependent manner, suggesting that specific, defined molecules in these substances are responsible for repelling ants. The exponential fit for concentration versus repellence supports that these molecules could be acting on specific ant receptors with first-order kinetics. A first-order reaction is also referred to as a unimolecular reaction as it depends only on the concentration of one reactant; it is quantitatively described by an exponential relationship. Thus, our finding that repellence is an exponential function of concentration suggests that there may be sensors in ants, such as receptors, that recognize specific molecules in the test substances. Such a mechanism could be responsible for the observed repellence effect. Lastly, we determined that, when combined, cinnamon oil and lemon juice performed synergistically.

Interestingly, cinnamon oil was more effective than lemon juice, whereas lemon juice was more potent. These contrasting observations suggest that the molecules in lemon juice responsible for its ant-repelling power operate at very low concentrations, but that, overall, the compounds in cinnamon oil have a greater ability to repel ants. By applying the Bliss Independence Model, a tool employed in pharmacology to quantify the interaction of two toxins, we determined that the combination of cinnamon oil and lemon juice was synergetic in repelling ants. Based on our observations, we conclude that all of our hypotheses should be accepted.

The apparatus we constructed was robust enough to quantify ant-repellence of a diverse array of substances with varying physical and chemical properties and over a range of concentrations. This two-dimensional arena could also be used to test the ability of substances to repel other non-aerial organisms. Indeed, by inverting the paradigm, i.e., using the time to reach the ring from the center, this apparatus could also be used to determine attraction of test substances.

Several intriguing questions emerge from this study. For example, which molecules in cinnamon oil and lemon juice are responsible for repelling ants? To what extent is the acidic nature of lemon juice responsible for its repellence and could other acids serve a similar function? What mechanisms do these compounds trigger in ants to elicit repellence? Given that several molecules have been reported to repel multiple arthropods (3), could cinnamon oil and lemon juice repel other pests?

In conclusion, we have demonstrated that lemon juice and cinnamon oil, which are non-toxic household products, are effective in repelling ants. The results of this study could be used to create eco-friendly, non-toxic, and relatively inexpensive ant repellents. Future investigations could also unravel ant odor receptor biology. Our findings add to the growing body of evidence that the invasion of various pests can be controlled by household products that do not carry the attendant harmful effects of commercial pest control chemicals.

METHODS

Ant repellence by household substances

Red ants (Carolina Biological Supply Company) were placed, one at a time, in the center of a 12-inch stainless steel pan, inside a 6-inch ring containing one of the following test items: chalk (Crayola), fresh ground cinnamon (Deep Foods), ginger powder (Deep Foods), lemon juice (Kroger), rosemary powder (Kroger), table salt (Morton Non-Iodized), and turmeric powder (Deep Foods); or one of the negative controls: water and grapeseed oil (Trader Joe's). Each substance was deposited "free-hand" in the shape of a ring 5 mm wide and 1-2 mm in height. The surface was cleaned with isopropyl alcohol between trials and with soap and water or acetone between groups. The time taken by each ant to exit the ring was measured with an iPhone StopWatch. The experiment was stopped at 240 seconds and repeated 6–8 times.

Lipid-soluble versus lipid-insoluble components of cinnamon and lemon

Cinnamon bark (Deep Foods) was ground to powder (Jura-Capresso spice grinder), and its fat-soluble components were extracted by heating it in grape seed oil (Trader Joe's) on an electric stove and filtering it through a sieve when cool. Dilutions (0.5–100%) of cinnamon oil and freshly squeezed juice of lemons (Kroger) were prepared in grape seed oil or water, respectively. Red ants were placed, one at a time, inside a 6-inch ring of various concentrations of cinnamon oil, lemon juice, or controls in the center of a 12-inch stainless steel pan. The time taken by each ant to exit the ring was measured. The experiments were stopped at 240 seconds and repeated 6–12 times.

Combination of cinnamon oil and lemon juice

The potencies (ED50: Median Effective Dose 50%) of cinnamon oil and lemon juice were calculated using Microsoft Excel. Combinations of cinnamon oil and lemon juice at equal potency concentrations (2xED50; 1/2xED50; 1/8xED50; 1/32xED50) were prepared. Red ants were placed inside a ring of these cinnamon oil and lemon juice combinations, or a ring of combined controls (grape seed oil and water). The time taken by each ant to exit the ring was measured. The experiments were stopped at 240 seconds and repeated 6–12 times.

Statistical analysis

Means and standard errors of the mean (SEM) were calculated for each group and normalized by subtracting the average control group time. Groups were compared using Student's t-test, and P values were obtained using MS Excel. Mean differences were considered statistically significant if $P < 0.05$.

Median times to leave the ring were assessed using Kaplan-Meier analysis (http://in-silico.net/tools/statistics/survivor) to generate survival curves, and log-rank tests were

performed to determine *P* values. Scatter plots were fit by exponential trend-lines and coefficients of determination (R^2) were determined (MS Excel regression analysis).

The efficacy of a substance was calculated as the ratio of observed inhibition (the longest mean time taken to exit the ring in the presence of a substance at its best concentration – mean time taken to exit the ring in the presence of control) to theoretical maximal inhibition (240 seconds – mean time taken to exit the ring in the presence of control). The ED50 values were calculated by plotting a log-linear curve. The half-maximal value of inhibition was identified on the y-axis, and the ED50 for this point on the exponential fit line was identified on the x-axis.

The interaction between two substances was analyzed using the Bliss Independence Model (8). Expected Additivity and Combination Index metrics were calculated according to these formulas:

Expected Additivity = Inhibition (Substance 1) + Inhibition (Substance 2) – [Inhibition (Substance 1) x Inhibition (Substance 2)]

and

Combination Index = (Expected Additivity / Observed Inhibition)

with the following interpretations:
Combination Index < 1 : Synergistic
Combination Index = 1 : Additive
Combination Index > 1 : Antagonistic

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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.

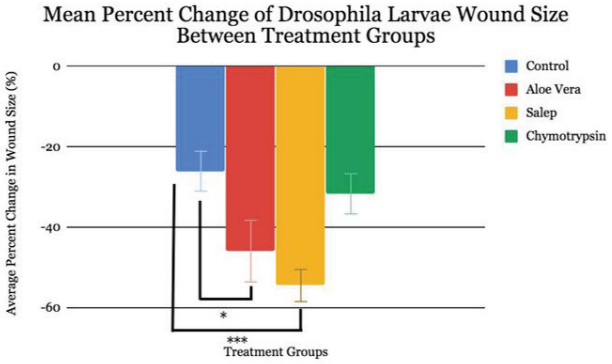

Figure 1. Average percent change in wound size among the four treatment groups: Salep, *Aloe vera*, alpha-chymotrypsin, and the control group. *n*=20 for all groups. Wound size is measured in percent change of wound size from puncture to 6 hours afterwards. The graph shows Salep with percent decrease 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

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%

significant.

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.

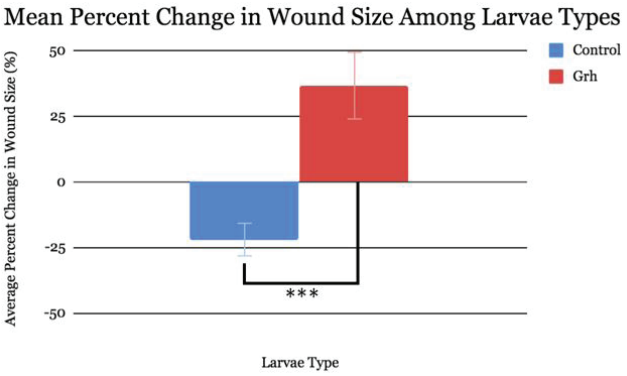

Figure 2. Average percent change in wound sizes among larvae types: wild type and grh mutant. n=10 for all groups. Wound size is measured in percent change of wound size from puncture to 6 hours afterwards. Additionally, the Standard Error margins have been displayed for each group with the grh mutant 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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Article

Giving Teens a Voice: Sources of Stress for High School Students

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ABSTRACT

Teen stress is a pressing health issue in the United States. Consistent, long-lasting stress can weaken the immune system and wear down physical reserves, leaving teens susceptible to negative mental and physical health outcomes. Recent legislation in the state of Virginia (HB1604/SB953) mandates the revision of the Health and Physical Education curriculum to incorporate standards of learning (SOL) that recognize mental health and the important connections between physical and mental health. The aim of our project was to create a survey that allows teens to have a voice in the conversation about revising the health SOL in response to student-reported stress and support factors. Using convenience sampling, high schoolers (n = 332) were invited to participate in an anonymous online survey. To identify factors contributing to teen stress levels, participants were asked about their demographics, high school courses and activities, free time, and perception of the support they receive. Stress levels were then evaluated using Cohen's validated 4-item Perceived Stress Scale. Regression and correlation analysis findings suggest that a student's gender, homework level, amount of free and sleep time, perceived parental pressure, and family encouragement of relaxation predicted their perceived stress. Students who felt guilty taking time off or who worried about violence had higher stress levels than other teens, indicating that students' emotions play a role in their stress perceptions. Results from this study can help school communities identify sources of teen stress and inform the development of educational instruction that helps students successfully reduce and cope with stress.

INTRODUCTION

Teen stress is a pressing health issue in the United States. According to the American Psychological Association's survey Stress in America: Are teens adopting adults' stress habits?, teens report higher average levels of stress than adults (5.8 vs. 5.1 on a 10-point scale) during the school year (1). In fact, 27% of teens reported their stress levels as 8, 9, or 10 on a 10-point scale during the school year, which indicates a high level of stress (1). Stress is the body's response to a challenge. Humans react to stress by activating their central nervous system and setting off a chain of physiological responses that prepare a person to perform well under pressure (2). Long-lasting stress can weaken the immune systems and wear down physical reserves. When this happens, teens are susceptible to negative mental and physical health outcomes (2).

In March 2018, The Virginia General Assembly (392 Code of Virginia §22.1-207) passed legislation in the form of HB1604/SB953 that "requires health instruction to incorporate standards [of learning] that recognize the multiple dimensions of health by including mental health and the relationship of physical and mental health" in an effort to promote student health and well-being. The Virginia Department of Education (VDOE), which is responsible for reviewing and revising the standards of learning (SOL) to comply with the law, has proposed a working timeline for review of the SOL for Health and Physical Education in Virginia. The rationale for collecting survey data from Virginia high school students is 1) to identify the factors that correlate with stress in this population, and 2) to inform the conversation about how the Health SOL might be revised to include information on targeted coping mechanisms to reduce teen stress levels. The aim of this research is to give teens a voice in the conversation about stress factors with the hopes that their collective voice will inform targeted solutions that address teen stress. While the VDOE has indicated that they will solicit public comments for the SOL revisions in 2019, students and other stakeholders who do not closely follow state legislation and curriculum changes are unlikely to be aware of the mandated revisions or participate in the public comment period. Additionally, to our knowledge, no students are represented on the committee that is advising the VDOE on the Health SOL. By collecting and synthesizing data regarding students' experiences with stress and communicating study outcomes to the SOL review committee, we make it possible for the voices of the students (n = 332) who participated in our study to be considered in the curriculum revision.

Prior research indicates that there are multiple negative outcomes of teen stress, including depression, fatigue, unhealthy eating habits such as skipping meals or overeating, reduced self-perceptions of academic ability, physical pain, suicidal ideation, and social withdrawal (1, 3-5). Teens report that school is a considerable source of stress and that this stress impacts their performance at home, at work, and at school (1, 6, 7). One in ten teens reports that stress contributes to lower grades and 20% of teens say they neglect their work or school responsibilities due to stress (1).

Given the negative repercussions of stress, it is important to understand specific sources of stress in order to develop programs that help reduce stress and alleviate negative outcomes. This project was designed to address two research questions. First, we asked whether there are specific factors that are correlated with increased stress levels in high school students from Virginia. Second, we asked whether there are certain factors that help combat stress.

Results from this research indicate that student stress is multi-faceted. More homework and less time for other activities such as relaxation and sleep contributed to increased perceived stress levels. Furthermore, findings indicate that families play a role in perceived stress. Students who felt more parental pressure regarding their school performance reported higher stress, while family encouragement of student relaxation time was associated with reduced stress levels. Finally, it was evident that inner emotions shaped the perception of stress. Worrying about violence and feeling guilty about taking time off were tied to perceived stress levels. We propose that school communities should consider our research findings to inform the development of educational instruction that helps students successfully reduce and cope with stress.

RESULTS

In order to investigate factors that contribute to teen stress, we sent an anonymous online survey to high-school aged youth in Virginia. The survey asked students to answer questions about their demographics (e.g. gender, race), high school courses and activities, free time, and their perceptions of the support they receive from friends and family. Student stress levels were measured using the validated four-item Perceived Stress Scale (8). Data collection lasted two weeks, resulting in a sample of n = 332 completed surveys.

Descriptive Statistics

The majority of the sample attended specialized academic magnet school programs and students overwhelmingly attended public schools **(Table 1)**. Public school recruitment aligns with this project's purpose of adding to the conversation about mandated SOL revisions for the Virginia public school system. While the sample's racial statistics were roughly reflective of the total population of Virginia Beach City Public Schools (VBCPS), the population of Asians was higher in the study (19.6% vs. 6.5% overall) and black students were underrepresented (7.2% vs. 19.1%) **(Table 1)**.

Stress and Stressor Variables

We ran summary statistics for independent and dependent variable responses **(Table 2)**. Students in the sample were involved with extracurricular activities and homework. Three in ten students (29.6%) participated in extracurricular activities for between one and five hours each week, and half of the students (49.5%) reported more than 11 hours of weekly extracurricular participation. A quarter of the students (25.3%) reported doing two hours of homework per night, one in five (20.8%) reported three hours, and another quarter (24.8%) reported doing more than three and a half daily hours of homework. More homework time was significantly correlated with reduced sleep time ($r = -.225$, $p < .01$). One in five students (22.8%) reported sleeping five hours or less per night. Another third (33.4%) of students reported sleeping between five and six hours nightly.

	N	%
Grade in school:	**332**	
9	53	16.0
10	123	37.0
11	133	40.1
12	23	6.9
Gender	**332**	
Male	162	48.8
Female	166	50.0
Gender nonconforming	4	1.2
Race	**332**	
Black	24	7.2
White	215	64.8
Asian	65	19.6
Hispanic	15	4.5
Multiracial	13	3.9
School type	**332**	
Public	329	99.1
Private	3	3
Specialized school program	**308**	
Academic magnet	227	73.7
STEM program	7	2.3
International Baccalaureate	20	6.5
Arts program	10	3.2
Other	5	1.6
None	39	12.7

Table 1. Participant Characteristics

In addition to low sleep times, students reported that they had very little time to relax. Nearly three-fourths (72.0%) of surveyed students responded that they had "none" or "a little" time each week to relax with no responsibilities. Moreover, two-thirds (65.1%) reported feeling guilty "fairly often" or "very often" when they were relaxing, feeling as though they should be doing something productive instead. Another quarter (23.5%) of teens reported that they "sometimes" felt guilty while relaxing. Some of these feelings may have been related to family support. Not all families were likely to encourage teens to take time for themselves. More than four in ten teens (44.6%) said that their families "never" or "almost never" encouraged them to take time for themselves to relax and enjoy life. One in three students (33.7%) said that their families "sometimes" encouraged relaxation times, and one

in five said parents were encouraging "fairly" or "very often."

	n	Mean	St. Dev.
Potential Stressors			
Extracurricular activity[a]	331	1.60	1.43
Homework (hrs/night)	318	2.71	1.44
Sleep (hrs/night)	324	6.19	1.16
Peer inquiries (0=never, 4=very often)	332	3.33	.793
# Confidants	321	4.46	3.48
Relax time (0=none, 3= a lot)	332	1.15	.711
Parental pressure (0=never, 4=very often)	332	2.42	1.07
Commute time (mins)	322	32.05	20.08
Encourage relaxation (0=never, 4=very often)	332	1.73	1.03
Feeling guilty (0=never, 4=very often)	332	2.84	1.09
Worry of violence (0=never, 4=very often)	332	1.09	.94
Outcome			
Perceived stress[b] (possible range 0-16)	332	7.90	3.40

Table 2. Description of Subjects in Terms of Study Variables
Note. Potential stressors were entered into the equation in the order listed.
a. 1 = 1-5 hrs; 2 = 6-10 hrs; 3 = 11-15 hrs; 4 = 15-20 hrs; 5 = >20hrs
b. Higher scores indicate increased perceived stress

Our data show that other people show interest in students' academic performance. Almost all respondents (97.6%) indicated that other teens ask them personal questions about their grades and performance on tests, quizzes, and standardized tests. More than half (51.2%) were asked about performance by their peers "very often," and another third (33.4%) "fairly often." In addition to their peers' inquiries, students reported that they felt pressured by their parents about their academic performance. Almost half (47.7%) of those surveyed felt parental pressure "fairly often" (28.9%) or "very often" (18.8%). Another third perceived this pressure "sometimes" (33.7%). Perceived parental pressure was negatively correlated with family encouragement of relaxation ($r = -.294$, $p < .01$).

Other people play an important role in students' lives as well. Poor family relationships and family members' high expectations are also related to increased teen stress (10). Having social support provides a buffer that can modify an individual's response to events that are potentially stressful (11). Students were asked about the number of people they felt they could honestly and openly talk to about their lives. Student responses ranged from 0 (4.2%) to 20 (1.8%). Three in ten teens (30.8%) reported having one to two confidants. Another four in ten (44.5%) reported having between three and five confidants, and the remainder (24.7%) reported having between six and twenty people to whom they could talk openly. Notably, the reported number of confidants was positively correlated with the amount of time students had to relax each week ($r = .159$, $p < .01$) and with the amount of family encouragement of relaxation ($r = .125$, $p < .05$). Number of confidants was inversely related to teens' reported feelings of guilt while relaxing ($r = -.148$, $p < .01$). Future work is needed to further explore the specific relationships among these variables.

Students were asked about how often they worried about violence in their schools or homes. While 7 in 10 students (72.3%) students reported "never" (28.6%) or "almost never" (43.7%) worrying about violence, 2 in 10 (20.2%) reported worrying "sometimes" and a small but important number of students (7.5%) worried about violence "fairly" or "very often." Worry about violence was negatively correlated with sleep time ($r = -.290$, $p < .01$) and number of confidants ($r = -.176$, $p < .01$).

The dependent variable in this study was perceived stress, which was measured using Cohen's validated 4-item Perceived Stress Scale (PSS-4). Possible stress scores ranged from 0 to 16, with higher scores indicating more stress. The mean score (7.90) was at the scale's mid-point (8), with a standard deviation (3.40) showing considerable variation in teen reports of perceived stress **(Table 3)**. More than a third of teens (34.6%) scored 10 or higher on the scale, and a considerable number (n = 54; 16.3%) scored in the upper quartile (12-16) **(Table 3)**. A similar proportion (n = 55; 16.6%) scored in the lower quartile (1-4) **(Table 3)**. No student reported feeling zero stress.

Score	N	%	Cum %
1	7	2.1	2.1
2	13	3.9	6.0
3	13	3.9	9.9
4	22	6.6	166
5	29	8.7	25.3
6	43	13.0	38.3
7	29	8.7	47.0
8	34	10.2	57.2
9	27	8.1	65.4
10	38	11.4	76.8
11	23	6.9	83.7
12	18	5.4	89.2
13	21	6.3	95.5
14	7	2.1	97.6
15	6	1.8	99.4
16	2	.6	100.0

Table 3. Scale score totals for perceived stress, the dependent variable

Hierarchical Regression Analysis

The aim of this study was to determine which predictors contributed to students' perceptions of stress. Since the literature supports a difference between genders related to stress (10, 12), gender was entered into the model first, followed by the remaining predicted stressors. Adding gender to the model yields $R2 = .143$, $F(1, 301) = 50.269$, $p < .01$ **(Table 4)**, confirming prior research showing a difference in perceived stress between genders (10,12). Gender was coded male (0), female (1), and nonconforming (2). The positive regression coefficient for gender ($\beta = .221$; **Table 4**) indicates that being female or gender non-conforming is associated with increased stress (vs. being male). With the remaining stress predictors added to the model, $R2 = .355$, $F(11, 290) = 10.09$, $p < .01$, an indicator that the entered predictors improved the predictive capacity of the model by 23.7% ($\Delta R2$

= .237) **(Table 4)**. Beta weights for the individual predictors supported our hypotheses that increases in homework time (β = .106), parental pressure (β = .163), guilt (β = .121), and worry (β = .203) would predict higher stress levels **(Table 4).** Our hypotheses that commute time (β = -.008), extracurricular activity time (β = -.085), and number of confidants (β = -.069) would predict stress were not supported by the model **(Table 4)**. While the hypothesis relating increases in peer inquiries into a student's performance to increased stress was not supported (β = .091, p = .059), the low p-value indicated it approached significance as a predictor of stress **(Table 4).** As hypothesized, reductions in sleep (β = -.136), relaxation time (β = -.146), and family encouragement to relax (β = -.103) were predictive of increased stress **(Table 4)**.

DV: Perceived Stress		
Predictor	**ΔR^2**	**B**
Step 1	.143	
Gender[a]		.221**
Step 2	.237	
Extracurricular		-.085
Homework time		.106*
Sleep time		-.136**
Peer inquiries		.091
# Confidants		-.069
Relax time		-.146**
Parental Pressure		.163**
Family Encourag.		-.103*
Guilt		.121*
Worry		.203**
Commute		-.008
Total R^2	.380**	
F	50.269**	

Table 4. Regression of Perceived Stress on Hypothesized Stressors
Note. Beta weights listed are from the full model (through step 2)
aGender was coded male (0), female (1), nonconforming (2)
* p < .05 ** p < .01

DISCUSSION

Teenage years are filled with potential sources of stress, and teens' physical and mental health may be negatively impacted when they are exposed to high stress levels. This study, inspired by the first author's personal experiences with and observations of high school stress, provides insight into teens' perceived stress levels. On a perceived stress scale of 0 to 16, the mean score of students in this study was near the midpoint (7.90, s = 3.40). Because the PSS is not a diagnostic tool, there are no score ranges to indicate specific stress levels (e.g. low/medium/high). However, normative data allows us to compare our study results to other populations' perceived stress levels. In a sample of U.S. adults (n = 2,387), the mean score for the PSS-4 was 4.49 (s = 2.96) (13). While the norming study did not include children, the findings indicate that perceived stress decreases with age, with 18-29 year-olds reporting the highest stress levels (13). A recent study of English adults (ages 16-85) found a mean PSS-4 score of 6.11 (s = 3.14), with younger adults again experiencing higher stress levels (14). A small subset (n = 22) of the English sample aged 16-17 had a mean PSS-4 score of 6.91 (s = 2.89). The U.S. high school students in our sample experienced higher stress levels than average adults at the time of our survey. On average, students in our study scored 39.2% higher than the adults in the U.S. normative sample and 29.3% higher than English adults. While comparison to the younger U.K. subset is limited without additional descriptive statistics (e.g. student status), students in our study scored 13% higher than that population. This information, alongside increasing rates of teen depression and anxiety (15) and the fact that suicide is the third leading cause of death in 15-19 year-olds (16), is concerning. More work is needed to establish normative data regarding teens' perceived stress levels, yet the stress levels reported in this study warrant attention and remediation.

This project builds on prior work in teen stress research by exploring peer and parental pressures as predictors of stress. The aim of this study is to give teens a voice in the conversation surrounding state SOL revisions, and to explore specific factors that might predict stress. Project results indicate that physiological (sleep and relaxation), social (parental pressures and family encouragement), and emotional (worry and guilt) factors contribute to high school students' perceptions of stress. Targeted programs to educate students and families about stress, including contributing factors and skills to cope with stress, might lead to reduced stress levels and more positive health outcomes.

Adolescence is a time in life when individuals are vulnerable to peer pressure and want to fit in with a group (17, 18). The variable concerning peer inquiries approached significance as a predictor in this study. Peers' questions about academic performance may make students feel pressure to perform at a high academic level or to hide or misrepresent grades and scores that they feel are below expected levels. Either of these behaviors might contribute to student stress. Providing students with appropriate responses to peer inquiries about academic performance and promoting a culture of confidentiality and privacy among both teachers and students in academic settings might reduce student stress levels.

Only 1 in 10 students (11.4%) in this study reported getting the 8-10 hours of nightly sleep recommended to promote optimal health for teenagers aged 13-18 (19). Teens who follow these sleep recommendations are likely to have improved attention, learning, and memory. They also experience better quality of life and overall well-being than their peers with less sleep (19). To promote these outcomes, schools might consider revising the levels of homework and extracurricular activity time of their students. For example, teachers could coordinate test schedules for blocks of students in order to avoid giving students multiple exams at the same time. Coaches might consider students' time

commitments (e.g. academic obligations, commute or activity bus times) when scheduling practices. Standards of learning might be revised so that students are taught the importance of making time for sleep and relaxation in their schedules, as well as the importance of not over-committing themselves at the expense of much-needed rest and relaxation.

More than a quarter of students in this study (27.7%) expressed concern about school or home violence. Research shows that student experiences of persistent fear and anxiety can impact their learning and problem-solving capabilities (20). As such, the survey respondents who reported feeling worried about violence may be at risk for negative health and social outcomes. School officials should follow best practices in assuaging fears of school violence and implement programs that encourage and allow students to report their concerns about violence to trusted adults both inside and outside of the school system.

Interventions to counteract stressors are not limited to student approaches. Like other school systems, VBCPS offers parent workshops on topics relevant to their children. Potential topics for future workshops should include information about student stress, contributing factors, and ways to reduce stress and promote teens' coping mechanisms. Parents should understand the role that they play in creating stress for their teens when they pressure them about their academic performance. Likewise, they should know the importance of encouraging their teens to have a healthy school-life balance, and should encourage their high school students to take time for self-care, rest, and relaxation. When parents and students are informed about stress, its impacts, and ways to reduce it, they will be better equipped to manage teen stress levels.

Limitations

This research was designed to maximize validity, yet no study is without limitations. Survey respondents were not selected at random but recruited through social media. Convenience sampling resulted in a sample that was overly skewed toward academic magnet programs and not representative of the entire population of Virginia students. This is likely a result of the fact that the study's first author attends a public-school academic magnet program and was responsible for recruitment. As such, study results are not generalizable to the entire population of Virginia high school students but to students who attend specialized academic programs in Virginia public schools.

The dependent variable in this study was perceived stress, measured using the 4-item PSS scale. A limitation of the scale is that its predictive ability is limited to one or two months, so it may not accurately predict longer-term stress exposure (8). Future studies should focus on longitudinal measures to capture the outcomes of long-term high school stressors.

Since study participants were self-selected, only teens with certain characteristics or qualities may have chosen to participate, which creates sampling bias. Using an online-only survey may have created sampling bias as well, since teens active on social media may differ in some ways from their peers who rarely or never use social media. Another study limitation is self-report bias, since students may purposefully or inadvertently have mischaracterized their habits and perceptions. Lastly, the study's correlational design does not establish causal relationships. The direction of the variable relationships discussed in this paper is a topic for future research.

METHODS

Survey Development

The 23-item survey was developed based on a literature review, the first author's personal experiences, and anecdotal information collected via informal peer interviews and conversations. Prior research with teen populations indicates that sleep and homework are correlated with stress (1, 6, 7). Questions about extracurricular activities and commute time were added to gauge how these variables might correlate with sleep and relaxation time. The question about school violence was added in response to national news coverage of student reactions to recent school shootings (e.g. Stonewood Douglas High School), as well as to recent violent incidents involving students in or around local Virginia schools.

As a gap exists in the literature concerning the effect of peer pressure in academic settings as it relates to perceived stress, a question was added to gather data that would help fill that gap. We measured stress using Cohen's validated four-item version of the Perceived Stress Scale (PSS), which asks students about their thoughts and feelings over the last month. The PSS, which was used with permission, is sensitive to chronic stress and to stress that students perceive concerning upcoming circumstances (8), which is important when measuring high school stressors like homework (chronic stress) or exams and school violence (upcoming circumstances). The PSS is a self-report scale that measures the survey respondent's feelings about their own psychological stress. For example, questions ask about how often in the last month students felt confident handling their problems, or whether they perceived things to be "going their way." Responses ranged from never (0) to very often (4), with resulting range of scores from 0-16. The survey was hosted on the Research Electronic Data Capture (REDCap) system.

Sampling and Recruitment

This study used a cross-sectional, descriptive, correlational research design. We collected data from a purposive convenience sample of individuals in 9th-12th grades in Virginia using an online survey. The project was approved by the Ocean Lakes High School Math and Science Academy's Scientific Review Committee. No computer IP addresses were collected, and researchers were not able to identify study participants. We posted the survey link on social media (e.g. Facebook, Snapchat) and emailed friends of the first author (P.C.) to request participation. As expected,

snowball sampling occurred as individuals shared the link through personal emails and social media accounts. Because it is impossible to know how many people saw the link, we could not calculate a response rate. Data collection lasted two weeks and a total of 397 responses were recorded in REDCap. There were 65 incomplete surveys, resulting in a final sample of n = 332 high school students. Power analysis reduces the risk of Type II errors (false negative outcomes) and increases the statistical conclusion validity of a study (21). Using G*Power 3.1, an a priori power analysis using power level (1 – β = 0.95), significance level (α = .05), and a medium estimated population effect size (Cohen's f2 = 0.15), calculated a required sample size with 12 predictors of n = 178 (22).

Data Analysis

Data entries were cleaned prior to analysis. We recoded string entries that were entered in numeric variable fields, e.g. "six" was recoded to 6. Items in the Perceived Stress Scale were reverse coded as necessary, and a total stress score was calculated by summing responses to individual scale item values. Missing values were determined by Little's MCAR to be missing at random ($\chi 2 = 208.342$, df = 255, p = .985). No variable had more than 5% of cases missing values. Univariate outliers are cases with extreme values for one variable that may distort statistical analysis (23). Two respondents reported that they had 30 people with whom they could talk honestly about their lives. These two outlying cases had standardized scores more than 3 standard deviations above the mean (i.e. Z score > 3.29) and were recoded into the next highest value (20) plus one. Multivariate outliers indicate cases with an unusual combination of scores on two or more variables, and these cases have inflated Mahalanobis distances (19). Mahalanobis distances were generated using linear regression analysis and no value exceeded the $\chi 2$ critical value, indicating no multivariate outliers. SPSS bivariate correlation analysis indicated no problems with multicollinearity (r > .70) between continuous variables.

We entered the cleaned data into SPSS v24 for correlation and linear regression analyses. Because prior research shows significant variances in perceptions and levels of stress between boys and girls (10,12), gender was entered into the linear regression model in step one to control for its effect before investigating other stress-related variables. Eleven variables (extracurricular hours, homework hours, sleep hours, peer inquiries, number of confidants, relaxation time, perceived parental pressure, family encouragement, guilt, worry about violence, and commute time) were entered in step two of the regression analysis (in the listed order) to test their ability to predict stress levels.

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Article

Varying growth hormone levels in chondrocytes increases proliferation rate and collagen production by a direct pathway

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SUMMARY

The purpose of this project was to test whether growth hormone directly or indirectly affected the rate at which cartilage renewed itself. Growth hormone could exert a direct effect on cartilage or chondrocytes by modifying the expression of different genes, such as c-myc proto-oncogene, which in return increases the proliferation rate of the cells. In contrast, an indirect effect comes from growth hormone stimulating insulin-like growth factor. The results from this research supported the hypothesis that growth hormone not only increases proliferation rate but does so using the direct pathway. Out of the different levels of growth hormone that were tested, it was found that 0.300 µg/ml had the greatest effect since those samples had the highest collagen concentrations and reached confluency in T75 cell culture flasks in the least amount of time. This research can be used in the medical sciences for people who suffer from joint damage and other cartilage-related diseases, since the results demonstrated conditions that lead to increased proliferation of chondrocytes. These combined results could be applied in a clinical setting with the goal of allowing patient cartilage to renew itself at a faster pace, therefore keeping those patients out of pain from these chondrocyte-related diseases.

INTRODUCTION

Chondroblasts are the unmatured version of chondrocytes, or cartilage cells. These cells, chondroblasts, secrete the matrix that makes up cartilage. Eventually, these cells become embedded in this cartilage, mature and stop matrix production. The extracellular matrix that these cells produce is extremely important because it is what allows cartilage to serve its functions within the body, which include providing structural integrity, protecting the ends of bones that meet at joints, and making up body parts like the ear and nose. In order to fulfill these roles, cartilage is dependent on the amount of water that is found in its fibers. The protein aggrecan is only found in cartilage and is responsible for producing the high water content that is located in it; the water levels in cartilage can be up to 75% at any given time. Cartilage holds these aggrecan molecules by using collagen fibers, these fibers have a very high strength and they use this to trap the aggrecan inside the cartilage (1).

Growth hormones are the factors in the human body that stimulate this cartilage growth, mostly via indirect pathways. The growth hormones influence the chondrocytes indirectly through the use of insulin-like growth factor-I (IGF-I). IGF-1 is a protein-peptide hormone whose main purpose is supporting cellular division and growth (2). IGF-I is secreted by the liver and contributes to most of the effects caused by growth hormones. Although this model of indirect stimulation has been the main mechanism proposed to explain how growth hormones influence chondrocytes and the growth of cartilage, there have been a few studies suggesting that growth hormones may also directly stimulate chondrocytes. One way this could occur is through increasing the expression of c-myc proto-oncogene (*MYC*) (3). Proto-oncogenes are genes that encode for many proteins that control aspects of cell growth and proliferation, and include genes such as p53 (4). The increased expression of this gene, and other genes similar to it, would be induced directly by the growth hormone.

Previous research has demonstrated that, under normal conditions, approximately 1 mg of growth hormone (5) is released to all body parts where it is needed. For this project that daily amount of growth hormone was divided among an approximate amount of all the places this growth hormone travels in a day, which came to about 0.250 µL.

The first assay used is a cell counting assay, this type of method is used to quantify the amount of viable cells in a sample. This is important because one has to know the amount of cells expected in the outcome of the experiment to perform the test with accuracy and to receive accurate results. There are two main types of cell counting assays, manual and automated. Automated cell counting uses image cytometry, which is used to analyze individual adherent cells (6). On the other hand manual cell counting is the method used in this experiment and consists of using the human perception of the cell when counting. For this project the manual cell counting method was used due to limitations in budget and accessibility.

The second assay used is a Collagen IgG assay, a form of the Enzyme-linked immunosorbent assay or ELISA. This specific type of ELISA kit tests for collagen type II antibody in plasma and serum. The reason for the use of this assay is due to collagen type II being a gene product of some pathways that the growth hormone should directly affect. This means that where there is an increase in the concentration of Collagen it corresponds to an increase in the expression of those genes.

From this background research it was concluded that the cell counting assay was needed to support the hypothesis

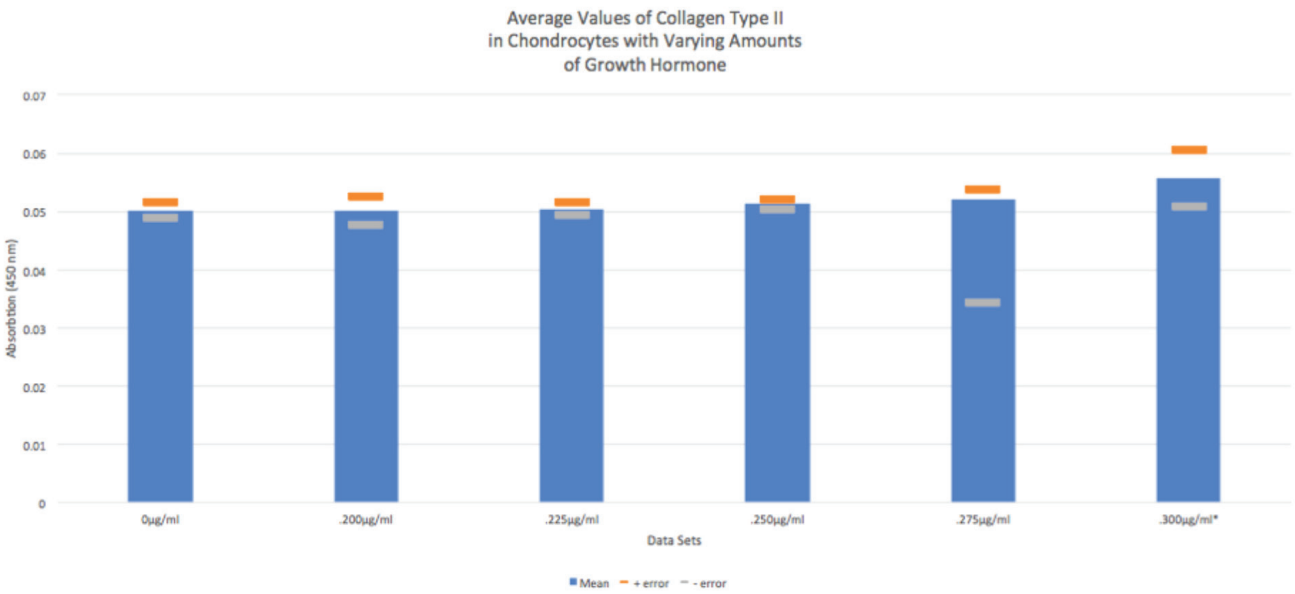

Figure 1. Average values of type II collagen in chondrocytes with varying amounts of growth hormone. Chondrocytes were treated with varying levels (0.200, 0.225, 0.250, 0.275, 0.300, 0 µg/mL) of growth hormone and concentrations of collagen were measured based on absorbance (450 nm). Each bar in the graph represents the average of ten trials. Error bars represent the margin of error for each data set. Experimental groups were compared via a t-test and asterisks (*) represent $p<0.05$.

of the growth hormone increasing the cell proliferation in chondrocytes. It was also concluded that the collagen IgG assay kit was needed to support the hypothesis stating that growth hormone uses the direct pathway of increasing c-myc proto-oncogene expression to increase the cell proliferation. With those assays it was drawn that increasing levels of growth hormone increased chondrocyte proliferation as well. It was also shown that this increased proliferation uses the growth hormone's direct pathways as collagen concentration increases in correspondence to growth hormone level increase.

Therefore, the purpose of this study was to determine whether increased levels of growth hormones could directly increase the rate that cartilage renewed itself through the expression of *MYC*. If this direct effect is indeed occurring, it was expected that when varying concentrations of growth hormone (0.200, 0.225, 0.250, 0.275, 0.300, 0 µg/mL) are added to chondrocytes, the chondrocytes that have a concentration of growth hormone at 0.250 µg/ml added will have the greatest increase of cell proliferation and collagen production. Additionally, it was expected that if the varying levels of growth hormone are added to samples of chondrocytes, then the growth hormone will have a direct effect on the increase of cell proliferation and collagen production in the chondrocytes.

RESULTS

Chondrocytes were treated with different concentrations of growth hormone, after which collagen levels were measured via an ELISA-based Collagen IgG assay. Samples that were treated with increasing concentrations of growth hormone showed an increasing collagen concentration (**Figure 1**). This suggests that growth hormone is able to use a direct pathway to increase collagen production, rather than solely relying on the indirect pathway.

The statistical significance of the data sets can also be seen in this figure. One of the data sets that has the most statistical significance in correspondence to the control of 0 µg/ml of growth hormone is the sample with 0.300 µg/ml of growth hormone. This sample had a *p*-value less than 5% or 0.05 and as seen in the figure it is the only sample that is noticeably different than the control.

Even with 0.300 µg/ml being the only statistically significant treatment group, the hypothesis stating that growth hormone has a direct effect on the proliferation of chondrocytes is still supported since there is an increase in the concentration of type II collagen.

A cell counting assay was also performed to quantify the number of viable cells in each sample and gain insight into the effect of growth hormone on cell proliferation. Similar to the findings in the previous experiment, treatment with higher concentrations of growth hormone led to increased cell proliferation (**Table 1**).

DISCUSSION

The second hypothesis, that growth hormones have a direct effect on collagen production, was supported with the outcome from the Collagen IgG Assay, since as more growth hormone was added to chondrocytes, the concentration of collagen increased. Although only the samples treated with the highest concentration of growth hormone showed collagen levels that were statistically significantly higher than

Table 1. Number of cells per mL for varying levels of growth hormone across three trials

Growth Hormone (µg/ml)	Trial 1	Trial 2	Trial 3
0.000	800,000	812,500	700,000
0.200	850,000	875,000	862,500
0.225	900,000	1,000,000	912,500
0.250	1,025,000	1,080,000	1,000,000
0.275	1,137,500	1,100,000	1,125,000
0.300	1,250,000	1,187,500	1,225,000

the untreated condition, the positive correlation between growth hormone concentration and collagen amount was consistent across all samples.

However, the first hypothesis was not supported, since 0.250 µg/ml of growth hormone was not the amount that induced gene expression the most (seen through increased collagen levels). It was expected that the results from the collagen IgG assay would produce a bell-shaped curve, with the concentration of collagen increasing until a certain point (hypothesized to be 0.250 µg/ml of growth hormone), and then decreasing until it got to 0.300 µg/ml. This, however, was not the case and the point at which growth hormone has the greatest effect on collagen production and cell proliferation has not been found yet.

The chondrocytes used in this project (murine-derived) took on average four days to reach confluency. However, when growth hormone was added, the average dropped to one day for the cultures that had 0.300 µg/ml of growth hormone added. Growth hormone also had an impact on the proliferation rate with the other samples; for example, the samples treated with 0.250 µg/ml of growth hormone took an average of two days to reach confluency (compared to an average of four days for control samples).

One of the difficulties encountered during this study was finding an affordable source of chondrocytes that would leave a budget for other materials. The cells that were used were sourced from an outside lab. Towards the end of the study, some of the cells were contaminated; this was most likely due to factors in the lab or an error when the protocol was carried out. Since this only happened toward the end of the project and it was the same protocols being repeated since the beginning, it was most likely an environmental factor that caused the contamination.

The research conducted in this study could be applied to the medical side of science for individuals that suffer from joint disorders or malfunction. This is because the results from this research show that growth hormone has a direct effect on the cell proliferation of chondrocytes. This suggests that using growth hormones locally for regenerating cartilage may be more effective than originally assumed, since growth hormone does not necessarily have to go through the secondary messenger of insulin-like growth factor. Our results also showed that growth hormone increased the rate at which chondrocytes reproduced or proliferated, which could be applied to increase the speed at which cartilage renews itself. This increase in efficiency and rate of renewal could guide therapies for these cartilage disorders or malfunctions, since pain occurs at sites where cartilage has worn away, causing bones to grind against each other. Increasing the rate that cartilage renews itself could help produce this cartilage faster and therefore decrease the pain from where the cartilage was lost.

Future studies could test chondrocyte samples with growth hormone concentrations that are higher than 0.300 µg/ml in order to find the point at which the effect that the growth hormone has on chondrocytes plateaus and then eventually starts to decrease. Completing this bell curve and then finding the highest peak of that curve would help to optimize the effect that growth hormone has on chondrocytes. Ultimately, this would show the level at which growth hormone could be added to cartilage and have the greatest effect on renewing or regenerating the cartilage that was lost.

METHODS

The chondrocyte cell line was murine-derived from the European Collection of Authenticated Cell Cultures.

Cell Culture Medium

In a Biological Safety Cabinet (BSC) 40 mL of DMEM was added to a 50 mL sterile test tube. 0.4 mL of Penicillin Streptomycin, 0.8 mL of L. Glutamine and 4 mL of heat inactivated Fetal Bovine serum was then added to the test tube. For the cells treated with growth hormone, growth hormone (ThermoFisher Scientific, 200 µg/ml) was added to the medium for a final concentration of 0.200 µg/ml, 0.225 µg/ml, 0.250 µg/ml, 0.275 µg/ml, or 0.300 µg/ml.

Cell Counting Assay

Sterile cell culture technique was maintained and cells were grown at 37C. Cells were checked under a microscope for any signs of bacterial or fungal contamination. Existing media was removed and flasks were washed with 2-5 mL of phosphate buffer saline (PBS). 5 mL of culture medium was added to the cell culture flask and the cells were detached using a sterile cell scraper. Cells were centrifuged for 5 minutes at 1200 RPM and then the supernatant was removed. Cell pellets were then resuspended with 5 mL of culture medium, and 0.5 mL was taken and transferred into a sterile Eppendorf tube. Cells were diluted in Trypan Blue in a 1:5 dilution. 100 µL of the Trypan Blue/cell suspension solution was taken and carefully pipette into the counting well of a hemocytometer and then the cells were viewed under a 10X microscope. The cell concentration was found by taking the average number of viable cells in the four sets of 4x4 grids and multiplying that by 10000 to get the number of cells per

milliliter. This was then multiplied by 5 to correct from the 1:5 dilution of the Trypan Blue. This assay was replicated three times for each of the data sets in the experiment.

Collagen IgG Assay Kit: ELISA Buffer Preparation

10 mL of ELISA Buffer Concentrate (10X) was diluted with 90 mL of UltraPure water.

Wash Buffer Preparation

5 mL of Wash Buffer Concentrate (400X) was diluted to a total volume of 2 L with UltraPure water. 1 mL of Polysorbate 20 was then added to the solution.

Mouse Anti-type II Collagen Polyclonal IgG Standard Preparation

The contents of the Mouse Anti-type II Collagen Polyclonal IgG standard was reconstituted with 1 mL of 1X ELISA buffer. The solution was mixed gently and labeled Solution #1. Seven clean test tubes were obtained and labeled #2 through #8. 500 µL of 1X ELISA Buffer was aliquoted into tubes 2-8. 500 µL from Solution #1 was transferred into tube 2 and was mixed gently. Next, 500 µL from tube #2 was transferred into tube #3 and mixed gently. This process was repeated for tubes #4 through #7, and tube #8 had no antibody added to it.

Anti-Mouse IgG/HRP Conjugate Preparation

A working solution was created by the addition of 0.6 mL of Anti-Mouse IgG/HRP Conjugate to an 11.4 mL Assay Buffer (12 mL total).

Plate Preparation and Reading

100 µL of the standards was added to the appropriate well on the Bovine Type II Collagen Precoated ELISA Strip Plate. The plate was then covered with a 96-well cover sheet and incubated for two hours at room temperature on an orbital shaker. The wells were then emptied and rinsed completely four times with the wash buffer made previously (for complete wash, the wells were filled completely and emptied; after the last rinse, the plate was gently tapped on an absorbent paper to collect residual wash buffer). Next, 100 µL of the diluted Anti-Mouse IgG/HRP Conjugate was added to each well on the plate and the plate was covered and incubated for one hour at room temperature on an orbital shaker. The wells were then emptied and rinsed four times with wash buffer. 100 µL of TMB substrate solution was added to each well of the plate and the plate was covered with a 96-well cover sheet and incubated for 15 minutes at room temperature in the dark. After this time the development of the plate was read at 650 nm and 100 µL of stop solution was added to the plate when Solution #1 had a value of 0.5 through 0.6. The underside of the plate was wiped with a clean tissue to remove fingerprints, dirt, etc. and the plate was read at a wavelength of 450 nm. This assay was replicated with ten trials with each of the six data sets in the experiment.

Statistical Analysis

First, the means of each of the data sets were obtained (0 µL, 2 µL, etc.). With that information, the standard deviation of the different data sets was found. A t-test was then performed to assess the statistical significance of the data sets with the *p*-value found. Finally, the margin of error was calculated for each of the data sets. All statistical analysis was conducted using Excel functions.

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Article

Majority and minority influence in teenagers for different social dilemmas

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SUMMARY

The influence of others on changing one's views is magnified when living in society since cooperation and identification within a social group is necessary for someone to feel accepted. In a previous study done in our high school two years ago, teenagers did not show conformist behavior in non-ambiguous situations (1). We wanted to evaluate conformity using social dilemmas to continue this previous research. We hypothesized that teenagers would follow others' influence—changing their initial opinion to belong to the group—particularly with increasing ambiguity of the dilemma. Forty-two high school students were tested by confronting them with three different social dilemmas. The initial position of the students after we presented the dilemmas was identified. Then, students were asked to discuss the dilemma out loud, expressing their initial opinions. Two "confederates" per group, who were previously asked in private to argue against the majority's opinion, voiced their contradictory opinion. Afterwards, students were asked again for their positions to see if their opinions had changed. We found variations in the proportions of students that changed their initial opinions depending on the dilemma. Furthermore, we found that both majority and minority influence could be responsible for changing the student's initial position. This change was dependent mainly on moral arguments given by the majority or minority and not by the size of the group. Therefore, we were unable to confirm our initial hypothesis that teenagers would show conformist behavior to feel part of a group, since conversion by minority influence, rather than conformity by majority influence, prevailed.

INTRODUCTION

For humans, being part of a group is important because it provides us identity, security, and structure, and satisfies our needs for affection, care, and belonging (2). Changing your opinion in support of the group's opinion is known as conformity behavior and occurs as a mechanism developed by individuals to feel part of a group (3).

There are two forms of social influence that affect individuals: majority influence and minority influence (4, 5). Causing minorities to conform is accomplished by majority influence while changing the majority to agree with minorities is recognized as minority influence (4). Majority influence is responsible for conformity and public compliance, whereas minority influence results in conversion (5). In the social influence context, conversion refers to the process of changing your point of view to a different one that implies a true change in your opinion (6). Both majority and minority influence are important for society. Majority influence allows morals, religion, or legality within social norms to regulate and harmonize the coexistence of people in a certain society; there are even essential standards almost universal in all cultures to support peaceful life (7). Nevertheless, minority influence is also important as a form of social change, since it usually involves a personal shift in opinion and is often viewed as a more creative form of social change (4).

Changing individual points of view to a majority position is due to two assumptions: the first is that majority judgment gives information about reality and therefore are probably correct. The second, that individuals want to be accepted and avoid disapproval (3). But exposure to minority viewpoints makes individuals consider different perspectives and encourages flexible thinking (8). Minority influence can cause a conversion of individual points of view by two reasons: the first is the consistency of judgments by a minority showing a clear view of reality, and the second is by an unwillingness to yield or compromise concerning the position (9).

Another important factor that can influence individuals to change their position is the degree of security or certainty an individual has on its answers. The more difficult the task or the more ambiguous the stimulus, individuals are more likely to look to others as sources of information regarding appropriate courses of action and show conformist behavior (10). Experiments in social psychology have shown that most participants changed their opinions when faced with opposing opinions in a greater number or when faced with hierarchy or authority, even in cases without arguments that endorsed those different opinions (3).

A study that investigated the different contributors for majority or minority influence found that the differences between majority and minority influence are not only due to group size and prevailing opinions of the majority, but also needed to consider the context and exposure to minority views (11). When people attend to more aspects of a situation, they reexamine and can take better decisions and minority views, therefore, raise greater thought about the issue (11).

In a previous study done in our high school two years ago, teenagers did not show conformist behavior in non-ambiguous situations (1). In this cited study, teenagers were

presented with the Solomon Asch visual test and a simple math test; these tests were done with the help of actors known as confederates, representing majority influence, that were asked to say the wrong answer out loud before the participant gave their answer. It was expected that teenagers changed their minds due to confederates' influence but this was not observed, and teenagers kept their answers (1). The authors indicated that one possibility for observing non-conformity was that the situations tested were no-ambiguous so majority influence was less likely to change individuals' answers (1). As a follow-up of this study, we wanted to evaluate conformity in social situations rather than non-ambiguous tests.

To evaluate majority and minority influence on changing individual opinions in different complex social dilemmas, this research asked whether teenagers would follow others' influence to belong to a group and whether they would change their initial opinions in a social situation, particularly when social dilemmas became more ambiguous. The results indicated that changing individuals' opinions depended not only on the controversy degree of the social dilemma but also on moral arguments presented by majority and minority influences rather than the size of the group.

RESULTS

To evaluate majority and minority influence on teenagers' judgment in social dilemmas of different complexity, high school students were confronted with three social dilemmas with different social reasoning complexity. After confronting them with the situations they had to show their opinion. When discussing it out loud in the group, two pre-selected confederates voiced their opinion against the majority. The students could change or stay with their decision for each social dilemma.

The first dilemma was presented in the form of a short video that showed a family who shows discrimination against a man of different ethnicity while waiting on a bench in a clinic (12) The second dilemma presented was also shown in a short video in which a policewoman was risking her own life to save a young man trying to commit suicide (13). The third dilemma presented was a hypothetical description in which the students witnessed a bank robbery by a man and later found out that all the money stolen was donated to an orphanage (14).

The initial positions for all of the presented social dilemmas showed a higher percentage of students being against the presented conflicts (**Table 1**).

We argue that the more similar the percentages of the two opposite positions (in favor or against), the more ambiguity or controversy the social dilemma held for the students. The testing order was first the discrimination dilemma, second the stop suicide dilemma, and third the bank robbery dilemma. The order of controversy, from less to more controversial, using the difference in proportions between in favor or against in each dilemma was first the discrimination dilemma, second the bank robbery dilemma, and third the stop suicide dilemma (**Table 1**).

Social dilemma	% initial in favor	% initial against	Testing order	Controversy degree
Discriminate	14	86	1st	Less
Stop suicide	36	64	2nd	Medium–High
Report robbery	21	79	3rd	Medium

Table 1: Initial position, testing order and degree of controversy for the different social dilemmas. The initial positions for the presented social dilemmas showed a higher percentage of opinions going against by the students. The order in which social dilemmas were presented to students was: first, the discrimination case, second, the stop a suicide attempt, and third, the report a bank robbery. The order of controversy of dilemmas, from less to more controversial was: first the discrimination dilemma, second the bank robbery dilemma, and third the stop suicide dilemma.

The change in initial position due to majority influence or minority influence after confederates voiced their arguments depended on the social dilemma (**Table 2**).

	Initial position	Not change poisition	Change by majority from in favor to go against	Change by minority from in favor to go against
Dilemma 1: Should a family discriminate against appearance?				
Against	36	32	-	4
In Favor	6	1	5	-
Dilemma 2: Should a policeman stop a suicide attempt?				
Against	27	18	-	9
In Favor	15	13	2	-
Dilemma 3: Should you report a bank robbery even if the money goes to an orphanage?				
Against	33	7	-	26
In Favor	9	7	2	-

Table 2: Initial position and change in position for the different social dilemmas according to majority or minority influence. The change in the initial position depended on the social dilemma. The discrimination dilemma had fewer students changing their initial position, and the change in opinion was almost the same due to majority or minority influence. For the suicide dilemma, the proportion of students changing their opinion showed some increase, and most of the changes in opinion were due to minority influence. The bank robbery dilemma presented the higher proportion of students changing their initial position, most students changed their initial position from being against to go in favor of reporting the robbery, persuaded by minority influence.

For the discrimination dilemma, 21% of students changed their initial position, and the influence in changing students' own opinions was almost the same by majority (5 persons changed) or minority groups (4 persons changed). For the suicide dilemma the proportion of students changing their

own opinion increased to 26%, but most of the changes in opinion were by minority influence (9 persons changed) that convinced students to go in favor of stopping a suicide; the majority influence to go against stopping the suicide was much less (only 2 persons changed). For the bank robbery dilemma, 67% of students changed their initial position from being against reporting the robbery to go in favor of reporting the robbery. In this case, almost all students who changed their initial position (26 out of 28) were persuaded by the confederates' minority and not by the majority opinion.

More students kept their initial opinion for the least controversial issue (discrimination) and the influence by majority or minority was almost the same (**Figure 1**). But more students changed their initial opinion for the more controversial issues (suicide and bank robbery). Minority influence was more important for changing students' initial opinions for the bank robbery dilemma than for the suicide dilemma (**Figure 1**). Changing or not initial opinion and changing their opinion by majority or minority is dependent on the social dilemma presented, ($p < 0.0001$, chi-squared test; **Figure 1**). Of the 42 high school students participating in this experiment, 10 never changed their initial opinion and were not influenced by others (24%) and only 1 student changed his initial opinion all 3 times, going with the minority influence for all 3 dilemmas (**Tables 1–2**).

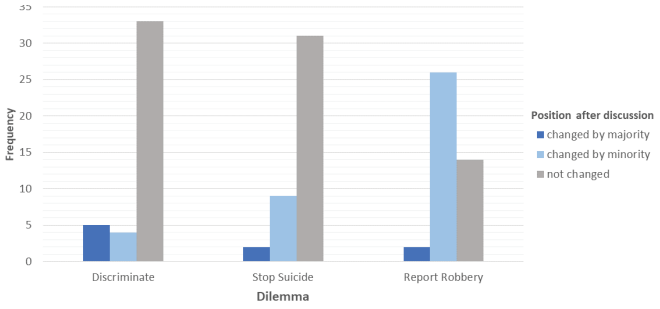

Figure 1: Change in position for each dilemma after discussion, as influenced by majority or minority (confederates). The less controversial discrimination dilemma showed the lower proportion of opinions being changed by social influence; as the dilemma became more complex for the bank robbery and the suicide dilemma, fewer students kept their initial position and this change in opinion was dependent on the moral arguments presented by minority or majority influence and not the size of the group (chi-square test, $p < 0.0001$).

DISCUSSION

This research was done as a follow up of the research done in the same high school two years ago, where teenagers did not show conformist behavior in non-ambiguous situations (1). In this cited study, using visual line tests and simple math tests, students were expected to change their initial answer by majority influence, but only 9% of teenagers showed conformist behavior, changing their initial answers for both tests (1). Authors argued that this change in expected behavior could be due to several factors, including cultural changes, group identification, and more individualism observed in teenagers in present societies, but also that the tests used were non-ambiguous and presented no dilemmas, therefore adolescents did not display conformist behavior (1). The authors suggested continuing this study using more ambiguous situations for testing social influence (1). Therefore, we wanted to evaluate conformist behavior as well as minority influence in more complex social situations as a follow-up research.

Our first expectation was that more students would change their initial position as the complexity of the dilemma increased. This was based on the fact that the identity of an individual is formed by all personal values and group values, including religion or moral standards that mark the type of decisions that an individual has (15). Since all students who participated were teenagers, we thought they did not have a strong criterion established to defend their opinions in a discussion and therefore would be influenced to change their initial position, but this was not always the case. Many students kept their initial opinion on the discrimination and suicide dilemmas but most of them changed their opinion in the bank robbery dilemma (**Figure 1**).

During the discussions after the cases were presented, it was interesting to witness the social phenomenon that appeared in the groups. We could see how people felt insecure and rejected from their ideas to the point of changing position for what they considered better and stronger arguments, mainly presented by the confederates that represented the minority influence. The conversion produced by a minority implies a real change of judgments or opinions (6) rather than compliance.

The proportion of students that changed their initial position varied with the complexity of the dilemma (**Tables 1–2**). Nevertheless, this change cannot be attributed only to conformist behavior due to majority influence or to increased ambiguity of the dilemma. A possible better explanation is that the change in opinion in more complex dilemmas was a consequence of minority influence due to conversion (5).

In the discrimination situation with low complexity (**Table 1**), only 21% of students changed their original position; of the students who changed their initial opinion, half did so by majority influence and half by minority influence (**Table 2**). In this scenario, it was harder for the confederates representing the minority to present valid arguments to convince students to accept discrimination, since there was very little ambiguity in this social situation, but some students were still influenced by the unexpected arguments of the confederates (for example, family was protecting the child from contagious diseases since they were in a clinic). For students that changed their initial position for being in favor of discrimination to being against discrimination, they did it by majority influence (comments included the realization by others that discrimination is never right, and there is no justification to discriminate based on appearance), thus showing conformist behavior. The video about discrimination had a low level of contradiction, as it was almost obvious to predict the posture participants would take;

the proportion of students that changed their opinion was much lower than the proportion of students that maintained their own judgment (**Figure 1, Table 2**).

The most controversial situation was the suicide dilemma (**Table 1**). Of all students, 26% changed their initial position, and most of them changed it from being against stopping the suicide to being in favor of stopping the suicide, influenced by the minority (**Table 2**). The difference in the amount of behavior guided by majority influence is related to the size of the social group: the larger the group of one opinion, the greater conformity is observed (16). Nevertheless, in this case, the difference in group size between the majority group and the minority group was less than for the other dilemmas (**Table 2**) making it more difficult to expect majority influence. Also, the complexity of the problem influences the change in position: the greater the difficulty in the task, the greater the conformists' behavior (17). However, this was not the case in this research, and minority influence by conversion prevailed over majority influence by conformity. This change in opinion favoring stopping suicide by minority influence can be explained by arguments presented by the minority that had higher moral values, indicating that it is more ethical to save a life than to ignore the situation, and that suicide is a complex issue. Thus, even when the majority was against stopping the suicide initially, the arguments of the majority were not enough to cause conformist behavior, and minority influence prevailed for students that changed their initial opinion.

Initially, 64% of students believed that suicide is an individual decision and should not be stopped by others, but after the discussion, only 45% of students kept this opinion. This initial thinking could also indicate the prevalence of individualism and the increasing absence of fellowship and solidarity in young generations (18).

The bank robbery dilemma, with a medium complexity level (**Table 1**), was the one that presented the highest proportion in opinion change (67%), with an overwhelming majority of students changing their initial position by minority influence. This indicates conversion of opinions when the minority, supported by confederates, presented stronger arguments compared to the arguments of the initial majority. Four major factors that give the minority its power: being consistent in expressing the minority opinion, being confident about the correctness of the views presented, being unbiased and reasonable when presenting ideas, and resisting the social pressure of the majority to change the minority views (6). The initial position of the majority of high school students being against reporting the bank robbery (**Table 2**) could be explained by a position of teenager rebellion against authority and societal norms, which in some degree is expected during adolescence (19), even more, if this rebellion is with a cause that seems fair. The change in opinion in the bank robbery dilemma from not reporting it to reporting it was mainly influenced by minority confederates (**Figure 1**) convincing students with stronger moral arguments and presenting a different point of view not seen initially by the students (the end does not justify the means, money in the bank does not belong to the bank but belongs to other people, and so on). Also, confederates representing the minority were very effective in presenting their opposite points of view and were unwilling to change their position, thus increasing the odds of influencing students.

Although teenagers were influenced to change their initial opinions, and the proportion of change varied depending on the different dilemmas presented, most of the changes were due to minority influence rather than majority influence. Therefore, we were unable to confirm our initial hypothesis that conformist behavior will prevail in teenagers when presenting with ambiguous social dilemmas. When teenagers changed their initial position, it was mainly by conversion when presented with different points of view and not by conformity to feel part of a group. All high school students knew each other, so maybe they were not intimidated by the group size, and therefore conformity was not a determinant, because students already felt secure about belonging to the high school group. For future research, we suggest comparing different generations to evaluate if majority and minority influence changes according to age, assessing majority and minority influence with persons that do not know each other, comparing if there are gender differences, and evaluating if individualism and rebelliousness in teenagers play an important role in initial points of view for different social dilemmas.

MATERIALS AND METHODS

We evaluated the influence of majority and minority on individual judgment in three social dilemmas. A total of 42 volunteer students (both genders) from 15–19 years old from Tecnologico de Monterrey high school Campus Cuernavaca, Mexico, were chosen randomly to participate in a psychological experiment in reason judgment. The 42 students were divided into 3 groups of 14 students. In each group, there were additionally two "confederates" (also high school students) integrated, who acted as a minority and who were previously instructed to take an opposite posture to the majority posture in each statement; the 14 students did not know the position of the 2 confederates. These two persons were not included in the analyzed data. We informed all student volunteers that they would receive individual formats in which they should anonymously write their answers for the experiment. Three situations of controversial social issues that went from a basic reasoning level (almost obvious answers) to a more complex one, were shown to the sixteen students, fourteen subjects and two confederates, with two rounds of questions for each one. The first dilemma was shown in a two-minute video where a family showed discrimination against a man of different ethnicity while waiting on a bench of a clinic (12) and the students needed to decide if they were in favor or against the family's attitude. The second dilemma presented was a two-minute video in which a policewoman risked her

own life to save a young man trying to commit suicide (13), and the students needed to decide if they were in favor or against stopping the suicide. The third dilemma presented was a hypothetical description presented in text form: "We are witnesses of how a man robs a bank. However, we observe that the thief does not keep the money, but instead gives it to an orphanage that lacks the resources to support the orphans who live in it. We can report the robbery, but if we do, it is likely that the money that the orphanage can now use to feed and care for the children must be returned." The students needed to decide if they were in favor or against reporting the robbery (14).

After each social dilemma was presented to the students, we paused to ask students if they were in favor or against the social dilemma presented. Then, each participant filled out an individual form with an honest and personal judgment. After the 14 participants finished with the first part of the experiment, we asked the students to discuss out loud for 3 minutes their opinions of the social situation. The participants' posture was announced, and the two confederates adopted an opposite posture to the group to affect the position of the majority of the students. Then all students debated for another 2–3 minutes and we asked the students to write their position again, in the anonymous format, and explain why they did or did not change their initial position in an open question. We conducted the same process for all three dilemmas. At last, the answers were collected and analyzed.

The data were analyzed using chi-square test of independence, with a significance level of 0.05 and the chi-square test function from Microsoft Office Excel, 2016.

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Article

Specific transcription factors distinguish umbilical cord mesenchymal stem cells from fibroblasts

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SUMMARY

Now at the forefront of scientific research, stem cells play a crucial role in regenerative medicine and cell therapy. Although noted for their great promise in understanding organismal development and potential as a treatment for various diseases, stem cell research and applications have been limited by ethical and political concerns. However, recent research regarding induced pluripotent stem cells (iPSCs) has revolutionized the principle of stem cell-based treatment, especially since certain ethical controversies are no longer applicable. In 2009, scientists discovered that differentiated somatic cells could be induced to their stem-cell state by expressing transcription factors specific to self-renewal and potency. Since iPSCs are derived from one's own somatic cells, they bypass ethical and political concerns. Our objective is to further investigate whether specific protein markers, inherent to stem cells and their properties of self-renewal and potency, can be used to identify umbilical cord mesenchymal stem cells (UC-MSCs). We compared fibroblasts as a control cell type because of their similar physical structure to stem cells and their lack of self-renewal and potency-specific markers. We cultured both cell lines and measured protein levels of four selected factors (β-actin, Klf4, Nanog, and Sox2) using western blot techniques. Our results revealed that these selected proteins were expressed exclusively by UC-MSCs and not by fibroblasts, successfully demonstrating that specific protein markers can be used to distinguish UC-MSCs.

INTRODUCTION

Stem cells are cells with the unique potential to differentiate, or specialize, into cell types with various specific functions. For example, while a retina cell of the eye or a β-cell of the islets of Langerhans can only divide into retina cells or β cells respectively, a stem cell has the potential to differentiate into a muscle cell, neuronal cell, etc. Stem cells can be divided into three large subgroups – embryonic stem cells (ESCs), adult stem cells (ASCs), and induced pluripotent stem cells (iPSCs) (1). Our investigation focused on umbilical cord mesenchymal stem cells (UC-MSCs). Mesenchymal stem cells (MSCs) are multipotent ASCs that are able to differentiate into cells of a mesodermal origin: osteoblasts, chondrocytes, and adipocytes (2).

Two properties essential to a stem cell include self-renewal and potency (3,4). Self-renewal describes the process of cell replication specific to stem cells. Through self-renewal, stem cells undergo replication while maintaining pluripotency, thus increasing the number of stem cells. Moreover, stem cells have potency, the ability to specialize into different types of cells. The potency of stem cells ranges from totipotent, meaning the cells are able to differentiate into over 200 cell types, to unipotent, meaning the cells are able to differentiate into only one type of cell, depending on where the stem cells were extracted and their stage of development.

To manage and ensure the potency and self-renewal of the cell, stem cells express specific proteins that maintain its immature state (5). The expression of certain proteins in differentiated, mature cells causes these cells to revert into undifferentiated stem cells. Using this principle, Shinya Yamanaka pioneered iPSC technology and discovered that four transcription factors could be used to generate pluripotent stem cells from mature, differentiated somatic cells. Specifically, Yamanaka successfully derived iPSCs from mature fibroblasts by introducing pluripotency-associated "reprogramming factors" – Nanog, Sox2, Klf4, and cMyc– into this somatic cell type (6).

In essence, expressing these protein markers can cause a differentiated, somatic cell to revert into a stem cell, regaining its potency and self-renewal capabilities. Our study involves three of these reprogramming factors (Nanog, Sox2, and Klf4) due to issues of availability and access. Nanog is a transcription factor that maintains pluripotency in ESCs by repressing the expression of cell determination factors. Sox2 is also a protein essential in maintaining the potency of undifferentiated stem cells (7). Klf4, or kruppel like factor 4, is involved in the proliferation, apoptosis, and reprogramming of somatic cells (8). In ESCs and MSCs, Klf4 is an indicator of stem cell capacity. Because all abovementioned factors contribute to stem-cell like properties, we hypothesized that these four factors would be present in all stem cells, including UC-MSC. As these factors have, in effect, given somatic cells the characteristics of a stem cell, it is possible to deduce that these four factors are present in all stem cells, including UC-MSC.

The aim of our experiment was to determine whether we could distinguish UC-MSCs from fibroblasts on the basis of protein marker expression. We chose fibroblasts as a control cell type because of their similarity in morphology to that of UC-MSCs, in order to prevent conclusions resulting from differential physical appearance. Furthermore, we used

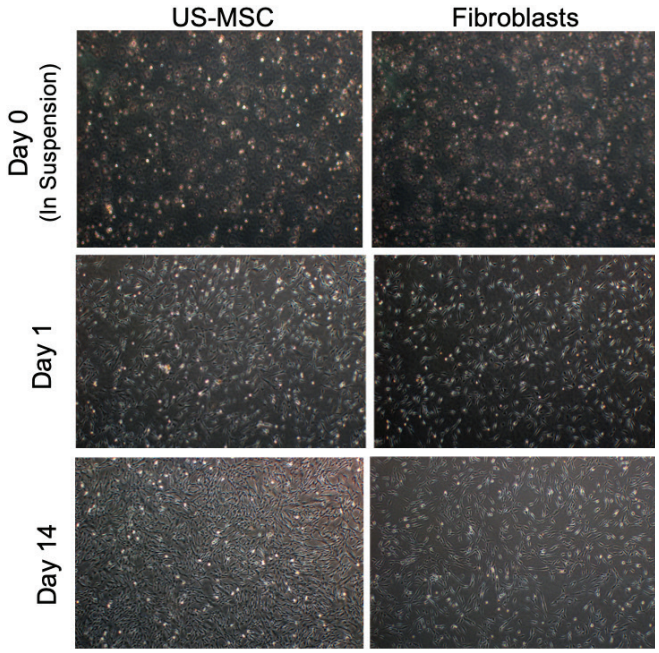

Figure 1. Growth of UC-MSC and Fibroblast Cell Lines. Representative images of UC-MSCs (left) and fibroblasts (right) were imaged using a microscope at 20x magnification at Days 0, 1 and 14 of culture. After 24 hours (Day 1), it is apparent that the cell lines have settled.

β-actin, a housekeeping gene present in both UC-MSCs and fibroblasts, as a loading control to ensure that success of the methodology.

UC-MSCs are pluripotent cells, meaning that they express proteins necessary for self-renewal and potency, the two key aspects that define a stem cell. On the other hand, a fibroblast should not need proteins for such functions, because they are specialized cells. Therefore, we hypothesized that self-renewal and potency-specific protein markers would be expressed in UC-MSCs, but not in fibroblasts, as they lack stem cell specific properties such as self-renewal and potency.

In conclusion, our results from these experiments revealed that only UC-MSCs expressed Klf4, Nanog, and Sox2 and both cell types expressed β-actin. This has important implications regarding methods of differentiating between different cell lines without varying physical characteristics.

RESULTS

We performed our experiments on cultured UC-MSCs and fibroblast cells. To ensure the health of UC-MSCs and fibroblast cells and make sure they were growing appropriately, we maintained consistent observation and collection of qualitative data. We demonstrated successful proliferation of both cell lines throughout the process of cell culture and cell banking using microscopes to observe the proliferation rates of cells (**Figure 1**). We cultured both cell lines for two weeks before we lysed cells for protein analysis (**Figure 1**).

We initially observed and compared the results of western blot with literature reading and band values, and subsequently compared protein markers' expression in UC-MSCs and fibroblasts

Western blot of Klf4 was conducted to examine its presence in both UC-MSCs and fibroblasts. (**Figure 2A**). Typically, Klf-4 has a band weight of approximately 56 kDa, as per the manual outlined on Cell Signal (CST), where the antibodies were purchased. UC-MSCs expressed Klf-4, while fibroblasts did not. Average band weights for Sox2 antibodies are approximately 35 kDa. The western blot results of UC-MSCs confirms the literature value of band weight, while fibroblasts did not express Sox2 (**Figure 2B**). Although not present in fibroblasts, Nanog is present in UC-MSC and reinforces the literature weight of Nanog, approximately 40 kDa (**Figure 2C**).

Western blot of β-actin, a housekeeping gene present in both UC-MSCs and fibroblasts, served as a positive control to ensure that the methodology of the investigation was conducted without error. (9) We found expression of β-actin, appearing as a band weighing 40 kDa, in accord with the value suggested by the CST manual (**Figure 2D**). Because β-actin was present in both cell types and of similar band intensities, the experiment's methodology was accurately completed.

DISCUSSION

Based on the expression levels of β-actin and three transcription factors, Nanog, Sox2, and Klf4, specific to self-renewal and pluripotency of stem cells, we concluded that the UC-MSCs express the protein markers that were used to develop iPSCs. As expected, fibroblasts, being specialized cells, expressed only β-actin, a housekeeping gene, and showed lower levels of expression of the self-renewal and pluripotency related markers.

There were some instances where human error could have impacted the results. For instance, the blotting paper of western blot may have had imperfections, such as small bubbles, that might have prevented the effective migration of proteins across the membrane. Another source of error may have been the amount of time the western blot was conducted. Due to constraints with the facility, we performed the gel electrophoresis overnight, in which a lower voltage was used for a longer period of time. The voltage or time may not have been sufficient for proteins to completely travel down the gel. Moreover, fibroblasts expressed minute amounts of Nanog. It could be hypothesized that fibroblasts would express little to no Nanog as it is a differentiated cell. Fibroblasts may contain trace amounts of Nanog as it has been shown that fibroblasts contain some UC-MSC like behavior (10). A potential source of error may have been during the experimentation, where pipettes may not have been completely sterile when transferring fibroblast and UC-MSC cell lysate. However,

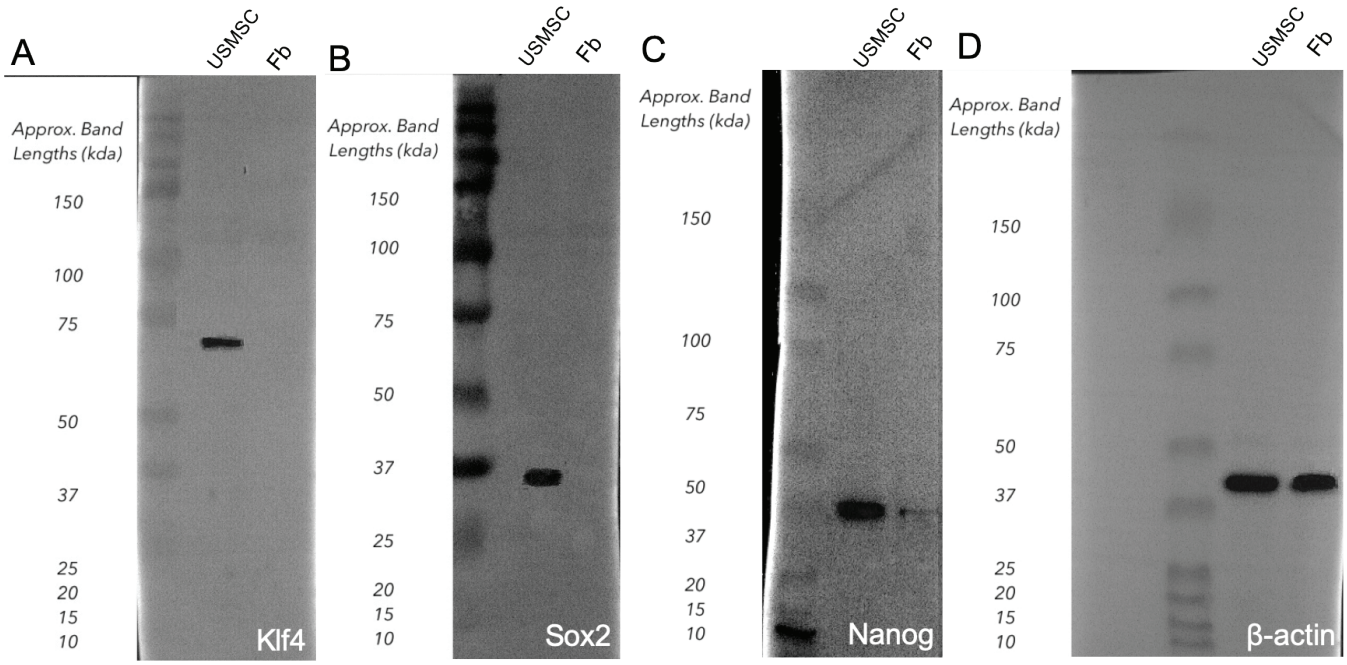

Figure 2. Comparison of protein levels of selected factors between US-MSC and fibroblasts. Protein levels in US-MSC and fibroblasts (Fb) were assessed by Western blot. (**A**) Klf4 is present in UC-MSC but not fibroblasts, confirming the hypothesis. (**B**) Sox2 is present in UC-MSC, but not fibroblasts affirming our hypothesis. (**C**) Nanog was expressed in UC-MSC, but not fibroblasts. (**D**) β-actin served as a control and similar band intensities of the same weight were observed.

given that predicted results were successfully obtained, we concluded that these aspects did not have a substantiable impact on results.

The experimental results suggest that analyzing protein expression using western blot is an effective technique of differentiating UC-MSC from other cell types. Therefore, western blotting proteins specific to a certain cell line seems to be a reasonable method of distinguishing certain cell lines.

Future studies can be conducted related to this experiment and field of study. The effect of stem cell age or time in growth medium on expression of self-renewal and potency factors could be examined. For example, do older stem cells express less transcription factors? This may have implications on future research, where younger stem cells are used to fully express the self-renewal and potency related characteristics of stem cells. The expression levels of potency transcription factors in other cell types, such as embryonic stem cells, could be examined as they are pluripotent and have a wider range of potency. Although likely legally restricted in many nations, this question presents an interesting study of pluripotency in different types of stem cells.

METHODS

Cell Culture

Umbilical cord mesenchymal stem cells and fibroblasts were cultured for two weeks at 37°C and 5% CO_2, allowing for proliferation to obtain the desired cell number for protein analysis. UC-MSCs were obtained from the CHA BioTECH, where there was a source of different cell lines.

Cells were grown in α-MEM containing fetal bovine serum (FBS), penicillin-streptomycin (P/S), and β-FGF2 (concentration 10 µg/mL).

Vials of cells were thawed in a 37°C water bath, centrifuged for five minutes at 1500 rpm, and the supernatant was removed. This process was repeated as necessary to ensure that only the pellet remained and all the supernatant was removed. Subsequently, the pellet was resuspended and added to a T-175 flask with media. To ensure successful cell seeding, a microscope with 40x magnification was used.

Cells from the flask were removed by first aspirating the pre-existing media and washing the cells using PBS. Then, 1X Trypsin-EDTA was added to the flask, which was placed in an incubator for one to two minutes. Fresh media was added to the flask and its contents were transferred to a new 15 mL tube. The tube was centrifuged at 1500 rpm for five minutes, and the supernatant was removed. A 9:1 ratio of FBS and DMSO was used to resuspend and transfer the cell pellet from the 15 mL tube to a vial. Vials were stored in a freezing box at -80°C for 24 hours and then placed in a liquid nitrogen tank.

Western Blot

The western blot analysis was performed to determine the presence of specific protein markers in UC-MSCs and fibroblasts cell lines. The process was split into three main steps – SDS-PAGE, antibody staining, and detection. To

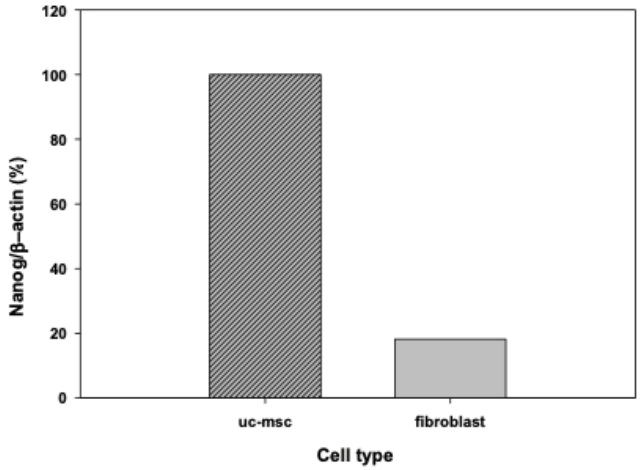

Figure 3. Ratio of Nanog to β-actin in UC-MSC and Fibroblasts
A considerably higher presence of Nanog was found in UC-MSC than in fibroblasts.

lyse the cells and extract their proteins, RIPA lysis buffer was added to the cell vials from the previous step and centrifuged for 15 minutes. The cell lysates were then placed on ice to minimize the effect of proteolysis and denaturation. Subsequently, SDS PAGE was conducted. In this particular experiment, an overnight electro transfer was used. Proteins were transferred from the gel to a polyvinylidene difluoride solution for greater accessibility to the antibodies.

Following SDS PAGE, primary and secondary antibodies were incubated for one hour at room temperature and washed using 3% BSA and TBST. The antibodies and proteins were placed on a microplate shaker for one hour, to ensure that the western blot was completed successfully. Finally, we added a detection solution (CST) to the membrane and used chemiluminescence to detect protein bands with a CCD camera. All antibodies used include anti-Nanog (CST), anti-Sox2 (CST), anti-Klf4 (CST), anti- β-actin (CST).

Data Analysis

We used ImageJ to quantify the intensity of each protein band. As observed in **Figure 3**, which represents the graphical data numerically, the expression of Nanog (as a ratio of Nanog: β-actin) in UC-MSCs and fibroblasts was 100:18. Therefore, expression of Nanog was considerably higher in UC-MSCs than in fibroblasts.

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