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Article

Evaluating Biomarkers and Treatments for Acute Kidney Injury in a Zebrafish Model

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SUMMARY

Coronary Artery Disease (CAD) is the leading cause of death in the United States. Despite the prevalence of CAD, there have been few clinical advancements to treat this disease, and mortality due to CAD is rising. Cohort studies have shown that 81% of Acute Kidney Injury (AKI) patients in the renal fibrosis stage later develop CAD. In this investigation, we aimed to create a cost-effective strategy to treat AKI and thus prevent CAD in this patient population. To achieve this aim, we conducted a three-pronged study in a model of *Danio rerio*. In the first phase, we tested whether AKI is induced in *Danio rerio* upon exposure to the environmental toxins arsenic, aristolochic acid, and cadmium. In the second phase, we evaluated nitrotyrosine as an early biomarker for toxin-induced AKI in *Danio rerio*. Nitrotyrosine levels increased 3.5 times its baseline after induction of AKI using arsenic, aristolochic acid, and cadmium. In the third phase of this study, we evaluated 4 treatments of renal fibrosis, the last stage of AKI, because it is a gateway connecting AKI and CAD. Upon conducting a Picro Mallory Trichrome Stain to assess the effects of these treatments on renal fibrosis, we found that the compound SB431542 was the most effective treatment (reduced fibrosis by 99.97%). The approach to treat AKI patients, and potentially prevent CAD, described in this investigation is economically feasible for translation into the clinic in both developing and developed countries.

INTRODUCTION

Coronary Artery Disease (CAD) is the leading cause of mortality in many developed countries and a major burden in developing countries. Despite the prevalence of CAD, there have been few clinical advancements to treat this disease, and mortality rates due to CAD are expected to rise (1). Recent cohort studies suggest that there is a correlation between kidney injury and cardiovascular diseases (2). Notably, 81% of Acute Kidney Injury (AKI) patients go on to develop CAD, indicating a strong correlation between renal and cardiovascular diseases. Thus, treating AKI effectively may prevent progression to CAD, as well as lower cardiovascular mortality rates. There are two main issues related to current treatments for AKI. The first issue is the most commonly used AKI biomarker, serum creatinine, may be inadequate. Serum creatinine is considered the gold standard for evaluating kidney function, yet existing literature suggests that it is a late biomarker of kidney injury and that it is highly unreliable (3). Serum creatinine increases only after about half of kidney function is lost (4). This biomarker also fluctuates significantly with meat consumption and anabolic agents, resulting in inaccurate diagnostic test results (5). The second issue related to treating AKI is a lack of therapeutics. Most patients are treated with hemodialysis but this is not an optimal option as it exacerbates reactive oxygen species presence and debilitates daily activities.

As a result, I conducted a three-pronged study in a model of zebrafish (*Danio rerio)* to induce, detect, and treat AKI. Zebrafish were utilized only during their invertebrate stage, until 168 hours post-fertilization, at which time they are deemed vertebrates. The simplicity of the zebrafish kidneys, structural similarities to that of humans, and similar glomerular filtration processes of the zebrafish make them a good model organism to conduct renal studies (6).

In the first phase of my project, I used the three most universal pollutants in the world – arsenic, aristolochic acid, and cadmium – to induce AKI in *D. rerio* (7-9). These pollutants are infamous for their carcinogenic effects and induction of respiratory issues, so I decided to investigate their effects on renal function (10-14**).** The induction of AKI by pollutants has not been tested, therefore we hypothesized that if AKI was induced through the pollutants, then these pollutants would also induce post-injury fibrosis.

In the second phase of my project, we evaluated a novel biomarker for pollutant-induced AKI. In researching potential biomarkers, we wanted to identify an early intervention biomarker to detect the presence of renal injury before significant damage occurs. We investigated the potential of nitrotyrosine, a compound that marks oxidative stress, as a marker of the pathogenesis and progression of AKI. We hypothesized that if AKI is induced through the environmental pollutants arsenic, aristolochic acid, and cadmium, then nitrotyrosine levels will significantly increase.

In the third phase of my project, we evaluated treatments that target the last stage of AKI, known as renal fibrosis. Fibrosis involves the damage-induced scarring of healthy tissue and the inappropriate accumulation of connective tissue that occurs after injury (15). It impacts most, if not all, vital functions of the kidney. No FDA-approved drugs have been developed to target renal fibrosis, and there are no clinical trials evaluating treatments to target renal fibrosis. It is imperative to therapeutically target this stage of injury because it is the

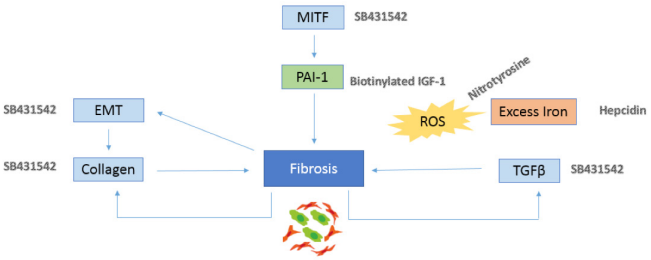

Figure 1. Four treatments were devised to inhibit different pathways of fibrogenesis such as excess iron production, transforming growth factor Beta, and collagen buildup.

progression stage from Acute Kidney Injury to higher mortality linked diseases, such as CAD as shown previously (16). evaluated the effects of four therapies – Hepcidin, SB431542, Biotinylated IGF-1, and IV-fluid – on fibrosis (**Figure 1)**.

For treating AKI, I used Hepcidin as an iron chelator, a compound that modulates excess iron levels in the human body. Excess iron can lead to reactive oxygen species generation, cellular death, apoptosis, or fibrosis (17). All four are harmful to the kidneys, so quantifying Hepcidin found naturally in the body could have mitogenic effects.

Additionally, I investigated biotinylated insulin-like growth factor (IGF-1). IGF-1 has proven to have several mitogenic properties such as stimulating cell proliferation and cellular regeneration (18). However, IGF-1 readily diffuses through tissues, an undesirable quality as localized targeting of the kidneys is more effective (19). Streptavidin is a homotetramer that has binding zones for four biotin molecules. There are multiple benefits of using such a delivery system. Streptavidin's versatility allows it to survive in many conditions within the human body such as extreme pH levels. Its counterpart, biotin, can easily be conjugated to bind with other molecules (20). The two compounds have high affinity binding due to Van der Waals interactions and morphological complementarity between the binding pocket and biotin conjugate (21). Davis et al. applied this delivery system to treat myocardial infarctions and reported 28 days of sustained delivery of IGF-1 in cardiomyocytes (22). This novel approach has not been tested in current renal research and thus holds promising data in renal treatments.

Another treatment I tested was SB431542, a novel molecule designed for inhibiting transforming growth factor beta 1-induced collagen 1α1 (collagen 1 alpha 1), production of fibronectin, and epithelial to mesenchymal transition (EMT), which are all interconnected with fibrogenesis (23). EMT is prominent during pathological conditions; when renal fibrosis sets in, about 36% of new fibroblasts come from local EMT (24). SB431542 can prevent such excess aggregation of collagen and extracellular matrix buildup and alleviate fibrogenesis.

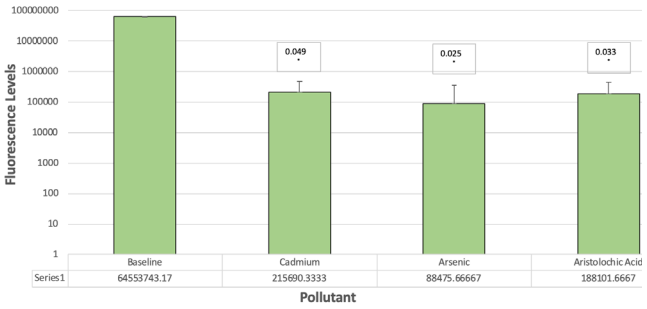

Figure 2. According to the PT-Yellow assay, the pollutants lowered fluorescence levels extensively.

The positive control I administered was IV fluid as it is currently used in treatments to replenish fluids and balance electrolytes (25). From these four treatments, I hypothesized that if post-injury fibrosis is present and treated with biotinylated Insulin Growth Factor-1 complexed with streptavidin, SB431542, Hepcidin, or IV fluid, then SB431542 will ameliorate the presence of fibrosis with the greatest efficacy.

Results

In order to confirm that arsenic, cadmium, and aristolochic acid induce AKI, we performed fluorescence analysis with PT-Yellow. PT-Yellow is a substance absorbed by the proximal renal tubules in the zebrafish, and it labels kidney function in relation to fluorescence emissions (26). Labeling of the proximal tubule correlates with levels of blood supply to the kidneys, such that lack of labeling (low absorption of the dye) correlates with reduced glomerular filtration rate and decreased kidney function. We used fluorescence analysis on ImageJ to measure PT-Yellow labeling. Decreases in kidney function were quantified by marking decreases in labeling in pollutant zebrafish embryos, in comparison to control embryos. Kidney filtration was impaired after exposure to pollutants, which supported the first hypothesis that the pollutants induce AKI (**Figure 2**). Of the three pollutants tested, arsenic caused the most extensive damage, lowering fluorescence levels by 99.68%.

Next, I verified nitrotyrosine's potential as a biomarker by

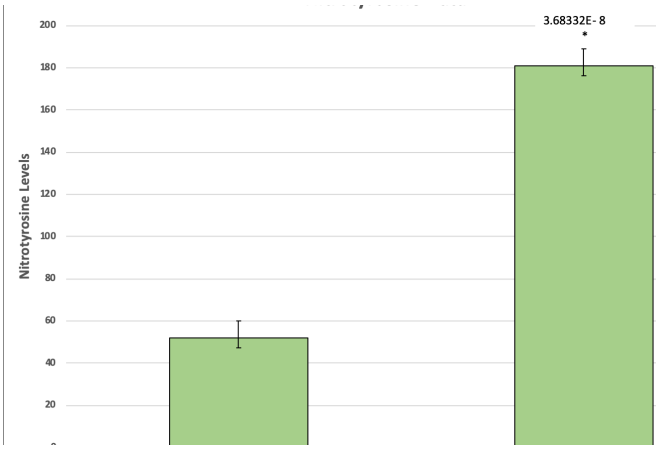

Figure 3. The Reactive Oxygen Species Detection Solution shows a direct correlation between the disease and nitrotyrosine levels (248.08% increase). A two tailed t-test proved a significant *p*-value of 3.68332E-8.

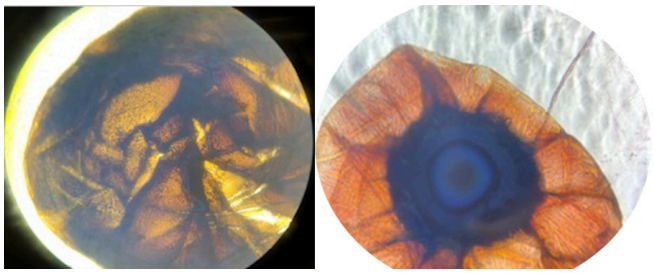

Figure 4. The picture on the left is a Picro Mallory Trichrome Stain conducted on a negative control embryo whereas on the right, the embryo is induced with fibrosis. Here, there are concentric rings of blue that is characteristic of collagen 1alpha1 presence. This is also the blastoderm region of an embryo where Epithelial to Mesenchymal Transition (EMT) occurs. However, the same degree of blue regions cannot be found in an embryo without fibrogenesis induced.

using a reactive oxygen species (ROS) detection solution. Single electron reduction between the ROS detection solution and nitrotyrosine produces chemiluminescence (27). A luminometer then measured levels of luminescence as a result of nitrotyrosine presence. "Nitrotyrosine levels were significantly increased by 248.08% following pollutant-induced (two-tailed student's t-test, *p*-value = 3.68332E-8; **Figure 3**)." Thus, we determined that there is a strong correlation between the proposed biomarker and AKI.

A Picro Mallory Trichrome stain was utilized to colorimetrically detect collagen 1alpha1 (COL1A1), an accepted marker of fibrosis. When stained samples were analyzed, we observed blue areas in the samples (**Figure 4**). The picture on the left of **Figure 4**, the control, did not have the concentric blue rings whereas the sample on the right did, indicating localized patterns of COL1A1 and therefore higher degrees of fibrosis. We used the Picro Mallory Trichrome stain to conclude that Cadmium, Arsenic, and Aristolochic Acid induced fibrosis by measuring COL1A1 (**Figure 5**).

In order to test the effect of four potential treatments (hepcidin, SB435142, biotinylated-IGF1, and IV fluid) on fibrosis, we measured COL1A1 levels after treating *D. rerio* at various concentrations of each. 0.004M of SB435142 reduced fibrosis with the greatest efficacy, specifically by 99.96 5%. 0.003M and 0.0019M of SB435142 lowered levels by 99.927% and 99.923%, respectively (**Figure 6**). Thus, the

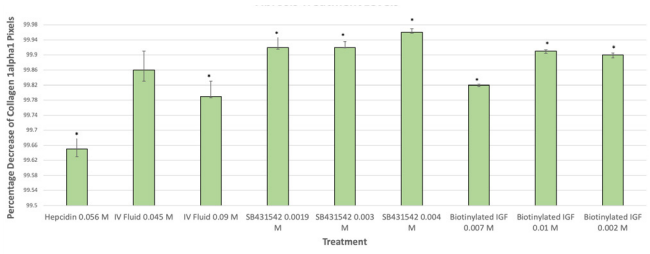

Figure 6. The Picro Mallory Trichrome stain showed an average decline in fibrosis levels for SB431542 at 99.97%. A two tailed t-test proved a significant *p*-value of 0.014.

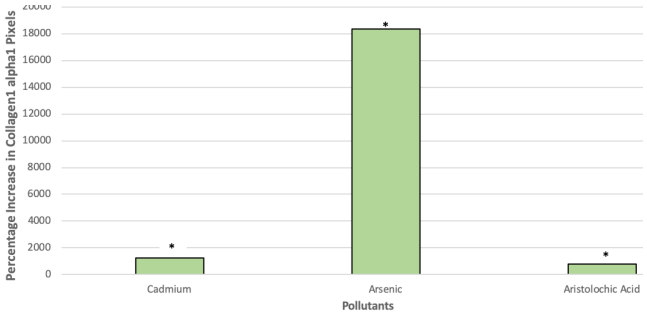

Figure 5. The Picro Mallory Trichrome Stain supported that the pollutants significantly compounded fibrosis levels. A two tailed t-test proved a significant p-value of 0.0126.

third hypothesis was supported that SB431542 was most effective in treating fibrosis (two-tailed student's t-test, *p*-value = 0.014).

The three other treatments also hold potential as biotinylated IGF-1, Hepcidin, and IV Fluid lowered fibrosis by 99.913%, 98.5%, and 98.53% respectively. Interestingly, the zebrafish treated with SB431542 also survived for a longer amount of time. Compared to samples that were not treated, addition of SB431542, biotinylated IGF-1, Hepcidin, or IV fluid extended average life span by 58%, 42.5%, 33%, and 10%, respectively.

Unexpectedly, when renal fibrosis was induced, the zebrafish also exhibited 'fibrous' eye shapes (**Figure 7**, right). These 'fibrous' eye shapes were recurrent throughout the data and are markers of microphthalmia-associated transcription factor (MITF) upregulation, which is consistent with existing literature (16). These peculiar eye shapes are only present in AKI-induced zebrafish, not in wildtypes such as the zebrafish **(Figure 7**, left**),** indicating that the upregulation in melanocytes is a result of renal damage. This observation can reshape research with diseases characterized by drastic increases in MITF, such as melanomas and chronic eye diseases, to refocus on renal injuries as a possible root cause.

Discussion

Through statistical analysis, fluorescence averages, and COL1A1 calculations, we concluded that the results

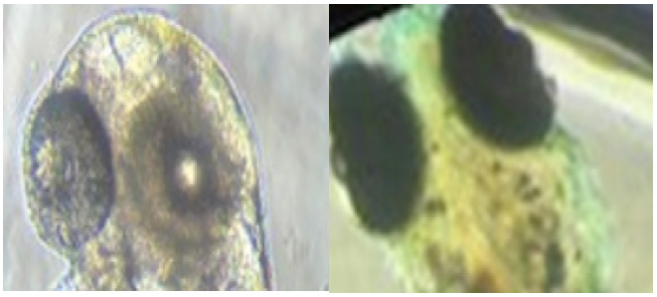

Figure 7. The fish on the left is a wildtype zebrafish with normal eye shape and MITF expression. On the right is an AKI induced zebrafish that exhibits a peculiar phenotypic eye shape known as fibrous eyes due to an increase in MITF expression.

supported each of the three hypotheses. Arsenic, cadmium, and aristolochic acid were shown to induce AKI, nitrotyrosine was shown to be a biomarker for AKI, and SB431542 was most effective in alleviating fibrosis.

The PT-Yellow assay served as a proof-of-concept that the environmental pollutants arsenic, cadmium, and aristolochic acid function as nephrotoxins and induce AKI, because they significantly lowered fluorescence. For example, arsenic levels dropped by 99.68%. Constant exposure to this water contaminant can cause substantial nephrological damage urging social and legal reform to ensure that the issue of pollution will be mediated for their nephrological impacts.

The PT-Yellow test also determined each treatment's ability to restore kidney function, measured by increases in fluorescence levels. Hepcidin, IV fluid, SB431542, and biotinylated IGF-1 were able to increase fluorescence rates of pollutant-induced samples by 384.7%, 209.4%, 654.5%, and 494.5%, which restored fluorescence levels to 89%, 99%, 97%, and 88% of the negative control's levels, respectively.

The ROS detection solution identified nitrotyrosine's robust presence with a 248.08% increase after AKI had been induced. This supports the first hypothesis because of a *direct* correlation between the proposed biomarker and AKI. Further research should establish that the nitrotyrosine increase is mediated by kidney damage.

The substance's easy detection, quantification, and lack of fluctuation compared to serum creatine affirm its potential in clinical treatments. Additionally, serum creatinine only elevates by 50% on average between 4 hours (baseline measurement) to 27 hours (28). Thus, the tested biomarker in this experiment is more strongly correlated with AKI and increases in a shorter time span.

Furthermore, the Picro Mallory Trichrome Stain supported the third hypothesis which predicted that SB431542 would be most effective in treating fibrosis. The higher concentrations of this solution were more effective- 0.004M decreased the collagen levels by 99.965%. Further studying of the pathways SB435142 inhibits, summarized in **Figure 1**, can open up new paths for developing AKI treatments.

Biotinylated IGF-1 also holds promising value in fibrosis amelioration. IGF-1 diffuses through tissues easily, which is not optimal considering the size of the zebrafish pronephros. In order to combat this characteristic, a biotin-streptavidin complex was used as a delivery mechanism for IGF-1 due to their high-affinity binding.

Additionally, we demonstrated that the pollutants not only induced AKI, but they also induced post-injury fibrosis which was, until now, not directly supported (**Figure 3**)**.** Furthermore, the data collected reconceptualized why there are such high mortality rates associated with CAD. Further testing may prove that pollutants could be a factor in the etiology of AKI and potentially CAD. Thus, AKI prominence correlates to an increase in CAD mortality, furthering the need for comprehensive treatment for AKI.

By observing the recurrent 'fibrous' eye shapes, two conclusions were deducted: first, that the kidneys regulate a multitude of processes throughout the body, reinforcing the importance of integrative strategies while treating diseases such as CAD, and second, that studies on diseases characterized by drastic increases in MITF (such as melanomas and chronic eye diseases) could refocus research on possible underlying renal complications and bring about advancements to both fields.

A shortcoming was due to the large sample size, combinations of the treatments were not feasible. Each of the four treatments were able to target a specific aspect of AKI effectively, however, combining them may produce better results. Synergistic reaction combining these treatments may be the path to effectively treat the pathways, so future research can include a matrix of different combinations.

Additionally, the ability to administer these treatments may be limited on a patient to patient basis. For example, Hepcidin cannot be administered in large quantities to patients who have iron deficiency, nor can IGF-1 be prescribed to diabetics due to the risk of hypoglycemia.

However, the current treatment, dialysis, is associated with nosocomial infections, ROS generation, and many other secondary issues. Nevertheless, dialysis is not effective in targeting fibrotic pathways because it only targets blood filtration. Additionally, it costs up to $72,000 annually to patients (28), and if CAD is subsequently developed, coronary artery bypass grafts costs between 70,000 to 120,00 dollars (29). All treatments utilized in this experiment, however, are readily accessible and cheap for translation into clinical applications for patients in both developing and developed countries.

These discoveries can function as catalysts, catapulting CAD research into renal fields. The results obtained may be transferred from bench to bedside insofar as the detection and utility of the therapy plans can fit into existent treatment structures yet produce more desirable results.

Methods

All procedures utilizing the three pollutants required a respirator and appropriate personal protection (proper shoes, safety goggles, gloves, and a lab coat). The pollutants were handled under a chemical fume hood to limit contact and inhalation. All trash generated was discarded in a chemical waste container in the Biohazard trash. All zebrafish embryos were used within the first 168 hpf while they are considered invertebrates. A total of 940 embryos were used; 6 per condition induced.

The pollutants were dissolved in sterile, distilled water. For dose titrations, embryos were collected, randomly divided into groups, and exposed to water containing pollutants at appropriate concentrations- 50 pbb of arsenic, 60 ppb of cadmium, and 35 pbb of aristolochic acid.

PT-Yellow was used to determine whether or not the pollutants induced AKI. 0.5 mL of PT-Yellow was added to 1.5 ml of embryo medium. After taking pictures with a fluorescent compound microscope equipped with a digital camera, images

were input into ImageJ to quantify integrated density and area. Corrected Total Cell Fluorescence (CTCF) was determined by the following equation: CTCF=Integrated Density - (Area of selected cell X Mean fluorescence of background readings).

Nitrotyrosine detection started at 48 hpf. For the ROS assay, 5 ml of ROS-detection solution was prepared by dissolving a generic ROS-sensitive probe stock solution in Hank's Balanced Salt Solution. Embryos were incubated in the dark for 15 minutes at 28°C. At the end of the incubation time, the solution was removed and washed twice with 2 ml of Hank's Balanced Salt Solution. Embryos were transferred to a black well plate and put into a luminometer.

To prepare a working solution of biotinylated IGF-1, streptavidin needed to be bound to this biotinylated growth factor. The compound was mixed with streptavidin in a 1:1 molar ratio and incubated for one hour in order for complete binding at each site. For SB431542, 0.0019M, 0.003 M, and 0.004M was isolated and added to 1.5 ml of zebrafish medium. For the next treatment, Hepcidin, 0.056M was added to 1.5 ml of zebrafish medium. The positive control, IV fluid had 0.045M and 0.09 M were added to 1.5 ml of zebrafish medium. The treatment stage started at 48 hpf as the kidneys start to form at 40 hpf.

For treated embryos, fixation started at 50 hpf, whereas for control samples, this process started at 48 hpf to account for treatment administration time periods for the fixation process, every 3 embryos were fixed with 4% paraformaldehyde and PBS. After leaving them overnight at 4°C, they were washed twice in PBS, 5 min each, at room temperature. Embryos were transferred to vials with 100% methanol and then cooled to -20°C for 30 minutes. After bringing them back to room temperature, they were immersed for 5 minutes in 50% methanol in phosphate buffered saline with Tween (PBST), and then 5 min in 30% methanol in PBST. The embryos were rinsed twice in PBST for another 5 min, and fixed for 20 min in 4% paraformaldehyde in phosphate buffered saline. A final rinse of PBST was administered twice for 5 minutes each.

After primary fixation protocols, a Picro Mallory Trichrome Stain was used to detect fibrosis by marking COL1A1 presence in samples. Embryos were placed on glass slides and submerged in saturated picric acid solution overnight. A wash with tap water (3 changes of running water for 2 minutes), then distilled water for 30 - 60 seconds was conducted. Then, Picro Mallory Solution A stained the embryos for 2 minutes. Excess dye solution was removed and slides were transferred drectly into Picro Mallory Solution B for 30 minutes. Slides were washed with 0.5% glacial acetic acid, and were subsequently dehydrated with 100% alcohol. To quantify the fibrosis levels, ImageJ was used with the RGB plugin (30).

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Article

The Effects of Altered Microbiome on Caenorhabditis elegans Egg Laying Behavior

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SUMMARY

Studies suggest the importance of the gut microbiome in human health and disease. However, the human microbiome is complex, and simpler models are needed to better understand the interaction between gut microbiota and the body. We developed a simple microbiome model system using the worm *Caenorhabditis elegans* (*C. elegans*). We hypothesized that if wild-type *C. elegans* feeds on bacteria other than lab standard *E. coli* OP50, there will be a change in its gut microbiome, thus influencing egg retention behavior. Compared with the control, the worms fed *Comamonas* bacteria retained more eggs than the worms fed *Bacillus* ($p < 0.001$). Therefore, when wild-type *C. elegans* is grown on varying bacteria, a change in gut microbiome may explain the differences noted in egg retention behavior. The *C. elegans* model created in this study is a simple representation of the more complex human-microbiota interaction occurring in our bodies. An interesting application of this model is finding out to what extent host response to various medications is affected by the microbiome and whether this can be used to guide a personalized approach to treating diseases.

INTRODUCTION

Caenorhabditis elegans (*C. elegans*) is a small and clear roundworm with a short lifespan. In nature, it is found in soil and rotting fruits and vegetables where it feeds on bacteria (1). Due to its simplicity, fast development, and sharing 40% genetic similarity with humans, *C. elegans* has served as a simple model system for various diseases found in humans (1). The human gut microbiome, a collection of microorganisms that live in our intestines, has in recent years been a focus of much research due to its potential role in our health, development, and tendency to get ill. Microbes in a healthy human adult are estimated to outnumber human cells by a ratio of 10:1, and the total number of genes in the microbiome exceeds the number of genes in the human genome by a factor of at least 200 (2). It is possible that gut microbiota can also influence the response to medications such as cancer therapies, and this may be the next frontier to be explored in humans when developing more effective cancer treatments (3). The human microbiome and its interaction with the host is very complex and much more research is needed in this arena (4, 5).

C. elegans and its simple bacterial diet provides a useful model to study host-bacteria interactions in a more controlled fashion. The type of bacteria eaten by the worm may change its rate of growth, as well as the ability to resist environmental stressors such as osmotic stress, and high or low temperatures (6). The microbes ingested by this worm appear to lead to a variety of effects that are beneficial to the worm apart from their role as mere food (7). It appears that live, metabolically active bacteria are needed to achieve the positive health benefits for the worm. Fluorescently tagged bacteria that were eaten by the worm have been visualized colonizing the gut and staying in the intestine to form the worm's microbiome (8). Most experiments with *C. elegans* do not take into consideration that a diverse worm microbiome may impact worm responses to experimental stimuli. More needs to be known about the effect of worm microbiota on its health and behavior.

C. elegans are self-fertilizing hermaphroditic worms. During its life cycle, *C. elegans* passes from egg stage through four larval stages before reaching adulthood, all within two to four days. One worm can, on average, produce 300 offspring over a 3-day period (4-10 eggs laid per hour). At any point in time, usually 10-15 fertilized eggs are retained within the uterus for several hours before being laid. The number of eggs in the uterus is a function of both the rate of egg production and the rate of egg laying. An intact motor and neural circuit is necessary for the egg laying process to be successful (8).

The egg laying behavior of *C. elegans* is influenced by a variety of factors including environmental stressors such as overcrowding, availability of food, temperature, and availability of sperm. If the environment is not favorable for egg laying—for example, there is not enough food or the food is harmful—the worms retain the eggs longer until the environment becomes more favorable (1). Egg retention (also called the egg in worm assay) was chosen for this study because it is a relatively easy assay to observe in the lab and results in a large sample size over a short period of time.

The purpose of this study was to better understand the host-microbiome interaction by developing a simple microbiome model system to determine the impact of different gut microbiota on *C. elegans* behavior. We hypothesized that if wild-type *C. elegans* is allowed to grow on bacteria other than the standard strain of *E coli* (OP50) normally used in labs, there would be a change in the worm's gut microbiome that could affect egg retention behavior. While altering the *C. elegans* gut microbiome by allowing it to feed and develop on three different strains of bacteria, we observed worms' egg retention capacity. Our study showed that, indeed, egg retention of the worms differed according to the bacteria they were raised on and presumably incorporated in their gut.

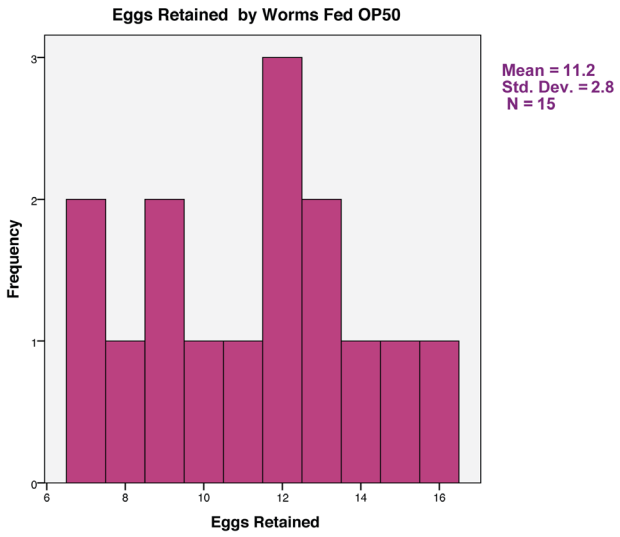

Figure 1. Number of eggs retained by worms fed OP50, using the egg retention assay. The *y*-axis indicates the number of worms that retained eggs at each specified count. The mean number of eggs retained by worms fed control *E. coli* was 11.2 ± 2.8 eggs.

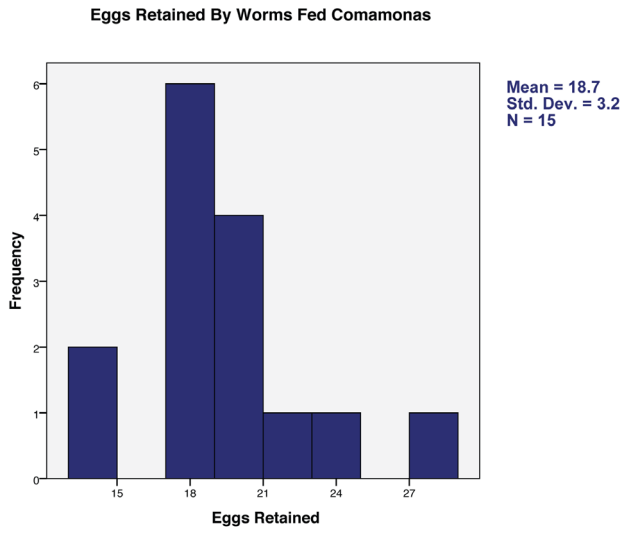

Figure 2. Number of eggs retained by worms fed *Comamonas* sp., using the egg retention assay. The *y*-axis indicates the number of worms that retained eggs at each specified count. The mean number of eggs retained by worms fed *Comamonas* sp. was 18.7 ± 3.2 eggs.

RESULTS

The bacteria chosen in this experiment have been shown to alter the gut microbiome and are non-toxic to *C. elegans* worms (9). We chose *E. coli* OP50 as the control bacteria since *C. elegans* worms are traditionally fed this bacteria in the lab. The two experimental bacteria used in this study are found on rotting fruits and vegetables in nature where they are eaten by *C. elegans*; thus they were good choices for further exploration (9). The experimental bacteria are different enough from *E. coli* OP50 that they likely would have an effect on the microbiome and egg retention behavior, but not so different as to be lethal to the worms (9). It was important to select bacteria that would not be lethal to *C. elegans*. If the chosen bacteria were toxic food sources, then any changes noted in egg retention may have been due to their lethality rather than the potential impact of the bacteria on their microbiome.

E. Coli OP50	*Comamonas*	*Bacillus*
15	14	11
9	18	5
14	27	10
11	21	11
12	19	8
12	18	7
8	18	6
7	19	9
13	19	2
16	17	6
7	19	10
13	18	14
9	14	13
10	17	6
12	23	9

Table 1. Number of eggs retained by C. elegans grown on three different bacterial strains

Summary Statistics	*E. coli* OP50 N = 15	*Comamonas* N = 15	*Bacillus* N = 15
Mean ± SD	11.2 ± 2.8	18.7 ± 3.2	8.5 ± 3.2
Mode	12	18	6
Median	12	18	9
Min-Max	7-16	14-27	2-14

Table 2. Summary statistics for egg retention by worms grown on three different bacterial strains.

The constant variables in this study included the amount of agar in the Petri dish, the age of the worm, the temperature at which the worms were maintained, the amount of time exposed to the type of bacterial lawn), and the strain of *C elegans* (wild-type). The independent variable was the type of bacterial lawn the worms were allowed to feed on (*E. coli* OP-50, *Bacillus megaterium*, or *Comamonas* sp., DA1877) The dependent variable studied was the egg retention of adult worms. The control group consisted of worms that were fed and raised on the standard laboratory *E. coli* OP50. Fifteen worms were studied per bacterial strain. Worms were allowed to grow on their respective bacterial strains from egg stage until adulthood.

Table 1 shows the number of eggs retained by bacterial strain for each worm trial. We also calculated statistics

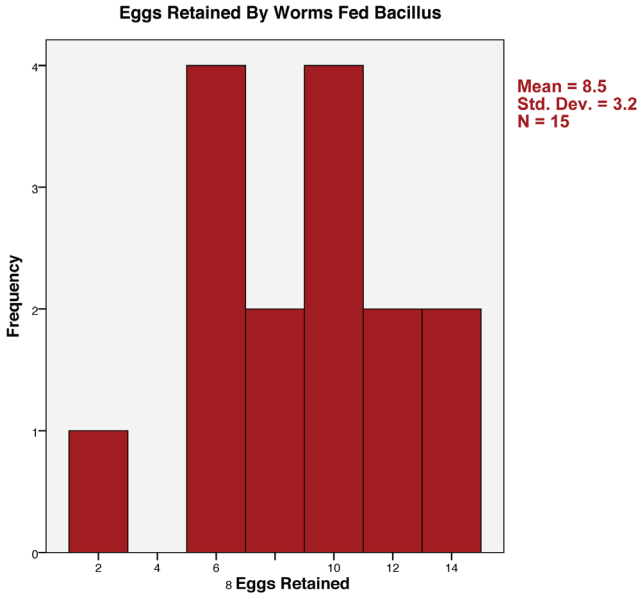

Figure 3. Number of eggs retained by worms fed *Bacillus*, using the egg retention assay. The *y*-axis indicates the number of worms that retained eggs at each specified count. The mean number of eggs retained by worms fed *Bacillus* was 18.7 ± 3.2 eggs.

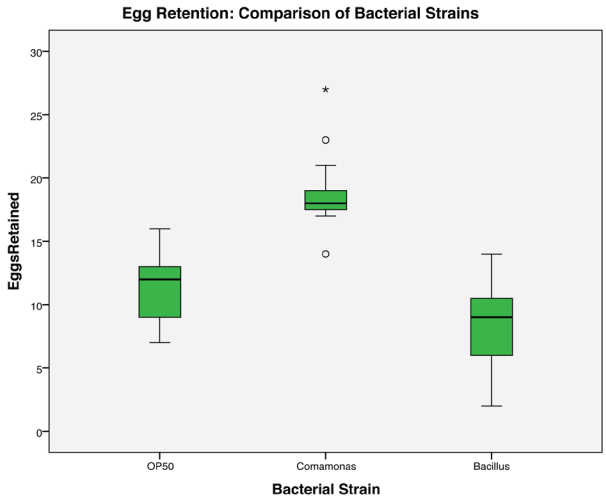

Figure 4. Comparison of eggs retained by worms grown on three different bacterial strains. *E. coli* OP50 was the control bacteria. Experimental bacteria were compared to *E. coli* OP50. A three-way comparison was made using one-way ANOVA. There was a statistically significant difference in the mean number of eggs retained when comparing *Comamonas* and *Bacillus* to *E. coli* OP50 (p <0.001). Worms that grew on *Comamonas*, sp, retained more eggs than the control, and those grown on Bacillus retained less eggs than the control.

including the mean, median, mode, and standard deviation of egg retention for each bacteria strain (**Table 2**). The mean number of eggs retained by worms fed control *E. coli* was 11.2 ± 2.8 (**Figure 1**), the mean eggs retained by worms fed *Comamonas* sp. was 18.7 ± 3.2 (**Figure 2**), and the mean eggs retained by worms fed *Bacillus megaterium* was 8.5 ± 3.2 eggs (**Figure 3**). Worms grown on *Comamonas* sp retained more eggs and worms grown on *Bacillus megaterium* retained fewer eggs than *E. coli* OP50, both statistically significant differences as measured by *t*-test (p < 0.001; **Figure 4**).

DISCUSSION

The study demonstrated that when *C. elegans* worms were allowed to feed and develop on different bacterial cultures, there was a change in egg retention behavior. In previous research on *C. elegans*, fluorescently tagged bacteria eaten by the worm were visualized within the transparent body as colonizing the gut (8). There are far more bacterial cells in *C. elegans* then worm somatic cells, a relationship similar to what is seen in humans. In addition, worms that are fed dead bacteria or nutrients that contains no bacteria have shorter lifespans (8). Together, these findings suggest that bacteria are more than just a food source for the worm and indeed need to be metabolically active for the worm to thrive, indicating an important interaction between the worm host and its bacteria.

Usually 10-15 eggs are retained in the worm at any given point in time. In our study, worms that were raised on *E. coli* OP50 fell into this range. However, worms that grew up on *Comamonas*, sp. retained significantly more eggs than the expected, and worms that grew up on *Bacillus* retained fewer. The worms were raised on the bacteria since they were eggs, thus controlling the length of exposure to the bacteria and making it highly likely that the only gut microbiome present was what they ate (*i.e.* the bacteria on the plate). The environmental stressors were kept constant for all worms to prevent bias in egg retention capacity.

Although this experiment shows that changes in bacterial exposure, and in all likelihood worm microbiome, impacts worm behavior, the study was not designed to analyze the worm microbiome or determine whether these differences lead to changes in offspring viability and longevity. It is possible that the experimental bacteria cause differences in egg production rate or egg laying rate, either of which could lead to the differences seen in egg retention. The exact mechanism by which the microbiome affects worm egg retention requires further study.

The host-microbiome model created in this study is a simple representation of the more complex human-microbiota interaction occurring in our bodies. Observing the impact of different bacteria on host behavior, as accomplished in this study, is the first step in understanding the pathways and genes used by bacterial cells in influencing host cells. Eventually studies are needed to find ways to change these pathways to control or treat disease. These studies need to first occur in animal models before they can be applied to humans. In humans, the gut microbiome may play a role in diseases such as asthma, inflammatory bowel disease, obesity, diabetes, and even anxiety (2). Gut microbiota may also play a role in changing individual responses to treatment. For example, some human studies have shown that individual variations in response to certain lipid-lowering drugs may

be due to the role that person's gut microbiome plays in the metabolism of the drug (4). Interestingly, early studies on the *C. elegans* microbiome suggest that different species of bacteria in *C. elegans* impact its response to cancer drugs, with some cancer drugs being more effective than others depending on the type of bacteria that resides in the worm's gut (3). An interesting application of this host-microbiome model is finding out to what extent host response to various medications is affected by the microbiome and whether this can be used to guide a personalized and more precise approach to treating disease in humans. In the future, it may be possible to alter the gut microbiota to enhance response to therapies or boost the body's own immune system.

When *C. elegans* feeds and grows on different bacteria, there is likely a change in its gut microbiome. We observed differences in egg retention behavior depending on what bacteria the worms were allowed to feed and grow on. Sophisticated gene sequencing analyses are needed to better outline host-microbiome interactions and determine the mechanism underlying our observations noted in this study.

MATERIALS AND METHODS

Worm Maintenance

3 cm nematode growth media (NGM) plates for worm growth were prepared by pouring NGM onto sterile petri dishes and allowing them to cool and set over 24 hours. *Escherichia coli* OP50 and the other two experimental bacteria (*Bacillus megaterium* and *Comamonas* sp.) were cultured and allowed to grow overnight at 37°C. A sterile pipette was used to seed each NGM plate with 100µl of bacterial culture and the bacteria were allowed to grow on the plates for 24 hours. Worms were placed on the plates and allowed to develop and lay eggs.

Age Synchronization

Ten young, fertile adult worms were picked using a worm pick and transferred to NGM plates freshly seeded with *E. coli* OP50 or experimental bacteria. The worms were allowed to lay eggs for 60–90 min. The parents were removed and the plates were incubated at 20 °C for 48 h to obtain L4 larvae.

Egg Retention (Egg in Worm Assay)

A 20% bleach solution was prepared, then a 10 µl drop of bleach solution was added to fifteen distinct locations on a 96 well plate. Fifteen age-synchronized adult worms from each experimental and bacterial lawn were picked, washed with M9 Buffer, and placed on a clean agar plate containing no bacteria. Then the worms were transferred into each bleach drop, one worm per well. The worm cuticle was allowed to dissolve for 10 min or until the worm burst open, expelling the eggs. The eggs were then counted under a dissecting microscope and the results were recorded. [Protocol adopted from Gardner, *et. al.* (10)]

Data Analysis and Statistics

Statistical software, SPSS version 12 was used to compare the egg retention of experimental bacterial strains with control, using the student *t*-test. A three-way comparison was made using one-way ANOVA. For all comparisons, p-value < 0.05 was considered statistically significant.

Safety Measures and Risk Assessment

All standard lab safety measures were followed, including the use of protective eyewear and safety aprons, and gloves. Sterile techniques were used when handling the bacteria. All bacteria used were BSL-1 bacteria. There were no other risks associated with this study.

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Article

Modulation of planaria regeneration by Resolvin D1 and the omega-3 fatty acid precursor 17-hydroxy docosahexaenoic acid

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SUMMARY

Omega-3 fatty acids (FA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) have long been consumed as medical supplements for their far-reaching health benefits, ranging from alleviating asthma symptoms to mitigating kidney and inflammatory diseases like colitis and diabetes. These fatty acids produce a variety of lipid mediator metabolites including maresins, protectins, and resolvins, and are essential for aiding the resolution pathways in inflammation. However, their roles in regenerative responses are relatively unknown. We decided to investigate the effect of Resolvin D1 (RvD1) in *Dugesia dorotocephala* regeneration, as RvD1 is the most widely studied lipid mediator. We found that regenerating planaria released significantly higher amounts of RvD1 in water than control and that *D. dorotocephala* could synthesize their own RvD1 from the omega-3 FA precursor 17-hydroxy docosahexaenoic acid (17-HDHA). We also observed that both RvD1 and 17-HDHA enhanced regeneration responses in planaria by using eye scoring and a modified cell metabolism assay (MTT). We conclude that planarians may utilize similar biosynthetic pathways to mammals in producing resolvins and that resolvins hold immense potential in enhancing regenerative responses in tissues.

INTRODUCTION

Omega-3 fatty acids (FA) are essential for regulating many homeostatic bodily functions such as the contraction of arterial walls, hormonal regulation, controlling anti-inflammatory responses in chronic ailments such as arthritis and asthma, and modulating many homeostatic responses (1). Humans rely on procuring these fatty acids from their diets because humans are unable to synthesize these omega-3 fatty acids (2).

Chronic deregulated inflammation is the causal factor for some of the most common human diseases such as asthma, colitis, arthritis, diabetes. In the last decade, numerous mediators formed through the metabolism of omega-3 fatty acids have been implicated in the resolution of inflammation (3). Maresins are a specific type of lipid mediator formed through the metabolism of the DHA (docosahexaenoic acid) branch of omega-3 fatty acids. In 2009, it was found that maresins are a class of potent, anti-inflammatory mediators that enhance the resolution phase of inflammation in a mouse model of peritonitis (4). This was the first time that maresins and other similarly structured lipid mediators were shown to play a key role in orchestrating tissue homeostasis, inflammation resolution, wound healing, and host defense (4). A study observing the effects of maresins on regeneration in planaria found that maresins enhanced or sped up regeneration in planarians after surgical incision and reduced "neuropathic pain" in mice (5). In planaria specifically, the study used immunofluorescence to quantify the amount of regeneration occurring at the severed ends of the cut planaria (5). This is the only published evidence of a role for omega-3 fatty acid-derived lipid mediator in planarian regeneration.

RvD1 and its precursor 17-hydroxy docosahexaenoic acid (17-HDHA) are a sister branch to the maresin lipid mediator and are also derived from DHA (6). A study in 2013 found that the external administration of resolvins does not disrupt homeostasis. Rather, it resolves acute inflammatory pain and chronic arthritis (7). In other words, in addition to inhibiting or blocking inflammation, resolvins incite the resolution of inflammation and promote the return to homeostasis (3). To our knowledge, no published study observing the effects of RvD1 and its precursor, 17-HDHA, on planaria exists.

Planaria are a unique model for studying regeneration because they are able to regenerate fully even when large portions of their body are removed, and they fully regenerate within 7 to 21 days. Planaria regenerate new tissue around a wound site, form blastema through epimorphosis and repair existing tissue through morphallaxis (8). New technologies, such as RNA interference, have been used to study the planarian stem cells and genome on a molecular level, which has revealed profound similarities between several parts of the planarian genome and genes that cause diseases in humans (9). Planaria's rare regenerative abilities are dependent on their large population of somatic stem cells. Both planaria and vertebrates share the functionally conserved Wnt/β-catenin and BMP signal transduction pathways for axis polarity, presenting an opportunity to research pluripotent stem cells in planaria to better understand mammalian disease and development and to better analyze relevant molecular processes in humans (9). Further research on planarian's highly-flexible central nervous system (CNS) may also reveal the machinery involved in activating axon regeneration and circuitry reformation in planaria, which may be critical in understanding activation of regeneration in mammals (9).

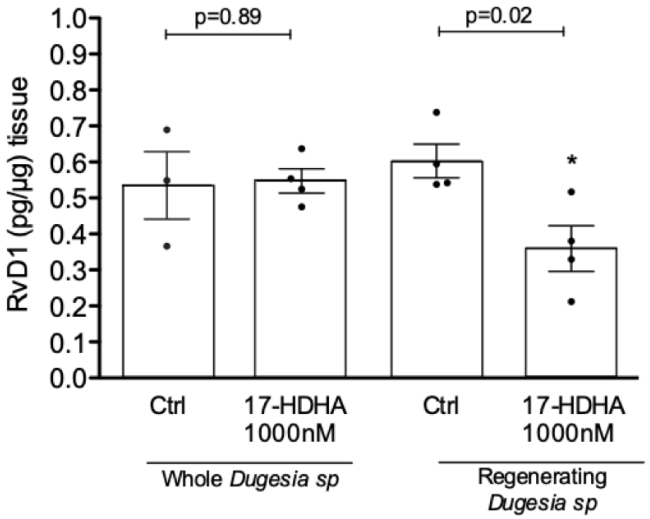

Figure 1. Tissue RvD1 levels in whole versus regenerating planaria. Planaria lysates in PBS were analyzed 3.5 days post-surgery. Each dot represents 2 planaria. Regenerating group represents 2 planaria (2 heads and 2 tails). Data is normalized to µg proteins per sample. Mean ± SEM. Student's t- test was performed between control (Ctrl) and experimental groups. *p<=0.05.

In this study, we measured planarian's regenerative responses with supplementation of RvD1 and 17-HDHA using an eye scoring index and a modified MTT assay. Additionally, we wanted to investigate if planarians could synthesize RvD1 from 17-HDHA. In this study, we show that planarians have the biosynthetic machinery to produce RvD1 from 17-HDHA. and that plameria might be able to respond to RvD1 if given exogenously.

RESULTS

Tissue and water RvD1 levels in regenerating planaria

We first investigated if *Dugesia dorotocephala* can biosynthesize RvD1 from the omega-3 fatty acid precursor 17-HDHA. Secondly, we also looked at short-term and long-term RvD1 production by *D. dorotocephala* incubated with 17-HDHA. Concentrations for RvD1 were chosen between .001 nM and 100 nM because effects of RvD1 are typically observed at nanomolar concentrations (1). Since RvD1 is formed from 17-HDHA, we used a 10 to 100 times higher concentration for 17-HDHA.

RvD1 from water samples harvested from planarian environment exhibited a significant increase in regenerating planaria compared to whole planaria incubated in 17-HDHA for 3.5 days (student's t-test, p=0.0002). While both control groups (whole & cut planaria) exhibited the same baseline RvD1 level of 0.0975 pg/mL (SEM: 0.002), 17-HDHA supplementation resulted in an increase in RvD1 production of up to 2.900 pg/mL (SEM +/- 2.185) in whole planaria and up to 14.28 pg/mL (SEM +/- 1.789) in regenerating planaria (**Figure 2**).

Tissue RvD1 levels exhibited no significant difference between planaria incubated in ethanol control or 17-HDHA for 3.5 days, when normalized to protein levels. However, a significant decrease in tissue RvD1 levels was observed in regenerating planarian tissue, where tissue RvD1 levels were 0.35 pg/µg protein (SEM +/- 0.06) in 17-HDHA supplemented planarians compared to 0.60pg/µg protein (SEM +/- 0.04) in ethanol-supplemented planarians (**Figure 1**).

17-HDHA time and dose response in planarian RvD1 production

We next studied the effect of increasing the 17-HDHA dose by 10-fold (10000 nM or 10 µM) and measured RvD1 production in water at both a shorter time point (2 days) and a longer time point (8 days). After 2 days, the concentration of RvD1 in the water of planaria incubated with 1 uM 17-HDHA was 1.45 pg/mL (SEM +/- 1.150), while planaria incubated in 10 µM of 17-HDHA did not produce detectable RvD1. A significant increase in RvD1 production was observed, however, after 8 days of incubation. Planaria incubated in 1 µM of 17-HDHA produced 45.53 pg/mL (SEM +/- 3.535) of RvD1 (student's t-test, p<0.05) , while planaria incubated in 10 µM of 17-HDHA produced 56.57 pg/mL (SEM +/- 11.63) of RvD1 (**Figure 3**).

RvD1 dose response measured through planarian eye scoring index.

An eye scoring index was created to categorize planarian regeneration into five stages based on the formation of photoreceptors in planaria tails post-surgery (**Figure 4**). Previous studies showed that optic regrowth and functional recovery were consistent despite injury type or differences in metabolic rate due to starvation (10). Therefore, eye scoring was utilized as an independent surrogate marker of

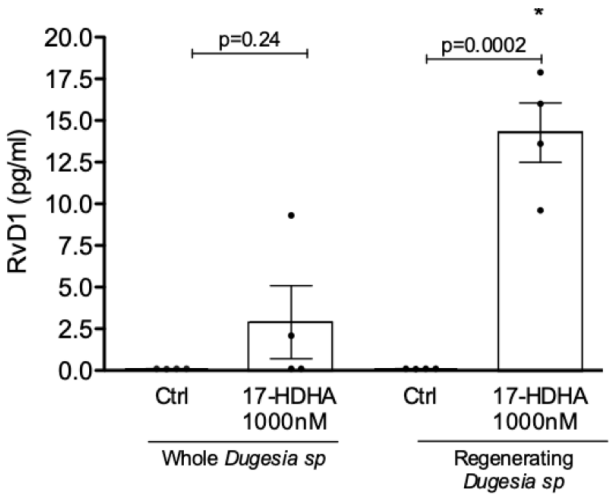

Figure 2. Water RvD1 levels in whole versus regenerating planaria. Water RvD1 levels in whole vs. regenerating planaria. RvD1 levels in water (planaria habitat, 500 µl) were analyzed 3.5 days post-surgery. Each dot represents 2 planaria. Regenerating group represents 2 planaria (2 heads and 2 tails). Data is mean ± SEM. Student's t- test was performed between Ctrl and experimental groups. *p<=0.05.

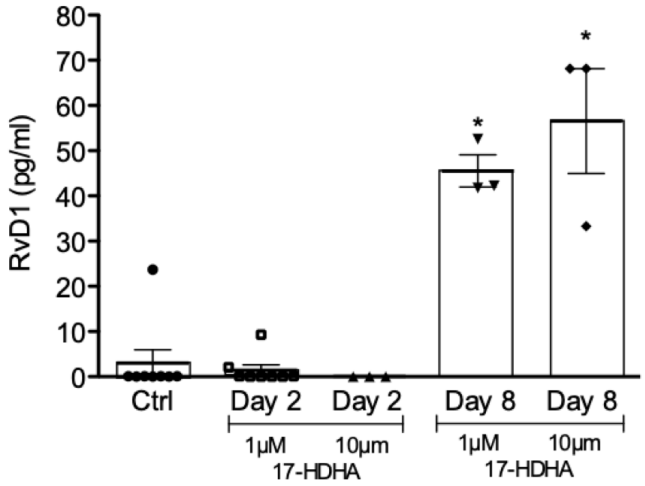

Figure 3. Water RvD1 levels in whole planaria 2- and 8-days post incubation with 17-HDHA. Water samples from planaria habitat water (1000 µl) were analyzed 2 days and 8 days post-incubation with 1 or 10 µM 17-HDHA. Each dot represents 1 planaria. Regenerating group represents 2 planaria (2 heads and 2 tails). Data is mean ± SEM. Student's t-test was performed between Ctrl and experimental groups. *p<=0.05.

regeneration, and we additionally scored the planarians' eyes that included several stages of complete eye formation.

The eye scoring index was developed to study the planarians' regeneration response to three dosages of RvD1 (0.01 nM, 0.1 nM, and 1.0 nM) at both a shorter time point (2 days) and a longer time point (8 days). After 2 days, the eye score of planaria incubated in 0.01 nM of RvD1 was 0.3333 (SEM +/- 0.3333), while planaria incubated in 0.1 nM and 1 nM of RvD1 did not show detectable eye formation. After 8 days of incubation, increased development of photoreceptors in planaria tails were observed. Planaria incubated in 0.01 nM of RvD1 scored on average 1.750 (SEM +/- 0.3660), whereas planaria incubated in 0.1 nM and 1 nM of RvD1 had a mean score of 2.143 (SEM +/- 0.4592) and 1.429 (SEM +/- 0.4286), respectively, on the eye scoring index (**Figure 5**).

Comparison of planaria response to 17-HDHA and RvD1 using planarian eye scoring index

To measure the reliance of regeneration on type of lipid mediator (17-HDHA or RvD1) and the dose dependency of each drug, an experiment was conducted using the eye scoring index on the regeneration of planaria after incubation in control or 17-HDHA or RvD1 for 4 days.

The planarian eye scoring index was used to compare planarians' regeneration response to 1 µM of 17-HDHA and two dosages of RvD1 (0.01nM and 0.1nM). After 3.5 days of incubation, the eye score of planaria incubated in 1 µM of 17-HDHA was 2.583 (SEM +/- 0.1353), while the eye scores of planaria incubated in 0.01 nM and 0.1 nM of RvD1 were 2.167 (SEM +/- 0.2161) and 2.938 (SEM +/- 0.1133), respectively, on the eye scoring index (**Figure 5**). All three eye scores were greater than the eye score of the control group (ethanol-supplemented planaria), which was 2.083 (SEM +/- 0.2117) on the planarian eye scoring index.

Using a modification of the MTT assay to quantify planarian regeneration

The planarian eye scoring index provides a semi-quantitative measure of planarian regeneration. We next investigated whether a modification of the MTT assay could be used to analyze planarian regeneration in a quantitative way. The MTT Assay is a cell metabolism assay that uses the crystallization of the violet MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to measure cell viability. When read through a plate reader, a higher optical density (OD) correlates to a higher concentration of violet colored formazan crystals, indicating greater cell proliferation.

The MTT assay was first used to study whole planaria of different sizes (small, medium, and large). After 4 hours of incubation in MTT, whole planaria demonstrated a mean increase of 0.2768 in optical density, compared to control. The optical densities of small, medium, and large planaria were 0.534, 0.561, and 0.592, respectively (**Figure 6D**).

We next hypothesized that the presence of more regenerating surfaces would result in more formazan crystal formation, producing greater optical densities. The MTT assay was used to analyze various quantities of planaria stubs (laterally-cut segments of the planarian body with two regenerating surfaces) (**Figure 6A-C**). After 4 hours of incubation in MTT, regenerating planaria stubs demonstrated a mean increase of 0.0318 in optical density compared to control; the optical densities for 1 stub, 2 stubs, 3 stubs, and 4 stubs of planaria were 0.290, 0.347, 0.394, and 0.368, respectively (**Figure 6D**).

We then used the MTT assay to study regenerating planaria (incubated in spring water) over a time course. Planaria were assayed at 0 days, 2 days, 5 days, and 7 days post-surgery. The optical density of regenerating planaria was initially 243.85 (SEM: 4.41) 0 days after surgery. Optical density peaked at 314.60 (SEM +/- 42.46) 3 days post-surgery before returning to 270.87 (SEM +/- 27.31) 5 days

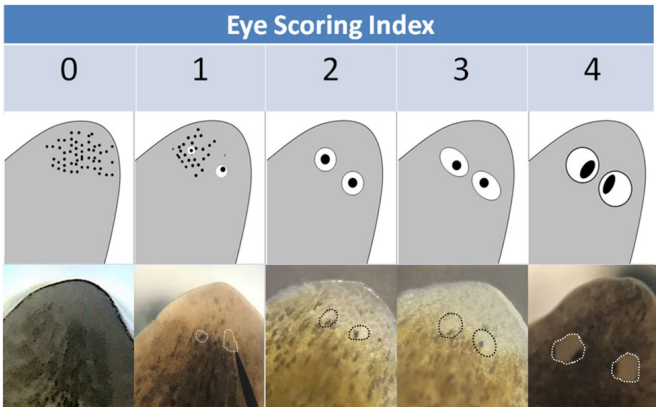

Figure 4. Planaria Eye Scoring Index. Representative images of score levels. Eye borders are demarcated with white dotted lines. Score 1 (planaria image) shows a pointer for one of the eyes.

post-surgery. At 7 days post-surgery, the optical density of regenerating planaria was 285.08 (SEM+/- 31.79) (**Figure 6E**).

Finally, we used the MTT assay to investigate planarian regeneration in response to 1 nM of RvD1. Optical densities of planaria incubated in RvD1 were normalized to baseline. The optical density of planaria incubated in RvD1 started at 1.428 (SEM +/- 0.5720) 0 days after surgery. Optical density decreased to 0.6601 (SEM +/- 0.1875) 2 days after surgery before reaching a peak optical density of 1.841 (SEM +/- 0.7827) 5 days post-surgery. The optical density of planaria incubated in RvD1 was 1.225 (SEM+/- 0.2761) 7 days after surgery. Planaria incubated in RvD1 had a net higher optical density across the 7-day time course. However, optical density of planaria incubated in RvD1 were 0.3399 and 0.0438 less than the optical densities of control planaria at 2 days and 3 days post-surgery (**Figure 6F**).

DISCUSSION

Few organs in the adult human body can fully regenerate after injury or disease, so regenerative medicine relies on the study of other species, such as planaria, to understand the signals that activate their healing processes (11). Planaria are often studied as a model regenerative organism because they are capable of regenerating large portions of their bodies from a small fragment to almost full recovery after injury or disease; they can also regenerate through spontaneous fission (8). Omega-3 fatty acids have been widely studied for their anti-inflammatory and pro-resolution properties, but little is known about omega-3 fatty acids in the context of regeneration. This study was aimed to investigate the possible role of omega-3 fatty acids in planarian regeneration, specifically RvD1 and its precursor 17-HDHA in *Dugesia dorotocephala*.

In this study, we observed an increase in RvD1 levels in planarian water samples in whole (unwounded) planaria incubated in 17-HDHA, but no change in tissue RvD1 levels between control and 17-HDHA-supplemented group was observed. This finding is exciting because this is the first time that *D. dorotocephala* are shown to have the biosynthetic pathways to synthesize RvD1 from 17-HDHA. In mammals, it is hypothesized that 17-HDHA is converted into RvD1 through enzymatic epoxidation by lipoxygenases. Therefore, further investigation is warranted to delineate the exact biosynthetic machinery in *D. dorotocephala* resolvin production. It is also likely that a variety of other lipid mediators (besides RvD1) are also biosynthesized by planaria through DHA and 17-HDHA, so a mass spectrometry-based approach could reveal other lipid mediators that are critical in orchestrating planarian regeneration. For example, we did not measure the production of Resolvin D2 (RvD2), so it remains to be seen if planaria can also produce RvD2 when supplemented with 17-HDHA. Moreover, further studies can investigate RvD1 production in planaria when supplemented with DHA, as DHA are one step upstream of 17-HDHA and are the earliest omega-3 precursor.

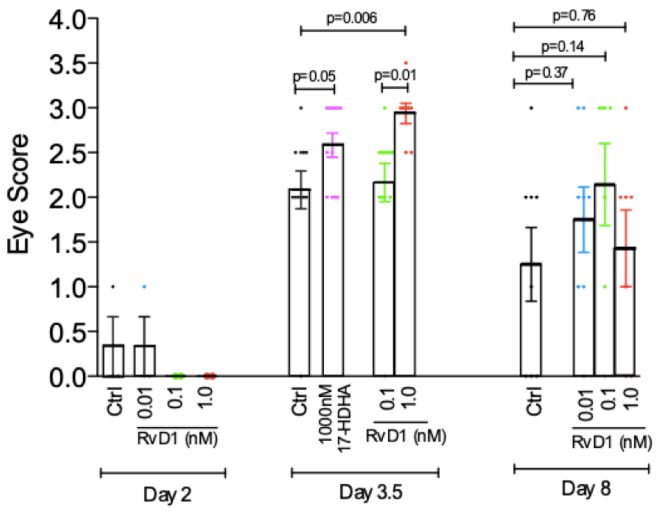

Figure 5. Resolvin D1 dose-response on planarian regeneration. Planarian regeneration was monitored and assayed using the eye scoring index (see Methods) in planarians 2, 3.5, and 8 days post-surgery. Each dot(n) represents one planaria; n≥7 for Day 8; n=12 for all groups of Day 3.5 except RvD1-1 nM where n=8; for Day 2, Ctrl n=3; 0.01 nM RvD1 n=3; RvD1 0.1 & 1 nM n=2. Data is mean ± SEM. Student's t-test was performed between the groups with shown *p*-value.

We observed that "regenerating" planarians had a higher release of RvD1 in the water compared to intact, unwounded planarians. This likely means that RvD1 biosynthesis is enhanced in regenerating planarians, suggesting that resolvins play a role in planarian regeneration. However, resolvins could also be a byproduct of an enzymatic upregulation that is not directly linked to planarian regeneration as planarians may not be exposed to 17-HDHA naturally. Additionally, we observed a decrease in RvD1 levels in planarian tissue samples, which showed increased RvD1 in surrounding water. This was surprising and could be the result of changes in RvD1 efflux or release from regenerating cells in the blastema. Investigating the precise pathway by which 17-HDHA and other lipid mediators diffuse through the planarian cells into their environment, how RvD1 is made and released into the environment, and which cells are responsible for RvD1 production, was beyond the scope of this study. Future studies requiring sophisticated microscopy and histological analyses can examine which genes, enzymes, and cell types are involved in resolvin biosynthesis in the planarian body after injury. Our results from the modified MTT assay indicated a high concentration of proliferating cells at the planarian blastemas, and future investigations could potentially explore resolvin production specifically within the planarian blastemas.

Irrespective of classifying the family or class of resolvins that could be produced by planarians (we only tested one species), one exciting future possibility is to alter regeneration in planarians that are exposed to water taken from DHA/17-HDHA-incubated planarians. This could further identify potential non-specific class effects of these molecules in

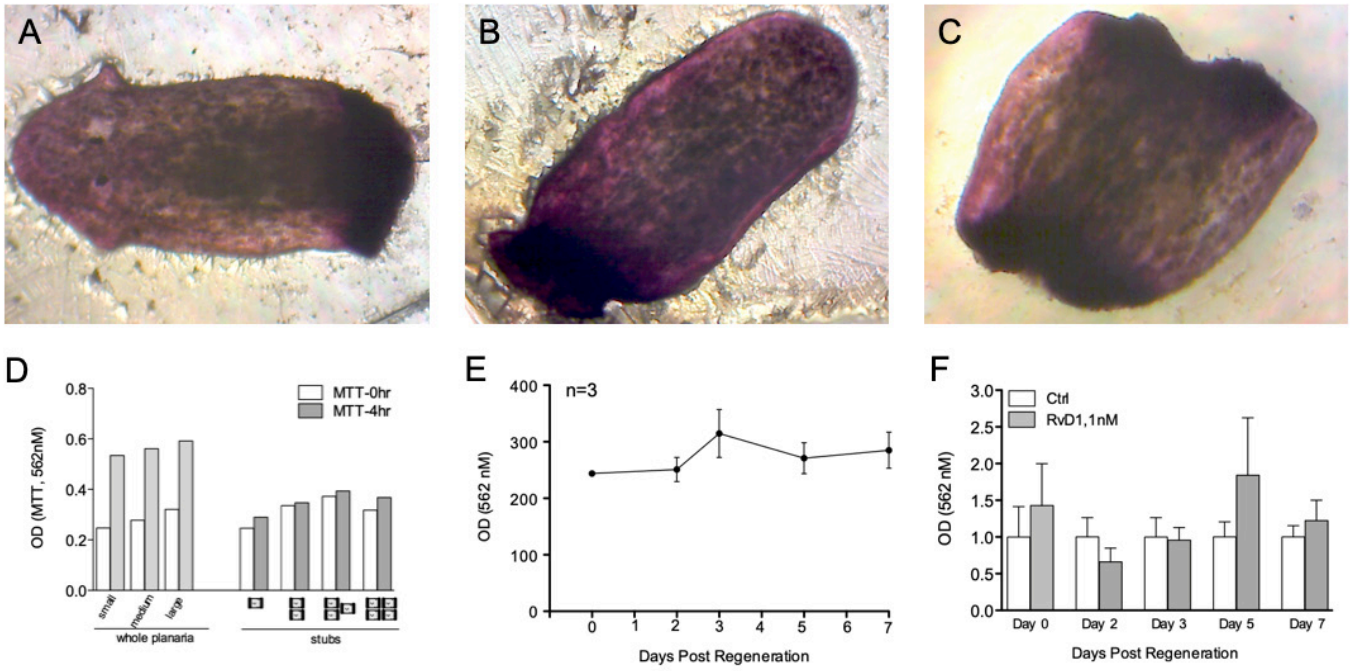

Figure 6. Planaria Regeneration assay based on a modified MTT method. A) Dissected planaria head after incubation in MTT for 4 hours; B) Planaria tail after incubation in MTT for 4 hours; C) Planaria mid-section stub after incubation in MTT for 4 hours; D) MTT assay readout (OD 562nm for formazan crystals) of different sizes of whole planaria and different number of planaria mid-section stubs after incubation in MTT for 4 hours, n=1 for each group; E) MTT assay readout (OD 562nm for formazan crystals) time course of regenerating planaria (head and tail) post-surgery ; n=3 per time-point. Data is Mean ± SEM. F) MTT assay readout (OD 562nm for formazan crystals) of planaria head and tails (each n = two heads and two tails) on Day 0 to Day 7 post-surgery. Day 0, n=9; Day 2, n=10; Day 3, n=10; Day 5, n=9; Day 7, n=13. Data is mean ± SEM.

regulating regeneration. In other words, autocrine regulation of mediators might influence planarian regeneration and could have implications in mammalian regeneration as well. Our study highlights an exciting possibility of mass production of bioactive lipids like resolvins from planarian. Currently, resolvins are produced by chemical synthesis. Just like having bacterial systems to produce biologics or antibodies for therapies, our system can be potentially improved for mass production of resolvins by planarians (regenerating). Planaria were successful in synthesizing RvD1 in this study, and further studies can be performed to confirm whether planaria can be used for the mass production of resolvin drug therapeutics.

In our studies, the eye scoring index was used for scoring planarian regeneration. Even though all eye scores in this investigation were all performed by one person, there is still a slight margin of variability in this assay, making it a moderately semi-quantitative approach. However, studying the time point of eye formation does provide an interesting benchmark to score planarian regeneration, and hence we used this to measure regeneration. One caveat of eye scoring assay is the time of the day used for observing eye formation. Due to barriers in experimental logistics, continuous monitoring of eyes could not be performed, hence the exact time-point of when eye formation reached our pre-developed scoring landmarks could not be identified precisely. Because of this disadvantage, we utilized a biochemical assay (MTT) to further shed light on regeneration index. The MTT assay was the empirically quantitative assay used in this investigation to measure regeneration index. While imaging planaria incubated in MTT, we observed that formazan crystals formed throughout the planarian body, more so during late stages of regeneration (5 and 7 days post-surgery) rather than at blastemas as previously observed (2,3, and 0 days post-surgery). Further investigation is needed to identify the exact cell types that are metabolically active in the planarian body post-surgery, which could be cells near the blastema or far away from the regenerating tissue. To our knowledge, we are the first to report a quantitative method of assaying planarian regeneration through MTT assay, which is widely used to analyze cell metabolism and toxicity in cancer biology. Further studies can also refine the modified MTT assay by titrating the MTT dilution (we used 1:10 according to the assay protocol), potentially examining the ideal concentrations, temperature, and environmental factors needed to enhance the sensitivity of the assay.

In a recent study, the investigators encapsulated aspirin-triggered RvD1 into a biodegradable biomaterial to investigate a model of sterile inflammation using local, sustained delivery of the drug (12). As expected, the scientists observed an increase in the accumulation of cell debris phagocytizing monocytes and macrophages, however they also observed a potent pro-angiogenic and vascular remodeling response of RvD1 in the tissue (12). This study provided a role of resolving

lipid mediators in differentiation and regeneration. While our study only observed oral ingestion/diffusion of 17-HDHA and RvD1 on planaria, future studies can examine the role of RvD1 in regenerative biology.

METHODS

Planaria care and dissection

Planaria species *Dugesia dorotocephala* was purchased from Carolina Biological Supply Company (Burlington, NC) and were placed in Arrowhead spring water (Nestle waters, North America). Planaria were fed egg yolk on a bi-weekly basis and water was exchanged every 3-4 days.

On the day of surgical dissection, a chosen number of planaria of equal or comparable size were first isolated into a separate container apart from the habitat. In all experiments, planaria were transported by plastic pipettes with blunt end of approximately 0.5 cm diameter. Planaria were dissected along their horizontal midline while free-swimming. We avoided dissecting them over cold surface, as they constricted from cold temperature making dissections difficult to perform with uniformity across the groups. Dissection was performed with a clean scalpel blade in one single strike/motion. After dissection, planaria heads were transferred back to a new habitat, while planaria tails were relocated to incubation in a 6 or 24-well plate in chosen control or experimental wells. For incubation of planaria in RvD1 or 17-HDHA-treated water in 6-well plates, we used approximately 500 µl – 4000 µl mineral water per well.

Protein Assay

We used the Bradford method, which uses Coomassie Dye for determining protein in planaria lysates. Planarians or regenerating planaria heads or tails were transferred into a glass dounce homogenizer, and lysates were prepared on ice using slow clockwise and anti-clockwise rotations. The lysates were then transferred to eppendorf tubes and saved at -20˚C until the day of use for assays. The Protein Determination kit was purchased from Cayman Chemicals (Ann Arbor, MI), and kit instructions were followed according to the company protocol. Protein Determination Assay reagent and Protein Determination BSA standard were prepared through serial dilution with UltraPure water. Protein BSA standard and the sample were first put into a 96- well plate before Assay Determination Reagent was pipetted into each well. The plate was incubated at room temperature for 5 minutes before measuring the absorbance at 595nm two times. Based on the standard values, a standard protein assay curve was plotted.

RvD1 ELISA

The RvD1 ELISA assay was used to measure RvD1 production in planaria tissue and water samples. The RvD1 Elisa kit was purchased from Cayman Chemicals, and was used according to company protocol. After defrosting at room temperature, ELISA buffer and the wash buffer were prepared by using UltraPure water to dilute (1X) reagents needed for the assay. A set of 9 standards were created through serial dilution where the first standard was 2000 pg/ml and the last standard contained 3.3 pg/ml of provided RvD1. Samples were thawed and spun down in a centrifuge at 4000 rpm (1800g). Standards and samples (50 µl) were transferred to 96-well plate with anti-serum and tracer, which was then covered in a film before incubating at 4˚C for 18 hours. After incubation, the plate was washed with wash buffer five times, incubated with Ellman's reagent for 90 min, and absorbance measured at 405 nm. The standard values were used to plot a standard RvD1 Assay Curve.

Eye scoring

Regenerating planarians were transferred one at a time (using a transfer pipet with a cut tip) onto a plastic surface. A droplet of water was provided for the planaria to swim freely and comfortably. Planarians were observed until a clear image of the eye was confirmed by the observer. In all the experiments, only the first author of this paper made the observations and scored the eye score to avoid any bias. Planaria eyes were scored on scale of 0-4 (**Figure 4**) based on the size and shape of the planarian eyes.

Modification of MTT assay

Planarians were first sorted according to lengths before grouping and dissection. In most experiments, similar sized planarians were used across the groups. In some preliminary experiments, groups were size-matched with equal number of large/mid-size/small planarians. Planaria were cut laterally right above the pharynx, and both head and tail portions were transferred to a 6-well plate using a cut transfer pipet and incubated in 4 mL of spring water or Resolvin D1. After an incubation period (either 0,2,3,5, or 7 days), 3.8 mL water (with/without RvD1) was aspirated out of the plate. The plate was tilted on a 30˚ angle and kept stable in the angle with planarians freely swimming in the 200 µl pool of water on the side of each well. Then, 20 µl of MTT solution was added in each well (1:10 dilution), and the planarians were incubated for 4 hours. Following the incubation, planaria were visually observed under a stereo microscope for capturing images and were then incubated with 200 µl of SDS solution (provided in the kit) for 12 hours at room temperature. Afterwards, the contents of each well were spun down briefly with a tabletop microcentrifuge to collect liquid to the bottom of the tube, and the solution was transferred into a 96-well plate and absorbance was measured at 562 nm. For each experiment, we also had a control group where planaria were incubated with MTT for a minute and then lysed with SDS. These control group values were used as baseline values (no formazan crystals), which were subtracted from 4 hour-incubation samples, to measure actual formazan crystal formation.

Note: Anuran Chatterjee is employed with AstraZeneca (Wilmington, DE) and AstraZeneca has not played any role in the study nor has provided any financial support for this study.

Dr. Chatterjee conducted the study on weekends outside his work hours with AstraZeneca.

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Article

The Effect of Statement Biased Popular Media Consumption on Public Perceptions of Nuclear Power

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ABSTRACT

This study assesses the effect of popular media consumption, as a function of education level, on the public's opinions about nuclear power. Opinions concerning ten statements about nuclear power were collected before and after participants read a news article in support of or in opposition to nuclear power. The purpose of the study was to determine the degree of malleability of people's opinions about nuclear power and whether this malleability increased or decreased with the subject's education level (high school students, college students, and college graduates). We hypothesized that, despite an evident agenda by the article's author, public viewpoints on nuclear power may fluctuate from their original standpoints after reading the article. The results indicate that participants, regardless of education level, were willing to change their opinions about nuclear power after consuming a single popular media article. After reading the media article, supporting nuclear power, participants showed more positive opinions about nuclear power. Conversely, participants who consumed the media article and were critical of nuclear power showed a more negative overall opinion about nuclear power. This indicates that respondents, regardless of education level, are open to modifying their opinions about nuclear power after consuming popular media, even if the media has a clear statement bias in support of nuclear power or against it.

INTRODUCTION

Public opinions concerning nuclear power took an abrupt negative turn after the Chernobyl disaster in 1986 (1,2). Public sentiment toward the benefits of nuclear power, however, began to display an increasingly positive rebound a year or so later (2,3). In the mid to late 2000s, public opinion had largely swung back in favor of nuclear power. Factors such as 32 years without a major nuclear incident, technological developments in nuclear safety, and an energy policy promoted nuclear power as a valuable component of the country's energy mix (4). Public opinion began to fluctuate based on media coverage and foreign affairs. Prior to the meltdown of the Fukushima Daiichi Nuclear Power Plant in Japan in 2011, nuclear power had gained an increase in public interest due to positive portrayal by the media and specific global referendums assessing support (5). In contrast, after the power plant's meltdown, the public's opinions of nuclear power again took a strong downturn (5,6) , thus ending what was perceived by many to be a resurgence in support for new nuclear construction (4). Public opinion surveys indicated that people's perceptions of the benefits of nuclear power did not shift after the Fukushima disaster. In contrast, public trust in the safety of nuclear power strongly declined, resulting in an overall decrease in support for nuclear power. In Australia, public support for nuclear power as a tool to combat climate change was at 42% in 2010, while only 30.5% of respondents believed that nuclear power should not be used to decrease the impacts of climate change. The same survey given in 2012, after the Fukushima disaster, showed a dramatic reversal in this trend. In 2012, 40.1% of respondents disagreed that nuclear power should be used to combat climate change, while only 34.4% of respondents agreed (4). A similar erosion in support of nuclear power after the Fukushima incident was also observed in Japan, China, Germany, Switzerland, Belgium, Italy, and many other countries (8-14).

Given the short time frame of the loss of public support for nuclear power after the Chernobyl disaster (2), we might expect public support for nuclear power to have recovered by now, due to the seven years that have elapsed since the Fukushima disaster. Changes in our society since the 1990s, however, may be responsible for the fact that this has not occurred. One of the main changes is the ease of access to information. The internet and the 24-hour news cycle provide a plethora of information that is accessible to the public at any time. The purpose of this study was to elucidate the degree to which the consumption of statement-biased popular media, either in support of nuclear power or against it, could affect people's opinions about the impact of nuclear power on society. Statement bias is when a media source, which ideally should convey an objective position that explores all facets of an issue, instead expresses a position that is clearly favorable or unfavorable towards a topic, offering only a partial perspective (15). Approximately half of respondents were presented with a news story that demonstrated statement bias in support of nuclear power (positively statement biased), and approximately half of respondents were presented with a news story that demonstrated statement bias against nuclear power (negatively statement biased). This approach aims to inform our understanding of the malleability of public opinion on this issue at an educational level. High school students,

college students, and college graduate's opinion fluctuation became measured between pre-test, and post-test.

These questions are of particular importance in the state of Georgia, where the construction of new nuclear reactors at Plant Vogtle in Waynesboro has generated significant controversy. The two new nuclear reactors are the first built in the United States in over 30 years and have generated a significant amount of both support and opposition from the public of Georgia. Though locally important, the new nuclear reactors at Plant Vogtle are also nationally and internationally relevant. These nuclear reactors are currently the only nuclear reactors under construction in the United States (16). The reactors represent a shift in priority away from generating cheap electricity and towards generating a higher portion of carbon-neutral baseload electricity (R. Just, personal communication, August 3, 2017). Culley, et al. (2010) discovered the presence of media framing, similar to statement bias, on the topic of nuclear energy in the case of Georgia's Plant Vogtle since 2010. The Augusta Chronicle and Atlanta Journal Constitution, two examples of popular media in Georgia, clearly present certain articles in a positive or negative view of nuclear power without defining the article as an opinion piece. Supposedly "unbiased media," specifically concerning Plant Vogtle, display serious opinionated pieces that are consumed by a mass audience. Some articles addressed carbon neutral arguments in both positive and negative spectrums, as it is a vital discussion in Plant Vogtle's development (17). This is an acknowledgement of the very real potential for future regulation of carbon dioxide emissions by the United States government (17, 18). The regulation of carbon dioxide is already a reality for many countries in Europe19 as well as globally (20,21,22).Some forward thinking companies are already planning for a future where such regulations exist in the United States. Nuclear power is one way that a larger portion of our electricity can be generated in a carbon neutral way while still providing sufficient electricity to meet demand (23). Moreover, the success or failure of the new nuclear reactors at Plant Vogtle may provide a lesson about the potential for nuclear power to provide viable solutions to the dilemma of maintaining, or even increasing, electricity generation while decreasing carbon dioxide emissions (5). We hypothesized that due to greater access to media and critical reading, the higher the education level, the less likely a participant would become susceptible to selection biased media. That is, how willing is the public to change their established opinions about nuclear power, based on exposure to a single news source intended to sway their opinions in one way or the other? Furthermore, how does the education level of the subject influence their susceptibility to this process? The higher the education level, the greater critical reading skills, allowing the reader to recognize the statement bias.

RESULTS

Study Design

Participants were asked to fill out a survey providing their level of agreement with ten statements related to the benefits and costs of nuclear power to society (pre-test) and then read one of two articles. The two articles used in this study were specifically selected because they present an obviously statement biased perspective either supporting nuclear power or opposing it. Of particular interest was whether the inherent statement bias in the articles would cause participants to question the credibility of the author of their assigned article as a trustworthy source of information. If the statement bias in the source caused participants to distrust the information they were consuming, their opinions in the pre-test and post-test should be extremely similar. If we can assume that more educated participants will have an increased awareness of the inherent statement bias, this type of critical analysis of the information and its source would cause a reduction in the impact of the news articles.

Figure 1. Results of a Repeated Measures Analysis of Variance (ANOVA) for time by treatment group interaction. .

Opinion Change Based On Article

To determine whether the changes in opinion among the respondents were significant, an ANOVA analysis was conducted to compare the six treatment groups across pre and post conditions. The six treatment groups were high school students who received the negatively statement biased article [HS (-)], high school students who received the positively statement biased article [HS (+)], college students who received the negatively statement biased article [Coll (-)], college students who received the positively statement biased article [Coll (+)], college graduates who received the negatively statement biased article [Grad (-)], and college graduates who received the positively statement biased article [Coll (+)].

HS (+), Coll (+), and Grad (+) groups showed a positive change in opinion from pre- to post-testing. In contrast, groups HS (-), Coll (-), and Grad (-) showed a negative change in opinion from pre- to post-testing (**Figure**

1, Table 1). To better understand the differences between the groups over time, the analysis indicated that there was not a significant difference between the six groups at pre-testing, $F(5, 145) = 1.56$ $p = 0.18$, $\eta^2_{partial} = 0.05$; there was, however, a significant difference between the groups at post-testing $F(5, 145) = 12.04$, $p < 0.001$, $\eta^2_{partial} = 0.29$ (**Table 2, Figure 1**). The main effect for time by itself was not significant $F(1, 145) = 2.38$, $p = 0.125$, $\eta^2_{partial} = 0.02$, but the main effect for treatment was significant $F(5, 145) = 4.08$, $\eta^2_{partial} = 0.12$. There is a significant interaction between time and treatment group, $F(5, 145) = 32.28$, $p < 0.001$, $\eta^2_{partial} = 0.53$, suggesting that the treatment groups are changing differently over time; because of the significant interaction, we focus on the interaction rather than the main effect for treatment group

ANOVA Analysis Education Level Differences

Post hoc tests indicated that the mean from the groups that received the negatively statement biased articles, HS (-), Coll (-), and Grad (-), were not statistically different from each other. They were, however, statistically different from the groups who received the positively statement biased article. Similarly, the mean from the groups that received the positively statement biased articles, HS (+), Coll (+), and Grad (+), were not statistically different from each other but were statistically different from the groups that received the negatively statement biased articles (**Figure 1, Table 2**).

	Group	Mean	Std. Deviation	N
Pre-test	HS (-)	32.90	8.02	21
	HS (+)	29.94	5.68	18
	Coll (-)	32.38	5.55	21
	Coll (+)	31	6.82	21
	Grad (-)	33.26	6.52	35
	Grad (+)	34.43	5.49	35
	Total	32.65	6.41	151
Post-test	HS (-)	29.1	8.91	21
	HS (+)	35.33	6.10	18
	Coll (-)	24.76	8.61	21
	Coll (+)	35.9	5.79	21
	Grad (-)	28.37	6.49	35
	Grad (+)	36.83	6.84	35
	Total	31.81	8.35	151

Table 1. Descriptive statistics by treatment group. Measures mean and standard deviation of each education level in pre-test and post-test. Total mean and standard deviation of all education levels is measured as well. N represents sample size.

The ANOVA analysis indicated that for groups HS (-), Coll (-), and Grad (-), the declines from pre-test to post-test were significant; the increases in scores from pre-test to post-test for groups HS (+), Coll (+), and Grad (+) were also all significant.

Two samples, students at a Georgia High School and a group of college students at Kennesaw State University display fluctuation in opinion change after reading a media article with often statement bias (**Figure 2, Figure 3**). The results of the survey for the college students did not appear to bear out the hypothesis that a higher level of education, and the corresponding expectation of stronger critical thinking skills, would result in a lower malleability of their opinions compared to the high school students (**Figure 4**).

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	p-value
HS (-)	HS (+)	-6.24*	2.29	.007
	Coll (-)	4.33	2.20	.051
	Coll (+)	-6.81*	2.20	.002
	Grad (-)	0.72	1.97	.714
	Grad (+)	-7.73*	1.97	.000
HS (+)	HS (-)	6.24*	2.29	.007
	Coll (-)	10.57*	2.29	.000
	Coll (+)	-0.57	2.29	.804
	Grad (-)	6.96*	2.07	.001
	Grad (+)	-1.5	2.07	.471
Coll (-)	HS (-)	-4.33	2.20	.051
	HS (+)	-10.571*	2.294	.000
	Coll (+)	-11.143*	2.204	.000
	Grad (-)	-3.610	1.971	.069
	Grad (+)	-12.067*	1.971	.000
Coll (+)	HS (-)	6.810*	2.204	.002
	HS (+)	0.571	2.294	.804
	Coll (-)	11.143*	2.204	.000
	Grad (-)	7.533*	1.971	.000
	Grad (+)	-0.924	1.971	.640
Grad (-)	HS (-)	-.724	1.971	.714
	HS (+)	-6.962*	2.071	.001
	Coll (-)	3.610	1.971	.069
	Coll (+)	-7.533*	1.971	.000
	Grad (+)	-8.457*	1.707	.000
Grad (+)	HS (-)	7.733*	1.971	.000
	HS (+)	1.495	2.071	.471
	Coll (-)	12.067*	1.971	.000
	Coll (+)	0.924	1.971	.640
	Grad (-)	8.457*	1.707	.000

Table 2. Post Hoc tests for the One-Way ANOVA at post-testing comparing the means of one group (I Group) to all other groups (J Groups). Measures mean, standard error, and p-value for each education level given a positive or negative article.

ANOVA tests conducted for analysis demonstrated that while the opinions of the 6 groups of respondents were not statistically different at the time of pre-testing, they had split into two clusters by the time of the post-test. The groups that received the positively statement biased article all showed a statistically significant increase in their overall opinion, and the groups that received the negatively statement biased article all showed a statistically significant decrease in their overall opinion (**Figure 1, Table 1, Table 2**).What is even more interesting is that the ANOVA tests revealed that there was no statistical difference in the overall opinion change at post-testing based on education level for the groups that received the same article. In summary, the respondents who received the positively statement biased article showed a similar change in their opinion regardless of whether they were high school students, college students, or college graduates. The same is true for the groups who received the

negatively statement biased article. This seems to disprove the initial hypothesis that an increase in education level would decrease the malleability of the respondents' opinions.

Survey Statement Trends

It is interesting that cost was the one specific issue that participants seem to link most closely to the overall value of nuclear power to society and its benefits to the future. Given previous studies that established a clear link between the positivity of people's opinions about nuclear power and safety concerns, it was surprising that the issue of cost seemed to be more important to our respondents than the issue of safety raised by Statement 2 "It is safe to live by nuclear facilities and reactors and Statement 10 "Nuclear power plants are unreliable" (4,6). This result may be due to the fact that one of the points of contention by the public concerning the new nuclear reactors being constructed in the state of Georgia

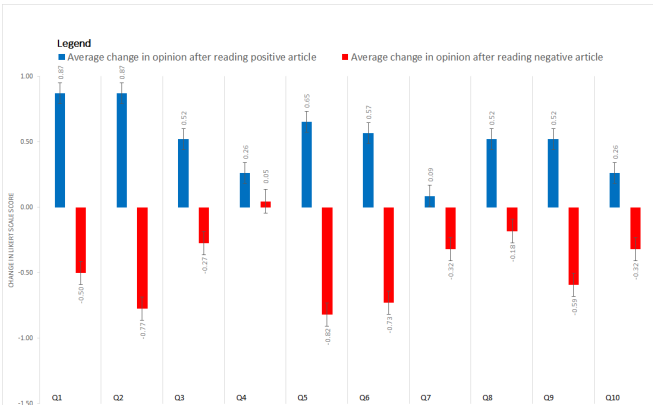

Figure 2. Positive article n=22. Negative Article n=22. Degree of opinion change for high school students after consumption of positive or negative popular media article about nuclear power. Values above or below each bar indicate the average change in opinion for each individual statement. The error bars represent standard error.

is the financial cost of the project, which the taxpayers are largely being asked to bear. This is especially frustrating to the public since cost overruns associated with the project have essentially doubled the cost of the project and delayed its completion by several years.

With the exception of Statement 4 "Many jobs have been created due to nuclear development" for high school students and Statement 8 "The U.S. should play a leading role in nuclear energy and safety standards" for college students and graduates, those who received the positively statement biased media article had a positive shift in their opinions, while those who received the negatively statement biased article had a negative shift in their opinions. While the degree of opinion change is generally lower in magnitude for college graduates than the results for the students, the changes in opinion that did occur were always in the direction of statement bias. It is also worth noting that there was no statistical difference in the opinions of the college graduates compared to the students who received the same kind of article. The opinions of college graduates after reading the negatively statement biased article were statistically equivalent to those of the high school and college students who read the same article, and the same was true for the graduates and students who read the positively statement biased article.

Regardless of education level, the clear presence of statement bias in the articles did not affect the participants' willingness to trust the information that they were being presented. It is also worth noting that the changes in opinion were greater than half a point in the Likert scale, which amounted to more than half a category, in most cases for both the high school and college students. Of the shifts in opinion recorded for high school students, 60% of them (12 out of 20) represent such a change. This trend was slightly higher for the participants who received the positive article (7 out of 10) than for those who received the negative article (5 out of 10). The college students showed a similar rate of change of opinion as the high school students (65% or 13 out of 20).

DISCUSSION

The participants who read the positive article showed more positive opinions about nuclear power, and vice versa for those who received the negatively statement biased article. Public opinion about nuclear power was generally quite malleable and susceptible to the influence of media statement bias. After reading a positively statement biased media article, respondents' opinions about nuclear power generally became more positive and vice versa for people who read a negatively statement biased media article. This was true across all levels of education surveyed.

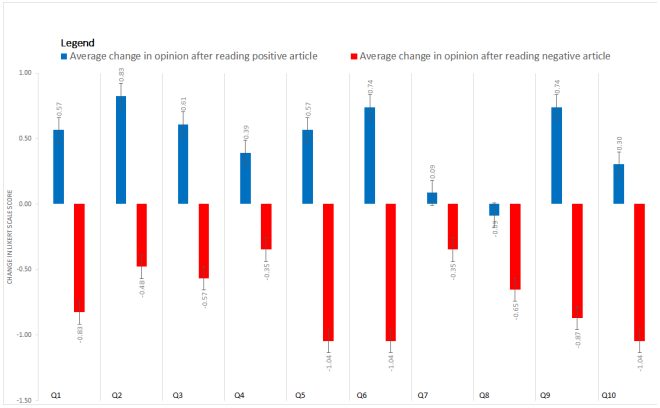

Figure 3. Positive article n=23. Negative Article n=23. Degree of opinion change for college students after consumption of positive or negative popular media article about nuclear power. Values above or below each bar indicate the average change in opinion for each individual statement. The error bars represent standard error.

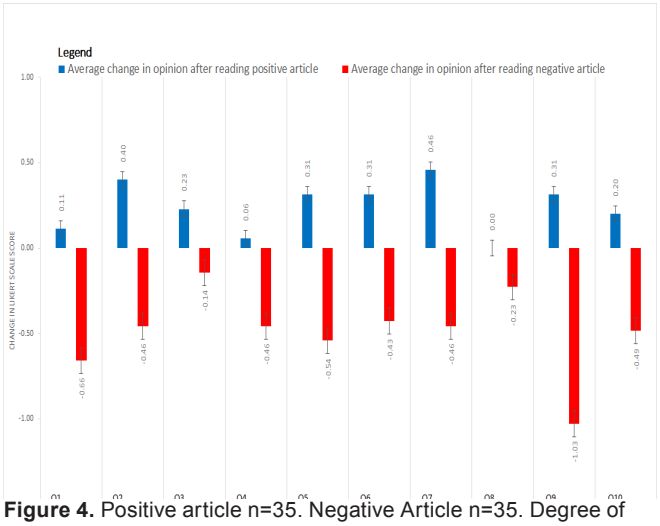

Figure 4. Positive article n=35. Negative Article n=35. Degree of opinion change for college graduates after consumption of positive or negative popular media article about nuclear power. Values above or below each bar indicate the average change in opinion for each individual statement.

Contrary to our initial hypothesis, a higher level of education did not result in a higher resistance to the media statement bias. College students and graduates showed a statistically similar level of susceptibility to statement bias, compared to high school students. All high school students in the study were at least 18 years of age, which may introduce some bias into the results. Not getting a representative sample of all high school students from 9th-12th grade may have skewed the results. The study also included college graduates (n=70) recruited either from teachers at the Georgia High School or graduates /instructors at KSU. This suggested that the college graduates were not any better able to identify media statement bias and limit its impact on their opinions than high school or college students.

Participants were not compensated for their time due to the nature of the study. The surveys for high school and college students were conducted in-person while the survey of college graduates was conducted online. This may also induce some bias in the results for the college graduates compared to the high school and college students.

The data suggests that high school students' opinions can become quite malleable and susceptible to modification after the consumption of a single media article about nuclear power. This seems to indicate that the inherent bias in the articles did not cause the college students to question the trustworthiness of the articles as a source of information either. Their opinions were just as malleable as those of the high school students. This is also reflected in the fact that, with the exception of Statement 8 "The U.S. should play a leading role in nuclear energy and safety standards", college students showed the same susceptibility to the influence of the media article statement bias.

Future studies could analyze the media's involvement in other environmental conflicts, such as genetically modified organisms, inorganic fertilizers, or microplastics, in order to investigate whether the same shift in opinion would occur if statement bias is present. A variety of controversial topics could be substituted in a repeated experiment. Another future study could use similar methods but include adults with doctorates as a group of participants, to test whether this highly educated sample has even greater resistance than college graduates. A sample containing only doctorates may be able to critically review the article presented to them more than those with lower levels of education. A participant with a doctorate may have seen more examples of statement media bias in their career. Moreover, a study could take place using middle school students and utilize basic language on energy in a survey. Most likely, the survey would have to be drastically different, but could uphold some of the same components from the original survey. Middle school students, who have learned earth and geological sciences, may lead to interesting fluctuation on nuclear opinions. Background on science and energy from classes may allow students to have a fascinating opinion on nuclear energy.

The statements most significantly impacted by the consumption of the statement biased media were 1 "Nuclear Energy is beneficial to society", 5 "Public money should be spent on nuclear reactors and facilities", and 9 "Nuclear energy is too expensive to be sustainable". Cost was a factor for which opinions remained malleable, even among college graduates. This shows that the concern most susceptible to media influence is cost and that people's opinions about this issue are correlated with their opinion of the overall benefit of nuclear power to society. The results of this study provide evidence that public opinion about nuclear power may be more open to change, with very little persuasion, than previously believed. Combatting climate change with alternative sources of energy, such as nuclear power, may emphasize nuclear energy's significant role both within the United States and within other countries to reduce carbon footprint.

The data suggests that high school students' opinions can become quite malleable and susceptible to modification after the consumption of a single media article about nuclear power. This seems to indicate that the inherent bias in the articles did not cause the college students to question the trustworthiness of the articles as a source of information either. Their opinions were just as malleable as those of the high school students. This is also reflected in the fact that, with the exception of Statement 8 "The U.S. should play a leading role in nuclear energy and safety standards", college students showed the same susceptibility to the influence of the media article statement bias.

This is an issue facing not only the United States, but also many countries around the world (24, 25). The ability of popular media to change opinions on nuclear power is an important component to determine how public opinion will inform the discussion taking place within governments on how to solve this difficult quandary. This study displayed how people's opinions about nuclear power are more easily

swayed by popular media than one might expect and that higher levels of education do not seem to moderate this malleability to a significant degree.

METHODS

Survey Data Collection

In early 2017, participants were asked to fill out a survey providing their level of agreement with ten statements related to the benefits and costs of nuclear power to society (pre-test). College students and high school students took surveys with paper and pencil. College graduates took an online survey that was delivered through email to all faculty of KSU's College of Science and Math, teachers at the Georgia high school, and some KSU graduates. The online survey was developed using Qualtrics, a web-based survey application, and displayed in a web browser. IRB approval was obtained from the IRB board at KSU prior to initiating this study (IRB Approval #19-213).

Survey Design

For the purposes of measuring the change in their opinions, statements 1, 2, 4, 5, 6, and 8 were denoted as "positive" statements about nuclear power and statements 3, 7, 9, and 10 were considered "negative" statements about nuclear power. These negative statements had their results reverse coded for clarity. The ten statements in the survey were: 1. "Nuclear Energy is beneficial to society." (positive); 2. "It is safe to live by nuclear facilities and reactors." (positive); 3. "Public support for nuclear power has decreased in recent decades." (negative); 4. "Many jobs have been created due to nuclear development." (positive); 5. "Public money should be spent on nuclear reactors and facilities." (positive); 6. "More nuclear facilities should be built in the future." (positive); 7. "Nuclear energy is not a solution for climate change." (negative); 8. "The U.S. should play a leading role in nuclear energy and safety standards." (positive); 9. "Nuclear energy is too expensive to be sustainable." (negative); 10. "Nuclear power plants are unreliable." (negative).

Participants' responses were recorded using a standard Likert scale with five options: Strongly disagree, Disagree, Undecided, Agree, and Strongly Agree. Each response was translated into a numerical value with Strongly Disagree having a value of 1 and each subsequent response increasing in value by 1 with a maximum value of 5 for Strongly Agree. The pre-test took approximately 3 minutes to complete.

After completing the pre-test, participants were then asked to read a popular media article about nuclear power. Participants were given one of two potential articles, which took approximately 2-3 minutes to read. One article was "Top 10 reasons nuclear power will be the key to America's energy future", published in The Hill, an American political newspaper. This article presents a clear statement biased positive viewpoint of nuclear power. The other article was "10 Reasons to Oppose Nuclear Energy", published by Green America, a national not-for-profit organization. This article presents a clear negatively statement biased viewpoint of nuclear power. The article given to each participant was selected randomly.

After reading the assigned article, participants were asked to complete the same ten-statement survey a second time (post-test). The post-test took approximately 3 minutes to complete. Including time to read the articles, the surveys took around 10 minutes to administer.

Participant Selection

The study included high school students from a Georgia high school (n=45) and college students at Kennesaw State University (KSU) in Kennesaw, GA (n=46). All high school students had received at least 11 years of formal education. College students, though not explicitly surveyed for grade level, ranged from freshman to senior year based on class rosters. High school and college students were recruited based on the willingness of the instructor to provide class time to conduct the survey. The pool of college graduates (n=70) was recruited either from teachers at the Georgia high school or graduates/instructors at KSU. College graduates sampled were a mix of Bachelor, Master's, and Ph.D. degrees. Fewer than five participants did not complete the study. Participants who did not complete both parts of the survey were not included in the analyzed data.

Statistical Analyses

Data were analyzed using the SPSS software. For analysis of pre- and post-test scores, composite scores for all items were created and analyzed. Comparison of six respondent sets were analyzed for opinion fluctuation based on statement bias from the given articles. The groups consist of high school students who received the negatively statement biased article [HS(-)], high school students who received the positively statement biased article [HS (+)], college students who received the negatively statement biased article [Coll(-)], college students who received the positively statement biased article [Coll (+)], college graduates who received the negatively statement biased article [Grad (-)], and college graduates who received the positively statement biased article [Grad (+)].

The impact of the consumption of the popular media article on participants' opinions was measured by subtracting the point values between the responses from the pre-test and post-test. For example, if a participant reacted to the statement "Nuclear energy is beneficial to society" with the response "Disagree" (2 points) in the pre-test, but changed their response to "Strongly Agree" (5 points) in the post-test, the change in their opinion would be recorded as +3. However, if a participant responded to that statement with "Agree" (4 points) in the pre-test but changed their response to "Undecided" (3 points) in the post-test, the change in their opinion would be recorded as -1. Thus, the average impact of reading the positive or negative article on the participants' opinions about nuclear power can be gauged.

Composite scores were calculated for pre-testing as

well as for post-testing. For the survey, the coefficient alpha was 0.85 at pre-testing and 0.90 at post-testing. Skewness and kurtosis statistics (using a cutoff of absolute value of two) for each of the six treatment groups for pre- and post-test survey composite scores indicated the data met the assumption of normality. Mauchly's test of sphericity and Levene's test of homogeneity of variance suggested equal variances between and within groups ($p > 0.05$).

Significance is measured with ($p< 0.05$) and a Bonferroni correction and an adjusted cutoff alpha of 0.05/6 = 0.008 (**Table 2**).

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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

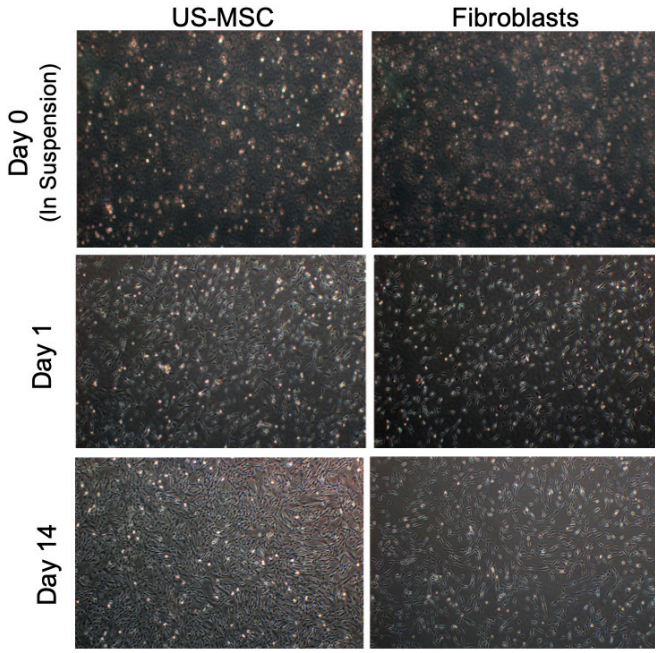

Figure 1. Growth of UC-MSC and Fibroblast Cell Lines. Representative images of UC-MSCs (left) and fibroblasts (right) were imaged using a microscope at 20x magnification at Days 0, 1 and 14 of culture. After 24 hours (Day 1), it is apparent that the cell lines have settled.

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,

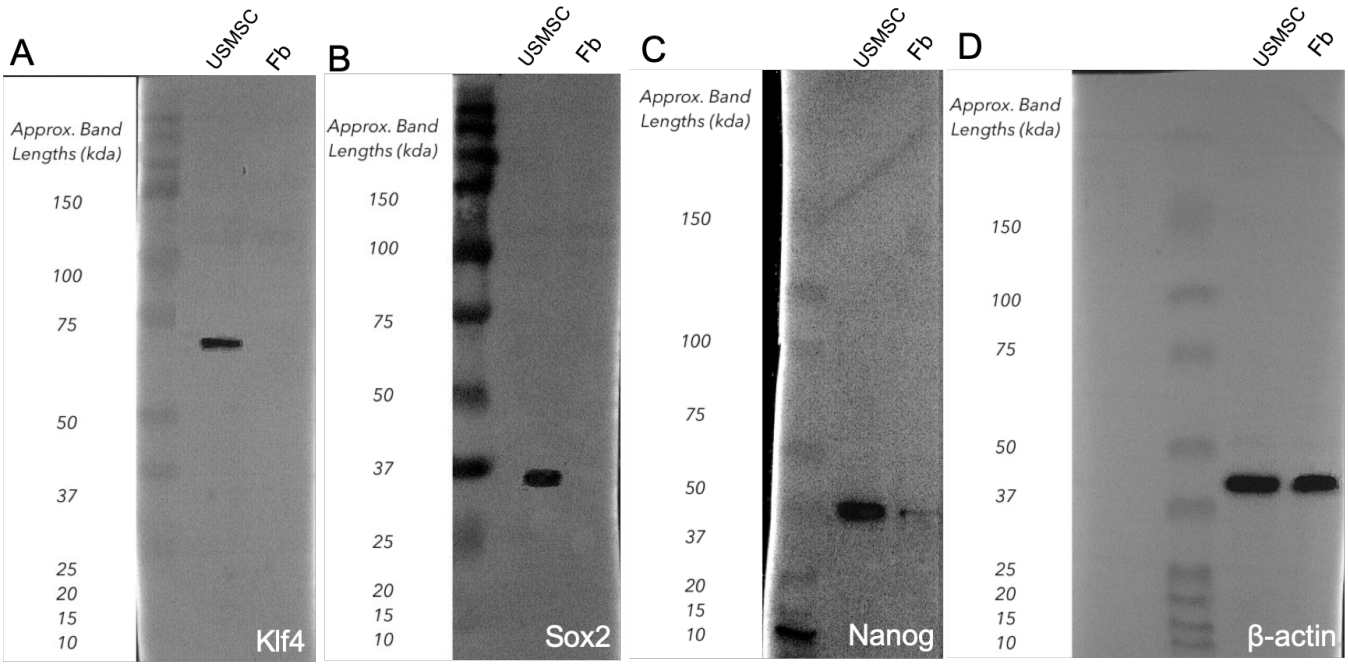

Figure 2. Comparison of protein levels of selected factors between US-MSC and fibroblasts. Protein levels in US-MSC and fibroblasts (Fb) were assessed by Western blot. (**A**) Klf4 is present in UC-MSC but not fibroblasts, confirming the hypothesis. (**B**) Sox2 is present in UC-MSC, but not fibroblasts 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

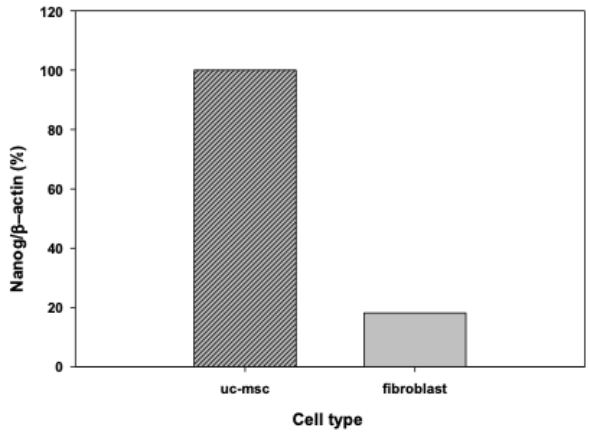

Figure 3. Ratio of Nanog to β-actin in UC-MSC and Fibroblasts
A considerably higher presence of Nanog was found in UC-MSC than in fibroblasts.

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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