

Reduced psoriasis skin irritation symptoms through the effects of Chinese herbal medicines on planarians

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SUMMARY

Psoriasis is a chronic autoimmune skin disease that causes skin inflammation. While its underlying causes are unclear, current treatments involve topical drugs and ultraviolet radiation, which risks skin cancer. In traditional Chinese medicine (TCM), psoriasis involves dual yin-yang symptoms, which complicates treatment from a solely modern medicine perspective, as most pharmacological treatments can only address one type of symptom, not both. According to TCM theory, psoriasis is believed to stem from issues in the liver and lungs: excess liver fire induces inflammation and malnourished lung yin compromises immunity. We hypothesized that compounds supporting the liver and lungs would decrease inflammation in *Dugesia dorotocephala* planarians. Our study analyzed the causes of psoriasis through TCM theory to develop a less toxic treatment from the active compounds of two TCM herbal remedies, wogonin from Liver FireClear pills and butylidenephthalide (BP) from LungVigor pills. We induced inflammation on the planarian membranes of *D. dorotocephala* and assessed the reduction of inflammation symptoms through a fluorescence toxicity assay following the application of wogonin and BP. Compared with the untreated control group, the highest concentrations of wogonin (0.20 µg/mL) and BP (40 µg/mL) reduced 97% and 98% of induced inflammation, respectively. Our findings suggest that the TCM herbal Liver FireClear and LungVigor pills may alleviate inflammation. Our results encourage future use of integrative medicine and TCM diagnosis theory to better understand the underlying mechanisms of psoriasis.

INTRODUCTION

Psoriasis is a chronic skin disease that affects over 125 million people worldwide (1). While its underlying causes are not clear, psoriasis is believed to be an autoimmune disease and involves common symptoms such as dryness, flakiness, peeling, and rashes — most notably red patches with a silvery-white coating called scale in plaque psoriasis — as well as inflamed tendons, itching, joint stiffness, plaque, and psychological conditions such as depression (2). There are seven primary types of psoriasis. About 80% of patients have plaque psoriasis, which involves raised, inflamed, red skin covered with silvery, white scales, and about 8% of patients have guttate psoriasis, which causes small, pink-

red spots on the skin (3). In humans, psoriasis is caused by the hyperproliferation of keratinocytes (or an abnormally high concentration of keratinocytes caused by certain cytokines) and the accumulation of activated T cells in the epidermis (3). Activated dendritic cells and macrophages, two types of phagocytes that present antigens, secrete cytokines like TNF-α and IL-23 that lead to the activation of keratinocytes in the blood, resulting in psoriasis (4). The expansion of autoreactive T cells is further promoted by antigen presentation to promote cytokines that activate keratinocytes (5). Imiquimod (IMQ), which induces psoriasis in mice, activates dendritic cells that ultimately lead to a buildup of keratinocytes in the blood and speed up the differentiation of skin cells in the epithelial barrier, resulting in psoriasis (6). Dendritic cells exposed to IMQ are known to increase keratinocyte accumulation by leading to a formation of microabscess, a localized collection of dead cells and other cells of the immune system (7).

Current treatments include mild to moderate topical formulas and severe systemic, oral chemical drugs and injections. While these are commonly used, some formulas can be extremely expensive, while others require high patient compliance (5). Ultraviolet radiation has become the major physical therapy for the treatment of psoriasis, but its remission period is short, and long-term application causes skin aging, skin cancer, and the increased risk of cataracts (8). Given the current risk factors involved in modern medicine treatment and the highly specific nature of psoriasis, safer and more accessible treatments are needed.

Traditional Chinese medicine (TCM) has a potential to offer alternative treatments that address these pitfalls and provide a long treatment cycle with little to no relapse. For example, some studies have shown that a decoction of TCMs could reduce the risk of recurrence and metastasis in colorectal cancer patients, therapy outcomes that can similarly aid in preventing psoriasis symptoms from recurring (9). TCM theory gauges diseases at a more holistic, whole-body level. In certain types of psoriasis, the redness indicates a “yang” symptom and the white flakes indicate a “yin” symptom; the presence of either type of inflammation indicates an excess of yin or yang in the body, which obstructs the yin-yang balance essential to maintaining proper function and avoiding sickness (10). Diseases that consist of both yin and yang-type inflammation are difficult to be resolved by solely modern medicine treatments, but with the combination of specific herbal formulas, their causes may be addressed through TCM (11).

According to the concept of qi in TCM, psoriasis indicates issues in the liver, lungs, and spleen (11). In TCM, qi, the body's life source and refined energy, is used to diagnose the conditions of organs and the spread of diseases and

symptoms around the body; each organ has a different type of qi responsible for maintaining balance (12). The TCM herbal formulas Long dan xie gan tang (LDXGT), marketed as Liver FireClear pills, and Bai he gu jin tang (BHGJT), marketed as LungVigor pills, are used to strengthen qi and reduce heat, which are mechanisms necessary for reducing common skin irritation symptoms of diseases like psoriasis (13).

In LDXGT, *Scutellariae radix* is a highly concentrated herb (14). Wogonin, a flavonoid from *S. radix*, is known to inhibit inflammatory activation of cultured brain microglia by decreasing levels of TNF- α and IL-1 β (15). In addition to *Rehmanniae radix*, the four most concentrated herbs in BHGJT are *Angelicae sinensis*, *Bulbus lillii*, and *Ophiopogonis tuber* (16). Butylidenephthalide (BP), a highly concentrated compound found in *A. sinensis* and BHGJT, is a plant metabolite that inhibits angiogenesis, the development of new blood vessels that induces inflammation leading to psoriasis (17, 18).

Studies have partially attributed the biomolecular activities and toxin removal abilities of certain TCM herbal drugs to the "Western" relationship and model between lipid peroxidation and inflammation (19). Previously, the TCM herbal drug xiao-chai-hu decoction has been shown to improve psoriasis-like skin lesions and inhibit inflammation cytokines in IMQ-induced mouse models (20). Previous studies have also shown that wogonin decreases in vivo expression of several inflammation-associated genes in a PCR mouse model (21).

We chose planarians (*Dugesia dorotocephala*) as the model organism because they are readily available at a low cost, easily cultured and maintained in a laboratory using spring water, potentially offering a low-cost alternative for skin inflammation tests (22). As freshwater-living flatworms found ubiquitously around the world, planarians are commonly used as a model in developmental and regeneration research for their abilities to regenerate independently (23, 24). They are invertebrate worms with a primitive brain that has features similar to those of the vertebrate nervous system (25). Previous studies have shown that certain planarian wound epidermis genes are required for blastema formation in tissue inflammation and regeneration (26). Although planarians lack keratinocytes and skin in general, these similarities make them one of the most suitable invertebrate models for skin inflammation testing with a focus on immunity and disease (27).

In our study, we induced inflammation by applying IMQ to the planarians. Treatment conditions involved inducing inflammation in the planarians in all groups, followed by adding concentrations of the two test substances (wogonin and BP) in the treatment groups. We added sodium fluorescein and immobilized the planarians so that levels of fluorescence could be measured under the microscope. Fluorescence directly correlated with levels of inflammation, so it was used as a marker for the amount of inflammation each planarian had. The amount of fluorescence was measured using the ImageJ software, which allowed for glowing areas, indicated in white compared to the darker gray background, to be selected manually.

We hypothesized that higher concentrations of wogonin and BP will alleviate the inflammation in planarians. Based on the observed decrease in fluorescence levels in *D. dorotocephala*, we reached the conclusion that both treatments of wogonin and BP are linked to lower amounts of inflammation. Our

results suggest that the immunofluorescence-planarian method is an accurate model for inflammation, and that wogonin and BP can potentially reduce inflammatory side effects in humans. Additional studies clarifying the effects of IMQ and both test (wogonin and BP) substances in organisms more closely related to humans are required.

RESULTS

To induce inflammation in the planaria, we used previous studies to determine concentrations of IMQ, which was used to induce inflammation in mice models (28). Subsequent trials with control and experimental groups with test substances only used the 5% IMQ solution. We conducted preliminary studies to ensure that the planarians can endure low DMSO (Heiltropfen) and ethanol (Flinn Scientific) concentrations of 1% each. We determined concentrations for both test substances by taking appropriate concentrations in previous mice studies and adjusting for the mass ratio between the mice and planarians (28).

To determine whether the wogonin and BP treatments affected inflammation induced by 5% IMQ, we treated the planarians with increasing concentrations of each test substance: wogonin was applied at 0.015 $\mu\text{g/mL}$, 0.036 $\mu\text{g/mL}$, 0.05 $\mu\text{g/mL}$, 0.15 $\mu\text{g/mL}$, and 0.20 $\mu\text{g/mL}$; BP was applied at 5 $\mu\text{g/mL}$, 7.2 $\mu\text{g/mL}$, 10 $\mu\text{g/mL}$, 25 $\mu\text{g/mL}$, and 40 $\mu\text{g/mL}$. After each planarian soaked in 5% IMQ for 10 minutes, we added the test substance immediately and let the planarians soak in it for 10 minutes. We used fluorescence microscopy to quantify levels of fluorescein accumulation and visualize increased inflammation levels such as those present in psoriasis. Ultimately, we used the area, mean, integrated density, and raw integrated density values to calculate the Corrected Total Area Fluorescence (CTAF) values, a measurement of fluorescence intensity. We used a one-way ANOVA and Tukey's Honestly Significant Difference (HSD) tests to assess statistical significance.

Changes in levels of fluorescein fluorescence were determined based on CTAF values. Based on the preliminary trials in this study, increased fluorescence levels indicate that the planarian membranes retained higher levels of sodium fluorescein due to decreased protection from the induced inflammation caused by the IMQ. When compared to the control (spring water and fluorescein dye), the 5% IMQ treatment group was visually brighter and consistently exhibited statistically significant increased levels of fluorescein fluorescence; planarians in the control group had an average CTAF value of 1.19×10^{10} , while those with 5% IMQ had an average CTAF value of 1.73×10^{11} ($p < 0.01$, Tukey HSD test, **Figure 1a**). When compared to the IMQ 5% treatment group, the wogonin treatment groups in low (0.015 $\mu\text{g/mL}$), medium (0.036 $\mu\text{g/mL}$), and high (0.05, 0.15, and 0.20 $\mu\text{g/mL}$) concentrations and the BP treatment groups in low (5 $\mu\text{g/mL}$), medium (7.2 $\mu\text{g/mL}$), and high (10, 25, and 40 $\mu\text{g/mL}$) concentrations exhibited significantly decreased levels of fluorescein fluorescence ($p < 0.01$, Tukey HSD tests, **Figure 1b-d**). The results of the one-way ANOVA test showed a p -value of 0.001 ($*p < 0.01$), thus indicating that there was a statistically significant difference between the average CTAF values among the treatment groups and the IMQ 5% group. The Tukey HSD test determined a p -value that was in further agreement with the 0.05 p -value given by the ANOVA test.

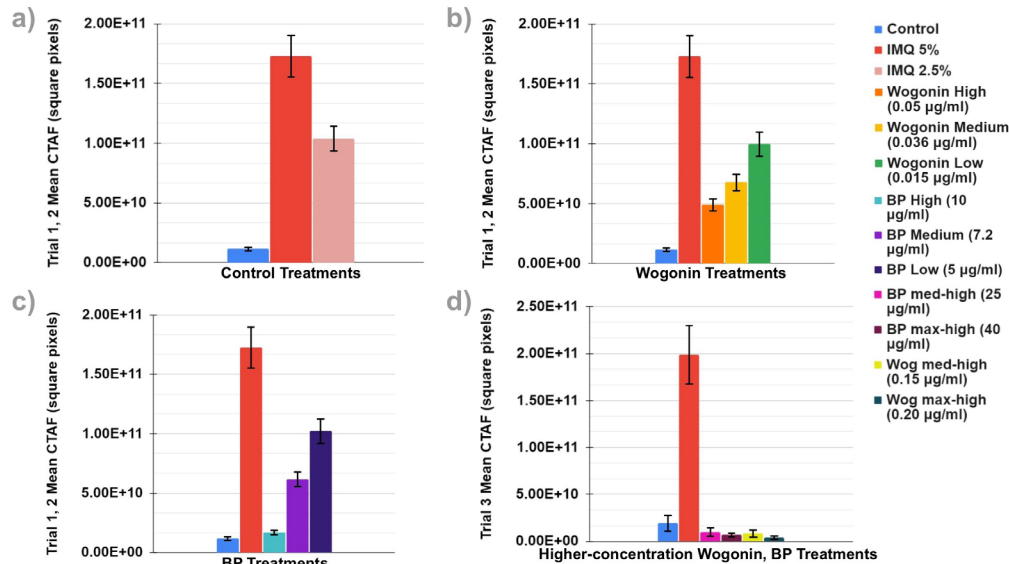


Figure 1. Mean Corrected Total Area Fluorescence (CTAF) after fluorescein exposure for four trials. One-way ANOVA, ** $p < 0.01$. Data shown as mean \pm 2 SEM. For a-c) 20 planaria were used in each treatment group with each group having 60 data points and for d) 10 planaria were used for each group and each group had 10 data points. **a)** Effect of IMQ to induce inflammation in planarians ($n=3$) vs control (fluorescein only). Planaria were grown under either control conditions, in 5% IMQ, or in 2.5% IMQ for 10 minutes. **b)** All tested concentrations of wogonin reduce IMQ induced inflammation. Planaria were grown under either control conditions, in 5% IMQ, in 0.036 $\mu\text{g}/\text{mL}$ wogonin after being soaked in 5% IMQ, or in 0.015 $\mu\text{g}/\text{mL}$ wogonin after being soaked in 5% IMQ for 10 minutes. **c)** All tested concentrations of BP reduce IMQ induced inflammation. Planaria were grown under either control conditions, in 5% IMQ, in 10 $\mu\text{g}/\text{mL}$ BP after being soaked in 5% IMQ, in 7.2 $\mu\text{g}/\text{mL}$ BP after being soaked in 5% IMQ, or in 5 $\mu\text{g}/\text{mL}$ BP after being soaked in 5% IMQ for 10 minutes. **d)** Higher concentrations of wogonin and BP reduce IMQ induced inflammation below the control level). Planaria were grown under either control conditions, in 5% IMQ, in 25 $\mu\text{g}/\text{mL}$ BP after being soaked in 5% IMQ, in 40 $\mu\text{g}/\text{mL}$ BP after being soaked in 5% IMQ, in 0.15 $\mu\text{g}/\text{mL}$ wogonin after being soaked in 5% IMQ, or in 0.20 $\mu\text{g}/\text{mL}$ wogonin after being soaked in 5% IMQ for 10 minutes.

Inflammation reduction effects of treatment groups of different dosages exhibited varying statistical significance levels compared with that of the control. Within the dosages of BP, the high and medium ($p < 0.05$, Tukey HSD test, **Figure 1c**) and high and low ($p < 0.01$, Tukey HSD test, **Figure 1c**) concentrations were statistically significant. Within the dosages of wogonin, the high and low ($p < 0.01$, Tukey HSD test, **Figure 1b**) concentrations were statistically significant.

DISCUSSION

Our findings suggest that the IMQ-fluorescence planarian model can be used as a rapid pre-screening method to inform more costly assessments of skin irritants and that the test substances wogonin and BP had significant, inflammation-reducing effects in *D. dorotocephala*. Preliminary trials comparing different concentrations of IMQ solution, representing different levels of induced inflammation in the planarian membranes, showed a direct correlation between the concentration of IMQ and fluorescence levels.

The data showed a decrease in fluorescence values, suggesting decreased inflammation levels as a result of increased wogonin and BP intake. The data demonstrated that the planarians that absorbed large amounts of either test substance had significantly decreased fluorescence intensities compared to those not introduced to the compound. More data from testing a broader range of doses is needed to better establish a direct correlation between increased administered dose and inflammation-reducing effect. Our data also suggests that the TCM herbal formulas, Liver

FireClear pills and LungVigor pills, may reduce inflammation symptoms present in psoriasis, though human studies can further confirm this.

Due to Biosafety level-1 (BSL-1) restrictions, there were a few key limitations. BSL-1 hindered experimentation with more comprehensive models involving vertebrates or cell cultures. Analysis of the test substances on a less comprehensive, invertebrate model such as planarians, as in the present study, poses differences with the more intricate systems of the human body involved in the development of pathologies such as psoriasis. Planarians do not have keratinocytes, skin, or the other immune cells necessary to induce psoriasis, so the model is limited in that its results may only be applied to the reduction of inflammation symptoms. Additionally, it is not known whether the planarians only topically absorbed the compounds, or whether they consumed and ingested it. In future studies with planarians, a positive control compound, such as a drug known to treat psoriasis symptoms when ingested orally, should be applied to see if planarians respond to it as expected. As a result of the biological differences between planarians and humans, the study's results can only be extrapolated to induced inflammation in planarian membranes, not psoriasis.

Light sensitivity, variability in planarian fluorescence, and microscope setup posed another key limitation. The computer software used to capture images of planarians under the fluorescence microscope, MicroManager, was not able to take photographs of complete planarians at a time, leading to variability in the differences in the area between

individual planarians. To boost their validity, future studies should evaluate another measure of inflammation apart from the fluorescence signal of fluorescein.

Testing the effects of IMQ and both test substances in organisms more closely related to humans, such as mice, would offer greater insight into their abilities to inhibit the key TNF and Th17 pathways to counter the buildup of keratinocytes in the blood (29, 30). Studies on planarians have also shown that TNF receptor associated factors play critical roles in cellular survival during tissue repair (31). Thus, the TNF pathway is worth future exploration to better establish a correlation between activity in the pathway and a decrease in the amount of keratinocytes in the blood. In addition, testing the prolonged effects of introducing the compound daily would be needed to confirm the safety and efficacy of both test substances.

If wogonin and BP are further tested, then both can be evaluated as potential treatment options that can be taken with topical medications, such as psoriasis cream, to alleviate symptoms of swelling and itchiness. Additionally, since wogonin and BP have antibacterial properties, further studies should examine the effects of more widespread and commonly used pharmaceutical antibiotics to establish a clearer comparison between the effects of Chinese medicines and modern medicines on their abilities to alleviate inflammation symptoms in psoriasis (32).

In this study, both wogonin and BP significantly reduced induced inflammation symptoms in the planarians. From preliminary trials, the data suggests that the planarian model can be used as a rapid pre-screening method to inform subsequent costly assessments of skin irritants. The study suggests that the TCM formulas Liver FireClear and LungVigor may reduce psoriasis inflammation symptoms. Our results should be verified in the future through human studies.

MATERIALS AND METHODS

Culturing *D. dorotocephala*

Planarians, *D. dorotocephala*, (Carolina Biological, Cat# 132970) were used to model psoriasis inflammation. Spring water (Crystal Geyser, Cat# 24514) was changed every other day to keep the living environment clean. We maintained planarians in groups of 20-40 in spring water and fed with three pea-sized portions of egg yolk (Kirkland Signature, Cat# 427381) once every week (33). We avoided feeding 24 hours before individual or small groups of planarians were chosen to be submerged in IMQ (Cayman Chemicals, Cat# 14956) or test substance solutions to avoid egg yolk in their bodies from interfering with fluorescence data.

Chemicals and Experimental Set Up

We made two concentrations of IMQ solution, 5% and 2.5% w/v, for the pretrial to assess the efficacy of the planarians and fluorescence as a skin inflammation model. We prepared the 5% IMQ stock solutions by combining 21.1 mg of IMQ (a whitish powder at room temperature), 38 mL of DMSO, and then 300 mL of spring water in a 500 mL Erlenmeyer flask after the IMQ powder completely dissolved in the DMSO. The 2.5% IMQ stock solution was similarly made by combining 10 mL of spring water with 10 mL of the premade 5% w/v IMQ solution.

Sodium fluorescein salt (Sigma-Aldrich, Cat# 518478) was used to make the 0.1% w/v fluorescein dye later applied for fluorescence in the worms (34). In a 150 mL Erlenmeyer flask, 0.1 g of sodium fluorescein salt and 100 mL of distilled water was mixed, then transferred into a separate container and wrapped in aluminum foil.

We conducted the first two trials with low, medium, and high concentrations of both test substances, wogonin (Cayman Chemicals, Cat# 14248) and BP (Cayman Chemicals, Cat# 34983), dissolved in ethanol, to examine consistency and replicability in data. The third trial raised the concentrations of the highest levels of both wogonin and BP. Wogonin was tested in concentrations of 0.015 µg/mL (low - first two trials), 0.036 µg/mL (medium - first two trials), 0.05 µg/mL (high - first two trials), 0.15 µg/mL (medium-high, third trial), and 0.20 µg/mL (max-high, third trial). BP was tested in concentrations of 5 µg/mL (low - first two trials), 7.2 µg/mL (medium - first two trials), 10 µg/mL (high - first two trials), 25 µg/mL (medium-high - third trial), and 40 µg/mL (max-high - third trial).

For the control treatment without inflammation, we soaked each group of 10 planarians in fluorescein only in an individual plastic chamber slide for 10 minutes (Figure 2). We first soaked each experimental group of 10 planarians in 10 mL of 5% IMQ for 10 minutes, 10 mL of the test substance (wogonin or BP) for 10 minutes, then 5 mL of sodium fluorescein for 5 minutes. The 2% agar was made by microwaving 2 g of laboratory grade agar (Flinn Scientific, Cat# A0013) and 100 mL of distilled water in 15-second bursts until boiling and left to cool in the refrigerator for about 5-10 minutes until lukewarm to the touch. After excess sodium fluorescein was sucked out to prevent the background from fluorescing, 2% agar was poured over the slide until its depth reached approximately 5-6 mm. The slide was placed in the refrigerator for 2 minutes to set and immobilize the planarians.

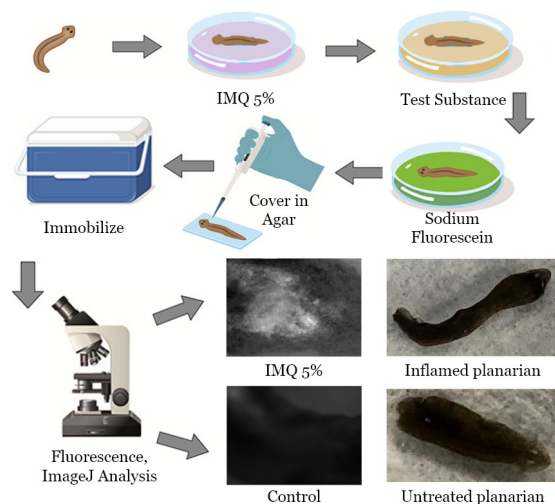


Figure 2. Flowchart of experimental procedure. Planaria were incubated in 5% IMQ for 10 minutes and then treated with wogonin or BP at different concentrations. Then they were exposed to sodium fluorescein for 10 minutes before having excess solution sucked out. The planaria were immobilized and covered in agar. Each slide of planaria was analyzed under a fluorescent microscope and the ImageJ program to measure the levels of fluorescence acquired under each treatment group.

Data Collection

The first two trials consisted of nine slides, each exposed to one of the nine following conditions: Control, IMQ 5%, IMQ 2.5%, BP low, BP medium, BP high, wogonin low, wogonin medium, wogonin high. The third trial consisted of five slides, each exposed to one of the five following conditions: Control, BP med-high, BP max-high, wogonin med-high, wogonin max-high.

Following a 24-hour exposure period to their respective experimental conditions, we captured ten fluorescence images per plate. To reduce experimental uncertainty, we ran two biological replicates for each of the nine conditions for the first two trials. This yielded 240 total images: 180 technical replicates for both test substances in the first two trials and 60 technical replicates per experimental condition for the third trial. Fluorescence was assessed microscopically 24 hours after chemical exposure through MicroManager-1.4 and ImageJ, an open-source image analysis software plug-in developed by the National Institutes of Health. We placed the slides in a dark room and took two photos per individual planarian using a Point Grey GRAS-20S4M-C microscope camera. For clearer imaging results, each slide was flipped over so that the planarians would be closer to the surface of the slide facing up.

Analyzing Fluorescence Data

To determine whether wogonin and BP treatment affected inflammation levels, we used fluorescence microscopy to measure aggregation levels of fluorescein, a synthetic organic photoactive dye. For fluorescence image analysis, the image FJI distribution of ImageJ software was used. To prepare the image for analysis and enhance the contrast to make the planarians more easily distinguishable from the background, we subtracted the background measurements (area and integrated density for the amount of fluorescence). ImageJ then outputted values including the area of the planarian, brightness levels, and the integrated density of the image, or the sum of the brightness of all the pixels inside the selected planarian outline.

We then calculated the CTAF values for each planarian using the formula:

$$CTAF = \text{Integrated Density} \times (\text{Average Area/Individual Area}) - (\text{Average Area for 10 planarians} \times \text{Background Fluorescence})$$

Next, we calculated the Background Fluorescence using the formula:

$$\text{Background Fluorescence} = (\text{Mean value of the background circle}) - (\text{Mean value of the selected portion of the planarian})$$

We further corrected CTAF values to account for the variation in area, as the planarians were too large to be captured wholly in one snapshot using the fluorescence microscope. For each individual planarian, we multiplied the individual integrated density value with a ratio dividing the average area value of the group of 10 planarians with the individual area value.

We determined the average CTAF values for each experimental group by adding all 10 calculated CTAF values then dividing by 10 (the number of data points per experimental group of one trial), so that there were nine average CTAF

values per trial to be compared amongst each other for the first two trials, and five values for the last trial.

Analyzing for Statistical Significance

We evaluated average CTAF values across each experimental group for statistical significance using ANOVA and Tukey HSD tests. We made statistical comparisons using a One-way ANOVA and post-hoc Tukey HSD Test (35). A *p*-value of less than 0.05 was taken as statistically significant.

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