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The Use of the Crude Extract of Immuno-Activated C. Elegans as Treatment for Inflammation

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ABSTRACT

Helminth Therapy is the practice of infecting oneself with parasites to cure ailments. The hypothesis was formed in the 20th century and experimentation began around 10 years ago. The main mechanism is the idea that infection causes a shift in immune response from Th1 (inflammation promoting) to Th2 (inflammation reducing) as the body uses Th2 response to fight against the helminth. However, self-infection comes with side effects. This study aims to exploit the anti-inflammatory response whilst minimizing the risks of helminth therapy. C. elegans contain the same secretory proteins responsible for responses in conventional helminths. Moreover, the malleable innate immune systems of C. elegans allow their genomes to be altered. By using bacterial and fungal pathogens (S. aureus, P. aeruginosa, and C. albicans. This change in genome alters the secretory proteins which help fight inflammation. Crude extracts of the immuno-manipulated C. elegans proteins can be obtained and used to cause the helminth Th balance switch without the negatives of being actually infected. Inflammation will be induced in zebrafish embryos via the use of TNBS and Dextran sodium sulfate; gut architecture, goblet cell count, and leukocyte migration will be used as measures of inflammation. Results show promise as C. elegans crude extract as a treatment for inflammation as well as support for immuno-regulating the C. elegans to fight specific types of inflammation. This study explores the experimental field of helminth therapy to validate it as an alternative inflammation treatment, especially in autoimmune diseases where the Th1 cytokine levels are so prevalent.

Introduction

Inflammation is a term which refers to the immune response of the body in response to foreign invaders. Inflammation helps to signal the immune system to fight foreign bodies, and heals damage. However, inflammation can also be bad for the body, causing chronic diseases like asthma and heart disease. Auto-immune diseases are especially linked with inflammation when the body creates an inflammatory response when there is nothing to respond to. Inflammation can be categorized into acute inflammation and chronic inflammation. It is safe to say that inflammation plays a large role in disease response. Inflammation is carried out in stages, the most important stage is the cellular changes stage. Within this stage leukocytes accumulate at the injury site, mainly neutrophils. Neutrophils are actively circulated in the blood and use chemotaxis to diffuse towards the injury site. When at the site, neutrophils ingest foreign bodies and are the most prevalent cells at the site for the first 24-28 hours. However, after this time frame the inflammation transforms from acute to chronic and macrophages take over. Macrophages are more mature neutrophils and are the marker of chronic inflammation. Chemical mediators such as cytokines also play an important role in mediating inflammation. While inflammation is vitally important for immune response, it can have negative effects such as arthritis, asthma, atherosclerosis, blindness, cancer, diabetes, autism and other mental illnesses. Even though inflammation is critical to remain healthy, unchecked inflammation can go on to cause these serious health detriments. Controlling inflammation levels can be key in treating these chronic illnesses. Cytokines are one of the most prevalent chemical mediators

associated with inflammation. Cytokines is a term used to describe any small protein which is a key modulator of inflammation. Cytokines are produced by leukocytes and are used to communicate with the immune system to determine the inflammatory response. Cytokine level moderation is a key factor in modulating inflammation levels. Lowering cytokine levels lowers inflammation as there are not as many cytokines to recruit an inflammatory response. Cytokines are often targeted in inflammation therapy for this reason (CDC). General inflammation medications act as broad immunosuppressants and depress the immune system in general. While this may work it can be toxic and leaves the body vulnerable to infections as the immune system is weakened. Helminth is an alternative therapy for many things including inflammation. Helminth therapy is the intentional infection of oneself by parasites to treat ailments. Common parasites used in helminth therapy are Trichuris suis, the pig whipworm, and the human hookworm Necator americanus. Traditional short-term helminth therapy includes the ingestion of eggs which will hatch in the cecum and colon where they will colonize. Short term treatment usually uses the parasite T. suis and has to be repeated every couple of weeks for efficient treatment. Long term traditional helminth therapy involves eggs being administered percutaneously where they will enter the vascular system and migrate to the lungs and small intestines. These infections can last for years and have rather severe side effects. (Helmby 2015) The basis of helminth therapy is the creation of an anti-inflammatory environment in the body which is caused by the parasite infection. When a person is infected their body mounts what is called a “type-2” cytokine response. A type-2 response that includes the secretion of IL-4, IL-9 and IL-13 as well as the secretion of mast cells, eosinophils and goblet cells. IL-4 is called the “prototypic immunoregulatory cytokine” as it regulates antibody production and inflammation on a wide scale, affecting many cells in different ways (Brown, Hural 1997). IL-13 functions very similarly to IL-4 and has been shown to have anti-inflammatory effects in vitro and in vivo (Vries 1998) .IL-9 has shown success in eliciting type 2 responses and reducing inflammation in chronic diseases such as arthritis (Rauber et al 2017). In Type 2 response there is granuloma formation which isolates the parasite and repairs damaged tissues. Furthermore, infection also causes immunoregulatory mechanisms to be triggered. This can cause the increase of T-cells, IL-10 levels, and TGF-b levels. IL-10 has been implicated in studies which identify it as a controlling factor of helminth infection pathology. Similarly, Higher T-cell levels have been shown to decrease inflammatory response. (Helmby 2015) This creation of an anti-inflammatory environment is what helminth therapy hopes to exploit. However helminth therapy is not seen as traditional in any fashion. Most medical professionals frown upon the practice. In fact, helminth therapy has never been officially prescribed in the United States. Moreover, the FDA has not approved the practice for use in the U.S. On the contrary, the therapy has a devout base who swear by it. Experimentalists agree that infections have caused relief from inflammatory disorders. However, they admit that the therapy can have side effects, especially in higher doses. This brings the issue with helminth therapy, the dangerous side effects. This has held back helminth therapy from becoming a mainstream form of treatment. Studies have emerged which utilize crude extract of worms for treatment as opposed to traditional infection. This method can eliminate the side effects of the actual parasite infection while keeping the anti-inflammatory aspects. A study used the crude extract of C. elegans (CEC) to treat asthmatic inflammation in mice. The study concluded that OP50 fed CEC was effective in treating the inflammation in the mice’s lungs. More specifically, the study found that IL-2 and IFN-y were found in lower quantities in treatment mice and IL-5 and IL-13 levels were highered. This produces an anti-inflammatory effect which was quantified by the level of inflammatory cells in the treatment groups. The study also identified IFN-y as a key cytokine involved in CEC’s role in inflammation. Moreover, another study identified that C. elegans play an important role in regulating th1 inflammatory response. (Kim et al 2012). This leaves open the possibility of further development of CEC for inflammation treatment. C. elegans are very cheap to mass produce and have a very wide array of commercially available mutants. This makes them a very good choice for the development of a treatment as millions of individual worms can be mass produced for incredibly cheap, meaning the price for the consumer will be cheap. The exact mechanism by which CEC has immunoregulatory effects is not explicitly known. It is widely accepted that its effects come from excretory protein interaction with mammalian systems, similarly to how live

helminths have effects. A proposed set of secretory proteins which may be responsible for CEC's effects are the C-type Lectin-Like Receptor (CLEC) proteins. CLEC proteins are assumed to play a role in C. elegans immune response. The innate immune system of C. elegans causes immune response to consist of wide scale genome changes in response to pathogens because they do not have a complex immune and nervous system. When inflicted with pathogens, C. elegans alter their genome to upregulate genes that code for CLEC. A study which assessed CLEC proteins in C. elegans found that clec-47 had cytokine-like properties when exposed to HEK 293 and Murine Macrophage cells. Clec-47 cells had proinflammatory effects by producing TNF-a, IL-1B, and IL-6. This provides evidence that Clec genes have cytokine-like effects when exposed to mammalian cells. The CLEC genes also have their function in C. elegans, they are crucial parts of the innate immune system. The genes are significantly affected by the introduction of pathogens to C. elegans, they are down and up regulated by the pathogens. For example, Clec-47 is induced by the introduction of P. aeruginosa (Pan et al 2021). This offers a mechanism for the immunoregulatory effects of CEC, through the interactions of these CLEC proteins and mammalian systems. Unfortunately clec-47 remains the only one of the CLEC proteins that were characterized in mammalian cells as it was chosen for its small size. However, it can be safely assumed that the rest of the CLEC proteins have cytokine-like effects in mammalian cells and are responsible for the immuno regulatory effects of CEC.

Candida Albicans, Pseudomonas aeruginosa, and *Staphylococcus aureus* are 3 pathogens which are relatively well studied in C. elegans. Studies have produced extensive data sets which detail the genome changes when C. elegans are infected with said pathogens. The data shows that CLEC genes are repeatedly affected by these pathogens, giving evidence that the change in expression of the CLEC proteins due to pathogens could correlate with differing levels of cytokine-like function when CEC is exposed to more complex organisms (Pukilla-Worley et al 2011, Irazoqui et al 2010). This leaves the question of how the regulation of these C. elegans genes can be exploited to interact with the mapk pathway and alter inflammation levels. Moreover, the bacterias *P. aeruginosa* and *S. aureus* have been shown to alter the genome of C. elegans in a way that 21% of the gene changes differ from the gene regulation in C. albicans infection. C. albicans and S. aureus pathogenesis are implicated with the conserved MAPK pathway while P. aeruginosa infection affects the QS (quorum sensing pathways), and the 2-component system gacA/gacS pathway. They can be used to identify how different infection types will have on the anti-inflammatory property of C.elegans Crude Extract. These pathogens can also be used to create the desired immuno-active environment in the C. elegans for extraction. Zebrafish are a great model organism for this study as they have immune response and inflammation systems which mimic human systems. They have cytokines which can be measured as well as macrophages and lymphocytes whose migration can be quantified. Assays have been developed to induce inflammation in zebrafish which can then be treated. The treatment of inflammation has applications across medicine as it can assist in treating chronic inflammation as well as localized acute inflammation. The researcher plans to use zebrafish embryos as a model organism to measure the effects of C. albicans, P. aeruginosa, and S. aureus treated C. elegans crude extract on inflammation. To accomplish this, zebrafish embryos will be used because of their relatively complex immune system and homologous cytokines. This allows for the induction of inflammation in various ways and to test treatment compounds in different ways. For example, the induction of Inflammatory bowel disease (IBD). The use of chemicals such as 2,4,6-trinitrobenzenesulfonic acid can be used to establish an inflammatory response in the rudimentary GI tract of the Zebrafish embryos. This can have a number of effects on the GI tract, causing architecture change and goblet cell migration. Fluorescent compounds can be digested by the Zebrafish, this will contrast against the GI tract walls so the architecture of the lumen can be observed. Moreover, alcian blue can be used to stained goblet cells to observe inflammation changes. NOS inhibitors can be used to ameliorate the IBD symptoms in the zebrafish and can be used as positive controls. The inflammatory response can be measured in part by measuring gene expression changes of inflammatory response controlling genes. The Stat1 and Stat6 pathways are one of these inflammatory response controlling genes. Stat1 and Stat6 are transcription factors which are involved in macrophage polarization, this is the differentiation between T1

response and T2 response. The Stat1 pathway is initiated by the reception of Th1 cytokines such as IFN-y, this results in the activation of the Stat1 transcription factor and thus the differentiation of Th1 macrophages as well as the suppression of Treg. (Banik) On the contrary, Stat6 is involved in Th2 polarization. Th2 cytokines such as IL-4 activate the transcription factor Stat6 which skews inflammatory response in the Th2 direction. This phenomenon mostly occurs when the body is fending off helminths, which is partly responsible for the anti-th1 inflammatory response from the body when facing helminths. (Maier) This dynamic can be used to measure the polarization of the inflammatory response cells, whichever response is dominant will have more expression of the respective transcription factor.

Purpose

The purpose of this study is to develop a novel and cost effective treatment for inflammation via the manipulation of the immune response of C. elegans.

Hypothesis

If C. elegans are exposed to *C. albicans, P. aeruginosa,* and *S. aureus* and are used as medication, then they will exhibit anti-inflammatory properties.

If the anti-inflammatory effects of Crude Extract from C. elegans exposed to C. *albicans, P. aeruginosa,* and *S. aureus* are compared then the Crude Extract from the C. elegans that were exposed to *C. albicans* will have the greatest anti-inflammatory effect.

Methods

C. albicans culture

1. Streak YEPD plate with C. albicans and incubate at 30 C
2. Inoculate a colony in 4ml YEPD media in test tube and shake at 30 C overnight

P. aeruginosa

1. Grow P. aeruginosa on agar plates
2. Inoculate in LB broth and shake at 37 C overnight

S. aureus

1. Grow S. aureus on agar plates
2. Inoculate in LB broth and shake at 37 C overnight

Crude Extract Preparation

Wash C. elegans away from the culture dish with M9 buffer; centrifuge at 1500 r/min in 1.5 ml EP tube at 4°C for 5 minutes; remove upper clear liquid; obtain C. elegans after washing precipitants three times. Put C. elegans in the ultrasonic cell disruptor (ultrasonic power: 200 W, ultrasonic time: 5 min); conduct sterilization via suction filtration to obtain crude extracts of C. elegans (C. elegans); prepare 50 μg/ml C. elegans solution with PBS solution;

Inflammation Induction

Intestinal

1. Larvae should be 3 d.p.f

2. IBD is induced by the addition of 75 µg/mL 2,4,6-trinitrobenzenesulfonic acid (TNBS; Sigma) to zebrafish medium
3. All IBD induction and rescue assays should be performed in 96-well mesh plates

Rescue (control)

50 µg/mL stock solution of S-methyl-L-Thiocitrulline acetate salt (Sigma) in embryo medium. From 3-5 d.p.f embryos should be exposed to 75 µg/mL TNBS solution, at day 5 the solution should be switched to 75 µg/mL TNBS and 25 µg/mL S-methyl-L-Thiocitrulline acetate salt until 7 d.p.f.

Alcian Blue Staining (Chernick)

Fixing Fish

Fix the specimens in 10% neutral buffered formalin overnight (16+ hours) at room temperature overnight on an orbital shaker and then move to 4°C until processing. Wash the specimens with 1X PBST twice

Alcian Blue Staining for Embryos

Fix the specimens in 10% buffered formalin overnight at room temperature or 4% paraformaldehyde (PFA) at 4°C overnight. Wash the specimens in 1X PBST twice. Stain in Alcian blue solution overnight at room temperature. Wash in 100% ethanol (EtOH) one time. Transfer through an ethanol series to 1X PBST. Bleach with hydrogen peroxide (3% H2O2 in 1% KOH) for 1 hour at room temperature or until the eyes of specimens become transparent (or very light grey).

Quercetin Staining

Rear fish in 0.05% quercetin for 2 hours. View under fluorescent microscope, record to document peristalsis patterns.

Histology

Anethazise by immersion in 0.2 mg/mL MS222

View on BX51 fluorescence microscope

Results

Alcian Blue Assay

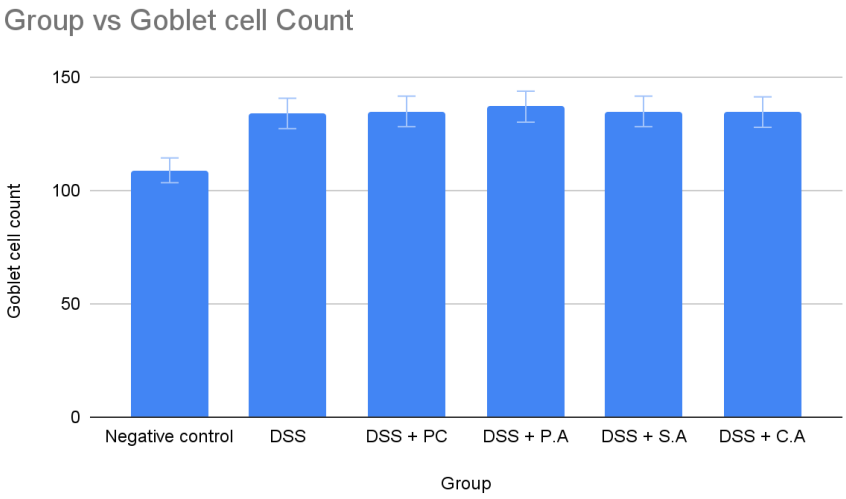

Figure 1. T-tests $p<0.05$

This assay was used to determine mucin production in response to inflammation and to effects of the CEC on the mucin production. The assay involves the staining of goblet cells with alcian blue and counting the amount of stained cells within the foregut. Results from this assay show that goblet cells were stained at a much higher rate in the DSS groups as expected. The difference between the negative control and the inflammatory groups was anticipated. However, the positive control and experimental groups did not have any significant effects on the goblet cells when compared to the DSS group.

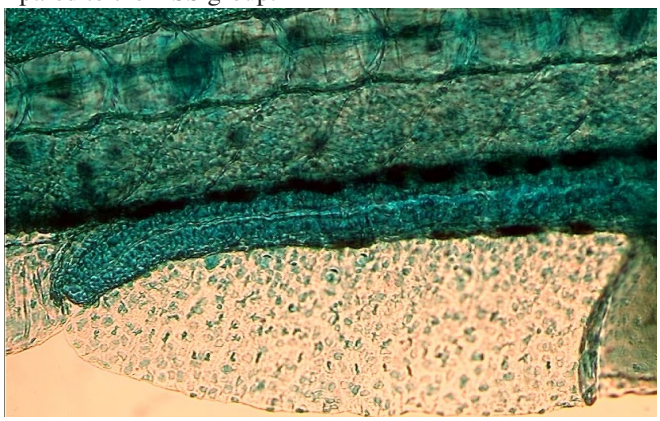

Figure 2. Alcian blue staining assay

Quercetin Staining Assay

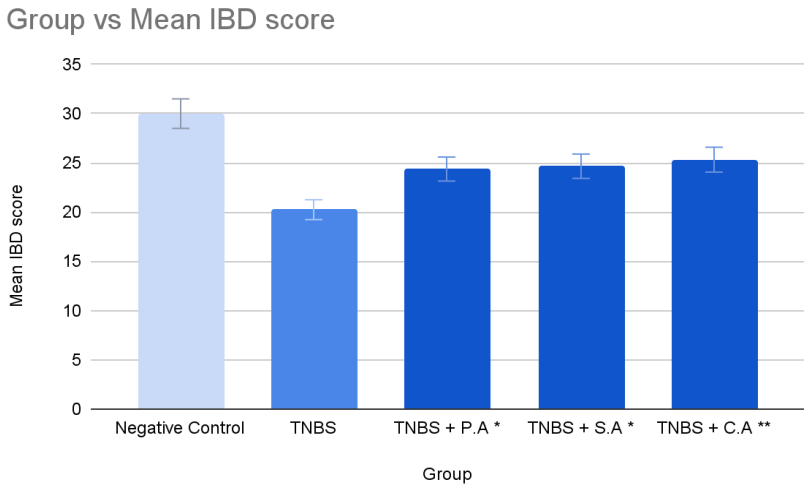

Figure 3. T-tests *p<0.05 **p<0.01

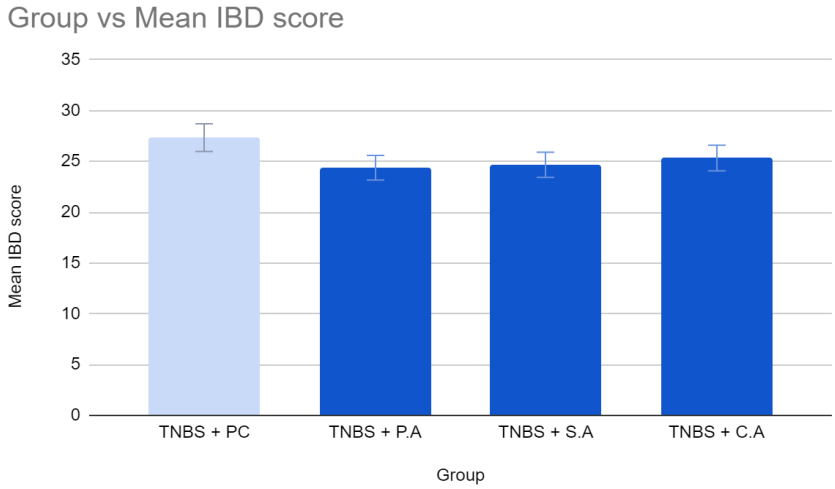

Figure 4. T-tests *p<0.05 **p<0.01

This assay was used to determine the effects of CEC on IBD gut architecture. TNBS was used to induce colitis-like inflammation in the gut of zebrafish and was co-administered with treatment groups. Quercetin, which is a fluorescent dye, was added to fish medium and the fish drank the dye, causing a fluorescent contrast within their gut. The fish are then observed and then rated on the "In vivo Observations of gut architecture" (Janowski 2010).Figure 3 shows the effect on the treatment groups on the mean IBD scores. In all 3 treatment groups the difference between the negative control and the TNBS groups were significant (*P<0.05,**P<0.01), suggesting that the CEC successfully ameliorated the IBD symptoms to an extent. The Candida Albicans exposed group had the most significance when compared to the TNBS group despite the fact that the difference between each experimental group was not significant. Figure 4 shows that the difference between the positive control and the experimental groups was not statistically significant, meaning that the experimental groups function statistically similarly to the positive control.

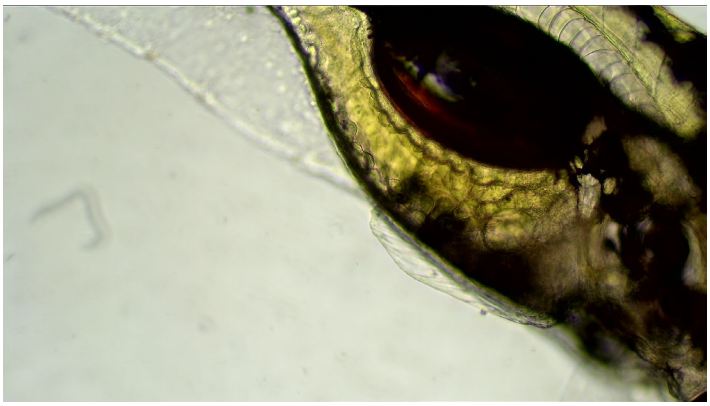

Figure 5. Light microscope view of quercetin staining assay, villi of gut walls are very visible

Fin Cutting Assay

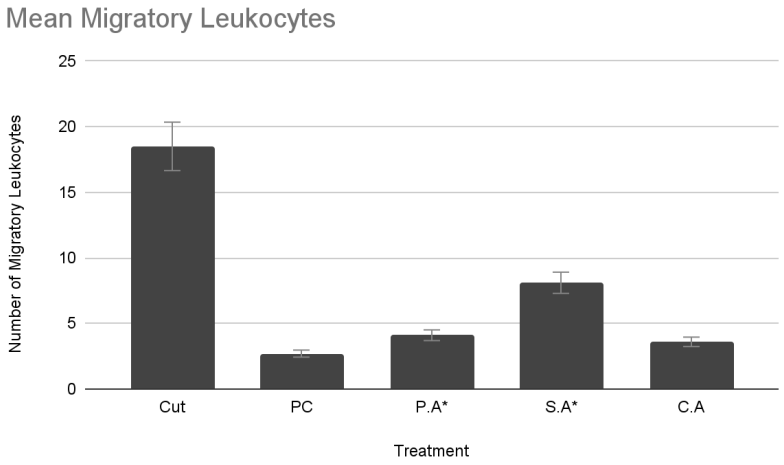

Figure 6. T-tests $p<0.05$

Figure 6 shows the effect of the treatment groups on the average number of migratory leukocytes in the fin cutting assay. The data shows that all 3 treatment groups significantly lowered the number of migratory leukocytes from the negative control (Cut). It also shows that the C.A group function significantly similarly to the positive control while the P.A and S.A groups did not.

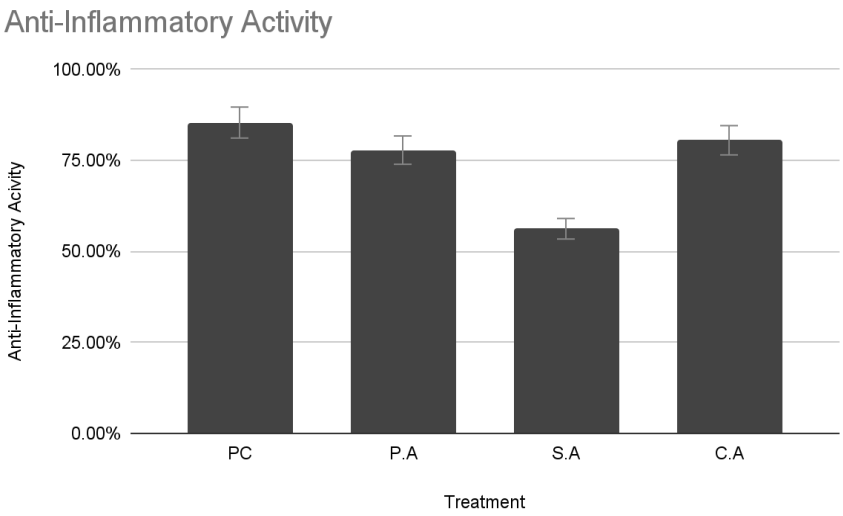

Figure 7. T-tests $p<0.05$

Figure 7 shows the anti-inflammatory activity of the experimental groups. This is calculated using the relative leukocyte migration values. The data shows that all of the treatment groups had at least 50% Anti-Inflammatory Activity with C.A having the most and S.A having the least.

Image 8. Light microscope view of positive control, notice low number of migratory leukocytes to the injury site

Conclusion

First hypothesis- If C. elegans are exposed to C. albicans, P. aeruginosa, and S. aureus and are used as medication, then they will exhibit anti-inflammatory properties.

In total, the first hypothesis was supported by the quercetin staining assay. The data showed both statistically significant differences between the negative control and the inflammatory group and no significant difference between the positive control. This indicates that the CEC was effective in functioning as an anti-inflammatory medication and performed on a similar level to an industry standard positive control. The fin cutting assay further supported the hypothesis, showing that the CEC treatments significantly lowered leukocyte migration to injury sites and exhibited similar anti-inflammatory properties to the positive control. The support of this hypothesis shows that the CEC can be effective as a treatment against inflammation. This not only supports previous literature but adds the component of the efficacy of immune-regulating the C. elegans to produce effective anti-inflammatory results. The different results across the fin cutting assay show how the different pathogens changed the anti-inflammatory effects and lends to the idea of immuno-regulating C.elegans before CEC extraction.

However, the first hypothesis was not supported by the alcian blue staining assay as there was no difference between the inflammatory group (DSS) and the experimental groups. But it should be noted that the positive control did not have its anticipated effect either and it may be due to an experimental design flaw.

Second hypothesis- If the anti-inflammatory effects of Crude Extract from C. elegans exposed to C. albicans, P. aeruginosa, and S. aureus are compared then the Crude Extract from the C. elegans that were exposed to C. albicans will have the greatest anti-inflammatory effect.

Moreover, the second hypothesis was also supported by the quercetin staining assay. Data showed that the C. albicans exposed CEC had the highest effect on lowering the inflammatory phenotype in the IBD models ($p<0.01$) when compared to the P. aeruginosa exposed and S. aureus exposed groups. The fin cutting assay also supports the second hypothesis as the C.albicans exposed CEC had the lowest number of migratory leukocytes of all the experimental groups as well as the highest anti-inflammatory effect. Again, the prevalence of the C. albicans treated group shows the effects of the different pathogens on altering the secretory proteins of the C. elegans.

Again, the alcian blue assay did not support the second hypothesis and the same possible experimental design flaw should be taken into account.

Application

Helminth therapy has always had a stigma around it despite the scientific evidence of it being an effective treatment regardless of the possible side effects. This study aimed to prove that when the side effects of helminth therapy are bypassed, it can be an effective inflammatory treatment. Fortunately, the results lend to this idea that C. elegans and other helminths can be used as a medication. The impact of this is enhanced by the fact that C. elegans can be continually grown and harvested if they were to be developed into a drug. A new culture of C. elegans can be purchased for around $10, and this culture can then be expanded for a relatively low cost; meaning that the development of a drug using C. elegans could be extremely cost effective due to low production costs. Furthermore, CEC offers an alternative treatment which differs from the functions of NSAIDs or steroids. The functional differences of CEC in its manipulation of th1 and th2 response gives another option for people with inflammatory disorders if NSAIDS or steroids are not effective. Also, the use of immunoregulation of the CEC which leads to different protein production levels can be tailored using different pathogens to fit disease phenotypes, while granted it will require further research it is another reason that CEC as a medication is so beneficial. By simply culturing C. elegans using different bacterias and fungi can change the function of the CEC, effectively being “customizable” to different diseases. Overall, the study hopes to destigmatize helminth therapy and transform it into a useful and impactful therapy tool.

Limitations

This study contained a few limitations. Firstly, the process of preparing worms for experimentation was meticulous and time consuming as the age synchronization is often unreliable and must be repeated until success. Furthermore, the C. elegans growth and reproduction was problematic in waiting for enough eggs to be laid for age synchronization. Moreover, zebrafish embryos were also very fragile and often died for no apparent reason. A big limitation was that the zebrafish could not survive a wide range of doses of the CEC, this meant that dose dependent variables would have been redundant as the fish would not survive the high doses. Another limitation was that DNA could not be efficiently extracted from the C. elegans and zebrafish to complete 3 qPCR assays. Without the chemical "proteinase K", it was not feasible to extract enough DNA for these qPCR assays. The assays would have looked for CLEC-47 in the C. elegans and would test for Stat1/Stat6 in zebrafish. This would have furthered evidence to support hypotheses and would have added assays besides staining assays. Also, without a microtome it was not possible to effectively measure the goblet cells from the alcian blue staining assays. Services were supposed to be supported by the University of Miami, but the microtome broke before it could be used to analyze the alcian blue samples.

Future Research

The goal of this study was to develop a novel and cost effective treatment for inflammation via manipulating the immune response of C. elegans. The study was effective in completing this goal, however there are additional steps which can take the study to the next level. Firstly, implementing more biochemical assays such as the aforementioned qPCR tests would shed light on the exact biochemical reasons for the data shown. Moreover, using a wider variety of bacteria/fungi and conducting a screening which uses similar principles as this study is essential to further the notion of immunoregulation C. elegans for alternative helminth therapy. Furthermore, a medicinal form of CEC would have to be developed for use in clinical trials and eventually as a medication. Ideally, this medication would contain a capsule which would trigger the immune system of the host similarly to a helminth. Then, when the body attacks the capsule, ideally within the stomach or intestines, the CEC can be released and the body will recognize and attack it similarly as it would a helminth. This would effectively mimic a helminth infection and give the intended anti-inflammatory effect. Future research could also explore the use of crude extracts of more traditional helminths as their secretory proteins are most likely more potent than those of C. elegans and they would offer a greater anti-inflammatory effect.

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Dextran sulfate, sodium salt; MSDS No. 9011-18-1 [Online]; ThermoFisher-Scientific: FAIR LAWN NJ.https://www.fishersci.com/store/msds?partNumber=AC611361000&productDescription=DEXTRAN+SULFATE+SODIUM+SA+100G&vendorId=VN00033901&countryCode=US&language=en