

Effects of Cordycepin on Regeneration in a Planarian Model

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## Abstract

Used widely in Traditional Chinese Medicine, the *cordyceps* mushroom has been shown to offer a plethora of health benefits. Aiding ailments from chronic fatigue to cancer, the major bioactive molecule, cordycepin, may also assist in tissue repair. To test these unsupported claims, planarian were cut in half to serve as a regenerative/wound healing model with cordycepin being administered at nine varying concentrations. Specific aspects of growth, being total length and blastema length, were observed over the 6 days following amputation. After analysis of variance, a significant interaction between segment and concentration was recorded with concentrations of interest corresponding to 1mM, 100uM, 100nM, 10nM, and 100pM on the 6th day of regeneration,  $F_{(8,287)}=3.305, p<.01, \eta^2=.0891$ . Differences in blastema on day 6 of regeneration were also observed,  $F_{(8,243)}=2.130, p<.001, \eta^2=.087$ , showing an increase in size in higher concentrations, 1mM and 100uM, and decreases in size when considering the lower concentrations of the experiment, 100nM, 10nM, and 100pM. The differential results indicate the possibility of different mechanisms of regeneration being influenced by cordycepin, but further biochemical testing is required.

## Introduction

### Cordycepin

In Western culture, the *cordyceps* fungus did not gain recognition until the 1993 Summer Olympic games when three female long distance runners from China broke five world records. The athletes were rumored to have been on a stringent daily regimen of *cordyceps* mushroom and turtle blood. The main bioactive ingredient of the *cordyceps* mushroom strain the athletes were using was subject to extensive studies following the results of the 1993 Summer Olympics; the molecule eliciting the most profound biological response in the *cordyceps* fungus was cordycepin, which had already been identified 30 years in advance (Kredich & Guarino, 1960). The molecule was discovered and isolated in the 1950's but did not attract much attention until the dramatic exposure attained in the Summer Olympics of 1993 (Cunningham et al., 1950). Though this molecule is relatively novel in Western civilization, Eastern cultures have been using a strand of *cordyceps* since the 15th century (Das et al., 2010). *Cordyceps* has been a cornerstone of Traditional Chinese and Tibetan medicines for hundreds of years, with reports of the mushroom being able to cure most any ailments ranging from chronic fatigue to cancer (Das et al., 2010). When referring to the literature and visiting studies of Western culture, the molecule cordycepin has shown promise as an immunomodulatory molecule, as an anti-inflammatory compound, and also as an anti-oxidant. The molecule has been subject to rigorous research in the field of cancer and has shown promise in cell cultures and animal studies (Tuli et al., 2013). Traditional Chinese Medicine emphasizes the use of the *cordyceps* fungus to increase rates of tissue repair, yet after revising the literature, no study has shown direct support of this statement. The lack of evidence, either supporting or rejecting this claim, has lead to the project

at hand of establishing whether or not cordycepin will have an effect on regenerative capacity of an organism, as will be experimentally demonstrated within a planarian model of regeneration .

### ***Cordyceps militaris***

*Cordyceps militaris* is one of the best-known entomopathogenic fungi found and collected within North America (Patel & Ingalhalli, 2013). *Cordyceps* is of Latin origin and translates as "cord" meaning club, and "ceps" meaning head; the translation is extremely literal and will become even more apparent once the process of infection is described in the latter part of this subsection. Generally, fungi are classified via the method of reproduction used, which represented within the species' spore distribution apparatus. *C. militaris* belongs to the phylum Ascomycota, meaning that spores are contained within a protective sac, known as an ascus (Patel & Ingalhalli, 2013). From these asci, ascospores are released into the surrounding environment once the breakdown of the ascus occurs. One can consider the *C. militaris* fungus to be an aerial assassin, as the pathogenic targets of the fungus include primarily winged insects. The *C. militaris* fungus principally infects insects and larvae belonging to the Lepidoptera order, consisting mainly of butterflies and moths (Zheng, et al., 2011). To outline the method of infection of the *Cordyceps* family, one would begin by describing the instance in which the spores of *C. militaris* lands upon the exoskeleton of an insect. From this point, the spores are presumed to be infective and begin growth into a mycelium, which is essentially an extensive network of fungal cells (Zheng, et al., 2011). The mycelium begins to secrete hydrolytic enzymes in order to break down the tough exoskeletal layer of the insect, consisting of alpha-chitin. Once inside the tough exterior, the mycelium now has access to the soft inner constituents of the insect, and thus the infiltration into the host tissues begins. The mycelium begins to replace the insect's tissue, slowly but effectively, in attempts to keep the insect alive for as long

as possible. This would be of evolutionary benefit in the sense that this mode of infection allows for maximum energy extraction from the biomass provided by the insect. Once the mycelium establishes itself within the insect substrate and begins energy and nutrient extraction via short absorptive anchors known as haustoria, the production of more spores begins, allowing the cycle to be repeated (Cunningham et al., 1950). After a period of mycelium growth and maturity, a specialized portion of mycelium, known as the sclerotium. The sclerotium suffers a great degree of interspecies difference as some may contain multiple red-pointed sclerotium, while others possess sclerotium that look like tiny white hairs (Zheng, et al., 2013). *C. militaris* gives rise to the characteristic orange mycological columns that appear to erupt from the insects' head and body. Within the sclerotium of any *cordyceps* species, the mature spores are held for release in their respective asci; but in conjunction to the spores, there are a plethora of mycological compounds found, including nutrients for spore vitality and various fungal metabolites, including the notorious compound, cordycepin. (Cunningham et al., 1950 ).

### **Planarian Anatomy**

To begin boldly, the planarian are one of the most fascinating organisms in terms of senescence, as they show virtually no signs of biological aging or telomerase degeneration (Tan et al., 2012). In addition to their asenescent nature, planarian possess the ability to regenerate all the cells found in their adult form, making them essentially immortal (Tan et al., 2012). One may question the introductory statement and reject the thought of a non-parasitic, freshwater flatworm being fascinating in the slightest, but all negative preconceived notions of flatworms should be thrown away this instant. As there are a multitude of different species of planarian which vary in terms of regenerative capacity and basic anatomy, they are an emerging choice of study in regeneration, cell biology, and pharmacology due to the extensive number of orthologous genes



that have been identified between the human and planarian genome (Reddien et al., 2005). It must be noted that *D. tigrina* are acoelomate in nature, in the sense that they lack an internal organ cavity. There is a small cavity referred to as the gastrovascular cavity, that is caudal to the pharynx and mouth of the organism, and is used for enzymatic catabolism of ingested particulate matter and distribution of nutrients throughout the entire planarian (Reddien & Alvarado, 2004). There is no respiratory system in the planarian; oxygen and carbon dioxide readily diffuse through the dermal layers following a gradient. In contrast to these systems, the planarian central nervous system patterning, even at the level of neurotransmission, possess a markedly similar pattern to that of the central nervous system observed in humans. (Reddien & Alvarado, 2004). One of the most convincing pieces of evidence for planarian and human neural congruity is the production of 0.1-5Hz oscillations that were recorded via electroencephalogram (Aoki et al., 2009). There exists a continuous waveform on the spectrum of electrical activity indicating that there exists a feedback loop or neural network in the simplistic planarian. Though the components of the nervous system are primitive relative to human systems, the planarian nervous system consists of a bi-lobed ganglionic component, just under the eyespots of the planarian. The bilateral masses are referred to as the planarian brain, which is the central mediator of the ladder like structure of the planarian nervous system which will be further described. In the planarian, two lateral nerve cords project from the primitive brain to the caudal portion of the planarian. These cords are accompanied by traversing nerves which connect the two cords, allowing for the essential circuitry to perform coordinated movements. (Sarnat and Netsky, 2002). The aforementioned eyespots are known as ocelli; these allow planarian to inaugurate locomotive action as a response to light, in a photophobic manner (Stevenson et al., 2010). Almost every single neurotransmitter found in the human brain are found in the planarian, including many of

the neurotransmitters implicated in major behavioural pathways seen in humans, such as serotonin, dopamine, acetylcholine, various opioids, and noradrenaline (Rangiah and Palakodeti, 2013). Needless to say, there a plethora of receptors required to sequester these neuroeffective molecules, which open the door to pharmacological research studies of endless capacity, including those involving cordycepin. In addition, drugs and other compounds are easily tested in planarian due to their constant dermal interface with the environment. It is assumed that a molecule in a solution will be regarded as being the same concentration within the planarian as per osmotic regulation and diffusion. Excretion of metabolic products is quite different in planarian, possessing their renal-equivalents in the form of flame cells, which are functionally tied to regulation of osmotic pressure and balance of ionic concentrations (Ruppert et al., 2004) The cellular processes of division and the requirements for adenosine triphosphate (ATP) formulated through aerobic respiration are homologous between both human metabolism, as well as planarian metabolism. Every living cell on earth requires energy in the form of ATP, so any implications arising from the activity of this molecule, or any of its various forms, within a cell will be pertinent to not only planarian, but humans as well. With these anatomical and physiological features in mind, the true value of planarian studies involving pathologies derived from metabolic or nervous system function are absolutely relevant, and at times provide more effective observation of cellular mechanisms (Reddien & Alvarado, 2004).

### **Conserved Pathways of Planarian Regeneration**

The planarian is rather simplistic in its anatomy, as described above, allowing for increased observational capacity of cellular mechanisms at work in the regenerative process. (Reddien & Alavarado, 2004). Though the planarian may be deemed primitive or simplistic, the use of planarian as a model for regeneration provides considerable translational benefit to human

regeneration. There are highly conserved pathways, found in cellular regeneration in humans, mediating planarian regeneration (Sater, 2011). These same pathways may be observed in all animals displaying bilateral symmetry, meaning that they not only possess a rostral and caudal aspect, but also a dorsal and ventral component (Ingham et al., 2011). These signalling pathways involve the canonical wnt pathway and the fibroblast growth factor (FGF) pathway, both of which are controlled by the hedgehog (hh) signalling pathway (Sater, 2011). The FGF pathway deals mainly with the wound healing process in the form of cellular signalling in the processes of differentiation and cellular proliferation. (Sater, 2011). The wnt signalling pathway is another evolutionarily conserved pathway present in planarian which consists of proteins which serve as signal transducers that can be found as receptors on the cell surface allowing for passage into the cell and cytoplasmic system (Nusse & Vamus, 1992). The canonical wnt pathway was initially characterized for its role in carcinogenesis, but it plays a much larger role in the cellular system as a whole. Accomplished via binding of  $\beta$ -catenin to the receptors, the initiation protein complex activation cascade occurs and ultimately results in a change in gene transcription and the consequential proliferation, cell migration and determination of anatomical axes within an organism (Nusse & Vamus, 1992). The hedgehog signalling pathway is active in embryonic and adult states and exists as a differential protein concentration in all tissues of the body. The differential concentrations allow for specific differentiation processes of the respective tissues and ultimately may be deemed the key regulator of development. The hedgehog protein controls the FGF pathway and the wnt pathway via modulation of gene expression (Sater, 2011). Another conserved pathway allowing for cellular proliferation is the TOR signalling pathway which is crucial in planarian for the blastema formation (Peiris et al., 2012). When interfered with, cellular proliferation is not entirely impaired, but blastema formation does not occur indicating

that the migratory and parts of the proliferative processes are blighted. It should be noted that the planarian is still able to grow in size, even without the formation of a blastema, leading to the distinct separation between whole planarian growth, and the presence and size of a blastema after amputation (Peiris et al., 2012). In planarian, these pathways in conjunction with the hedgehog pathway are crucial for the determination of anterior and posterior portions of the flatworm during regenerative periods, as well as the regenerative capacity of the planarian.

### **Planarian Model of Regeneration**

To give some appreciation for just how incredible the regenerative nature of the planarian truly is, these flatworms may be cut down to 1/279th of their initial size and will still grow back into a fully functioning worm. Planarian regeneration is undertaken in a four step process following a period of amputation or tissue destruction. (Reddien & Alvarado, 2004). In the initial step, there is a closure of the wound site through an extension of the ectoderm of the planarian. This extension of the outer dermal layer results in the closure of the wound and essentially allows the process of recovering the amputated tissue to begin (Reddien & Alvarado, 2004). After the wound response has been initiated by the aforementioned evolutionarily conserved pathways, the initiation of the blastema formation begins. The blastema may be characterized by a group of cells that aggregate in the region just beneath the amputation, which is clearly observable via microscopic analysis or sometimes can be so conspicuous as to be noticed by the naked eye (Baguna et al., 1989). The type of cells within the blastema are known as neoblasts, which are analogous to totipotent stem cells, possessing the ability to differentiate into any tissue, including nervous tissue, in the adult form of the planarian (Reddien & Alvarado, 2004). Neoblasts not only constitute 30% of the cellular population in the planarian, but these cells are also capable of migration to the site of the lesion, even though they contain very little cytoplasm

(Reddien & Alvarado, 2004). This seems counter-intuitive as a cell lacking cytoplasm would not be optimal on a mobility basis, but neoblasts have been shown to aggregate and differentiate rather rapidly as the planarian regeneration process takes approximately 10 to 14 days (Baguna et al., 1989). Therefore, neoblasts are the driving factor in the replacement of tissues which have been severed from the amputative process (Baguna et al., 1989). Although the entire process takes 10-14 days, the mitotic index, meaning the number of cells undergoing division in the planarian, reaches pre-amputation levels 5 days after. (Salo & Baguna, 1984). This is why most of the literature regarding regeneration looks at the first 5-7 days of the process as an indicator of regeneration. After this period of aggregation, the third regenerative step begins involving a period of proliferation that is largely concerned with cellular division in order to constitute the entirety of the tissue that is missing from the planarian post-amputation (Reddien & Alvarado, 2004). Once the proliferative period has supplied sufficient cellular material, the fourth and final step in planarian regeneration calls for differentiation of the totipotent neoblasts found within the blastema (Reddien & Alvarado, 2004). As the cells are totipotent, they are able to replace lost ganglia, epithelial tissues, or other tissue types within the planarian in a rather seamless fashion with absolutely no visual scarring taking place. (Reddien & Alvarado, 2004). In addition to this process, the head and tail possess differential criteria in terms of the determination of polarity as a signal for which tissue to regenerate. The head section requires an initiation period of depolarization in order to allow the appropriate signals to be sent to the neoblasts and the pathways involved in the regenerative response and wound healing (Beane et al., 2011). The tail section is much less specific in terms of membrane potential alterations post-amputation and is chiefly concerned with the four step process outlined above. This entire regenerative process is

predominantly dictated by the evolutionarily conserved pathways discussed in the former section. (Sater, 2011).

### **Molecular Structure of Cordycepin**

Cordycepin is also known as 3'-deoxyadenosine and may also be referred to as an adenosine analogue (Tuli et al., 2013). The molecular formula of cordycepin is  $C_{10}H_{13}N_5O_3$ , whereas adenosine is represented by the molecular formula  $C_{10}H_{14}N_5O_4$  (Tuli et al., 2013). It should come as no surprise that the molecular structure of cordycepin is strikingly similar to that of adenosine with the exception of cordycepin lacking a hydroxyl group, an oxygen and hydrogen atom, on the 3' position of the furanose ring of adenosine (Tuli et al., 2013). The molecular weight of cordycepin is 251.24g/mol; as it is of such low molecular weight and is so similar to the endogenous adenosine molecule, cordycepin is readily passed through the blood brain barrier (Tuli et al., 2013). This similarity of the molecule to adenosine also reveals some of the functional aspects of the molecule within a biological system, as is seen across multiple disciplines; structure dictates function.

### **Proposed Mechanisms of Action**

When referred to in the literature, cordycepin is known as a bioactive metabolite in the sense that cordycepin is able to elicit a biological response in organism (Tuli et al., 2013). It seems highly unlikely that the exact method of action of cordycepin within the cell will be known to the researcher until after analysis of results, but the potential mechanisms of cordycepin action may be deduced by analyzing the possible derivatives which may be formed when cordycepin enters the cell. Cordycepin acting within the cell may elicit effects in a broad range of cellular functions due to the chemical similarity to adenosine. It may be involved in a number of different cellular

functions in which adenosine participates in such as DNA and RNA implications, the possibility of acting as a neurotransmitter imitating adenosine, and it may be able to play a role in metabolic signalling in the form of cordycepin monophosphate (CoMP), and cordycepin triphosphate (CoTP) (Tuli et al., 2013). Once inside the cell, cordycepin may begin to interact with the cytoplasmic contents, such as phosphorylating enzymes. This interaction leads to the possibility of cordycepin being crafted into various degrees of phosphorylated cordycepin derivatives in the form of CoMP and CoTP. Due to the structural organization and analogous nature of cordycepin to adenosine, cordycepin is able to be readily phosphorylated. The enzyme that phosphorylates adenosine to form adenosine monophosphate, adenosine kinase, cannot discern between cordycepin and adenosine leading to the formation of cordycepin monophosphate (Klenow, 1963). Cordycepin triphosphate is formed by an enzyme known as ATP synthase found in the mitochondrial membrane and acting to provide the cell with its source of adenosine triphosphate (ATP). This enzyme is also unable to discern between adenosine and cordycepin and thus CoTP may be formed (Klenow, 1963). This indiscretion displayed by enzymes does not exclude the receptors within the planarian nervous system. Cordycepin is able to act on P1 receptors in a similar fashion to adenosine, leading it to be classified as an adenosine agonist (Tuli et al., 2013). P1 receptors may be found in both the planarian and human nervous system and bind purinergic compounds like adenosine to provide neuroprotective effects, as well as the inflammatory response (Palmer & Trevethick, 2007).

**Cordycepin Acting as Adenosine** If cordycepin were to act in its administered form, the most pertinent function that may be imitated by cordycepin would be elicited through binding to the A1 nervous system receptor. A1 receptor binding facilitates neuronal membrane hyperpolarization, which is a function of the neuroprotective nature of adenosine (Pavenstädt,

1994). Since the establishment of the anatomical axes is in part determined by depolarization, there may be signalling interference due to polarity dysfunction as a result of the hyperpolarization elicited by cordycepin (Beane et al., 2011).

There is also the possibility that the stimulation of regeneration may be a result of adenosine binding to an A2A receptor within the central nervous system of the planarian (Palmer & Trevethick, 2007). The A2A adenosine receptor mediates the inflammatory response to a physically injuring stimulus. This is again an evolutionarily conserved pathway known as the innate immune response (Palmer & Trevethick, 2007). With an anti-inflammatory response elicited via the A2A receptors, there may be an accelerated rate of healing observed at the site of amputation.

**Cordycepin Monophosphate Acting as Adenosine Monophosphate** The possibility of cordycepin acting as CoMP is legitimate and may cause an inhibitory effect on regeneration through activation of the adenosine monophosphate-activated protein kinase (AMPK) enzyme (Wang et al., 2010). This enzyme is essentially the metabolic master of the cell as it is the signalling center for cellular energy homeostasis. The enzyme is responsible for determining if energy consuming pathways are to be activated, or if energy producing pathways are to be activated. The inability of the AMPK complex to distinguish between cordycepin monophosphate and the adenosine monophosphate molecules lead to an agonistic effect elicited in the presence of cordycepin monophosphate (Wang et al., 2010). Due to the rise in levels of AMP due to cordycepin monophosphate activation of this enzyme, it would result in the inhibition of the TOR pathway and the activation of ATP-producing pathways (Wang et al., 2010). In a regenerative setting, the optimal cellular processes would be those consuming ATP in order to migrate, proliferate, and differentiate neoblasts into the missing tissue. The TOR



pathway is crucial for blastema formation within the planarian regeneration process (Peiris et al., 2012). TOR signalling is decreased under conditions where there is resultant AMPK activation which may impair the speed and efficiency of the regeneration.

**Cordycepin Triphosphate Acting as Adenosine Triphosphate** If cordycepin were to be entirely processed into an ATP analogue, the effects may result in the stimulation of regeneration via the increase of intracellular energy availability and through interaction with the AMPK complex. In a cell, the energy released from ATP does not so much concern the adenosine portion of the molecule as it does the high energy interphosphate bonds. These bonds are also known as phosphoanhydridic bonds which possess high energy molecules which are generally unstable (Kofman, 1975). This is why the cell prefers ATP as its primary source of energy currency as the high energy electrons in the bonds provide for energy that is released from the bond once it is broken. The ATP-mime, CoTP, may be able to provide the energy in the form of high-energy bonds that will aid in the energy-hoarding process of regeneration. In the presence of extracellular ATP, neuronal cells release growth factors, pertaining to neuronal tissue as well as other tissues present within an organism (D'Ambrosia, 2001, Erlinge, 1998).

In addition to all of these molecules acting individually, it should be noted that in a cell, molecules and enzymes are in constant motion and thus are always reacting with one another. This means that there may be rather differential effects based on the interaction between multiple pathways within one organism.

### **Present Study**

The purpose of the proposed study is to establish whether cordycepin will have an effect on regeneration, stimulatory or inhibitory. This will provide a quantitative analysis of the effects of cordycepin on tissue regeneration within a planarian model which may or may not be able to support the claims of increased tissue repair in Traditional Chinese Medicine. With the possible mechanisms of action listed above, the regenerative effects will also be investigated in terms of effects seen in neuronal tissues versus the other tissues of the planarian body, including the musculature, the dermis, and the gastrointestinal component.

### **Cordycepin and Regeneration**

There will be a robust effect on regeneration due to the multiple paths of possible interaction as outlined above in the proposed mechanisms of action of cordycepin. The profound mediation of regenerative processes that may be elicited by cordycepin lead to the conclusion of a strong effect, but the direction, being stimulatory or inhibitory, still remains unclear. As for the differences of regeneration as seen in neuronal tissue, the anterior component, or the various other tissues, the posterior component, there will be a difference in the regenerative capacities observed due to the energy requirements needed to regenerate an epithelial cell in contrast to a neuron. The neuronal dendrites, soma, and axon consist of a single cellular membrane; the length of these distal processes lead to a surface area much larger than a simple epithelial cell. The increasing complexity of organ architecture leads to differential cellular processes when damage occurs due to the energy required to replace them (Coletti et al., 2013). As epithelial cells are easily differentiated at a relatively low energy cost to the organism, they will likely be able to regenerate much faster than the head component of the planarian, which is responsible for

growing the complex neural component. From a purely speculative point of view, the hyperpolarization of the membrane via A1 adenosine receptors may lead to a planarian that is unable to regenerate the neuronal component of the flatworm, including the ganglia, ocelli, nerve cords and pharynx due to the interference in membrane signalling.

## Methodology

A total of 290 planarian, of species *D. tigrina*, were employed in this study to investigate the effects of cordycepin on regeneration in order to increase the power of the study. 15 planarian were assigned to each group, head or tail and subject to varying cordycepin concentrations.

## Procedure

The first variable that will be manipulated in this experiment is which segment of the planarian is subject to administration of the drug, the head or the tail segment, while the second variable will be the respective dosage of cordycepin administered to the planarian, as measured in mol/L. There will be 8 different concentrations administered to the planarian, other than the control; those concentrations will a control, corresponding to 0mM, while the other concentrations will be 1mM, 100uM, 10uM, 1uM, 100nM, 10nM, 1nM, and 100pM. How the measurement of planarian regeneration will occur will be through the measurement of two separate factors; the first being the entire length of the planarian segment, measured in centimeters, while the second pertains to the size and appearance of the blastema formation, measured in centimeters.

**Measurement of Total Length** The measurement of total length will be determined using a digital camera and ImageJ software and recorded daily. To do this, planarian will be pipetted from the 2mL Eppendorf tube, into a petri dish and placed on a grid background where a picture will be taken with a digital camera for analysis via ImageJ software. ImageJ is a picture editing program that allows the user to take an image of the planarian in a petri dish on a grid background of known length. The ability to measure the background grid and standardize that length allows the determination of exactly how many pixels are in the known length. With this

method of standardization, the translational capability to other parts of the picture is unparalleled, allowing for the measurement of the segment in centimeters.

**Measurement of Blastema Length** In order to observe the blastema, a digital camera and microscope were employed, along with the aid of capture software. The blastema measurements were taken on day 0, day 3, and day 6 with a light microscope and digital camera in order to monitor the progression of the blastema through the critical period of mitosis, being day 1 to day 5 (Salo & Baguna, 1984). No measurements of the blastema occurred on day 0, but images were documented to formulate a reference point. As the process of neoblast migration takes 6-24 hours to commence, meaning it would not be present in the image (Salo & Baguna, 1984).

**Drug Dosage and Determination** The concentrations that will be experimentally examined in the planarian will be the control with a concentration of 0mM, while the other concentrations will be 1mM, 100uM, 1uM, 100nM, and 1nM. In order to achieve these concentrations, a set of serial dilutions were run from a stock mixture produced using 25mg of cordycepin, purchased from Sigma-Aldrich, diluted in H<sub>2</sub>O. This range was derived by referring to the literature of cordycepin being tested in cell cultures in which significant results were obtained for anti-oxidant, anti-inflammatory, anti-cancer and immunomodulatory effects. The anti-cancer effects were observed in the upper range of the four concentrations, whereas the anti-inflammatory and other effects were elicited by concentrations approximately pertaining to the micromolar and nanomolar range (Tuli et al., 2013). In order to prevent burnout in the researcher, concentrations were staggered and the number of concentrations per trial ranged from four some weeks, and all nine experimental concentrations in other weeks.

**Regenerative Setting** In order to create a regenerative setting in the planarian, there must be an induced amputation just above the pharynx. Where the scalpel has cut through will result in two segments of the planarian, a head and a tail respectively. The blastema will form at the caudal end of the planarian head segment, whereas the blastema will be expected to form at the anterior portion of the tail segment. In this sense, the tail segment of the planarian will be required to grow back the complex neuronal component of the body, whereas the head segment will be responsible for regeneration of the less complex musculature, dermis, and gastrointestinal tissues. These segments were kept separately in the 2mL Eppendorf tubes that was labeled with their own unique label. The planarian were randomly sorted after decapitation and introduced into their respective solutions of various cordycepin concentration. 1mL of drug was administered every second day for a duration of 6 days. Administration of cordycepin occurred every second day in order to prevent overdose of planarian as the halflife of cordycepin is unknown, but the halflife of cordycepin triphosphate is 14.3 days, as derived from Perkin-Elmer MSDS sheets. On days when solutions were changed, 1mL of cordycepin solution was delivered via the extraction of the liquid and subsequent pipetting of new solution into the 2mL Eppendorf tube. During testing, the planarian were held in an environment with consistent ambient temperature corresponding to approximately  $21^{\circ}\text{C} \pm 2^{\circ}\text{C}$ . Planarian were also housed in a dark area, underneath a cardboard box, to avoid stress as they are naturally photophobic animals.

## Materials

Copius amounts of *D. tigrina* were purchased from Carolina Biological in order to facilitate experimentation. Transfer pipettes were used for the transportation of planarian from the petri dish to the 2mL Eppendorf tube, and vice versa. 25mg vials of cordycepin were purchased from Sigma-Aldrich and utilized as necessary. With the aid of a weigh-scale, accurate

to 4 decimal places, the proper amount of cordycepin was diluted in H<sub>2</sub>O and subject to an intensive mixing process, accomplished by a vortex, in order to create a stock solution. A micropipette was necessary to ensure accurate and efficient transfers in creating the next set of solutions by way of serial dilutions. All cordycepin solutions were made in 50mL centrifugation tubes. A digital camera was used to record the length of the planarian daily on a 0.5cm grid paper. A computer with ImageJ software was necessary for the analysis of the images obtained from the digital camera. A light microscope with capture technology was crucial in collecting the blastema data which will be discussed further.

**Light Microscopy** As planarian are translucent, light will pass through their bodies readily, essentially allowing for a greater contrast of the tissues within the planarian. As the blastema contains much less pigmentation in its undifferentiated state, the ability of the light to pass through the planarian is of great advantage to the researcher as it allows for contrast of tissues. Light microscopy allows for the intimate observation of living samples that would otherwise be unavailable to the naked eye. Light microscopy with the aid of a digital camera and capture software allows for the privilege of monitoring progression of blastema formation and the regenerative process as a whole.

## Results

All analyses were conducted using SPSS software for Windows (Version 22). After running descriptive statistics, there were no outliers. Two planarian of the 290 planarian employed for the study were decimated during testing as a result of handling error. For the variable of total length, homogeneity of variance has been violated meaning that great caution should be taken when considering the significance of the results. For the variable of blastema length, homogeneity of variance and normality were met. When considering the within-subjects effects, it should be noted that these are simply secondary areas of interest. The purpose of the study was to determine differences in regeneration as a function of concentration and segment meaning that emphasis should be placed on the between-subject effects.

### Total Length

**Within-Subjects Statistics** Total length was used as a gross measure of the growth experienced by the planarian over the course of the 6 day regenerative process. When analyzing the data, Mauchly's test indicated that sphericity has been violated ( $\chi^2_{(20)}=436.77$ ,  $p=.000$ ), meaning that the Greenhouse-Geisser correction must be used to allow for compensation of the violation ( $\epsilon=.606$ ). Repeated measures analysis of variance revealed a significant result of day, day and segment, day and concentration, and also a significant three-way interaction between day, segment, and concentration which will be explained further in the following sections.

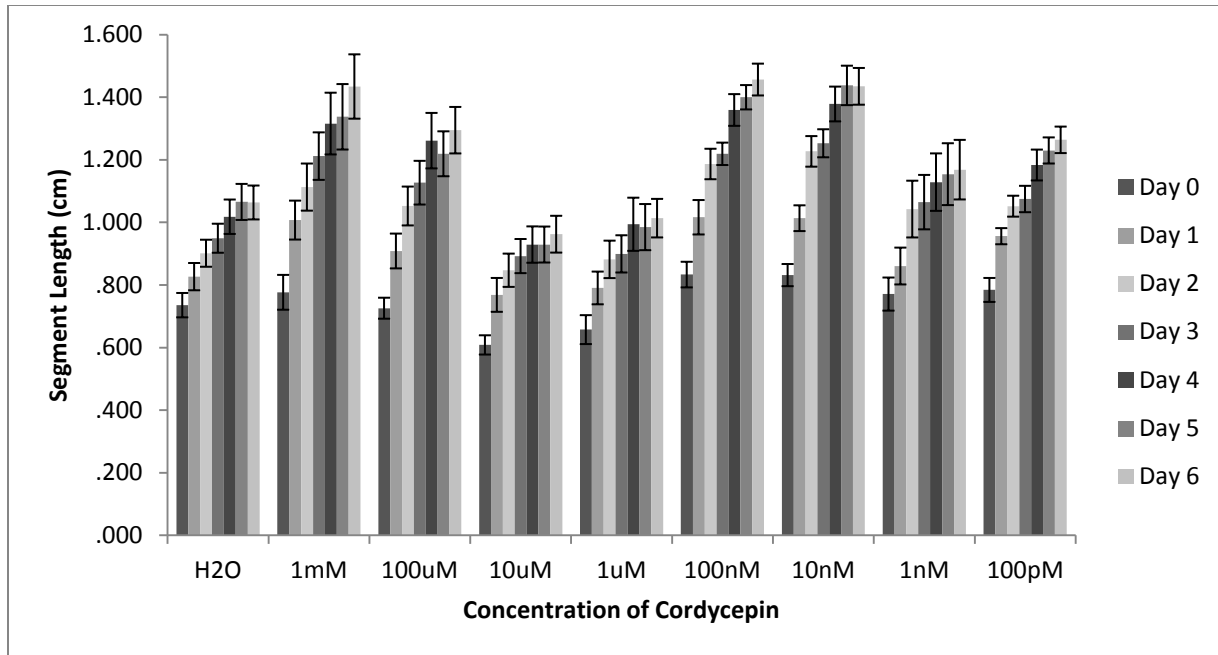
**Day** The results revealed a significant effect of day within-subjects,  $F_{(3.636, 160.59)}=514.705$ ,  $p<.001$ ,  $\eta^2=.656$ .

**Day and Segment** When considering the interaction between day and segment of the planarian, there are significant differences observed,  $F_{(3.636, 160.59)}=153.234$ ,  $p<.001$ ,  $\eta^2=.362$ .



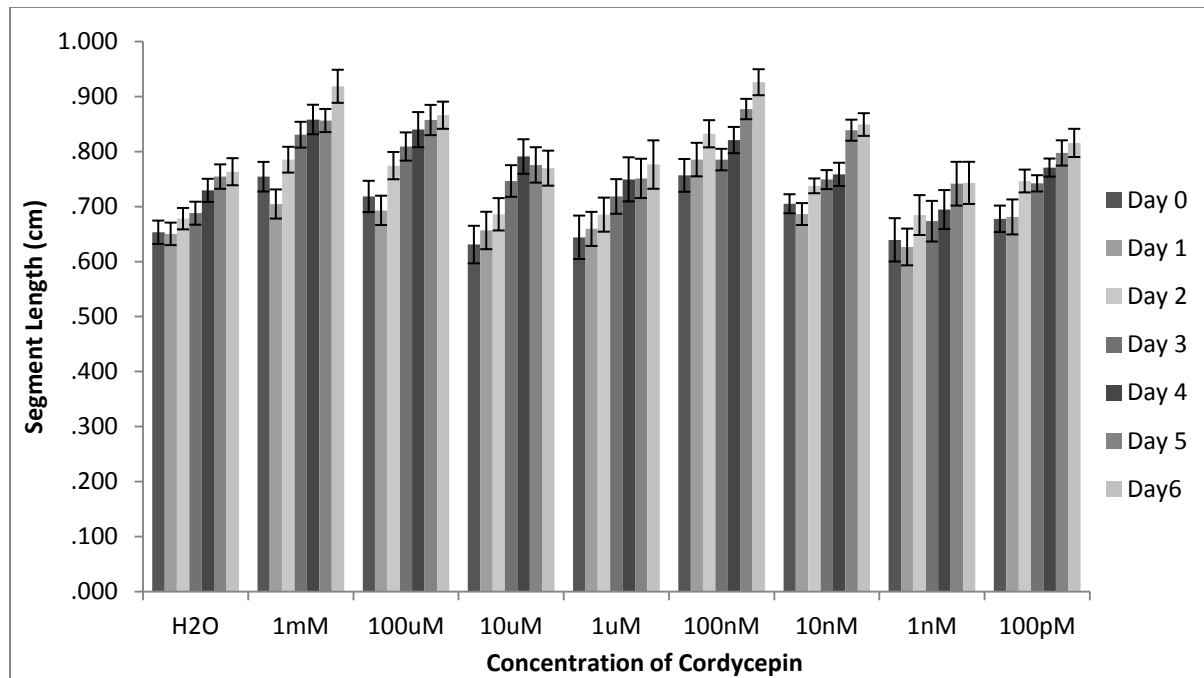
**Day and Concentration** Examining the interaction between day and concentration within-subjects unveils a significant result as well,  $F_{(29,089,981.746)}=3.912$ ,  $p<.001$ ,  $\eta^2=.104$ .

**Day, Segment, and Concentration** The three-way interaction is the real aspect of interest when it comes to the total length of the planarian segments. A significant interaction between day, segment, and concentration is present in the data,  $F_{(29,089,981.746)}=2,598$ ,  $p<.001$ ,  $\eta^2=.071$ . This means that total length is dependent on the day of regeneration, the type of segment regenerating, and the concentration of cordycepin the planarian have been subject to. The visualization of tail and head growth may be seen in *figures* 1, and 2, respectively. There was refrain from automated post-hoc analysis of the results to conserve power within the experiment. With the sheer magnitude of groups, the post-hoc Bonferroni correction would result in an alpha-level that is incredibly low, meaning an increase in Type 2 error. In order to identify significance in the data, visual inspection and analysis of standard errors of the mean will be the elected method of analysis for the data presented in all graphs.



*Figure 1.* Total length of cordycepin-treated regenerating tail segments over a 6-day period.

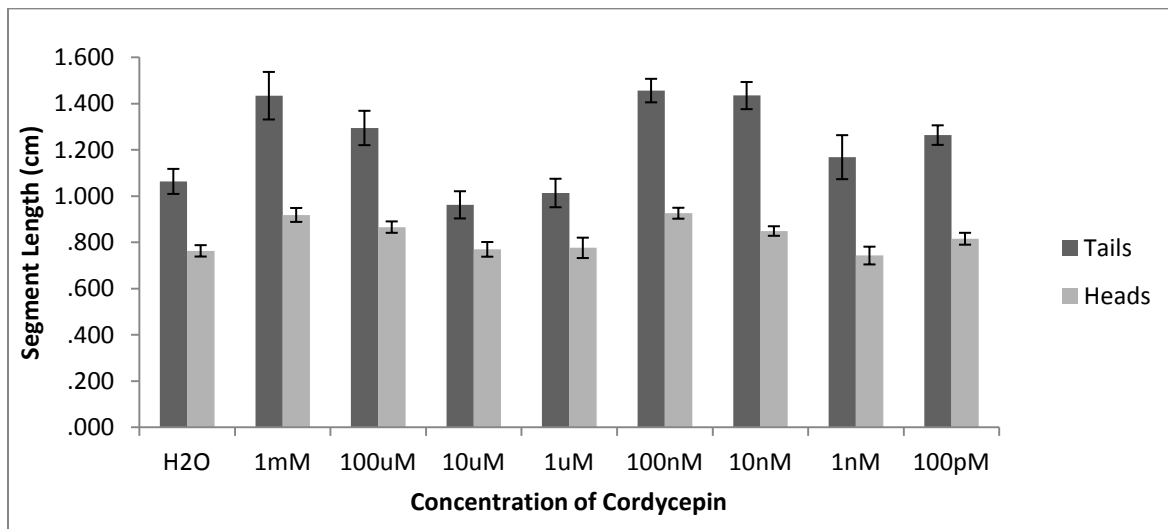
Figure 1 is the graphical representation of the change in segment length of cordycepin-treated regenerating tail segments over a 6-day period. Means are presented for tail segments in each experimental concentration for each day of regeneration with error bars representing the standard error of the mean. Visual inspection produces a clear effect due to the robust effect cordycepin exhibited on regeneration in planarian. These concentrations correspond to 1mM, 100uM, 100nM, 10nM, and 100pM as being significant from controls.



*Figure 2.* Total length of cordycepin-treated regenerating head segments over a 6-day period.

Figure 2 is the graphical representation of the change in segment length of cordycepin-treated regenerating head segments over a 6-day period. Means are presented for head segments in each experimental concentration for each day of regeneration with error bars representing the standard error of the mean. Visual inspection of the head and tail graph in conjunction show that there is a clear pattern of optimal concentrations eliciting effects in the regenerative model, but they seem to be of less magnitude in terms of segment length within the head sections. These concentrations correspond to the same as those found to be significantly larger from controls in the tail segments at 1mM, 100uM, 100nM, 10nM, and 100pM.

**Between-Subjects Statistics** In order to follow up on this interaction in a clear and concise manner, the data will be collapsed and the cumulative means for day 6 will be regarded as the areas of interest. Recalling the purpose of the study, the objective was to determine if cordycepin elicited differences in the regenerative process and to see if there was a difference in head or tail segments. Day 6 was chosen as it is indicative of the end of growth in regeneration and the return of cell division to baseline levels (Salo & Baguna, 1984). Using analysis of variance, it was revealed that a significant effect of concentration  $F_{(8,287)}= 11.503, p<.001, \eta^2=.254$ , and a significant effect of segment  $F_{(1,287)}=265.294, p<.001, \eta^2=.496$ . Further analysis revealed an interaction between-subjects  $F_{(8,287)}=3.305, p<.01, \eta^2=.0891$  when considering segment and concentration of cordycepin. This means that on the 6th day of regeneration, the total length is a function of the concentration of cordycepin and whether the segment is a head or a tail. In figure 3, the results are clearly seen when viewing the interaction between segment and concentration.



*Figure 3.* Interaction between segment and concentration on total length on day 6 of regeneration. Figure 3 is the graphical representation of the interaction between segment and

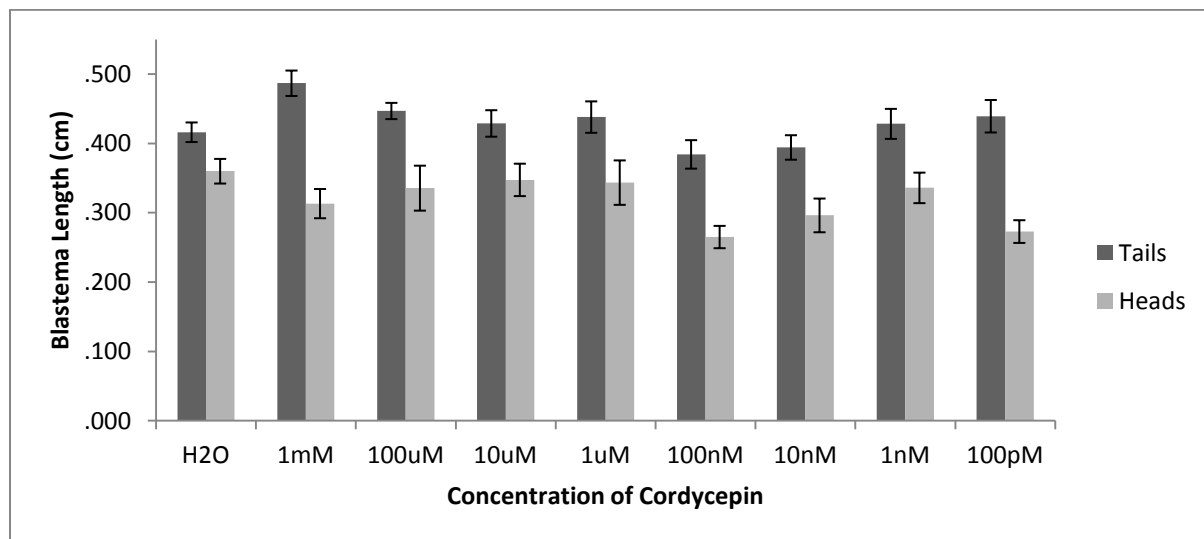
concentration on total length of cordycepin-treated regenerating heads and tails on day 6 of regeneration. There was refrain from post-hoc analysis on these results just as there was in the case of figures 1 and 2. Visual inspection of the means and error bars, represented by the standard error of the mean, shows that there are clearly specific concentrations driving the interaction in terms of total length. These concentrations correspond to the same as those found to be significantly larger from controls in figures 1 and 2 at 1mM, 100uM, 100nM, 10nM, and 100pM.

### **Blastema Length**

**Within-Subjects Statistics** In order to accurately acquire a flavour for the changes in regeneration registered at the cellular level, blastema data was collected throughout the course of regeneration. Maluchy's test of sphericity and tests of homogeneity of variance were shown to be non-significant, meaning no statistical corrections needed to be made. There was a significant effect of day,  $F_{(1,178)}=523.214$ ,  $p<.001$ ,  $\eta^2=.746$ , a significant interaction between day and concentration,  $F_{(8,178)}=4.023$ ,  $p<.001$ ,  $\eta^2=.153$ , as well as a significant interaction between day and segment on blastema length,  $F_{(1,178)}=20.567$ ,  $p<.001$ ,  $\eta^2=.104$ . There was no significant three-way interaction between day, concentration, and segment,  $F_{(8,178)}=1.345$ ,  $p=.224$ ,  $\eta^2=.057$ .

**Between-Subjects Statistics** Recalling the purpose of the study, the between-subjects aspect must be analyzed as it is the primary area of interest in terms of concentration of cordycepin and segment. After analysis of variance, it was revealed that there was no significance for the main effect of concentration on day 3 of regeneration,  $F_{(8,243)}=.470$ ,  $p=.876$ ,  $\eta^2=.021$ . There was a main effect of segment on the blastema length,  $F_{(8,243)}=41.854$ ,  $p<.001$ ,  $\eta^2=.190$ . The interaction between segment and concentration also was non-significant,

$F_{(8,243)}=.662$ ,  $p=.724$ ,  $\eta^2=.029$ . However, on day 6 of regeneration, there was a main effect of segment  $F_{(8,243)}=128.677$ ,  $p<.001$ ,  $\eta^2=.420$ , as well as concentration  $F_{(8,243)}=3.954$ ,  $p<.001$ ,  $\eta^2=.151$ . There was a significant two-way interaction present between concentration and segment on the blastema length,  $F_{(8,243)}=2.130$ ,  $p<.001$ ,  $\eta^2=.087$ , meaning that the concentration of cordycepin effected blastema length, dependent upon if the treated segment was a head or tail. The interaction has been graphed in figure 4. Just as was done with the variable of total length, day 6 will be the day of interest for purposes of clarity and explanation.



*Figure 4.* Segment and concentration interaction on blastema length on day 6 of regeneration.

Figure 4 is the graphical representation of the interaction between segment and concentration on blastema length of cordycepin-treated regenerating heads and tails on day 6 of regeneration.

There was refrain from post-hoc analysis on these results as explained previously. Visual inspection of the means and error bars, represented by the standard error of the mean, shows that there are clearly specific concentrations driving the interaction in terms of blastema growth.

These concentrations are similar to those found to be significantly different from controls in figure 3 at 1mM, 100nM, 10nM, and 100pM. Head segments treated with 100nM, 10nM, and

100pM all showed significant decreases in blastema length by day 6, whereas 1mM and 100uM treated tail segments showed a significant increase in blastema length by day 6.

In order to fully appreciate the results of the experiment, total length and blastema length must be considered almost as one cohesive variable. The significant results obtained by the above analysis have been summarized in table 1 below.

*Table 1.* Summary of blastema length and total length effects as a function of cordycepin concentration.

| Morphology | Segment | Concentration of Cordycepin |       |       |      |       |
|------------|---------|-----------------------------|-------|-------|------|-------|
|            |         | 1mM                         | 100uM | 100nM | 10nM | 100pM |
| Blastema   | Heads   | -                           | -     | ↓     | ↓    | ↓     |
| Length     | Tails   | ↑                           | ↑     | -     | -    | -     |
| Total      | Heads   | ↑                           | ↑     | ↑     | ↑    | ↑     |
| Length     | Tails   | ↑                           | ↑     | ↑     | ↑    | ↑     |

Table 1 clearly displays the significant interaction effects observed in figures 3 and 4. Using standard errors of the mean overlap as criteria for significance, 1mM, 100uM, 100nM, 10nM, and 100pM appear to be the optimal concentrations for effects on regeneration. Arrows indicate the directionality of the significantly different means when compared to those of controls upon visual inspection.

## Discussion

### Hypotheses

When referring to the initial hypotheses of the study, there was a robust effect on regeneration of stimulatory capacity and there was a significant effect of segment type on regeneration. There were significant increases in length at concentrations of 1mM, 100uM, 100nM, 10nM, and 100pM indicating that there are clearly optimal doses for cordycepin action on regeneration. As for the mechanisms proposed in the introduction, very few inferences and conclusions can be drawn from the data without further biochemical testing. Cordycepin acting as adenosine to interfere with regeneration is able to be ruled out. Every blastema viewed under light microscopy showed no morphological differences from control other than size. This would indicate that there was no interference in terms of membrane hyperpolarization preventing the formation of the head, as was previously hypothesized. However, cordycepin acting on different adenosine receptors cannot be entirely ruled out. Although polarity was not interfered with, it is possible that cordycepin interacted with adenosine receptors to elicit an anti-inflammatory response via A2A receptors of the central nervous system (Palmer & Trevethick, 2007). Further experimentation in terms of fluorescence-staining to determine receptor densities is required to know this for certain.

Through the activation of AMPK via action as cordycepin monophosphate (CoMP), the effect on regeneration was speculated to be inhibitory through the inhibition of energy consuming pathways and promotion of energy producing pathways. As previously mentioned, AMPK is the metabolic master and is regarded as the monitor for cellular process activation regarding proliferation and apoptosis. (Li et al., 2005). As regeneration is an energy-intensive process, the



inhibition of energy-producing pathways via AMPK activation was hypothesized to be detrimental. AMPK activation has been known to decrease signalling in the TOR pathway, which has been implicated in controlling the proper formation of the blastema (Peiris et al., 2012). Since the formation of a blastema was observed in all cases of regeneration and no concentrations produced planarian of significantly smaller size than controls, decreases in TOR signalling and inhibition of regeneration are not likely to have occurred. There still remains the possibility that AMPK activation may have resulted in different pathways pertaining to growth to become activated. AMPK is considered to be the dictator of metabolic homeostasis and can promote ATP consuming pathways, as well as ATP producing pathways (Wang et al., 2010). The ability of cordycepin to elicit activation of the AMPK has been thought to be the major pathway responsible for eliciting biological effects and may help to explain the total length increases observed in figures 1 and 2 (Wong et al., 2009). This will be further explored in the discussion of concentration effects on general growth, but more experimentation in the form of biochemical assays would be necessary in order to determine the pathways of activation.

Cordycepin acting as cordycepin triphosphate is a mechanism that is also unable to be confirmed or denied within the means of this study. If cordycepin were to mimic ATP, this would result in an increase in phosphate bonds that are able to be cleaved in order to produce cellular energy. In an energy-dependent process such as regeneration, this increase in energy availability may be a possible reason for the growth seen in the study. Extracellular ATP, or CoTP, will initiate the release of growth factors from cells which may also help to explain the robust effect of stimulation of regeneration (D'Ambrosia, 2001, Erlinge, 1998). A simple assay to test the ratio of ATP:ADP in the cells would allow for the discrimination of proliferating cells or

apoptotic cells (Bradbury et al., 2000). Higher ratios of ATP:ADP are indicative of proliferating cells, whereas higher ratios of ADP:ATP are indicative of apoptotic cells (Bradbury et al., 2000).

### **Within-Subjects Effects**

The within-subjects effects have been analyzed to provide validity to the study as planarian undergoing regeneration are expected to differ in length as a function of day. The between-subjects effects are the statistics of interest which will be discussed in depth in the next part of the discussion section.

**Day** The result of day being significant comes as no surprise as one would expect a difference in the total length of the planarian as a function of time, as regeneration and all metabolic processes require a temporal component (O'Neill & Feeney, 2014). The entire process of regeneration is energy-dependent, and the resultant morphological manifestation is rather rapid considering the process at hand. As mentioned previously, the mitotic index of the planarian levels off by the 5th day of regeneration (Salo & Baguna, 1984). Therefore, the total amount of growth peaks around day 5, as the planarian will possess the total number of cells for integration into pre-existing tissue (Salo & Baguna, 1984). After day 7, a period of tissue remodelling begins (Reddien & Alvarado, 2004).

**Day and Segment** It would be expected that the segment of the planarian would show differences in regeneration due to differential processes undertaken by each segment. The head segment is essentially responsible for the simple closure of the wound and regeneration of the apex that is physically characteristic of the planarian tail. However, the tail segment is responsible for regeneration of the complex nervous component of the worm, including ventral nerve cords, the pharynx, bi-lobed ganglion, as well as the specialized photoreceptors, also

known as ocelli. Intuitively, this would require much more in terms of metabolic requirements, as well as different processes in terms of differentiation of the neoblasts and incorporation of newly produced tissues (Lobo et al, 2012). The interesting thing about figure 3 is the apparent pattern of growth that seemed to be amplified within the tail segments. Since more cells are required to regenerate the neural component of the planarian, this may be the reason as to why the effects of cordycepin may have been more pronounced in the tail segments when compared to the head segments.

**Day and Concentration** This result would suggest that differences within each worm are a function of the time that is passing, as well as the concentration of cordycepin that they have received for the 6-day period. Due to the wide range of effects seen to be elicited by cordycepin, it should come as no surprise that there were significant differences observed in different concentrations. In the planarian, neoblasts undergo a period of migration, a period of division, and a period of integration meaning that concentration of cordycepin will differentially affect these active metabolic processes involved in regeneration (Reddien & Alvarado, 2004). The concentration of cordycepin will determine the method of action and which processes are influenced. Unfortunately, these questions cannot be answered indefinitely without further testing, but inferences may be made when utilizing the data collected from both growth variables of total length, and blastema length.

**Day, Segment, and Concentration** The three-way interaction present in the data pertaining to total length is dependent on the day of regeneration, the type of segment, and the concentration of cordycepin the planarian have been subject to. As segment and day have been rationalized, concentration is the next important component of this interaction which will be further discussed in the concentration effects on general growth. The total length displayed this

three-way interaction, whereas blastema length did not. This may be an issue with the amount of data that was collected for the blastemas. Differences may have manifested earlier on in regeneration that could have gone undetected, although significance was only seen on day 6 in this study.

### **Between-Subjects Effect of Concentration on Growth**

**Higher Concentrations** When referring to the literature, the effective concentrations of 100nM and 10nM have been used to elicit an anti-inflammatory response, while 1mM and 100uM have been the concentrations of choice in inducing apoptosis within cancer cells (Tuli et al., 2013, ). The anti-cancer concentrations from the literature seem to conflict with the results presented in table 1, but there is a possible rationale for the resulting increase in blastema size within the tail segments. When tail segments regenerate the larger neuronal component, the 1mM and 100uM concentrations of cordycepin may be preventing cell adhesion through AMPK activation, as has been shown in previous literature (Wong et al., 2009). When cells are prevented from adhering to their neighbouring structures, they begin to grow larger in size as a compensatory measure (Wong et al., 2009). Though neoblasts are not adhering to one another, the planarian ectoderm, or exterior membrane, is keeping the cells contained within the organism at the site of the wound. Constant exposure to cordycepin within the Eppendorf environment could have contributed to chronic AMPK activation through its monophosphorylated form, cordycepin monophosphate (CoMP). As there is immense overlap of biochemical pathways, activation of AMPK has been shown to interact with the MAPK, mitogen-activated protein kinase, pathway that is essential for cell function controlling proliferation of cells, differentiation, and mitosis (Kim et al., 2010, Orton et al., 2005). The chronic activation of AMPK may have prevented a period of MAPK signalling that is fundamental in halting

proliferation of neoblasts and beginning the differentiation of the blastema into the pre-existing tissues (Lobo et al., 2012). The possible interference of MAPK signalling would ultimately prevent differentiation and result in the steady proliferation of the neoblasts contributing to the increased blastema length observed in the 1mM and 100uM. This is purely speculative in nature and more testing in the form of a western blot protein analysis would need to be conducted to determine if the AMPK and MAPK pathways are in fact eliciting the morphological effects observed in this study.

**Low Concentrations** When referring to table 1, there is a significant decrease in the size of the blastema in concentrations of 100nM, 10nM, and 100pM, all while displaying a significant increase in the total length. From these results, it may be inferred that the number of cells in the blastema are decreasing as those within the existing tissues of the planarian are increasing. This would indicate that there is an increased efficiency in utilizing neoblasts within the blastema for incorporation into the previously established tissue. Therefore, the possibility of cordycepin eliciting an effect on the differentiation component of regeneration is an argument that should not be done away with too quickly. An alternate hypothesis contributing to the decrease in blastema length and increase in overall length may be seen through a differential interaction of the above mentioned AMPK and MAPK pathways elicited via lower concentrations (Li et al., 2005). MAPK is capable of inducing a plethora of cellular functions, including differentiation, providing a possible rationale as to what is occurring in table 1 (Orton et al., 2005). If cordycepin interacted with AMPK to promote a differential activation of MAPK, independent of the manner mediating the effects seen at 1mM and 100uM, it may provide for a logical basis to explain the decrease in blastema size, and increase in total size at 100nM, 10nM, and 100pM. Again, biochemical tests are needed to provide scientific backing to this statement. It should be noted

that 100nM and 10nM concentrations have been used to elicit anti-inflammatory and anti-oxidant effects in the literature (Tuli et al., 2013). With this anti-inflammatory and anti-oxidant action in mind, cordycepin may be utilizing these effects to seemingly increase the rate of regeneration. The beginnings of what looks like another optimal peak occur around 100pM. The multiple peaks in figure 3 may be indicative of differential activation of receptor subtypes, as similar results have been observed in the pharmacological literature using planarian (Murugan & Persinger, 2014).

### **Implications of Current Study**

Though this study will not save the world, every journey begins with one step. The first step of this study would be the contribution of quantification of cordycepin action in a model of planarian regeneration. The molecular weight of cordycepin is rather small at 251.24 g/mol, allowing for passage across the tight endothelial cells of the blood-brain barrier (Da Silva et al., 2011). This characteristic of cordycepin, coupled with the knowledge of anti-inflammatory action elicited by cordycepin may prove beneficial after insults to the brain (Tuli et al., 2013).

In the tail segments of cordycepin treated planarian, there was a significant increase in the size of the blastemas at 1mM and 100uM, as well as a significant increase in total length. There are two possible rationales for this result; there are alterations in the proliferative capacity of the planarian or the cells of the blastema are increasing in size. As 1mM and 100uM have been used previously in the literature to elicit anti-cancer effects, it seems unlikely that it is the process of proliferation that is largely being affected (Tuli et al., 2013). The rationale of AMPK and MAPK signalling interference discussed above may provide for a feasible theory to explain the apparent proliferation at the two highest concentration of cordycepin that were investigated, 1mM and

100uM. Alternatively, the effects of increased blastema size could also be explained by the ability of cordycepin to prevent cell adhesion causing the cells to grow larger in response.

Further testing to determine the mechanism of action is required.

In the head segments of the cordycepin treated planarian, there was a significant decrease in the size of the blastemas of planarian treated with 100nM, 10nM, and 100pM while the total length increased. This may be due to the anti-inflammatory action of cordycepin, ultimately accelerating the regenerative process and allowing for the rapid integration of the blastema into the pre-existing tissues. As inferred earlier in the discussion section from the results in table 1, the possibility of differentiation-promotion cannot be ruled out. Further testing is absolutely required to determine if cordycepin is aiding in differentiation or anti-inflammation.

### **Limitations and Future Directions**

**Liminations in Data Collection** A fair number of limitations existed in this study pertaining to aspects of data collection, as well as data analysis. The largest limitation in terms of data collection was the physical orientation of the planarian within the petri dish while trying to capture the image. Unfortunately, there is no planarian gurney or microscopic rack capable of straightening the worms out to a perfect 180° angle. Copious amounts of time and persistence were utilized in trying to capture an image with a planarian as straight as possible. Another limitation of data collection was the planarian mobility under the microscope. The unexpected movements would sometimes blur the picture due to the camera settings. A mixture of anticipatory action, patience, and incredible dexterity allowed for the tracking and capture of these worms under the microscope. Experimentation with exposure time, as well as a method of non-damaging immobilization, may prove useful in future research.

**Limitations in Experimental Design** In terms of limitations of the design, a slight alteration in the number of concentrations tested would have been beneficial for statistical analysis. The justification for having nine experimental concentrations was the exploratory nature of the study, as cordycepin studies have seldom been conducted using living organisms and the large focus has been on cell cultures (Tuli et al., 2013).

**Pathway Determination** The open-endedness of this study may be seen as a negative by some, but the foundation laid by this study has opened the door to a plethora of experiments. The first crucial experiment would include the determination of the pathways activated by cordycepin during the regenerative process. In order for this to occur, a western blot analysis of protein concentration could be run. As is the case with most processes of the human body, there needs to be an activation of some sort at the molecular level, as is the case with the previously discussed AMPK pathway (Wang et al., 2009). With proteins, activation comes in the form of phosphorylation; most proteins will become active under phosphorylation. The western blot could be used to contrast cordycepin treated planarian and controls in order to determine the amounts of proteins and their activated counterparts during the regenerative process. This would allow for a crystal clear look at the proteins, pathways, and processes activated in the presence of cordycepin specific to the regenerative process.

**Extended Observation and Pigmentation Quantification** The extended observation from 6 days to 14 days would be beneficial to observe the method of action cordycepin undertakes throughout the entire regenerative process. Cordycepin may elicit an unforeseen effect on the process of differentiation, but this is another aspect in which cordycepin has no supporting literature for. Another area of improvement for future experiments would be the observation of the blastema every day, rather than every third day. Differences may have manifested earlier on



in regeneration that could have gone undetected, although significance was only seen on day 6 in this study. The analysis of pigmentation within the collected blastema images may prove useful in the support of a differentiation based argument for the action of cordycepin in a regenerative model of planarian.

**Dosage Exploration** Though this experiment was exploratory, the exploration of concentration needs to be extended further into higher concentrations, as well as lower concentrations. As there exists significant peaks at the highest and lowest ends of the curve, 1mM and 100pM, this experiment was not successful in covering all active ranges of cordycepin. This means that more investigation needs to be done to discover the point at which cordycepin becomes ineffective to the organism in both the millimolar concentrations and femtomolar concentrations.

**Non-Regenerative Planarian Exposure** Not only does more exploration need to be done in determining a cordycepin cutoff point, there should also be exploration in the realm of non-regenerative planarian response to cordycepin. This experiment would aid in determining if the effects elicited by cordycepin are through pathways of regeneration, or through pathways of general growth that may be steroidal in nature as cordycepin has been shown to promote steroidogenesis at higher concentrations in the millimolar range (Tuli et al., 2013).

**Whole *Cordyceps* Mushroom Extraction** An aspect of Traditional Chinese Medicine that this study has neglected to consider is the multiple other constituents of the *cordyceps* mushroom. Certain compounds, such as vitamins and lipopolysaccharides, within the *cordyceps* mushroom are bioactive and able to elicit immunomodulatory effects that may further aid in the wound healing/regenerative process (Tuli et al., 2013).

## Conclusion

In conclusion, cordycepin displayed a robust stimulatory effect on regeneration, as well as differentially affecting the type of tissue that was regenerating in terms of the magnitude of growth. The same pattern of significant increase in total length existed within head and tail segments at 1mM, 100uM, 100nM, 10nM, and 100pM. There were different results in terms of the blastema length. 1mM and 100uM concentrations increased the size of the blastema in tail segments while the 100nM, 10nM, and 100pM concentrations decreased the size of the blastema in head segments. Cordycepin has been shown to possess anti-inflammation and anti-oxidant function, as well as interference in protein synthesis. With such a broad range of effects and possible mechanisms influencing regeneration, more experimentation in the form of biochemical assays needs to be completed order to determine the aspects of regeneration that cordycepin is interacting. Overall, this study lays a foundation for studying the wound-healing response and regeneration in planarian in the presence of cordycepin.

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