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Reactive Oxygen Species Production in Head Regeneration of Planaria from Cadmium Exposure

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REACTIVE OXYGEN SPECIES PRODUCTION IN HEAD REGENERATION OF PLANARIA FROM CADMIUM EXPOSURE

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Abstract

Exposure to cadmium, a pervasive neurotoxic chemical found in our environment, poses significant health risks, disrupting various physiological systems and fostering carcinogenesis. Commonly found in everyday items like cigarettes, batteries, and plastics, cadmium disrupts the equilibrium between reactive oxygen species (ROS) production and cellular repair mechanisms, culminating in oxidative stress and potential cell damage. In toxicological and pharmaceutical research, planarians offer valuable insight for understanding how exposure to chemicals akin to human scenarios can alter biological systems. This experiment addresses the effect cadmium chloride ($CdCl_2$) has on planarian regeneration and whether antioxidants can mitigate those adverse outcomes. Our study investigates the hypothesis that increasing exposure to 20 μM $CdCl_2$ progressively impedes cephalic regeneration through ROS production. However, introducing antioxidants N-acetyl-L-cysteine (NAC) and MitoTEMPO will alleviate such effects and facilitate normal head-to-tail regenerative polarity. Using *G. dorotocephala* as a model organism, a trunk fragment assay was conducted to evaluate cephalic regeneration over two weeks following exposure to six concentrations, 0 μM, 2.5 μM, 5 μM, 10 μM, 15 μM, and 20 μM $CdCl_2$, examining its toxic effect. Our findings revealed a concentration-dependent toxicity, with higher $CdCl_2$ concentrations resulting in hindered blastema, ocelli, auricle repair, and significant death. Cotreatments with antioxidants, well known for their ROS-neutralizing properties, exhibited partial mitigation of the adverse effects, fostering planaria regeneration to a degree despite $CdCl_2$ exposure. By delving into the intricate cellular and molecular mechanism governing this regenerative phenomenon, we can inform strategies for advancing regenerative medicine and contribute to scientific knowledge in shaping public health from the risks of heavy metal exposure.

Acknowledgments

I extend my heartfelt thanks to my mentor, Dr. Cassandra S. Korte, whose guidance and tenacious support have played a pivotal role in shaping my growth and accomplishments over the past few years. Her influence has been truly transformative, and I aspire to emulate her significance in the scientific realm one day. I express my sincere appreciation to my committee members, Dr. Erika Doctor and Dr. Alanna Lecher, for their passion and expertise in chemistry and statistical analysis, which allowed for obtaining accurate and valuable results. From providing graduate school interview practice over the summer to guiding me in enhancing my scientific communication skills, I thank Dr. Wayne Law, as he has been a crucial part of the steppingstones toward helping me meet the finish line and become a great researcher. A special thanks to Jonathan Newman and Anden Velez from Dr. Korte's Planaria Lab Group for their invaluable lessons in all things regarding planaria. I express my gratitude to Khelia Gihozo for her commitment to collaborative late-night sessions in the library, where we mutually upheld accountability while working on our thesis. To my family, your love and support have been my pillars throughout my time at Lynn University. A special shoutout to my cousin Maria Serpas for encouraging me to attend this university and steadfastly accompanying me through the last four years. I want to thank my aunt Martha Velasquez for being a comforting sanctuary during challenging times, becoming my home away from home. To my siblings Javier Jr. and Karina, my nephews Korey and Kenzo, and my parents, Javier and Maria – you are my unwavering number one supporters, and I am endlessly blessed for your presence in my journey.

Dedication

In profound gratitude and unwavering love, I dedicate this thesis to my parents, Javier and Maria, whose boundless support and encouragement have been my guiding lights throughout my academic journey. In moments of doubt and despair, it was their constant belief in me that fueled my determination to persevere. Their tireless work ethic and persistent commitment to my success have shaped my aspirations within the realm of science, inspiring me to reach for the highest standards and pursue my dreams with an unyielding passion. Everything I have achieved and aspire to achieve is a testament to their significant influence on my life. I am forever grateful for the foundation they have provided, and this dedication is a humble acknowledgment of the debt I owe to them for shaping the resilient person I am today.

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Abbreviations and Acronyms

ATP	Adenosine triphosphate
Cd	Cadmium
$CdCl_2$	Cadmium chloride
EtOH	Ethanol
GSH	Glutathione
IOS	Instant ocean salt
MT	Metallothionein
NAC	N-acetyl-L-cysteine
ROS	Reactive oxygen species
SEM	Standard error of the mean
SOD	Superoxide dismutase
TEMPO	Piperidine nitroxide
TPP+	Triphenylphosphonium

Reactive Oxygen Species Production in Head Regeneration of Planaria from Cadmium Exposure

Introduction

Cadmium (Cd), a stealthy invader among the elemental ranks, mimics the benign guise of calcium and zinc ions, yet harbors a perverse potency that disrupts both human and environmental stability. This heavy metal is naturally occurring in the earth's crust, typically generated as a byproduct of the extraction and refinement of zinc, lead, and copper ores. Cadmium exhibits a high degree of malleability owing to its inherent physical softness, rendering it easily formable. Its versatility stems from exceptional corrosion resistance and significant metalworking attributes, notably its low melting point, making it highly applicable across diverse industrial sectors (Unsal et al., 2020). Given its propensity to persist in the environment over extended periods of time, cadmium is detectable in various commodities such as tobacco, batteries, plastics, and even food. Its buildup can lead to organ dysfunction, respiratory and reproductive toxicity, neurological defects, and cancer as classified by the International Agency for Research on Cancer (Kalafatić et al., 2004).

Exposure to this well-distributed toxicant can disrupt cellular mechanisms, disturbing the equilibrium between the production of reactive oxygen species (ROS) and the cell’s capacity for detoxification and repair (Wu et al., 2011). Upon entering the cell, cadmium can transverse across the membrane into the cytoplasm via calcium channels, triggering its binding with crucial molecules such as glutathione (GSH) and metallothionein (MT) to mitigate intracellular and extracellular fluid levels. GSH functions to sustain cellular redox balance by scavenging free radicals and ROS while facilitating the detoxification of harmful compounds. Metallothionein is characterized by its high metal-binding affinity; it coordinates metal ion homeostasis and

alleviates oxidative stress. ROS, comprising highly reactive oxygen-containing molecules, can result in cell damage and apoptosis when present in excess, whereas moderate levels are necessary for maintaining cellular homeostasis or initiating cell signaling pathways (Gauron et al., 2013). Cadmium instigates the release of oxygen-rich free radicals, including superoxide anion ($\bullet O^{-}_{2}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($\bullet OH$), inducing oxidative stress to arise and set off a chain reaction culminating in lipid peroxidation, disruption of protein structures, impairment of antioxidant defense, and inflammatory response (Zhang et al., 2016).

Planaria, freshwater flatworms, are characterized by their elongated and flattened dorsoventral body structure, primitive nervous system, and singular digestive opening. This type of invertebrate model organism is commonly used in the laboratory setting due to its distinctive physiological characteristics, regenerative abilities, and susceptibility to various toxicants and pharmaceuticals. The discovery of regeneration in the 18th century sparked a wave of excitement among naturalists. This led to experiments on various animals to determine the prevalence of regeneration. This phenomenon remains one of the enigmas in biology due to its remarkable ability of planaria to regrow lost or damaged cells, tissues, and entire organs (Elliot & Alvarado., 2012). Planarian regeneration is a complex process that involves the activation of neoblasts, a form of pluripotent stem cells, to regrow lost tissue and organs within two weeks. Neoblasts exhibit impressive plasticity, capable of dividing and differentiating into myriad cell types, allowing planaria to regenerate lost anatomical structures following wound closure (Plusquin et al., 2012; Reddien, 2018). Blastema, a cluster of undifferentiated cells that can proliferate and differentiate into diverse cellular lineages, are vital for tissue regeneration. While ROS are implicated in this regenerative cascade, excessive ROS production can inhibit tissue regrowth by oxidative stress (Wu et al., 2012). Notably, exposure to elevated concentrations of cadmium

chloride ($CdCl_2$) have been associated with impeded blastema repair in planarians, underscoring the significance of antioxidants in counteracting the detrimental effects and fostering tissue repair. Antioxidants are crucial in mitigating oxidative damage by neutralizing ROS, stabilizing oxidative stress, and augmenting regenerative potential. Previous studies by Wu and colleagues have demonstrated that co-exposure of cadmium-treated planarians to the antioxidant N-acetyl-L-cysteine (NAC) conferred protection against cadmium-induced lethality, attributed to alleviated oxidative stress and reduced bioaccumulation (2014).

Due to its toxicological properties, cadmium can impede the absorption, transportation, and utilization of various other elements. Antioxidants derived from natural compounds such as NAC and MitoTEMPO act as scavengers for free oxygen radicals, curbing damage progression. NAC, a natural amino acid derivative containing an acetylated form of the thiol-containing molecule L-cysteine (Ermakov et al., 2021), directly interacts with hydrogen peroxide and hydroxy radicals by donating electrons, converting them to water. It enhances the enzymatic activity of superoxide dismutase (SOD) by reducing superoxide radicals into less harmful molecules (Unsal et al., 2020). It can also bolster the intracellular levels of GSH in fortifying the cell's defense against hydrogen peroxide and iron ion catalysis of the Fenton Reaction. The antioxidative properties of NAC offer promising avenues for mitigating free radicals, potentially minimizing the effects of metal exposure.

Mitochondrial oxidative metabolism is a major endogenous source of ROS, particularly superoxide, generated as a byproduct of oxidative phosphorylation (Le Gal et al., 2021). Consequently, the mitochondria become a primary target of cadmium toxicity, as cadmium binds to protein thiols in the mitochondrial membrane, leading to disruption of mitochondrial function, inhibition of the respiratory chain reaction, and production of ROS. MitoTEMPO, a compound

comprised of the antioxidant piperidine nitroxide (TEMPO) and lipophilic cation triphenylphosphonium (TPP+), is a unique antioxidant, as it is mitochondria targeted. TEMPO functions as a SOD mimetic, catalyzing the dismutation of superoxide, while TPP+ serves as a membrane-permeable cation that accumulates within the mitochondria, driven by the membrane potential (Du et al., 2016). MitoTEMPO is thus uniquely poised to reinforce cellular defenses against oxidative stress and strengthen mitochondrial integrity and function, perhaps allowing neoblast-driven regeneration to take place despite cadmium exposure.

While numerous flatworm species populate the biological landscape, not all exhibit the remarkable regenerative prowess observed in species such as *Schmidtea mediterranean* and *Dugesia japonica* (Owlarn & Bartscherer, 2016). These two species have emerged as prominent subjects in toxicity and regenerative research due to their tractable anatomy, predictable behavior, and well-characterized genetics, rendering them suitable to laboratory manipulation and maintenance. Much of the research regarding severe and acute cadmium-induced lethality has surrounded these two species, showcasing that high levels of exposure lead to increased oxidative stress. Research suggests distribution of cadmium has been documented in the cephalic regions of these planarians, proposing a heightened susceptibility of the head to cadmium toxicity relative to other body regions (Wu et al., 2014). However, limited data on the relationship between metal exposure and regenerative capabilities of other species, such as *Girardia dorotocephala*, emphasize the need for further exploration into this biological phenomenon.

Investigating the effects of high $CdCl_2$ exposure on *G. dorotocephala* and whether antioxidants can inhibit ROS accumulation presents an opportunity to elucidate critical parallels and distinctions. The research conducted in this study addresses the hypothesis that increasing

exposure to a maximum of 20 μM $CdCl_2$ will progressively inhibit cephalic regeneration by ROS production, however, introduction of NAC and MitoTEMPO will mitigate those adverse effects and promote restoration of the planaria's typical head regenerative polarity. By focusing on the intricate cellular and molecular mechanisms underlying this regenerative process, strategies for human application regarding advancing regenerative medicine can be applied. Enriching our understanding of behaviors and underlying mechanisms stemming from $CdCl_2$-induced toxicity is instrumental not only in improving scientific knowledge but also in informing public health initiatives aimed at mitigating the adverse effects of heavy metal exposure.

Materials and Methods

Reagents

The chemicals used include cadmium chloride ($CdCl_2$) from Ward's Science (West Henrietta, NY), absolute 200 proof ethanol (EtOH) from Fisher Science (Pittsburgh, PA), N-acetyl-L-cysteine (NAC) from ThermoFisher Scientific (Waltham, MA), and MitoTEMPO from Sigma-Aldrich (St. Louis, MO).

Animal Care

Girardia dorotocephala purchased from Carolina Biological Supply (Burlington, NC) and ranging in size from 2 mm to 13 mm, were maintained in glass culture dishes filled with instant ocean salt (IOS; from Instant Ocean, Earth City, MO) water and housed in a dark incubator at a temperature of 23°C. Preceding each trial, the flatworms were subjected to a feeding regimen of liver paste for four weeks, followed by a one-week starvation to establish a uniform metabolic status (Wu et al., 2011). To ensure optimal experimental conditions, the flatworms underwent three cleanings per week over the course of the four weeks, and recordings of their health were conducted to ensure reproducibility in each trial.

Planarian Treatments

A stock concentration of 20 mM $CdCl_2$ in IOS was prepared, and 2.5 µM, 5 µM, 10 µM, 15 µM, and 20 µM $CdCl_2$ were subsequently diluted in IOS water. For each concentration, two tail fragments were placed in each well of a 6-well dish purchased from Corning Incorporated (Kennebunk, ME) over a two-week period. Each treatment dish had thorough cleanings and IOS water replenishments every two days. With a total of three trials, a cumulative count of 216 flatworms were used for the dose-response effect of on $CdCl_2$-induced regeneration experiment.

The positive control involved solutions of ethanol to establish a baseline level of regeneration activity, as this exposure delays cephalic regeneration (Morris et al., 2021). The ethanol trials underwent identical procedures as the $CdCl_2$ solutions, using ethanol concentrations of 0.01%, 0.1%, and 1% (Appendix A). A total of 216 worms were used for this set of treatments.

The antioxidant treatments followed the same procedure as the $CdCl_2$ solutions, with additional exposure of regenerating flatworms to NAC and MitoTEMPO to assess potential rescuing effects. A 24-hour pretreatment with 10 µM NAC or 5 µM MitoTEMPO preceded $CdCl_2$ exposure. Post-amputation, tail fragments were arranged in a 6-well plate, with two fragments per well, and subjected to a co-treatment of 10 µM NAC or 5 µM MitoTEMPO with 5 µM $CdCl_2$ throughout a two-week regeneration period. A total of 192 flatworms underwent this treatment within the two trials.

Amputation Assay

Water was frozen in Petri dishes to reduce the motility of the flatworms, and to minimize direct contact between the ice and their bodies, all procedures were conducted atop a piece of filter paper placed on the frozen dish (Chan & Marchant, 2011). Using aseptic technique, the

flatworms were amputated by a scalpel halfway between the planarian anterior apex and the anterior end of the pharynx. Following amputations, the tail fragments were positioned in the wells of a 6-well dish containing respective treatments, while the heads were placed in a Petri dish and set aside for future lab use. For each trial, daily observations were conducted over a two-week period on each tail fragment, and a scoring sheet featuring various criteria for regeneration ability was used to monitor changes in each fragment. Additionally, worms were photographically documented to record their progress.

Regeneration Scoring and Imaging

The effects of $CdCl_2$ exposure in amputated planarians were observed on a stereo dissecting scope and imaged using an iPhone 14. Each flatworm was scored daily for two weeks based on the criteria established by Van Huzien and coauthors (2017) and Velez and colleagues (2020). Table 1 briefly outlines the scoring system for cephalic regeneration progress. A score of 0 indicated any occurrence of death during the two-week period. Fresh amputation of the planarian tail was denoted by score 1, while score 2 indicated wound contraction. Closure of the wound received a score of 3, followed by a score of 4 as the formation of a pale stump of tissue occurred. Figure 1 illustrates ocelli (eye) spot formation, represented by a score of 5, while the complete formation of two ocelli receives a score of 6. Auricle (chemoreceptor) formation is assigned a score of 7, and complete auricle formation on each side of the head is denoted by a score of 8. Partial pigmentation leads to a score of 9, and full pigmentation throughout the body receives a score of 10. Finally, a score of 11 is achieved when complete regeneration has taken place. This comprehensive scoring system evaluates the regenerative capacities of planarians under various experimental conditions, offering a crucial tool for studying the underlying mechanisms of regeneration.

Table 1:

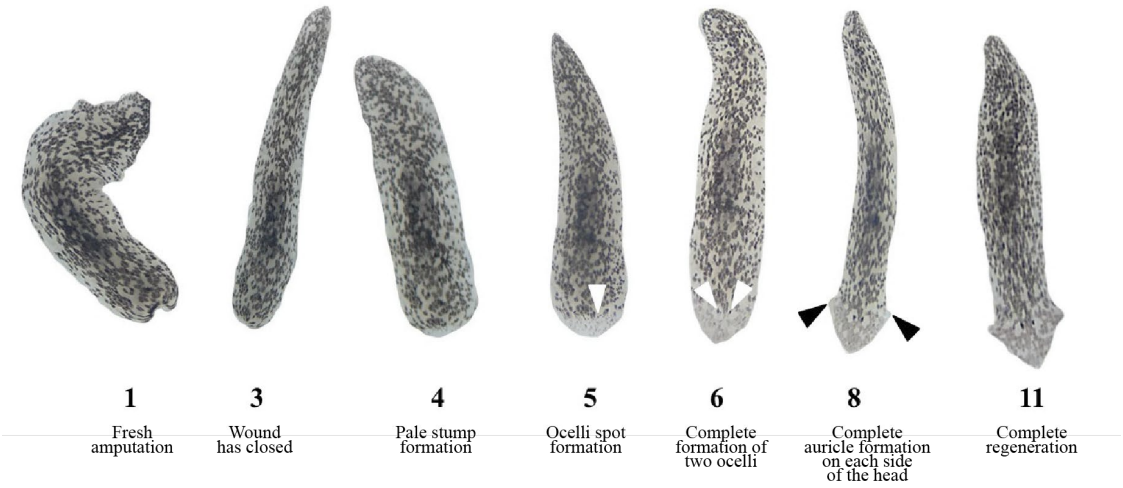
Planarian Regeneration Scoring Reference

Score	**Tail Fragments**
0	Death
1	Fresh amputation
2	Wound contraction has occurred
3	Wound has closed
4	Pale stump formation
5	Ocelli spot formation
6	Complete formation of two ocelli
7	Auricle formation
8	Complete auricle formation on each side of the head
9	Partial pigmentation
10	Full pigmentation in all of the body
11	Complete regeneration

Note. The table shows the different criteria for a complete planarian regeneration over a two-week period.

Figure 1:

Normal Planarian Regeneration



Note. The progress of cephalic regeneration on the same flatworm over 14 days. White arrows show ocelli formation and black arrows indicate auricle formation.

Statistical Analysis

Data from each experiment were analyzed using the Kruskal-Wallis Test and the Pairwise Test of Wilcoxon Rank Sum Test (Appendix B). These analytical tools were strategically employed to examine and interpret the results obtained. Both statistical analyses enabled discerning significant differences among multiple $CdCl_2$ treatments on day 14, identifying those with varying values in the regrowth of essential structures such as ocelli and auricle in average regeneration scores. Comparisons to each antioxidant focusing on day 14 were also obtained.

Results

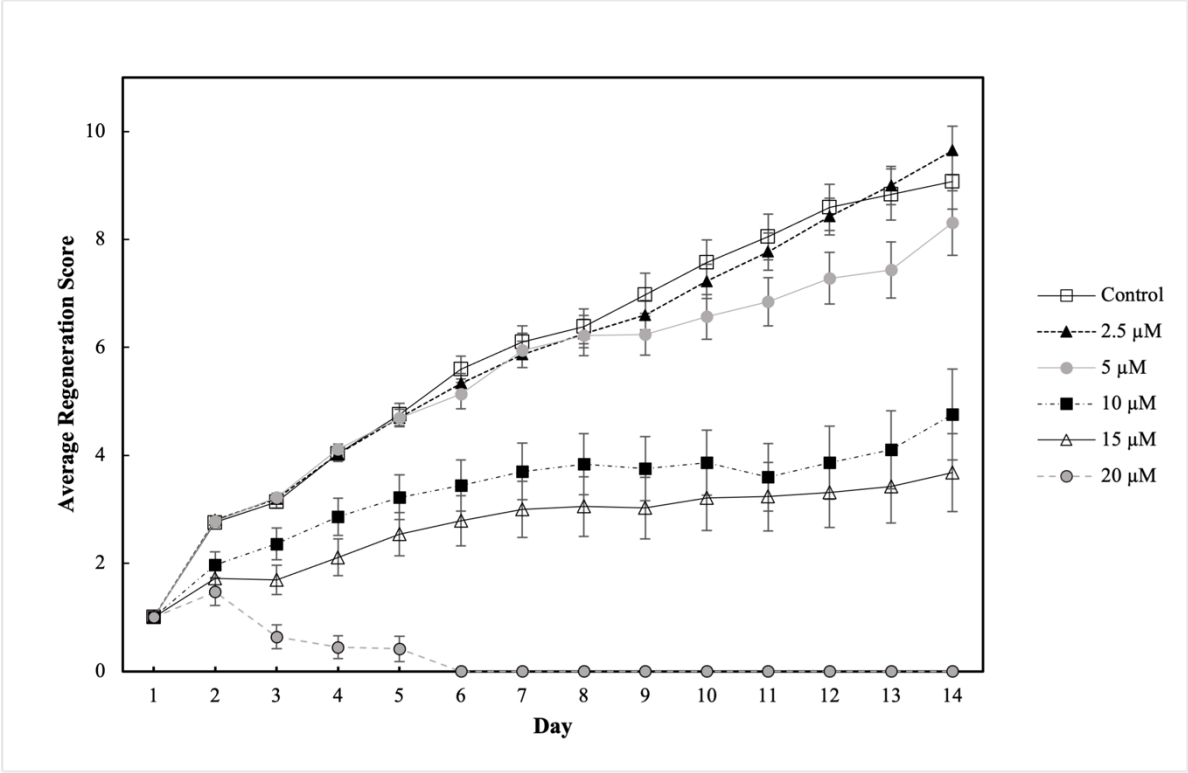
Planarian Regeneration Response to Various $CdCl_2$ Concentrations

Over the experimental period of two weeks, lower concentrations of $CdCl_2$, notably 2.5 µM, and 5 µM, demonstrated a minimal deviation from the baseline levels of the control in terms of average regeneration score, suggesting a relatively modest effect on blastema, ocelli, and

auricle repair processes (Figure 2). Despite having no treatment, the control group had an average score of 9.07, while 2.5 μM $CdCl_2$ had an average score of 9.65 on the last day with a total of two deaths (data not shown), and 5 μM $CdCl_2$ had an average score of 8.30 with a total of five deaths (data not shown). By day two, increasing concentrations of 10 μM and 15 μM $CdCl_2$ showed decreasing regeneration scores, indicating $CdCl_2$ toxicity-induced repair inhibition as 40 planaria died between both concentrations (data not shown). A few tail fragments did not make it past day two throughout the experiment, resulting in floating debris within their respective well (Figure 3C-E). The highest concentration, 20 μM $CdCl_2$, culminated in the death of all the planaria (n=36, data not shown) by day six of the experiment for all three trials.

Figure 2:

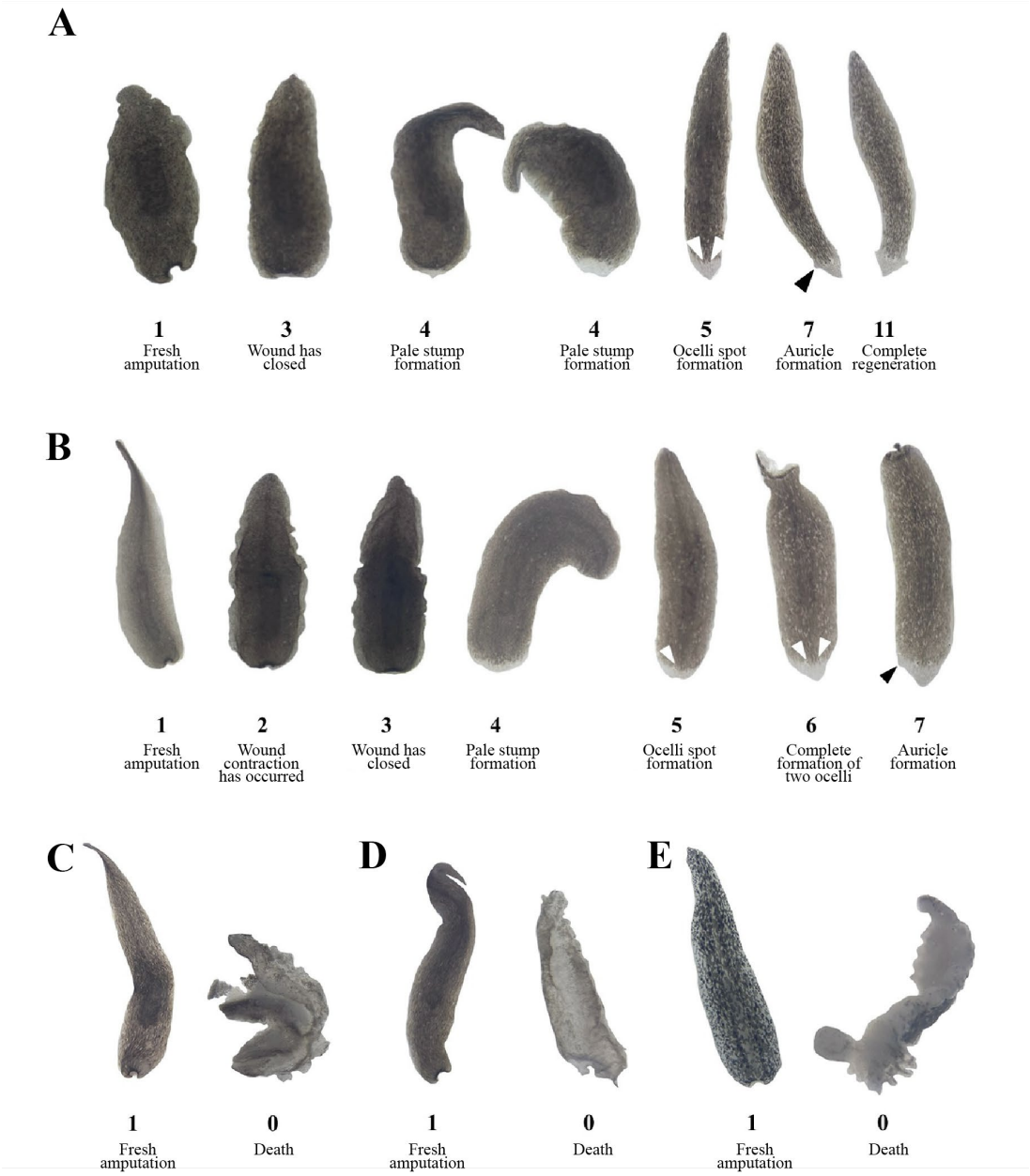
Effect of $CdCl_2$ Concentrations on Average Regeneration Score



Note. Planaria were exposed to IOS water as the control, alongside varying concentrations of $CdCl_2$ ranging from 2.5 µM to 20 µM over a two-week period. The data depicts the average ± SEM regeneration score for each treatment per day ($p < 0.0001$).

Figure 3:

Planarian Regeneration Score After $CdCl_2$ Exposure



Note. The progress of cephalic regeneration on the same flatworm per $CdCl_2$ treatment over 14 days. (A) Flatworm exposed to 2.5 µM $CdCl_2$. (B) Flatworm exposed to 5 µM $CdCl_2$ shows the dropping of its tail halfway through the two weeks. (C) Flatworm exposed to 10 µM $CdCl_2$ from day one and day two. (D) Flatworm exposed to 15 µM $CdCl_2$ from day one and day two. (E)

Flatworm exposed to 20 µM $CdCl_2$ from day one and day two. White arrows show ocelli formation and black arrows show auricle formation.

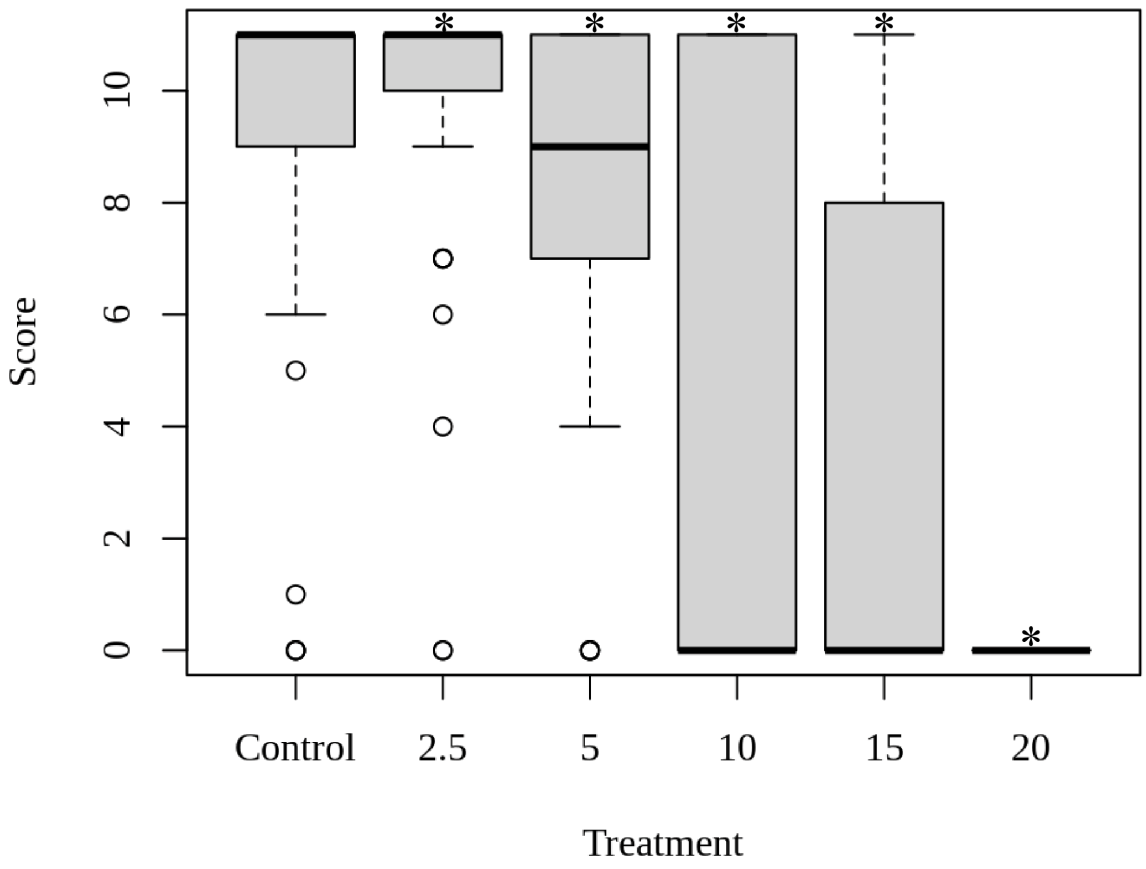
A Kruskal-Wallis Test was conducted to compare the effect of $CdCl_2$ on the regeneration score of the planaria on day 14 in 0 µM, 2.5 µM, 5 µM, 10 µM, 15 µM, and 20 µM $CdCl_2$ treatments (Figure 4). There was a significant effect of $CdCl_2$ on regeneration score at the $p < 0.05$ level for the six treatments [$H(5) = 106.91$, $p < 0.0001$]. Pairwise comparisons using the Wilcoxon Rank Sum Test with continuity indicated that the mean score for 10 µM $CdCl_2$ (4.8 ± 0.8), 15 µM $CdCl_2$ (3.7 ± 0.7), and 20 µM $CdCl_2$ (0 ± 0) treatments were significantly different than the control ($p < 0.001$). However, the 2.5 µM $CdCl_2$ (9.7 ± 0.4, $p = 0.98$) and 5 µM $CdCl_2$ (8.3 ± 0.6, $p = 0.93$) treatment did not significantly differ from the control group (9.1 ± 0.5).

Regarding the variations observed among increasing $CdCl_2$ treatment groups, the treatments with 10 µM $CdCl_2$ (4.8 ± 0.8), 15 µM $CdCl_2$ (3.7 ± 0.7), and 20 µM $CdCl_2$ (0 ± 0) showed significant differences compared to the 2.5 µM $CdCl_2$ treatment ($p < 0.001$). Conversely, the 5 µM $CdCl_2$ (8.3 ± 0.6, $p = 0.57$) treatment showed no significant difference compared to the 2.5 µM $CdCl_2$ treatment. Further analysis reveals the differences between the 10 µM (4.8 ± 0.8) and 5 µM $CdCl_2$ treatments (8.3 ± 0.6) were statistically significant ($p < 0.001$), indicating lower scores in 10 µM $CdCl_2$. Similarly, there was a significant difference observed between the 15 µM (3.7 ± 0.7) and 5 µM $CdCl_2$ treatments (8.3 ± 0.6, $p < 0.001$), as well as between the 20 µM (0 ± 0) and 5 µM $CdCl_2$ treatments (8.3 ± 0.6, $p < 0.001$), both suggesting lower scores in the higher $CdCl_2$ treatment groups. When comparing the 15 µM (3.7 ± 0.76) and 10 µM (4.8 ± 0.8) $CdCl_2$ treatments, no significant difference was found ($p = 0.80$), although a significant difference was observed between the 20 µM (0 ± 0) and 10 µM $CdCl_2$ treatments (4.8 ± 0.8, $p < 0.001$). Lastly, the comparison between the 20 µM (0 ± 0) and 15 µM (3.7 ± 0.76) $CdCl_2$

treatments also revealed a significant difference ($p < 0.001$). These findings underscore the effects of varying $CdCl_2$ concentrations on regeneration scores, highlighting the significance of higher $CdCl_2$ levels in influencing outcomes. Specifically, our results indicate that as $CdCl_2$ concentrations increase, there is a notable decrement in regeneration scores, emphasizing the adverse effects of elevated $CdCl_2$ exposure. However, it is noteworthy that lower $CdCl_2$ concentrations did not yield significant differences in regeneration scores, implying a threshold effect wherein higher $CdCl_2$ concentrations are necessary to elicit pronounced changes in blastema repair. This decisive insight from the $CdCl_2$ experiment steered our decision to choose the 5 μM dose for antioxidant treatments. By striking a balance between being slightly higher than 2.5 μM and remaining below concentrations associated with heightened mortality and impaired blastema repair, we positioned our approach safely within a middle ground.

Figure 4:

Day 14 Regeneration Score vs $CdCl_2$ Treatment



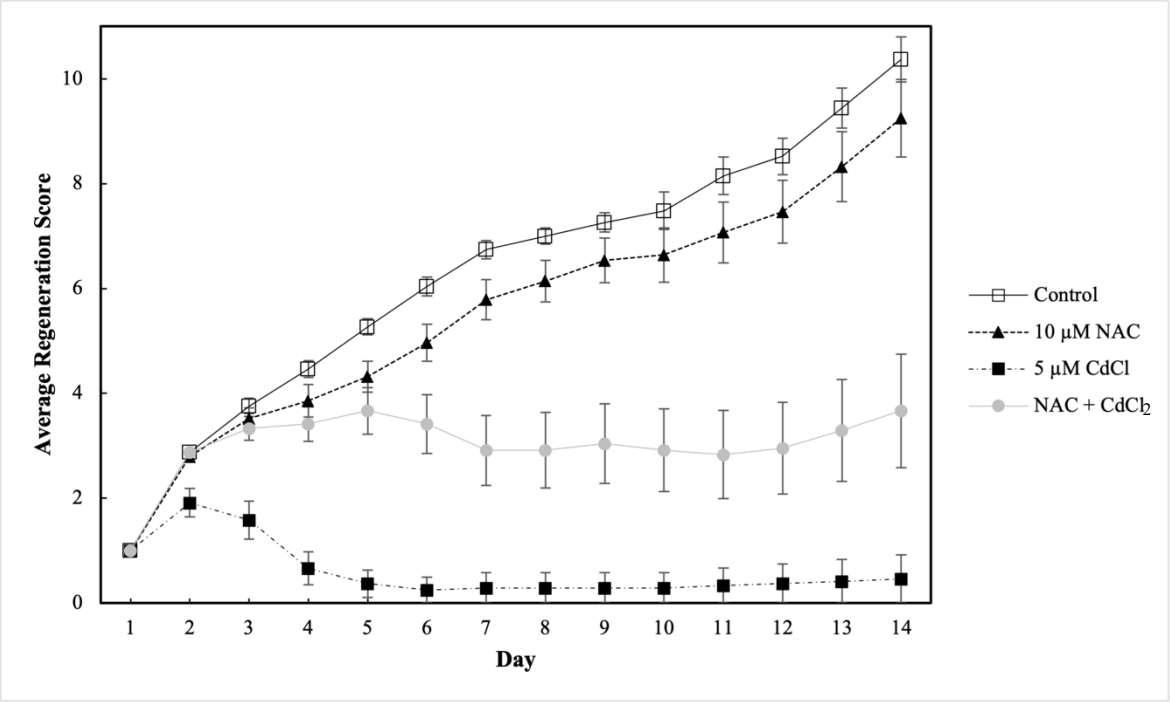
Note. Final regeneration scores of planaria between all six treatment groups (Control, 2.5, 5, 10, 15, 20 μM). Each boxplot presents the median scores derived from triplicate experiments, with a total of n=12-14 flatworms per dish across all six treatments. Whiskers depict the range of scores, while the interquartile range is delineated by gray boxes, with outliers represented by open circles. * indicates significantly different compared with the untreated control ($p < 0.001$).

Planarian Regeneration Response to NAC Antioxidant Treatment

Throughout the duration of the study, the control group consistently displayed the highest regeneration score, reaching an average score of 10.37. The treatment of 10 μM NAC alone on planaria exhibited an average regeneration score of 9.25 with a total death of 4 (data not shown). This suggests the potential facilitation of blastema repair by the antioxidant properties of NAC, with outcomes similar to those of the control group. The administration of 5 μM $CdCl_2$ alone had 23 deaths (data not shown) across both trials. It resulted in delayed regeneration, peaking on day two with an average score of 1.92 before declining to 0.29 by day seven and fluctuating around 0.46 by day 14 (Figure 5). The cotreatment of 10 μM NAC and 5 μM $CdCl_2$ maintained regeneration scores below maintenance levels, reaching nearly a score of 4 over the two-week period and total of 15 deaths (data not shown).

Figure 5:

Effect of NAC on Average Regeneration Score



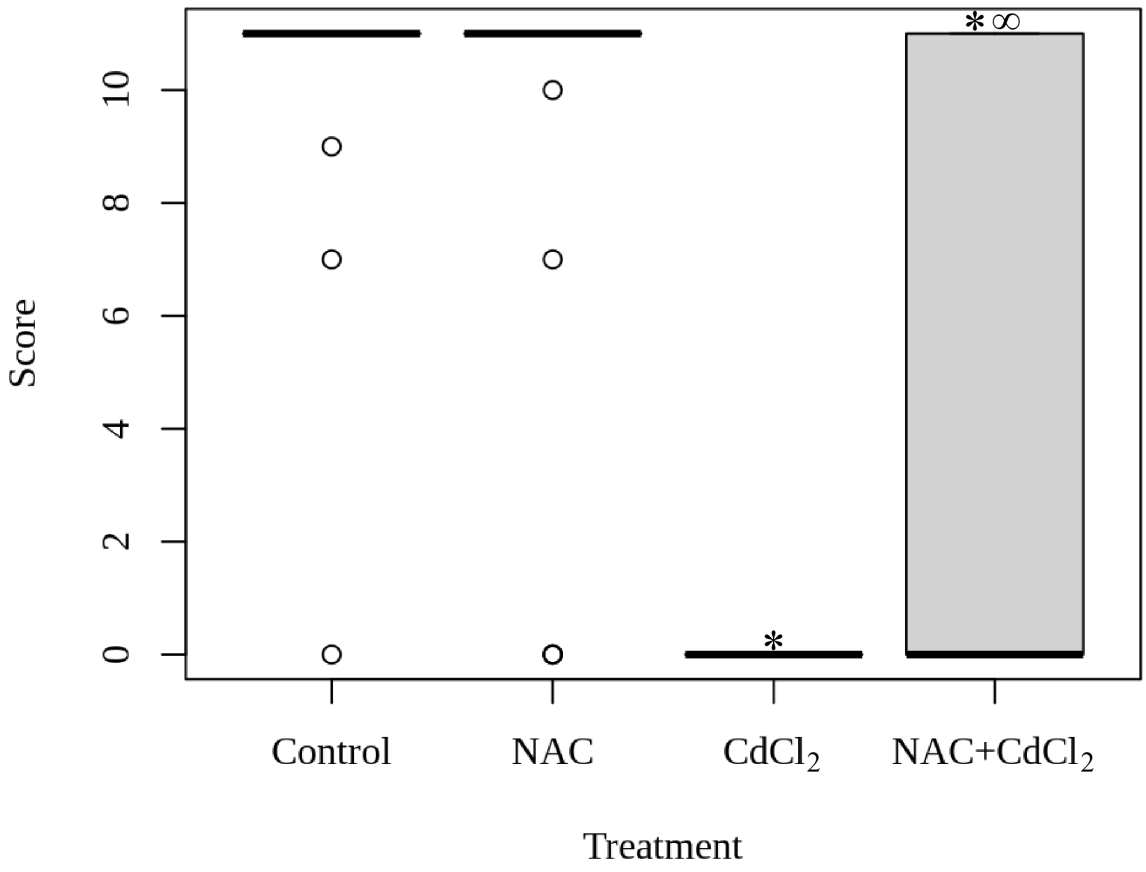
Note. For 14 days, planaria were exposed to IOS water as the control, 10 µM NAC, 5 µM $CdCl_2$, and a cotreatment of 10 µM NAC with 5 µM $CdCl_2$. The data depicts the average ± SEM regeneration score for each treatment per day ($p < 0.001$).

A Kruskal-Wallis Test was conducted to examine the effects of four treatments (control, 10 µM NAC, 5 µM $CdCl_2$, and a cotreatment of 10 µM NAC with 5 µM $CdCl_2$) on regeneration scores for day 14 (Figure 6). The differences among treatment groups were significant, $H(3) = 58.72$, $p < 0.001$. Pairwise comparisons using the Wilcoxon Rank Sum Test with continuity correction revealed substantial differences in the control and 5 µM $CdCl_2$ treatments ($p < 0.0001$) and the control and the cotreatment of 10 µM NAC with 5µM $CdCl_2$ ($p < 0.001$). However, no significant difference was observed between the control and 10 µM NAC treatments ($p = 0.28$). Additionally, the comparisons between 5 µM $CdCl_2$ and the 10 µM NAC

treatments ($p < 0.0001$), the 5 μM $CdCl_2$ and the cotreatment of 10 μM NAC with 5 μM $CdCl_2$ ($p < 0.01$), and the cotreatment of 10 μM NAC with 5 μM $CdCl_2$ and 10 μM NAC treatment ($p < 0.01$), also showed a difference in significance. The pairwise comparisons indicate the observed differences in regeneration scores among the various treatments underscore the importance of carefully considering the specific treatment combinations and their respective impacts on blastema repair processes.

Figure 6:

Day 14 Regeneration Score vs NAC Treatment



Note. Final regeneration scores of planaria between all four treatment groups. Treatments involved IOS water as the control, 10 µM NAC, 5 µM $CdCl_2$, and a cotreatment of 10 µM NAC with 5 µM $CdCl_2$. Each boxplot presents the median scores derived from duplicate experiments, with a total of n=12-14 flatworms per dish across all four treatment. Whiskers depict the range of scores, while the interquartile range is delineated by gray boxes, with outliers represented by

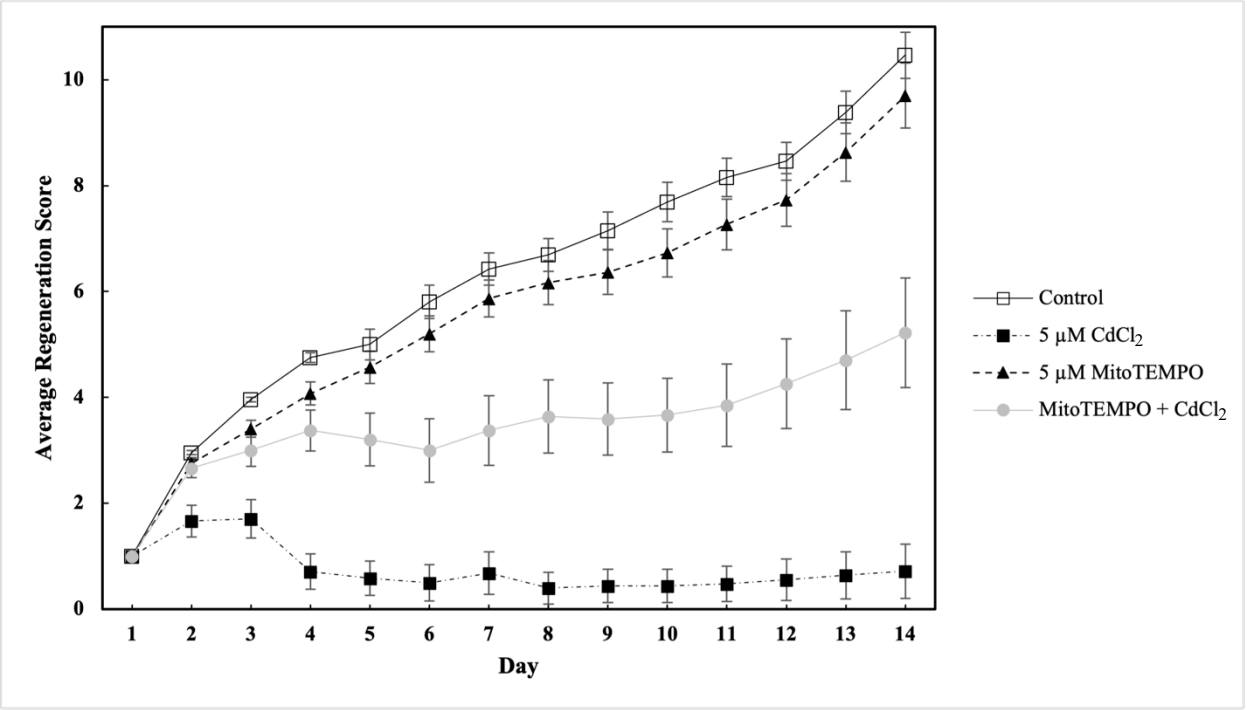
open circles. * indicates significantly different compared with the untreated control ($p < 0.001$). ∞ indicates significantly different compared with 5 μM $CdCl_2$ alone treatment ($p < 0.01$).

Planarian Regeneration Response to MitoTEMPO Antioxidant Treatment

The results depicted in Figure 7 illustrate the impact of MitoTEMPO on the average regeneration score within a two-week timeframe. The control had the highest regeneration score among all the experimental groups, with an average regeneration score of 10.46. The 5 μM MitoTEMPO group with only 3 deaths (data not shown) had a gradual increase in average regeneration score, reaching 9.7 over the 14 days. The 5 μM $CdCl_2$ group coupled a total of 23 deaths (data not shown) and demonstrated the lowest score, plummeting to 0.70 on day four and fluctuating between 0.58 and 0.72 until the last day. The cotreatment group of 5 μM MitoTEMPO with 5 μM $CdCl_2$ exhibited 12 deaths (data not shown) and a progressive rise in its regeneration scores to 5.22 throughout the duration of 14 days.

Figure 7:

Effect of MitoTEMPO on Average Regeneration Score



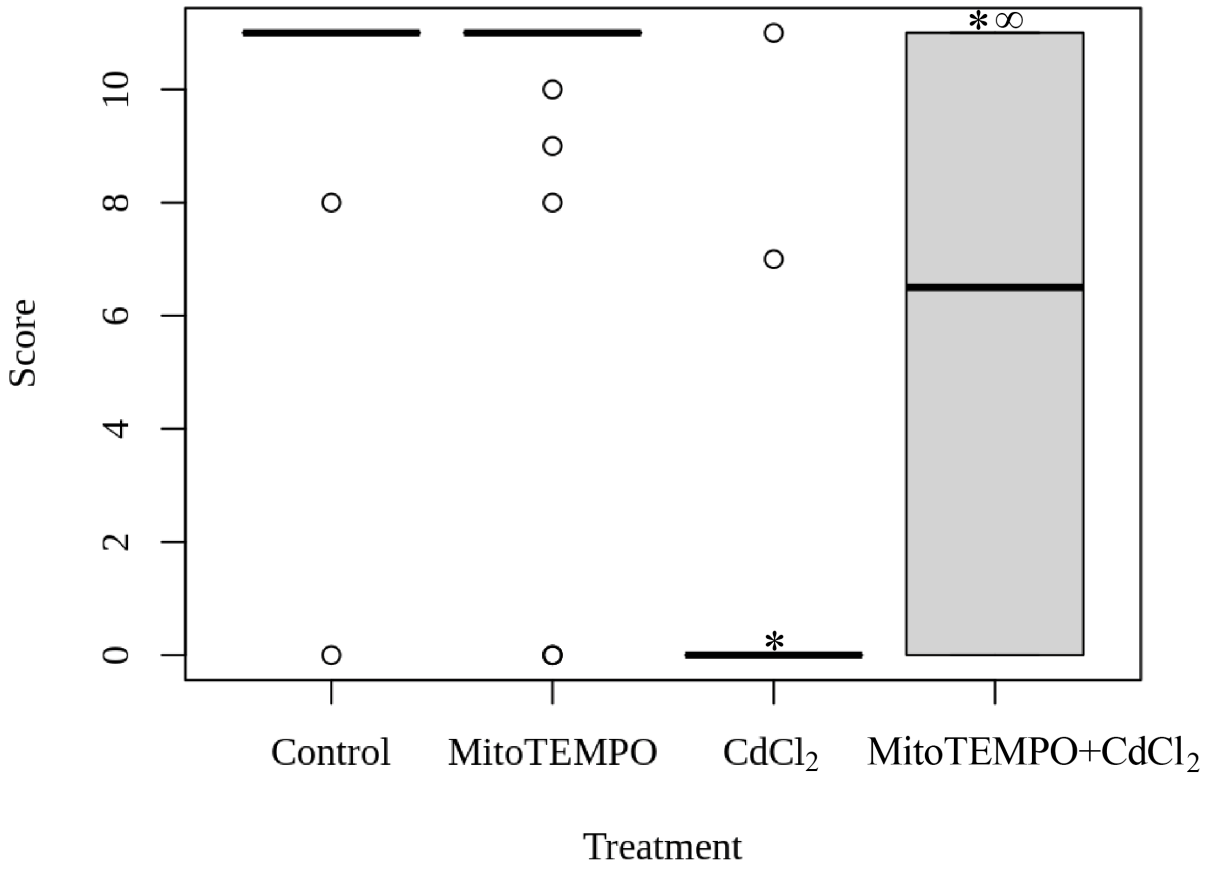
Note. Planaria were subjected to treatment with IOS water as the control, 5 µM MitoTEMPO, 5 µM $CdCl_2$, and a cotreatment of 5 µM MitoTEMPO with 5 µM $CdCl_2$. The data depicts the average ± SEM regeneration score for each treatment per day ($p < 0.001$).

A Kruskal-Wallis Test was conducted to examine the effects of four treatments (Control, 5 µM MitoTEMPO, 5 µM $CdCl_2$, and a cotreatment of 5 µM MitoTEMPO with 5 µM $CdCl_2$) on regeneration scores for day 14. The differences among treatment groups were significant, $H(3) = 56.98$, $p < 0.001$. Pairwise comparisons using the Wilcoxon Rank Sum Test with continuity correction revealed substantial differences in the control and 5 µM $CdCl_2$ treatments ($p < 0.001$) and the control and the cotreatment of 5 µM MitoTEMPO with 5µM $CdCl_2$ ($p < 0.001$). However, no significant difference was observed between the control and 5 µM MitoTEMPO treatments ($p = 0.21$). Additionally, the comparisons between 5 µM $CdCl_2$ and the 5 µM

MitoTEMPO treatments ($p < 0.0001$), the 5 µM $CdCl_2$ and the cotreatment of 5 µM MitoTEMPO with 5 µM $CdCl_2$ ($p < 0.01$), and the cotreatment of 5 µM MitoTEMPO with 5 µM $CdCl_2$ and 5 µM MitoTEMPO treatment ($p < 0.01$), also showed a difference in significance. The use of this antioxidant treatment, alone or in combination with 5 µM $CdCl_2$, appears to facilitate tissue repair to a certain extent (Figure 8), suggesting its properties may be contributing to modulating the cellular mechanisms involved in repair processes.

Figure 8:

Day 14 Regeneration Score vs MitoTEMPO Treatment



Note. Final regeneration scores of planaria between all four treatment groups. Treatments involved IOS water as the control, 5 µM MitoTEMPO, 5 µM $CdCl_2$, and a cotreatment of 5 µM MitoTEMPO with 5 µM $CdCl_2$. Each boxplot presents the median scores derived from duplicate experiments, with a total of n=12-14 flatworms per dish across all four treatment. Whiskers depict the range of scores, while the interquartile range is delineated by gray boxes, with outliers represented by open circles. * indicates significantly different compared with the untreated

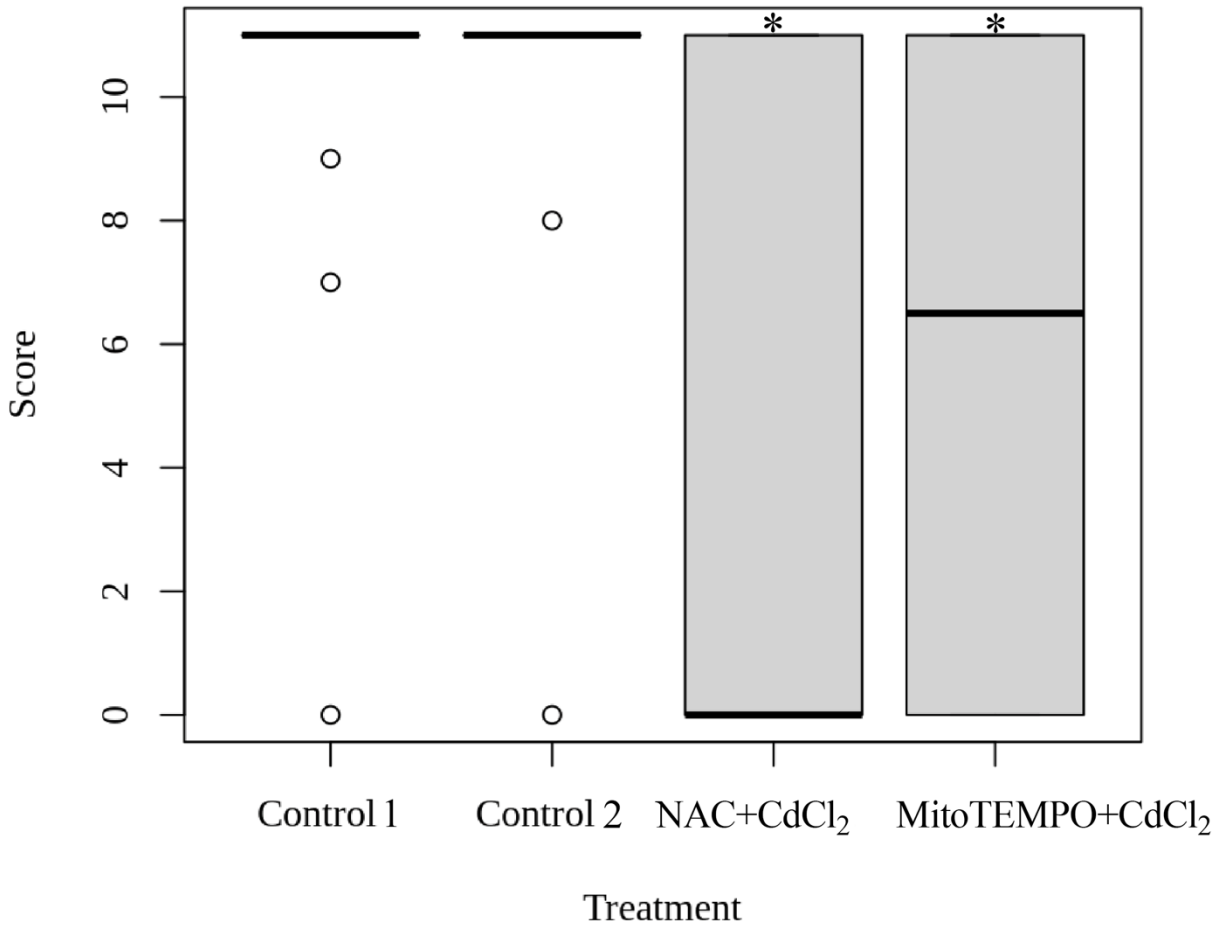
control ($p < 0.001$). ∞ indicates significantly different compared with 5 μM $CdCl_2$ alone treatment ($p < 0.01$).

Comparison of NAC and MitoTEMPO Treatment on Day 14 Regeneration Score

As a secondary analysis in response to comparable findings observed in our antioxidant trials, a Kruskal-Wallis Test with pairwise comparison using Wilcoxon Rank Sum Test was conducted to assess the comparative efficacy of NAC and MitoTEMPO treatments (NAC control, MitoTEMPO control, cotreatment of 10 μM NAC with 5 μM $CdCl_2$, and cotreatment of 5 μM MitoTEMPO with 5 μM $CdCl_2$). The Kruskal-Wallis Test revealed a statistically significant difference among the treatment groups, $H(3) = 36.02$, $p < 0.0001$. Upon prior findings, we've noted that subsequent pairwise comparisons indicated differences between the control and cotreatment groups of each singular antioxidant ($p < 0.001$). Analysis showed that the control groups from opposing treatments compared to both cotreatments showed significant disparities ($p < 0.001$). However, no statistically significant differences were found between the NAC control and MitoTEMPO control ($p = 0.70$) or between the cotreatments of antioxidants NAC and MitoTEMPO ($p = 0.59$). These outcomes show that while both NAC and MitoTEMPO exhibit partial efficacy in mitigating the effects of $CdCl_2$ exposure, they do not statistically differ, suggesting a potential therapeutic equivalence between the two antioxidants within this experimental context (Figure 9).

Figure 9:

Day 14 Regeneration Score Comparison of NAC and MitoTEMPO Treatments



Note. Final regeneration scores comparison of planaria between both antioxidant treatment groups. Treatments involved IOS water as control 1 for NAC and control 2 for MitoTEMPO, and cotreatment of 5 µM MitoTEMPO with 5 µM $CdCl_2$ and a cotreatment of 10 µM NAC with 5 µM $CdCl_2$. Each boxplot presents the median scores derived from duplicate experiments, with n=12-14 flatworms per dish across all four treatments. Whiskers depict the range of scores, while

the interquartile range is delineated by gray boxes, with outliers represented by open circles. * indicates a significantly difference compared with the untreated control ($p < 0.001$).

Planaria Dropping Tails

In the first set of treatments with varying concentrations of $CdCl_2$, the control group showed the highest number of dropped tails, resulting in eight. Treatments with increasing concentrations of $CdCl_2$ (2.5 – 20 µM) showed a decreasing trend in dropped tails, with the highest concentration resulting in no dropped tails, likely due to the toxic effect of the 20 µM $CdCl_2$ causing mortality at a faster rate (Table C1). During the treatments with the antioxidant of NAC, the control group had three dropped tails, whereas exposure to 10 µM NAC alone had four. Exposure to 5 µM $CdCl_2$ alone and the combined treatment of 5 µM $CdCl_2$ and 10 µM NAC resulted in no dropped tails (Table C2). In the treatment set of the antioxidant MitoTEMPO, the control group exhibited two dropped tails, while the treatment with 5 µM $CdCl_2$ alone resulted in one dropped tail. Exposure to 5 µM MitoTEMPO alone showed to have the highest amount of dropped tails (6), whereas the combined treatment of both 5 µM $CdCl_2$ and 5 µM MitoTEMPO had only two dropped tails for the entirety of the two weeks (Table C3).

Discussion

In the realm of environmental toxicology, cadmium stands out as a formidable threat, ranking seventh in the Top 20 Hazardous Substances Priority List, with human activities amplifying its omnipresence (Klaassen et al., 2009). $CdCl_2$ concentrations from 2.5 µM to 18 µM have been noted in studies to have the most impact on planarian regeneration (Calevro et al., 1997). This range of exposures assisted in narrowing down the optimal concentration suitable for assessing antioxidant responses. We began our experimentation at 2.5 µM $CdCl_2$ to adhere to established exposure parameters and extended the upper limit to 20 µM $CdCl_2$ to explore

potential thresholds beyond prior observations. Our findings identified a concentration-dependent toxicity as planaria responded to different $CdCl_2$ exposures. Lower concentrations of $CdCl_2$ delayed blastema, ocelli and auricle repair, and caused some death, however, as the concentration increased, we observed faster rates of mortality alongside continued delay in the repair processes. Extending our investigation to a concentration of 20 μM $CdCl_2$ revealed that concentration was too toxic for the planaria to successfully regenerate, let alone survive (Figure 2).

Following observations of $CdCl_2$- induced toxicity, we explored the potential mitigating effects of NAC and MitoTEMPO on planaria regeneration. Our results indicated that antioxidants provided partial rescuing effects in the planaria that survived. We can presume that the exposure to NAC aided in cephalic regeneration by SOD activity replenishing intracellular GSH levels (He et al., 2017), which enabled scavenging of ROS to lessen oxidative damage and preserve as much of the cell's integrity. We can propose that MitoTEMPO was able to target and scavenge ROS within the mitochondria, protecting the cell's mitochondrial functions for cellular energy production in efficient tissue regeneration. In addition to noting the potential contributions of chemicals to stress factors, speculation regarding the observed responses could also stem from the possibility of interspecies variations differing in sensitivity, variations impacted by mating seasons, or disruptions in signaling pathways.

While the mechanism governing anatomical restoration post-amputation varies among planarian species, *G. dorotocephala* emerges as a potential model for unraveling the relationship between metal toxicity and regeneration. The limited research on this specific species and its response to metal and antioxidant treatments necessitated this study. The regenerative capabilities of planaria offer a source of optimism in understanding and mitigating the impacts of

such toxicants, allowing the intricate interplay between morphallactic strategies and species-specific responses (Reddien & Alvarado, 2004) to stressors remain a focal point of inquiry. In our laboratory, we have consistently received planarian specimens identified as *G. dorotocephala*. However, our experiments revealed physical phenotype variations indicating potential speciation within our samples, with observed species differentiation confined to a single order of flatworms. These variations include differences in length, dimensions, auricle structure, and pigmentation patterns among the flatworms (Appendix D). Subtle yet significant variations in morphological traits may serve as key indicators of underlying genetic diversity and possible advantageous traits. Without precise identification beyond the assumed genus, it is plausible that these variations in species could impact their sensitivity and survival capabilities under specific exposures. Such variation could have influenced our experiments' regeneration scores and overall responses, with the species differentiation accounting for some of the observed variability in our results. Therefore, further investigation into the precise speciation of these planarian populations is warranted to better understand and interpret our experimental findings.

Instances of spontaneous "dropping tails," a form of asexual reproduction serving as a defensive tactic against unfavorable environmental conditions (Ward's Science, 2005), were observed among certain flatworms (Figure C1). While such occurrences are anticipated under exposure to toxic substances, it was unexpected to document the tail dropping within the control groups of all treatments (Appendix C), which was maintained in standard IOS water conditions post-amputation. This unexpected behavior underscores a significant deviation from the established literature, which primarily associates tail dropping with adverse water conditions rather than metal or any sort of chemical exposure. Remarkably, despite efforts to regenerate

their cephalic regions, fragments exposed to various $CdCl_2$ concentrations and antioxidant treatments exhibited repeated tail autotomy. No research has gone into full detail regarding dropping tails from metal toxicity or antioxidant treatment, hence considering that a signaling pathway was dysregulated.

Numerous signaling molecules are released promptly post-epidermal injury, and are crucial for wound detection and initiation of early responses such as mammalian immune cell recruitment. These molecules include extracellular ATP, ROS, and polyunsaturated fatty acids (Owlarn, 2017). Studies have demonstrated that disruptions in the in β-catenin levels signaling cascade, can result in planarian regeneration errors. It's noteworthy that this pathway may serve a pivotal function not just in regular injury response but also in triggering tail dropping. Inhibition of key components of this pathway, such as β-catenin and *wnt1*, can lead to dysregulated injury responses, consequently delaying neoblast formation and tail droppings due to the imbalance (Petersen & Reddien, 2011; Reddien, 2018). Despite possessing antioxidant properties to scavenge excess ROS, the administered concentrations of 10 μM NAC and 5 μM MitoTEMPO, whether alone or in conjunction with $CdCl_2$, failed to elicit the necessary molecular signals or expression for complete rescuing effects, potentially inducing the phenomenon where an already regenerating fragment underwent spontaneous tail dropping. Future research focused on elucidating the mechanisms underlying the observed spontaneous tail dropping phenomenon should be replicated under controlled condition to validate its occurrence and understand its dependence on the β-catenin signaling pathway. Subsequent experiments can manipulate key components of the pathway and use molecular analysis to examine expression levels of pathway-related genes and signaling molecules.

Despite their distinct mechanical properties, the observed similarities to the effects of NAC and MitoTEMPO on planarian regeneration raised intriguing questions regarding their potential therapeutic equivalence. Our secondary analysis, prompted by their comparable outcomes, suggests complex interplay within the biological system. While NAC acts as a direct radical scavenger, intercepting oxidative stress pathways, MitoTEMPO exerts its effects within the mitochondria, targeting mitochondrial ROS. However, given the interconnected nature of cellular processes, it is plausible that NAC's role as a GSH precursor could extend to indirectly impact mitochondrial function through cellular redox status, thereby contributing to the observed comparable outcomes. Active mitochondria are crucial for proper neoblast development in planaria, especially in regeneration and in meeting the increased metabolic needs during cell specialization (Haroon et al., 2021). Therefore, if GSH levels are elevated, it can impact the integrity of the mitochondria by inhibiting autocatalytic lipid peroxidation (Unsal et al., 2020). To comprehensively evaluate their therapeutic potential, further investigations incorporating preliminary trials exploring various concentrations, augmenting sample sizes, and mitochondria-targeted probe assays are warranted (Dikalov & Harrison, 2014). Such explorations would not only elucidate the robustness of these antioxidant effects but also delineate the precise utility and comparative efficacy of NAC and MitoTEMPO.

Planaria, being hermaphroditic, engage in both sexual and asexual reproduction to diversify genetic material and enhance species survival. The development of eggs within their bodies yields either "summer" or "winter" eggs, contingent upon ambient temperature (Ward's Science, 2005). Temperature variations serve as a primary environmental trigger for physiological adjustments promoting mating and reproduction in planaria, with reproductive organs typically manifesting during winter months and regressing in summer months. The shift

in temperature suggests a complex interplay of biochemical enzymatic reactions modulated by environmental stimuli (Nodono & Matsumoto, 2022), which could influence susceptibility to external influences like $CdCl_2$ concentrations. In addition to temperature fluctuations, it's crucial to consider the impact of environmental contaminants during planaria mating season. Environmental exposure during this sensitive period could influence reproductive success and overall population health. Heightened sensitivity to contaminates like $CdCl_2$ during mating seasons suggests a potential vulnerability to environmental stressors, which could ultimately contribute to species decline. The unexpected presence of winter eggs in July (Appendix E) among the flatworm batch utilized in the $CdCl_2$ trials highlights a fascinating correlation between the flatworm's reproductive capabilities and its capacity for regeneration despite toxic environments. Future research endeavors should aim to standardize trial conditions by conducting experiments concurrently and during consistent seasons to mitigate the heightened risk of mating season influences on sensitivity.

In summary, our experiment supported the initial hypothesis that increasing concentrations of $CdCl_2$, up to 20 μM, hindered blastema repair through the suspected mechanism of ROS overproduction. The antioxidant treatments with NAC and MitoTEMPO showed promise in mitigating ROS production, promoting cephalic regenerative polarity in *G. dorotocephala*. While these findings are encouraging, further research is warranted to explore the antioxidant mechanisms and their direct impact on ROS scavenging, shedding light on the relationship between oxidative stress and regeneration inhibition in planaria. To investigate ROS scavenging, one approach could involve employing direct imaging techniques such as fluorescence microscopy to visualize the dynamic interactions between ROS and neutralizing molecules within living cells or tissue. Biochemical assays could also be utilized to quantify

ROS levels and asses the efficacy of various scavenging agents in neutralizing ROS-induced damage. Our results underline the detrimental effects of $CdCl_2$ on regenerative processes to ongoing research in regenerative biology, offering potential avenues for the development of novel therapeutic interventions to mitigate toxicological impacts on regeneration, improving public health outcomes.

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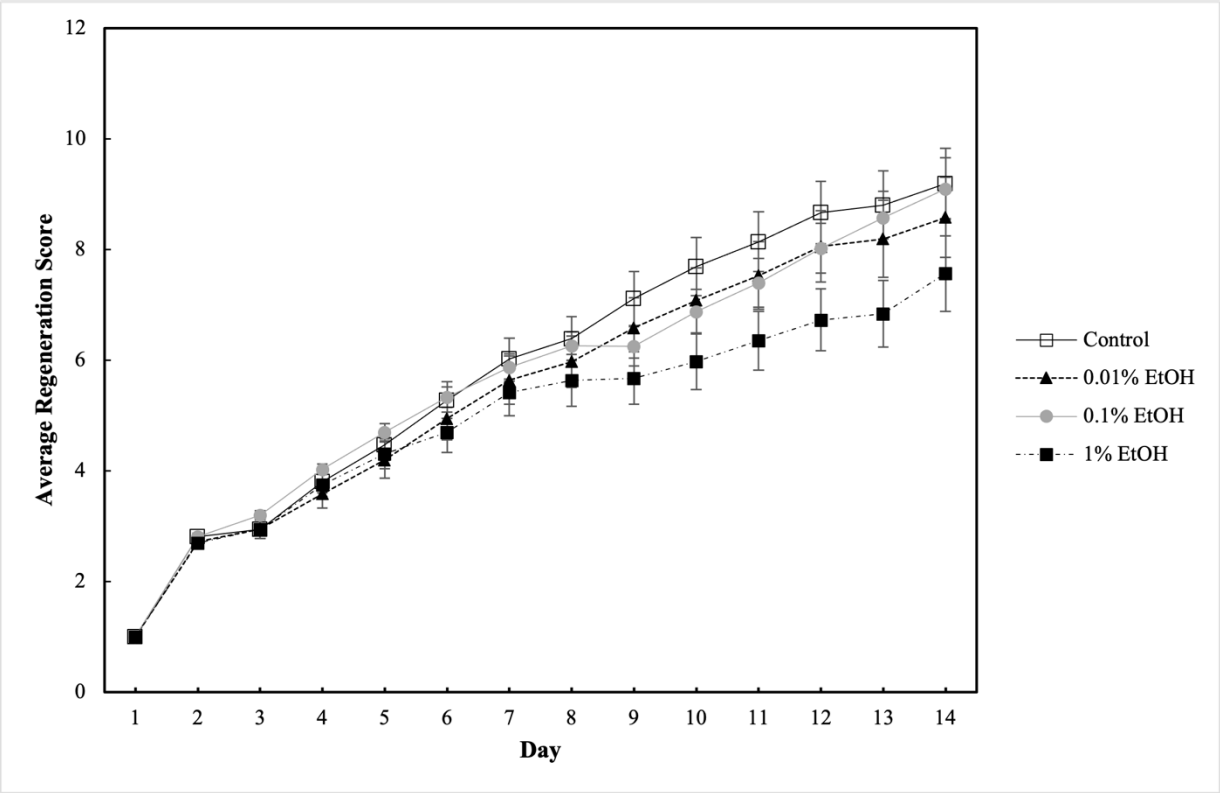
Appendix A

Figure A1:

Effect of Ethanol on Average Regeneration Score



Note. Planaria were exposed to IOS water as the control, alongside varying concentrations of EtOH ranging from 0.01% to 1% over a two-week period. The data depicts the average ± SEM regeneration score for each treatment per day.

Appendix B

Figure B1:

Day 14 Regeneration Score vs $CdCl_2$ Script Analysis

```
#Natalie Gonzalez
#Thesis Data
#Kruskal-Wallis Rank Sum Test for CdCl2 Data
#Spring Semester, 2024

#plot score versus concentration by treatment data as a boxplot
boxplot(Score~Treatment, data = D14CdCl2, ylab = "Score")

#conduct the Kruskal-Wallis test
testresults <- kruskal.test(Score~Treatment, data = D14CdCl2)

#print the test results
print(testresults)

#conduct a Pairwise Test of Wilcoxon Rank Sum Tests
posthocresults <- pairwise.wilcox.test(D14CdCl2$Score,D14CdCl2$Treatment)

#print the post-hoc results
print(posthocresults)
```

Note. An R script demonstrating the codes used for analyzing the effects of different $CdCl_2$ concentration treatments on regeneration score outcomes. Raw data pertaining to the regeneration score vs treatment on day 14 were inputted for statistical analysis using the Kruskal-Wallis Test, followed by the Pairwise of Wilcoxon Rank Sum Test.

Figure B2:

Day 14 Regeneration Score vs NAC Treatment Script Analysis

```r
#Natalie Gonzalez
#Thesis Data
#Kruskal-Wallis Rank Sum Test for NAC Data
#Spring Semester, 2024

#plot score versus concentration by treatment data as a boxplot
boxplot(Score~Treatment, data = D14NAC, ylab = "Score")

#conduct the Kruskal-Wallis test
testresults <- kruskal.test(Score~Treatment, data = D14NAC)

#print the test results
print(testresults)

#conduct a Pairwise Test of Wilcoxon Rank Sum Tests
posthocresults <- pairwise.wilcox.test(D14NAC$Score,D14NAC$Treatment)

#print the post-hoc results
print(posthocresults)
```

Note. An R script demonstrating the codes used for analyzing the effects of NAC treatments on day 14. Raw data was inputted for statistical analysis using the Kruskal-Wallis Test to assess overall treatment effects, followed by the Pairwise of Wilcoxon Rank Sum Test to evaluate specific treatment group differences.

Figure B3:

Day 14 Regeneration Score vs MitoTEMPO Treatment Script Analysis

```
#Natalie Gonzalez
#Thesis Data
#Kruskal-Wallis Rank Sum Test for MitoTEMPO Data
#Spring Semester, 2024

#plot score versus concentration by treatment data as a boxplot
boxplot(Score~Treatment, data = D14MitoTEMPO, ylab = "Score")

#conduct the Kruskal-Wallis test
testresults <- kruskal.test(Score~Treatment, data = D14MitoTEMPO)

#print the test results
print(testresults)

#conduct a Pairwise Test of Wilcoxon Rank Sum Tests
posthocresults <-
pairwise.wilcox.test(D14MitoTEMPO$Score,D14MitoTEMPO$Treatment)

#print the post-hoc results
print(posthocresults)
```

Note. An R script demonstrating the codes used for analyzing the effects of MitoTEMPO treatments on day 14. Raw data was inputted for statistical analysis using the Kruskal-Wallis Test to assess overall treatment effects, followed by the Pairwise of Wilcoxon Rank Sum Test to evaluate specific treatment group differences.

Figure B4:

Comparison of NAC and MitoTEMPO Treatment on Day 14 Regeneration Score Script Analysis

```
#Natalie Gonzalez
#Thesis Data
#Kruskal-Wallis Rank Sum Test for NAC+MitoTEMPO Data
#Spring Semester, 2024

#combine both antioxidant data sets
combineddata <- rbind(D14NAC,D14MitoTEMPO)

#focus on select treatment combinations
selectlevels <- c("ControlN","ControlM","NAC+CdCl2","MitoTEMPO+CdCl2")

#reorder the levels of treatment factor
combineddata$Treatment <- factor(combineddata$Treatment, levels =
selectlevels)

#plot score versus concentration by treatment data as a boxplot
boxplot(Score~Treatment, data = combineddata, ylab = "Score")

#conduct the Kruskal-Wallis test
testresults <- kruskal.test(Score~Treatment, data = combineddata)

#print the test results
print(testresults)

#conduct a Pairwise Test of Wilcoxon Rank Sum Tests
posthocresults <-
pairwise.wilcox.test(combineddata$Score,combineddata$Treatment)

#print the post-hoc results
print(posthocresults)
```

Note. An R script demonstrating the codes used for analyzing the effects of NAC to MitoTEMPO treatments on day 14. Raw data was inputted for statistical analysis using the Kruskal-Wallis Test to assess overall treatment effects, followed by the Pairwise of Wilcoxon Rank Sum Test to evaluate specific treatment group differences.

Appendix C

Table C1:

The Dropping of Tails in $CdCl_2$ Experiment

Treatment	Dropped Tails
Control	8
2.5 µM $CdCl_2$	4
5 µM $CdCl_2$	2
10 µM $CdCl_2$	1
15 µM $CdCl_2$	2
20 µM $CdCl_2$	0

Note. The total number of dropped tails within 14 days of the $CdCl_2$ treatment. Each treatment group, ranging from the control to various concentrations of $CdCl_2$ is listed along with the corresponding number of dropped tails recorded over three trials. Aside from the control group having eight dropped tails, the frequency of tails dropping generally decreases with elevated $CdCl_2$ concentrations due to a greater amount of fatalities.

Table C2:

The Dropping of Tails in NAC Treatment

Treatment	Dropped Tails
Control	3
5 µM $CdCl_2$	0
10 µM NAC	4
5 µM + 10 µM NAC	0

Note. The total number of dropped tails within 14 days of the NAC treatment. Each treatment group is listed along with the corresponding number of dropped tails recorded over two trials. The control group exhibited three dropped tails, while the 5 µM $CdCl_2$ treatment, with the exception of rapid death, and the combination of 5 µM $CdCl_2$ and 10 µM NAC showed no instances of dropped tails. The 10 µM NAC treatment recorded four dropped tails.

Table C3:

The Dropping of Tails in MitoTEMPO Treatment

Treatment	Dropped Tails
Control	2
5 μM $CdCl_2$	1
5 μM MitoTEMPO	6
5 μM + 5 μM MitoTEMPO	2

Note. The total number of dropped tails within 14 days of the MitoTEMPO treatment**.** Each treatment group is listed along with the corresponding number of dropped tails recorded over two trials. The control group and combination group of 5 μM $CdCl_2$ and 5 μM MitoTEMPO showed a dropping of two tails each, while the separate treatments experienced one (5 μM $CdCl_2$) and six (5 μM MitoTEMPO).

Figure C1:

Visualization of Planaria Dropped Tails

Note. A comparison of regenerative progress and spontaneous tail dropping in three flatworms. The top row displays the state of each flatworm once self-amputation occurred, while the bottom row depicts the appearance of the dropped tail.

Appendix D

Figure D1:

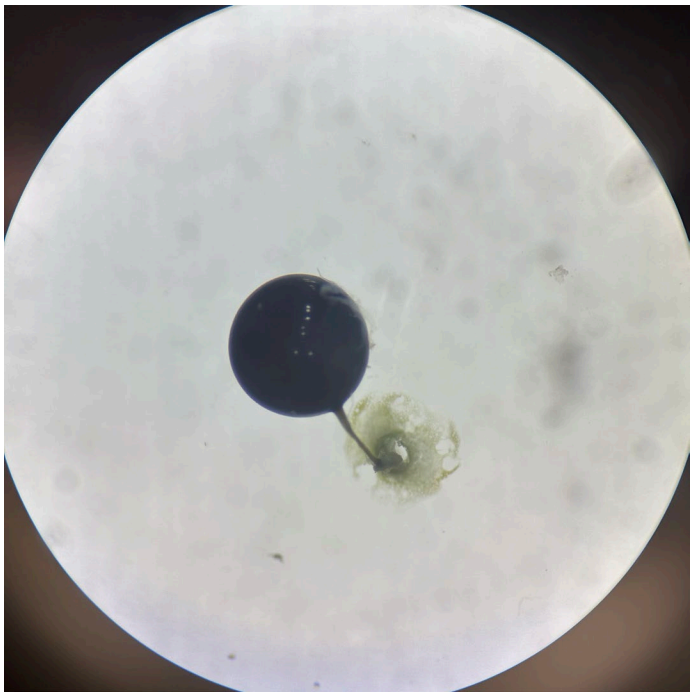
Planarian Species Differentiation

Note. The specimen diversity received from Carolina Biological Supply (Burlington, NC). (A) The flatworm displays predominately white coloration at the top with grey and black spots throughout, gradually clustering towards the tail. (B) An opaque-colored flatworm with adorned black tiger-like stripes throughout its long body. Faint ocelli and lack of auricles are noticeable. (C) A lengthy flatworm characterized by curbed auricles and a grey hue with scattered black and white dots across the body. (D) A typical *G. dorotocephala* flatworm with dark brown coloration. The tip of the head and its auricles are pointed out with a curbed structure. (E) A flatworm with a lengthy slender body, sharp and narrow auricles and head tip. Pigmentation shows a consistent distribution of brown and white spots throughout the body. (F) A flatworm with a pattern pigmentation of a rich brown hue and sparse white spots. The head tip and auricles are less pointed, featuring smaller curved edges.

Appendix E

Figure E1:

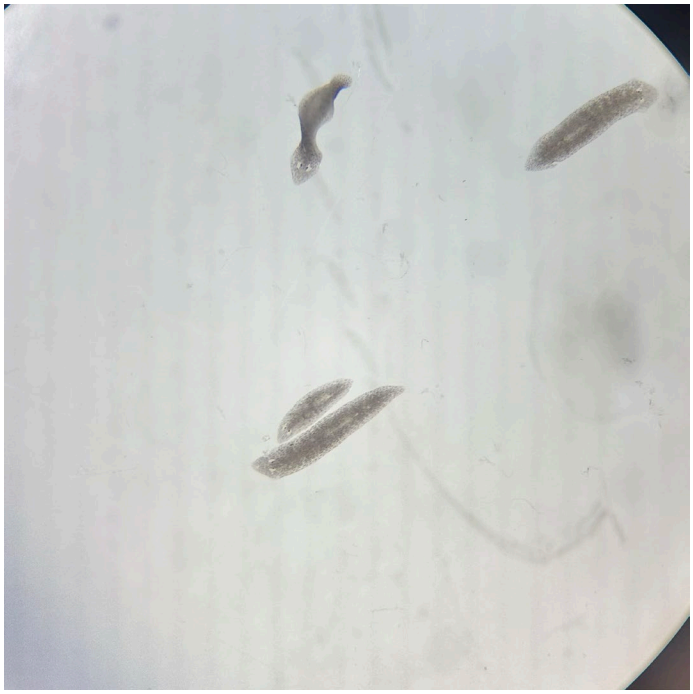
Planarian Winter Egg and Offspring



Note. An encapsulated dark brown casing with a peculiar stalk structure, featuring a small crust-like appendage at its tip. This was the very first winter egg to be laid in the Planarian Lab at Lynn University. July 5th, 2023.

Figure E2:

Planaria Hatchlings



Note. Upon hatching from their winter egg on July 19th, 2023, there were a total of four viable offspring observed. The newborn flatworms emerged as delicate, nearly transparent individuals and varied in length. Despite their small stature, the flatworms showed lots of movement around the culture dish.