Investigating Whether Thymol Pre-Exposure Improves Regeneration Capacity in Hydra vulgaris Following Hydrogen Peroxide (H_2O_2) Induced Oxidative Stress

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Abstract

Regeneration in Hydra vulgaris is driven by the proliferation and differentiation of interstitial stem cells. When exposed to 1 mM hydrogen peroxide (H_2O_2), a reactive oxygen species (ROS) that induces oxidative stress, Hydra experience DNA damage. This study investigated whether pretreatment with thymol, an antioxidant, is able to preserve the regenerative capacity of Hydra vulgaris under oxidative stress (Tsarkova, Elena, et al, 2023). It was hypothesized that thymol would mitigate oxidative damage and improve regeneration in the presence of H_2O_2. Hydra were starved for 7 days before experimentation. Five Hydra per trial were trisected around the mid-gastric region to induce regeneration. A toxicity test identified that 10μM thymol was the ideal non-toxic working concentration (Mann-Whitney U Test, $p < 0.01$, compared with 20μM). Four experimental groups were evaluated: a positive control (thymol), a negative control (H_2O_2), a control (Hydra Medium), and the treatment (H_2O_2+ thymol). Regeneration was assessed at 24, 48, and 72 hours using a standardized 0-5 regeneration scale. There was a statistically significant difference in mean regeneration scores between Hydra exposed with H_2O_2 and Hydra pretreated with H_2O_2 + thymol (Mann-Whitney U Test, $p < 0.01$). Hydra pretreated with thymol under oxidative stress conditions demonstrated improved regeneration compared to the 1mM H_2O_2 only group. These findings suggest that thymol protects regeneration by reducing ROS damage and preserving stem cell-mediated regeneration. Hydra vulgaris provides a cost-effective screening model for evaluating

compounds that protect regenerative capacity under oxidative stress and supports further investigation of thymol and related antioxidants in regenerative biology.

Introduction

Tissue regeneration is a critical biological process found in most organisms. While humans have limited regeneration abilities, *Hydra vulgaris*, a freshwater cnidarian, possesses extraordinary regenerative capabilities(Lovas, J. R., & Yuste, R., 2022; Vogg, M. C., Galliot, B., & Tsiairis, C. D., 2019). Hydra are capable of regenerating full bodies from just a small fragment of tissue due to the huge concentration of interstitial stem cells (Reddy et al., 2019). Highly conserved molecular signaling pathways, such as the Wnt/β-catenin signaling pathway, are the primary inducers for regeneration. Furthermore, the evolutionarily conserved trait of this signaling pathway allows it to play the same role in human wound healing and repair (Haval et al., 2020). Oxidative stress plays a key role in tissue and cellular regeneration. Oxidative stress occurs in the presence of excess reactive oxygen species (ROS). ROS are byproducts of cellular metabolism; they play important roles in cell signaling, immune responses, and wound healing (Kozlov et al., 2024). In particular, ROS act as signaling molecules to initiate the regenerative process, or more specifically, activate the Wnt/β-catenin signaling pathway (Kozlov et al., 2024). However, ROS can become harmful in large concentrations. When ROS levels become excessive, they can lead to cellular damage and inhibit regenerative processes altogether by impairing stem cell functions (Haval et al., 2020). This damage occurs because ROS are highly reactive molecules and often oxidize other cellular components to stabilize themselves, leading to molecular damage. To counteract this imbalance and restore cellular homeostasis, biological systems rely on antioxidant mechanisms to donate an electron to the unstable ROS molecule (Muscolo et al., 2024). However, as humans age, antioxidant systems

degrade and ROS can lead to neurodegenerative diseases (Manoharan et al., 2016). For example, in Alzheimer's disease, excessive ROS production contributes to neuronal damage, protein misfolding, inability to regenerate neuronal cells, and progressive loss of cellular function (Manoharan et al., 2016). One potential approach involves utilizing antioxidant compounds to effectively restore redox balance and reduce excess ROS concentrations. Thymol, a naturally occurring phytochemical, has been shown to have strong antioxidant and free-radical-scavenging properties in previous studies (Muscolo et al., 2024). Thymol was heavily explored and its biochemical properties as an antioxidant have been shown(Chroho, M., Rouphael, Y., Petropoulos, S. A., & Bouissane, L., 2024). However, its impact on tissue regeneration under oxidative stress has not been fully studied in regenerative organisms and on the conserved cell signaling pathways. This study will investigate whether thymol pretreatment will improve the regenerative capacity of *Hydra vulgaris* under oxidative stress induced by hydrogen peroxide. The null hypothesis states that there will be no significant difference between the mean regeneration scores of Hydra exposed to 1 mM of H_2O_2 with thymol pretreatment and Hydra exposed to 1 mM of H_2O_2 alone. The alternate hypothesis is that Hydra pretreated with 10μM thymol and then exposed to 1 mM of H_2O_2 will exhibit significantly higher mean regeneration scores following H_2O_2 exposure due to thymol's antioxidant properties that aid in reducing oxidative damage. To test this hypothesis, three experimental phases were conducted: a thymol concentration/toxicity test to determine a safe optimal concentration, an H_2O_2 validity test to confirm oxidative stress-induced regenerative impairment, and the primary treatment, which compared H_2O_2, Thymol, H_2O_2 +Thymol, and a control. Controlled variables across all groups included a 7-day starvation period to standardize metabolic state, trisection of the mid gastric region, equal group sizes of 5 Hydra per dish, a consistent rinsing mechanism

between treatments, and regeneration was assessed at identical timepoints using the same 0-5 scoring rubric.

Methods and Materials

Creation of Solutions:

To create the Thymol 50 μM stock solution, 7.51mg of thymol was dissolved in 0.1mL of 95% ethanol in a beaker. Then Deer Park natural Spring Water was mixed in and added to bring the total volume of the stock solution to 1L. Working solutions of 10μM, 20μM, 30μM, and 40μM were created by using 20mL, 40mL, 60mL, and 80 mL, respectively, and adding spring water to make the total volume 100mL. Each solution was stored in an airtight container and remade on a weekly basis. A 1mM H_2O_2 solution was prepared by diluting 4.5 mL of 3% hydrogen peroxide into 1 L of Deer Park natural Spring Water in a beaker. This solution was freshly prepared as needed due to its instability and short shelf life.

Thymol Toxicity/Concentration test:

All Hydra were sourced from Carolina Biological Supplies. Sixty *Hydra vulgaris* were randomly selected and starved for 7 days. They were then divided into six groups (n = 10 per group) and exposed to thymol concentrations ranging from 0–50μM (increments of 10) for 24 hours. Between all treatments, the Hydra were rinsed by placing them in a cell strainer and gently pouring water over them. All Hydra were trisected to isolate the mid gastric region(Figure 2), and redistributed into groups of 5 per dish with 2 initial trials at each concentration. After 24, 48, and 72 hours, regeneration was assessed based on Figure 1 and recorded. Due to toxicity and Hydra death at 40μM and 50μM, the subsequent trials(n=6) were conducted using concentrations from 0-30μM(increments of 10) using 40 Hydra with 5 per concentration.

Hydrogen Peroxide Validity Test:

Sixty *Hydra vulgaris* were randomly selected after 7 days of starvation, with 30 pretreated in hydrogen peroxide for 4 hours, and 30 serving as controls. Between all treatments, the Hydra were rinsed by placing them in a cell strainer and gently pouring water over them. All were then trisected to isolate the mid gastric region (Figure 2) , distributed in groups of 5 into 6 petri dishes per group (H_2O_2 and Control), and assessed for regeneration at 24, 48, and 72 hours based on the scoring rubric in Figure 1.

Effect of Thymol on Hydra Regeneration Under Oxidative Stress:

200 *Hydra Vulgaris* were randomly selected, with 100 pretreated in 10μM thymol for 24 hours, after which subsets were either immediately trisected following pretreatment or subsequently exposed to hydrogen peroxide for 4 hours before trisection or exposed to hydrogen peroxide for 4 hours alongside a non-thymol pretreated group; between all treatments, the Hydra were rinsed by placing them in a cell strainer and gently pouring water over them. All samples were trisected to isolate the mid-gastric region (Figure 2), distributed in groups of 5 across 10 petri dishes per condition (control, H_2O_2 only, thymol only, H_2O_2 + thymol). All were evaluated for regeneration at 24, 48 and 72 hours using the standardized scoring rubric (Figure 1). Experimental conditions were all constant during this time in a dark cabinet at room temperature, 22°C.

Score:	0	1	2	3	4	5
Relation to regeneration	No regeneration	Initial wound closure: Cut edges are beginning to close	Early Bud formation: A small mound appears at the location of the wound	Partial regeneration : The body column reforms	Advanced regeneration : Tentacles are mostly formed	Complete regeneration : Fully regenerated hydra

Figure 1. Regeneration Scoring Criteria for *Hydra Vulgaris*
Hydra regeneration was assesong using a 0-5 scaled based on the observable recovery following amputation. Scores were assigned per Hydra and averaged per petri dish
(created by researcher)

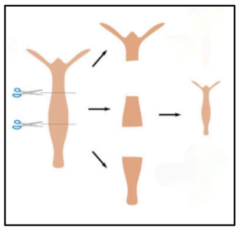

Figure 2. Model for Trisection Hydra to measure regeneration. Image adapted from Reddy et al. (2019)

Results

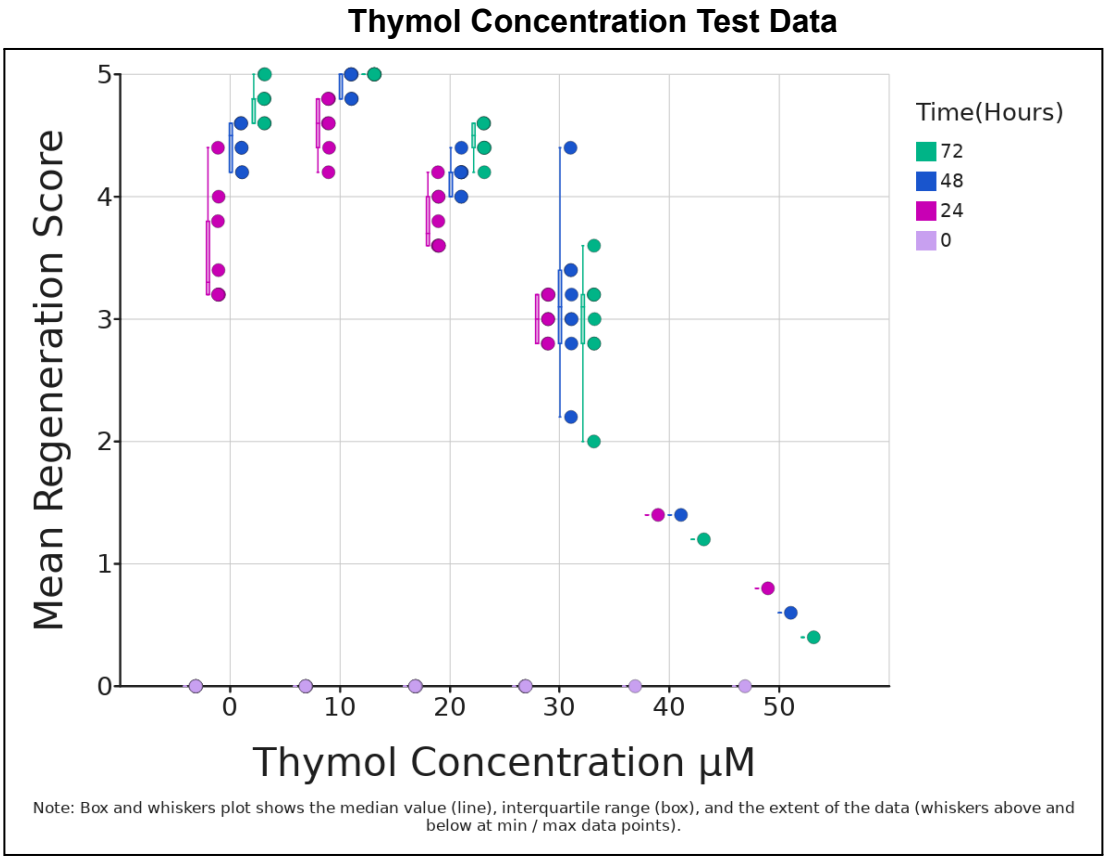

Figure 3. This shows the concentration-dependent effect of thymol on Hydra regeneration under oxidative stress. Regeneration scores increased over time across all groups. The 10µM demonstrated the most rapid and complete regeneration by 72 hours while 20µM-30µM showed

partial recovery with 20μM being more efficient. Higher concentrations 40-50μM, ended up being toxic to the Hydra. 40μM and 50μM killed the Hydra

(created by researcher in DataClassroom)

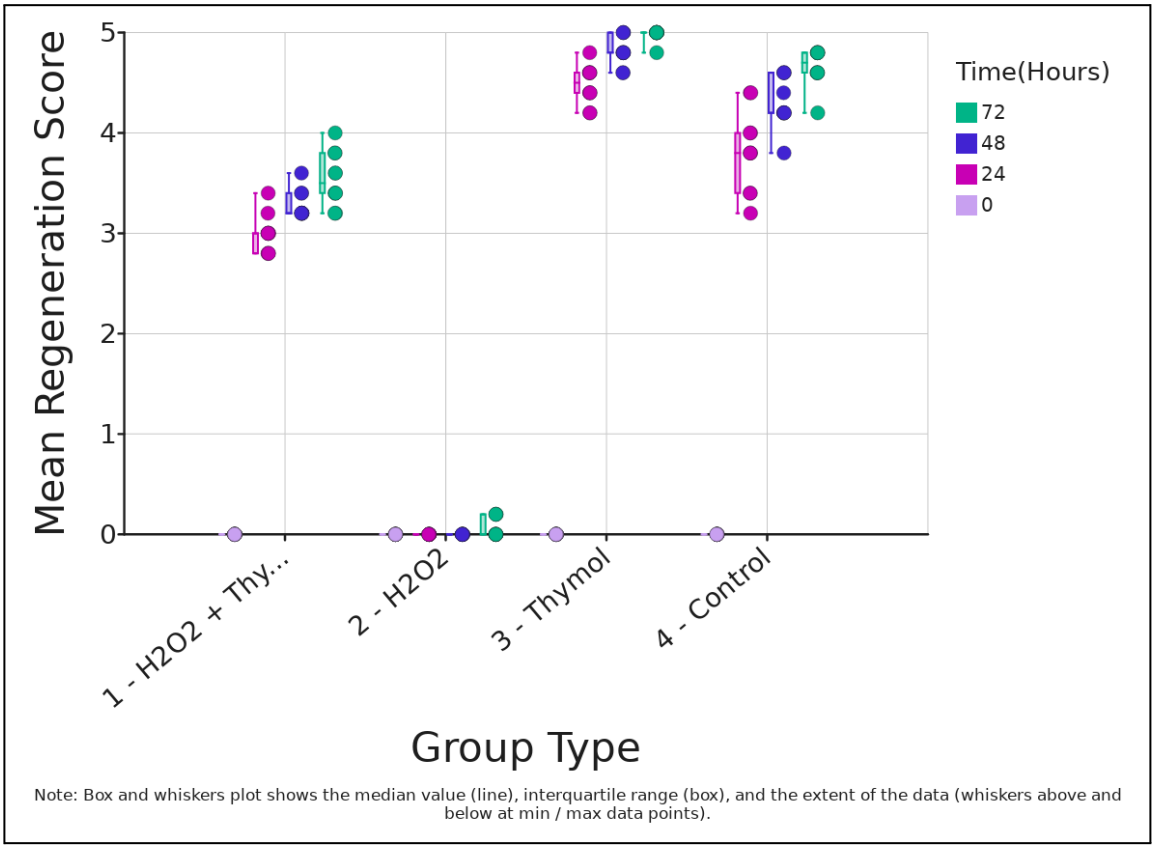

Figure 4. Analysis of regeneration in *Hydra vulgaris* across 4 treatment groups: 4 - control (no H_2O_2 no thymol), 3 - thymol only, 2 - H_2O_2 only, and 1 - H_2O_2 & thymol. Mean regeneration scores (0-5) were calculated solely from original cut fragments. H_2O_2 exposure suppressed regeneration when also pretreated with thymol the regenerative capacity was increased over the course of 72 hours.

(created by researcher in DataClassroom)

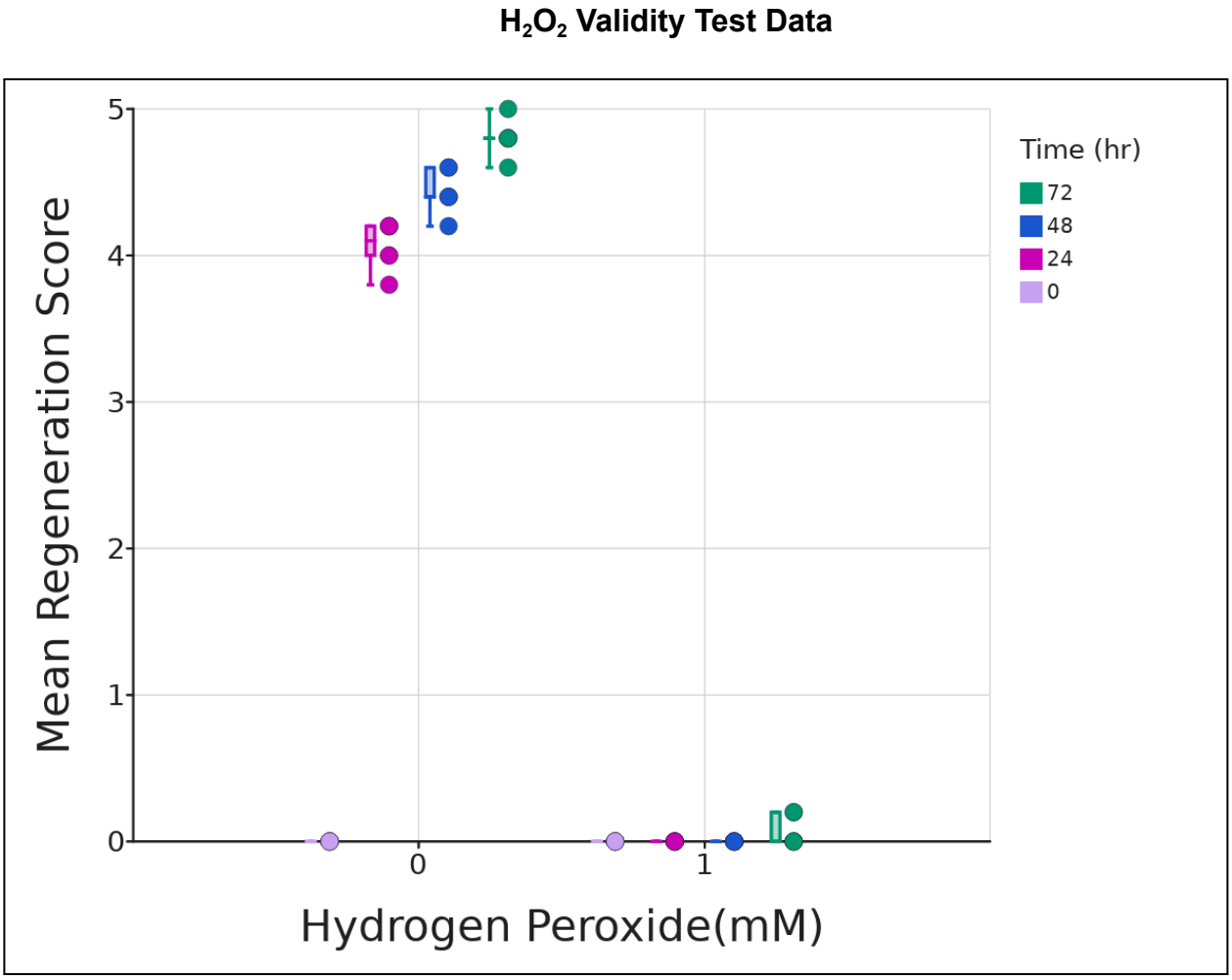

Figure 5. Validation of oxidative stress model in *Hydra vulgaris* using 1mM of H_2O_2. Hydra exposed to H_2O_2 showed suppression of regeneration across all timepoints (0-72 hr) while the untreated controls showed continued regeneration and achieved near complete regeneration by 72 hours. (created by researcher in DataClassroom)

Mann-Whitney U Test - Concentration Test

Time = 24 Hours	10μM	20μM	30μM
0μM	P < 0.01	P =0.16	P =0.01
10μM		P < 0.01	P < 0.01
20μM			P < 0.01
30μM			

Time = 48 Hours	10μM	20μM	30μM
0μM	P < 0.01	P = 0.01	P <0.01
10μM		P < 0.01	P < 0.01
20μM			P = 0.01
30μM			

Time = 72 Hours	10μM	20μM	30μM
0μM	P < 0.01	P < 0.01	P <0.01
10μM		P < 0.01	P < 0.01
20μM			P < 0.01
30μM			

Figure 6. This shows the results of the Mann- Whitney U Test comparing each concentration with each other. 40μM and 50μM were dropped because they were killing the Hydra

(created by researcher in DataClassroom)

Mann-Whitney U Test - Treatment Test

Time = 24 Hours	H_2O_2	Thymol	Control
Thymol + H_2O_2	P < 0.01	P < 0.01	P < 0.01
H_2O_2		P < 0.01	P < 0.01
Thymol			P < 0.01
Control			

Time = 48 Hours	H_2O_2	Thymol	Control
Thymol + H_2O_2	P < 0.01	P < 0.01	P < 0.01
H_2O_2		P < 0.01	P < 0.01
Thymol			P < 0.01
Control			

Time = 72 Hours	H_2O_2	Thymol	Control
Thymol + H_2O_2	P < 0.01	P < 0.01	P < 0.01
H_2O_2		P < 0.01	P < 0.01
Thymol			P < 0.01
Control			

Figure 7. This shows the results of the Mann- Whitney U Test comparing the mean regeneration score of each group. The purple region indicates there was a significant difference between the H_2O_2-only group and the H_2O_2 + Thymol groups

(created by researcher in DataClassroom)

Discussion

Our results indicate that exposure to 1 mM hydrogen peroxide significantly impairs head and foot regeneration in *Hydra vulgaris*(Figure 5). Hydra exposed to solely hydrogen peroxide consistently exhibited lowered regeneration scores in comparison to untreated Hydra with no pretreatment (Figure 5), which suggests oxidative stress disrupted stem cell function and tissue regeneration. In contrast, Hydra pretreated with 10μM of thymol prior to 1 mM hydrogen peroxide exposure demonstrated increased and improved regeneration under oxidative stress(Figure 4). Although these Hydra were still exposed to the same concentration of hydrogen peroxide, their regeneration scores were significantly higher. This suggests that thymol effectively preserved the regenerative capacity of Hydra even under excessive ROS concentrations. A Mann-Whitney U test comparing regeneration scores between the thymol + H_2O_2 group and the H_2O_2-only group revealed statistically significant differences at 24, 48, and 72 hours ($p < 0.01$) (Figures 4 and 7). Furthermore, the usage of a 10μM concentration of Thymol was found to be the best choice to promote regeneration without inducing toxicity. The concentration test results showed that higher thymol concentrations were associated with reduced regeneration and increased mortality, whereas 10 μM thymol maintained significantly improved regeneration outcomes compared to both higher concentrations and controls ($p < 0.01$) (Figures 3 and 6). Thymol is suggested to have reduced the oxidative damage during hydrogen peroxide exposure. The excessive accumulation of ROS can damage critical cell components such as DNA, proteins, membranes, etc. These are essential for cell division and differentiation to occur in the wound. Thymol scavenges ROS and limits oxidative damage; through its antioxidant properties, it may have helped to preserve stem cell viability and

efficiency in Hydra. Regeneration in *Hydra vulgaris*, as previously mentioned, is heavily dependent on specific signaling pathways such as the Wnt/β-catenin pathway (Chatterjee S & Sil PC, 2022). Oxidative stress may interfere with these pathways and impair proper wound regeneration. The addition of a thymol pretreatment, through its antioxidant properties, may have helped stabilize intracellular redox conditions and maintain the integrity of these signaling mechanisms. The increase in regeneration scores when exposed to Thymol suggests that it can play a vital role in restoring redox balance and in cellular regeneration as a whole. Overall, the data rejects the null hypothesis and supports the alternative hypothesis that thymol pretreatment at 10 μM significantly improves regeneration in *Hydra vulgaris* under oxidative stress conditions. These results are consistent with Haval et al. (2020), who demonstrated that 1mM H_2O_2 inhibited head and foot regeneration in Hydra by impairing the DNA repair mechanisms, and with Muscolo et al. (2024), who identified thymol's free-radical scavenging properties as a key mechanism for reducing oxidative cellular damage.

Evaluation

Experimental tests demonstrated that thymol improves regeneration under oxidative stress; several limitations and potential sources of error may have influenced the results. One of the biggest possible limitations was the qualitative nature of the study. It introduces subjectivity and bias without a clear set of guidelines to score on. Differences in interpretation can be problematic. Furthermore, despite attempts to standardize metabolic rates through a 7-day starvation period, Hydra regeneration could have been affected by size differences or the physiological state of individual Hydra. This added variability could have affected the mean regeneration scores. Additionally, thymol was dissolved in 0.1mL of 95% ethanol before dilution

into 1L of stock solutions. While this concentration is minimal, the absence of an ethanol-only control means its potential effect on regeneration cannot be entirely ruled out. Furthermore, regeneration scoring was not conducted blind to group assignment, meaning the observer knew which treatment each Hydra received during scoring. This introduces the possibility of observer bias influencing the qualitative regeneration scores. The sample size of 5 Hydra per dish also limits statistical power and may reduce the ability to detect small differences between each group.

Conclusion

The results shown by the experiment further strengthen the concept that Regeneration requires a tightly modulated balance in ROS. ROS bursts are crucial for regeneration, but also harmful at excessive amounts. Thymol is able to stabilize the unstable ROS and preserve homeostasis in the intracellular environment. It acts as a modulator by keeping ROS levels in check without eliminating the necessary ROS. The usage of hydra provides insight into the role oxidative stress plays in regeneration, tissue repair, and stem cells in more complex organisms due to the signaling pathways present being evolutionarily conserved. It helps contribute to a broader understanding of how redox reactions affect regeneration. Also shows how antioxidant based therapies can be developed to be applied and used in more complex organisms.

Future Work

Rather than using Regeneration as a measure of ROS levels, fluorescent oxidative stress markers could be utilized to directly quantify ROS levels. PCR(polymerase chain reaction) could also be used to quantify the expression of key signaling pathway genes, namely Wnt/β-catenin,

which would directly show the relation between ROS levels and antioxidant activity in the Hydra. This would further show and prove the protective effect of Thymol and how antioxidants play a role in preserving regeneration. Furthermore, it would be a quantitative measurement rather than qualitative, which would further support the results due to the lack of data collector bias or any other confounding factors during data collection. In addition to the fact that the Wnt/β-catenin pathway is found in humans, Hydra could be used as a pre-screening model for possible regenerative treatments due to the vast similarity between humans and Hydra regenerative processes.

These findings have broader implications for regenerative medicine as a whole. Thymol and other antioxidants have the potential to serve as therapeutic candidates for preserving cellular regeneration under stress conditions. Additionally, the use of *Hydra vulgaris* enables early-stage testing in an organism that shares conserved regenerative processes with humans, paving the way for future treatments.

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