The Effect of H_2O_2 on *Tetrahymena Thermophila* Population Growth Rates

Reeva Bhandal , Kasra Golchin

Abstract

Tetrahymena thermophila is a unicellular eukaryote that is commonly present in freshwater environments and has a relatively quick growth rate making it an ideal organism to utilize in laboratory settings. This study investigates the impact of hydrogen peroxide (H_2O_2) concentrations on *Tetrahymena thermophila* growth considering the higher H_2O_2 levels in freshwater compared to marine environments. Samples with H_2O_2 concentrations of 0mM, 0.1mM, and 0.3mM were prepared, and *Tetrahymena thermophila* growth was monitored by cell counting using a hemocytometer every three hours. Linear regression, one-way ANOVA, and Tukey-Kramer analysis were employed for data analysis. The study revealed a concentration-dependent decrease in *Tetrahymena thermophila* growth with increasing H_2O_2 concentrations. Significant differences were observed between the control (0mM) and 0.1mM ($p = 0.0334$), as well as 0.3mM concentrations ($p = 0.01$). However, no significant difference was found between the 0.1mM and 0.3mM concentrations ($p = 0.5611$), suggesting a potential threshold effect. The findings align with existing literature, indicating a concentration-dependent response in *Tetrahymena thermophila* growth. Our findings confirm our hypothesis that increasing hydrogen peroxide concentrations leads to a decrease in *Tetrahymena thermophila* growth rates, further research may be conducted to explore the complex cellular mechanisms affected by exposure to H_2O_2.

Introduction

Tetrahymena thermophila (*T. thermophila*) is a unicellular eukaryote called a ciliate that is present in aquatic environments (Orias et al., 2011). This protozoa is most commonly found in freshwater systems (i.e. rivers, streams and lakes) where they feed on bacteria and carry out a large variety of functions (Collins & Gorovsky, 2005). In comparison to other mammalian cells, *T. thermophila* is large in size ranging from 30 to 50 µm and it has a quick growth rate where the cells double every two to three hours under optimal conditions (Collins & Gorovsky, 2005; Ruehle et al., 2016). These characteristics make *T. thermophila* an ideal organism to investigate in a laboratory setting where they often live in a chemically defined media (Ruehle et al., 2016).

In natural water systems, hydrogen peroxide (H_2O_2) is produced and introduced through both biotic and abiotic pathways — it is found in freshwater systems at varying concentrations (Ndungu et al., 2019). Although H_2O_2 is a very stable species of reactive oxygen in natural waters and low concentrations are typically not harmful, higher concentrations can be toxic to aquatic life — including salmon species (Oluwatoyin-Sunday et al., 2020). Both *T. thermophila* and salmon live in freshwater environments for portions of their life and both feed on zooplankton thus connecting them in the food chain (Eggers, 1978). Identifying how H_2O_2 concentrations can impact *T. thermophila* can be used by future studies to identify how this can affect salmon species in this environment.

The objective of this research is to identify if hydrogen peroxide has an influence on the growth rate of *T. thermophila*. This investigation is important because *T. thermophila* and salmon coexist in the same freshwater environments where H_2O_2 concentrations have been found to be higher than marine environments (Ndungu et al., 2019). We hypothesize that if the concentration of hydrogen peroxide increases, then *T. thermophila* growth rate will decrease. Previous research has shown that increasing H_2O_2 concentration from 0mM to 0.5mM inflicts great damage on *T. thermophila* DNA making it almost unrecognizable (Lah et al., 2004). Additionally, a concentration of 1mM H_2O_2 completely inhibited *T. thermophila* growth leading us to test the following concentrations: 0mM, 0.1mM and 0.3mM (Errafiy et al., 2013). We decided to incubate the cells at 30°C as this temperature is within the range of conditions optimal for *T. thermophila* growth (Laakso et al., 2003). Thus, we predict that as the concentration of H_2O_2 increases from 0mM to 0.3mM, the growth rate of *T. thermophila* will be significantly impacted.

Methods

Preparation of Samples

The *T. thermophila* medium was set up by the lab technician the day before inoculating cultures. After receiving the stock solution of *T. thermophila*, an initial cell count was conducted to find the initial concentration. Our stock concentration was just below the recommended concentration to begin lab growth rate experiments so we did not dilute the stock solution of *T. thermophila* to obtain a working solution. If this was not the case and the concentration was above the recommended value, then it would have been diluted with a medium to reach the required concentration.

Hydrogen peroxide was diluted into concentrations of 0.1mM and 0.3mM with standard medium using micropipettes and added into their corresponding test tubes. The 0.3mM treatment tubes contained 2mL of 0.6mM H_2O_2 medium and 2mL of *T. thermophila* stock solution. The 0.1mM treatment tubes contained 0.65mL of 0.6mM H_2O_2 medium, 1.35mL of standard medium, and 2mL of *T. thermophila* stock solution. The control of 0mM H_2O_2 contained 2mL of standard medium and 2mL of *T. thermophila* stock solution. Each treatment level was replicated three times. After adding everything to the test tubes, they were moved to the 30°C incubator to allow the cells to grow. A flame was used to sterlize when working with the *T. thermophila* stock solution.

Counting the Cells

The cells were counted across four days and every three hours, giving us the following samples with the unit of time (t) being hours: t = 0, 3, 6, 16.5, 19.5, 22.5, 40.5, 44.5, 66. Counting was stopped once the stationary phase was reached. For each sample, 100µl of *T. thermophila* culture from the test tubes and 10µl of fixative was pipetted into the

labelled counting tubes (Eppendorf tubes) to prevent the cells from growing further. A flame was used to sterlize when working with the *T. thermophila* cultures.

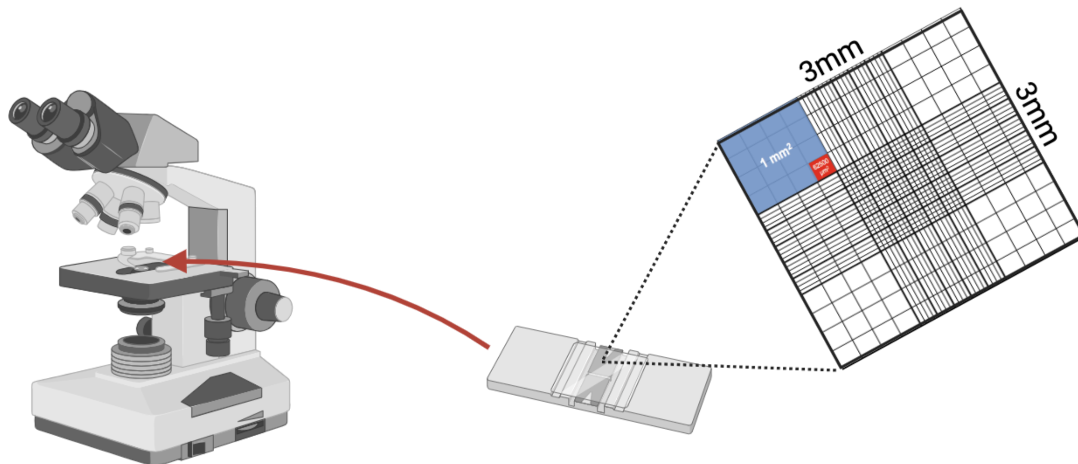

Figure 1: Depiction of hemocytometer grid under the microscope showing both the 1mm x 1mm and the 3mm x 3mm squares. Visual created with Biorender.com.

A hemocytometer was used under a microscope to count the cells; cells touching the top and left borders were counted, cells touching the bottom and right borders were not counted. Samples were mixed with a micropipette before pipetting 20µl of the sample and discharging it under the coverslip of the hemocytometer. For the initial cell count and t = 3, 6 samples, the largest square - 3mm x 3mm - was counted to obtain the initial concentration. For the rest of the samples, the 1mm x 1mm medium sized squares were counted until 100 plus cells were reached on the counter. Between counting different samples, the hemocytometers were sprayed with ethanol and wiped with a kimwipe. Each sample was counted twice, then we proceeded to find the average between both counts, this was the number used for the calculations to find the concentration of cells. Calculations to identify cells/mL concentration were computed and recorded in a table immediately after counting the sample of cells.

Calculation

To analyze the effects of different H_2O_2 concentrations (0 mM, 0.1mM, 0.3mM) on the growth rate of *T. thermophila*, the concentration of cells for each trial needed to be calculated. To calculate the concentration of cells the following calculation was used when counting 1mm x 1mm sized squares:

$$\frac{number\ of\ cells\ counted}{number\ of\ boxes}(5 \times 10^3)(1.1)$$

Data analysis

After a complete dataset was collected, a linear regression was performed for each replicated to obtain an slope. The resulting slopes were then categorized according to the levels of treatment, and a one-way ANOVA test was conducted to generate a p-value using Graphpad Prism. Subsequently, a Tukey-Kramer analysis was performed to identify significant differences between each treatment level. Furthermore, the slopes obtained from the linear regression for each replication were grouped by treatment level, and a scatter plot was generated to visually represent the data.

Results

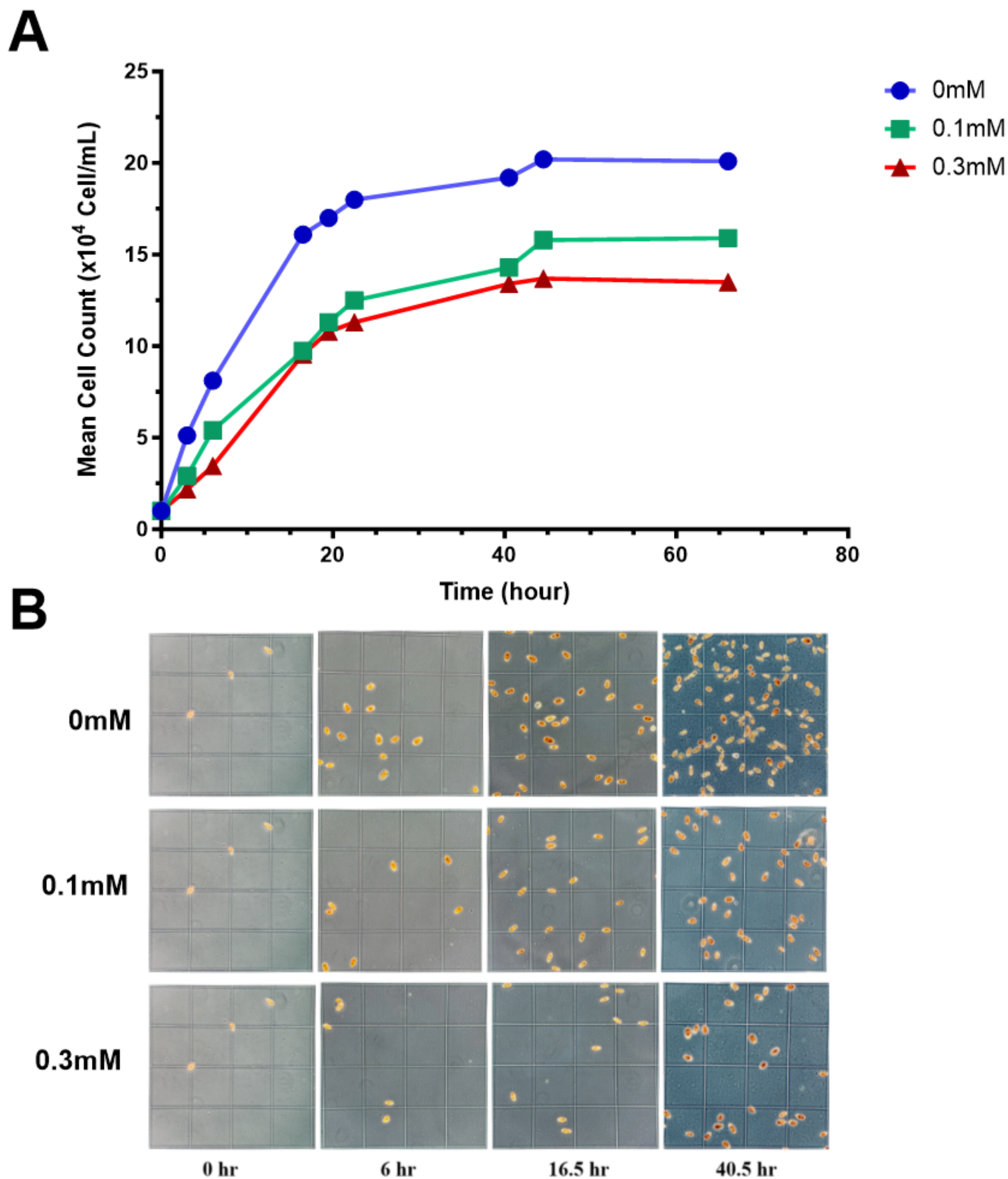

Figure 2: **A)** Mean cell counts plotted over a period of time (hr) for 3 different concentrations of hydrogen peroxide (0 mM, 0.1mM, 0.3mM). **B)** Light microscopy image of hemocytometer taken at different time intervals. Each row is an indication of different hydrogen peroxide concentration (0mM , 0.1mM, 0.3mM) used to incubate *T. thermophila*. Each oval shaped unit in the images counts for a singular organism.

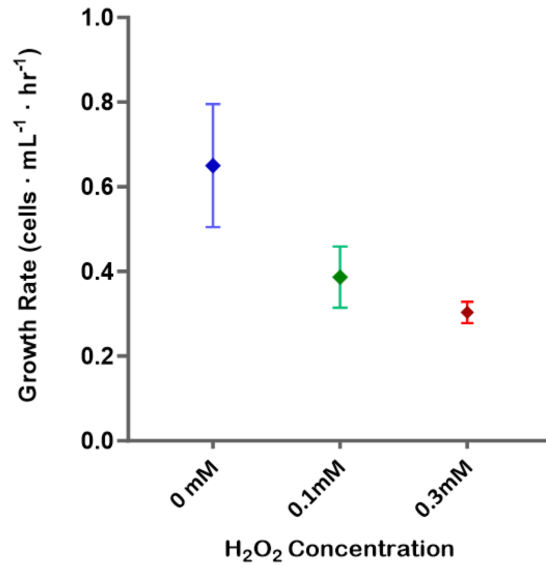

Figure 3: Scatter plot graph of growth rate over 3 different H_2O_2 concentrations. The mean of each H_2O_2 treatment is shown by rhombus. One way ANOVA test revealed a p-value of 0.001 ($p<0.05$) among the means and Tukey-Kramer test revealed a p-value of 0.0334 among 0mM-0.1mM, p-value of 0.01 among 0mM-0.3mM, and p-value of 0.5611 among 0.1mM-0.3mM as well as r^2=0.7846.

Quantification analysis in Figure 2 panel A highlights that *T. thermophila* was found to have a slower growth under H_2O_2 condition and it revealed that there is reduction in cell growth as the H_2O_2 concentration increases. This outcome can be seen visually in the panel B. In figure 3, a one-way ANOVA was conducted on the slopes obtained from the linear regression analysis of each treatment group, revealing a statistically significant difference among the means of the concentration ($p = 0.001$, n=9 biological replicates). The coefficient of determination (r^2) was calculated to be 0.7846, indicating a high degree of correlation between the concentrations of H_2O_2 and the observed effects on *T. thermophila*. Subsequent Tukey's multiple comparisons test was performed to distinguish any specific differences between each individual concentration using p-values . The results showed a significant difference in the growth rates between the 0mM and 0.1mM concentrations ($p = 0.0334$) as well as between the 0mM and 0.3mM concentrations ($p = 0.01$). However, there was no significant difference found between the 0.1mM and 0.3mM concentrations ($p = 0.5611$).

Discussion

In this study, our primary aim was to investigate the influence of H_2O_2 concentrations on the growth rate of *T. thermophila*. Given the results of the one-way ANOVA and Tukey's multiple comparisons test (Figure 3), we can confidently assert that the growth rate of *T. thermophila* is significantly affected by different concentrations of H_2O_2. Tukey test performed on the mean growth rates suggests that exposure to 0.1mM and 0.3mM concentrations of H_2O_2 led to significant reduction in *T. thermophila* growth when compared to the control group (0 mM). This outcome was proven as the p-values obtained were significantly low ($p<0.05$). However, no significant difference was found between the 0.1mM and 0.3mM concentrations ($p = 0.5611$) which possibly indicates a threshold effect where the impact on *T. thermophila* growth diminishes beyond a certain concentration. In addition, our predictions were substantiated as the data in Figure 2 demonstrated a notable decrease in growth rate with increasing H_2O_2 concentrations as the quantification analysis revealed a clear trend of slower growth as the concentration of H_2O_2 increased.

In comparing our results to existing literature, our study demonstrates a concentration-dependent response in *T. thermophila* growth, aligning with previous research such as Lah et al. (2004), which observed substantial DNA damage in *T. thermophila* at a concentration of 0.5mM H2O2. While Lah et al. focused on higher concentrations, our decision to explore the range up to 0.3mM is consistent with their findings. Furthermore, Derdak et al.'s (2022) exploration of H_2O_2 concentrations (0.1mM to 1.0mM) mirrors our findings, reinforcing a decrease in *T. thermophila* cell count with increasing H_2O_2 concentration. Additionally, our results align with Errafiy et al.'s (2013) observations, revealing significant differences between control (0mM) and experimental groups (0.1mM

and 0.3mM). This consistent pattern across studies enhances the reliability and validity of our conclusion regarding hydrogen peroxide's inhibitory effect on *T. thermophila* growth.

The absence of a significant difference between the growth rates of 0.1mM and 0.3mM concentrations seen in Figure 3 suggests the idea of a specific limit or a potential threshold effect. Beyond a certain concentration, additional H_2O_2 may not exert a proportional impact on *T. thermophila* growth. This finding adds depth to our understanding of the dose-response relationship, emphasizing the need for future studies to explore concentration thresholds in more detail. Although our examination of growth rate offers a comprehensive perspective, it does not explore the complex cellular mechanisms affected by varying concentrations of H_2O_2. To achieve a more thorough understanding of *T. thermophila's* reaction to oxidative stress, future research should explore these specific cellular processes.

Conclusion

In summary, our data aligns with our prediction that as hydrogen peroxide increases, the growth rate of *Tetrahymena thermophila* decreases. Significant differences between control (0mM) and experimental groups (0.1mM and 0.3mM) imply H_2O_2 inhibits growth, while the lack of difference between 0.1mM and 0.3mM suggests a potential threshold effect. Further research should be undertaken to pinpoint the specific concentration threshold and unravel the underlying molecular mechanisms governing Tetrahymena's response to oxidative stress.

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