

# The effect of caffeine on the regeneration of Brown Planaria (*Dugesia tigrina*)

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## SUMMARY

Planaria are considered the most primitive form of cephalized animal with similarity to vertebrate nervous systems. They possess nearly every neurotransmitter present in most mammals, including dopamine. Planaria are known for their extraordinary ability to regenerate from a small tissue fragment, making planaria an ideal model to study the nervous system. Planaria have pluripotent somatic stem cells known as neoblasts. Following amputation, neoblasts migrate to the wound site. They then give rise to a mass of new tissue called the blastema. Cells in the blastema differentiate over a period of several days to replace missing body structures. Previous research compared scoring methods of neurotoxicity on the neurological, locomotive, and morphological functions of planarian. Higher levels of dopamine have been found in regenerating planaria, indicating that dopamine may have a role in regeneration. Caffeine enhances dopamine signaling in the brain. Therefore, this study aimed to explore the effect of caffeine on the regeneration rate of planaria. In this study, twenty-one planaria were exposed to two concentrations of caffeine. The heads were amputated, and regeneration was recorded by digital photography. The study showed that the highest dosage of caffeine accelerated the regeneration rate of the planaria in comparison to the lower dosage and control. The planaria treated with the lower caffeine dosage also regenerated in less time than the control specimen. This study is evidence that a high dose of caffeine accelerates planaria regeneration and implicates caffeine as a possible treatment to stimulate the regeneration process.

## INTRODUCTION

Degeneration, or death of nerve cells, results in movement and mental functioning problems. These symptoms, commonly observed in Parkinson's patients, are a result of the dopaminergic neuron death in the substantia nigra region of the brain. As the disease progresses, motor skills and mental acuity decline. The National Parkinson Foundation estimates that over 10 million people worldwide suffer from Parkinson's disease. With 60,000 people diagnosed annually in the United States, it is estimated that by 2020 more than 1 million Americans will be living with Parkinson's disease (1). It is generally accepted by the scientific community that neurogenesis is evident in many areas of the brain, including the hippocampus (2). However, the ability to regenerate neurons

varies amongst species and diminishes most significantly in the human brain as it matures. Given the hippocampus and striatum govern long-term memory and cognitive skills, respectively, evidence of the ability to regenerate nerve cells, although limited, in these regions is a promising area for continued research that may benefit patients suffering from neurodegenerative diseases (2).

Since research suggests that neurons in humans may regenerate, the use of models with regeneration capabilities may be useful for the study of neurodegenerative diseases like Parkinson's. The central nervous system of planaria consists of a bi-lobed brain connected to a pair of nerve cords that extend ventrally from the head (3) and has extensive regeneration capabilities. In fact, all parts of the planarian can regenerate, even from a fragment as small as 1/279th of the organism's original size (4). Regeneration in planaria is caused by pluripotent stem cells distributed throughout the body, which differentiate into all cell types (4). High levels of dopamine have been extracted from regenerating planarian, suggesting that dopamine has a role in the regeneration process (5). In addition, planaria share with vertebrates all the major developmental signaling pathways of cells (4). Regions of the planarian genome have been identified as having significant similarity to human disease-related genes (3). These commonalities, along with ease of maintenance and cost-effectiveness of husbandry, make planaria an excellent test model. By studying the regeneration process in this system, we can learn how to positively impact neurogenesis.

Caffeine is an alkaloid compound commonly found in coffee, tea, and cacao and is used medicinally as a stimulant and diuretic. Caffeine enhances dopamine signaling in the brain by antagonizing adenosine receptors (6). Studies of planaria treated with caffeine solutions have had varying results. In a study at East Tennessee State University, researchers found that levels of caffeine greater than 0.01 M were toxic to planaria after three days of exposure to the treatment (7). Measures of survivorship in this study also showed that after three days, 60% of the planaria died in the 1000  $\mu$ M solution, while 50% died in the 100  $\mu$ M solution (7). All planaria survived at a caffeine concentration of 10  $\mu$ M (7). Another study suggested that a 10  $\mu$ M caffeine treatment accelerates the stages of regeneration starting with blastema development, growth, and differentiation when compared to a control treatment of spring water (8). Because planaria react to their environmental conditions using chemical, mechanical, and light sensory neurons, data can be collected on these cells to assess the effect of treatments.

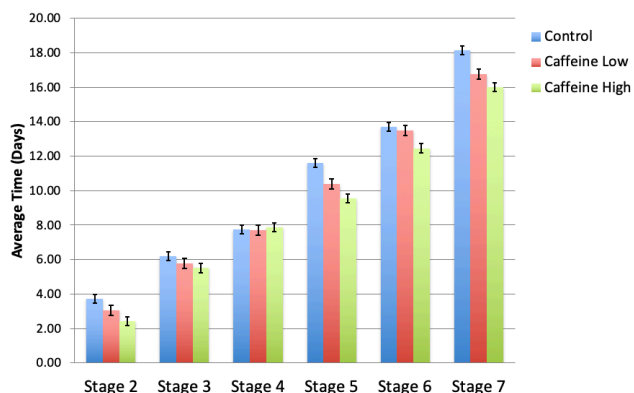
The goal of this research was to determine the ability of caffeine to accelerate the regeneration of pluripotent planarian cells. Considering dopamine has been found in high levels during planaria regeneration and caffeine enhances dopamine signaling, the hypothesis is that the caffeine treatment will decrease the time for planaria to regenerate after amputation. For the purpose of this study, amputation was performed immediately below the head to separate the bi-lobed brain from the ventral nerve cord. The number of days from amputation to full regeneration was assessed through visual inspection. The purpose of the research is to investigate the usage of caffeine as a treatment to stimulate regeneration.

## RESULTS

We measured the time to full regeneration of amputated planarian in three experimental groups: control, low caffeine dose (30  $\mu\text{M}$ ) and high caffeine dose (60  $\mu\text{M}$ ). To measure the rate of regeneration, the head of each planarian was amputated to separate the bi-lobed brain from the nerve cord that extends ventrally from the head. Every 24 hours, a photo was taken of each planarian, the photo was analyzed, and the stage of regeneration was recorded. The elapsed time (in days) required for each specimen to reach each stage of regeneration was recorded. The average elapsed time (in days) was calculated for each stage for each experimental group (Figure 1).

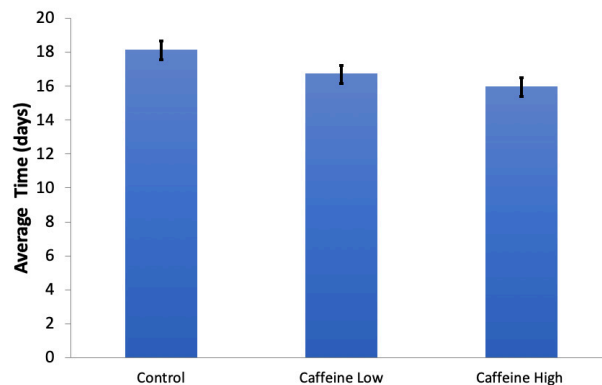
The results indicate the high caffeine treatment accelerated the time (16.00 days) to reach full regeneration in comparison to the low caffeine (16.74 days) and control (18.15 days) groups. The high caffeine group accelerated the time to reach all regeneration stages, except stage 4. Planaria treated with low caffeine reached all regeneration stages in less time than control specimens

The average time to full regeneration for each experimen-



**Figure 1. Average time to reach each regeneration stage by experimental group.**

Each planarian was inspected to determine the stage of regeneration. The average for each experimental group by stage was calculated and the standard error determined. The high caffeine dose accelerated all regeneration stages except stage 4. The low caffeine dose accelerated the regeneration in stages when compared to the control group.



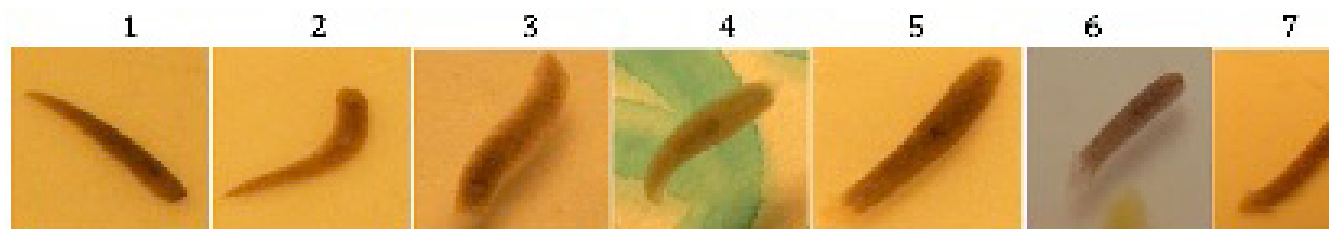
**Figure 2. Average time to reach full regeneration by experimental group.** Each planarian was inspected to determine the final stage of regeneration, stage 7. The planaria in the high caffeine group reached stage seven in the shortest period of time of 16.0 days  $\pm$  1.0699 (60  $\mu\text{M}$ ;  $p=0.0225$ , one-way ANOVA); low caffeine in 16.74 days  $\pm$  1.3495 (30  $\mu\text{M}$ ;  $p=0.1894$ , one-way ANOVA), and control in 18.15 days  $\pm$  1.549.

tal group was calculated by taking the duration in days from amputation to the first observation that a planarian reached stage 7. Planaria in the high caffeine group regenerated more quickly than planaria in the control group (Figure 2). The average regeneration time for the high caffeine group was 16 days, 2.15 days shorter than the control time of 18.15 days. This reflects an 11.8% increase in elapsed regeneration time ( $p=0.0225$ ). No difference was found in the average regeneration time between the control and low caffeine treatment groups ( $p=0.1804$ ). The difference in regeneration time between time high caffeine and low caffeine groups was 0.74 days, or 4.4% increase in elapsed regeneration time. This difference was not statistically significant ( $p=0.6777$ ).

As studies have shown, caffeine may have a positive or negative effect on planaria depending on dosage. Previous studies indicated fatalities at a dosage level of 0.01 M. This study compared the dosage of 30  $\mu\text{M}$  to 60  $\mu\text{M}$  of caffeine and found that the higher dosage accelerated planaria regeneration without fatalities.

## DISCUSSION

The results support the hypothesis that a caffeine treatment stimulates regeneration by decreasing the time required for planaria to regenerate following amputation below the bi-lobed brain. Each planarian was amputated below the head and visually inspected to determine that the amputation was completed within the guidelines. The regeneration stage was assessed based on the presence of developmental milestones for each stage of regeneration, as documented in the material and methods section. Based on the average time it took each planarian to reach stage 7 (full regeneration), Planaria in the low caffeine group (30  $\mu\text{M}$ ;  $p=0.1804$ ) did not regenerate more quickly than those in the control group. However, the planaria treated with a high dose of caffeine (60  $\mu\text{M}$ ;  $p=0.0225$ ) regenerated in 11.8% less time and 2.14 fewer days as compared to the control group. As planaria regene-



**Figure 3. Planaria regeneration score reference.** Representative images of planaria taken in an elongated position every 24 hours in macro mode.

rate completely in 16-18 days, this represents a substantial increase in regeneration rate. These findings also suggest that regeneration in planaria was affected by the caffeine dosage, as only the higher dosage (60  $\mu\text{M}$ ) of caffeine had caused a significant increase in the rate of planaria regeneration.

Caffeine, a widely consumed psychoactive substance, effects the nervous system; it enhances dopamine signaling by slowing down dopamine reabsorption in the human brain. Previous studies support that planaria have dopaminergic receptors in their nervous system, and high levels of dopamine have been extracted from regenerating planaria. (9) Planaria absorb chemicals such as caffeine by epithelial diffusion or intake the chemicals through their pharynx. This has been demonstrated in pharmaceutical toxicology testing utilizing spectrophotometry (10). Caffeine is metabolically active in the planarian, as it has been found to increase planarian motility in a concentration-dependent manner, a behavioral effect consistent with findings in vertebrates (6).

Planaria maintain a large population of pluripotent stem cells. This study suggests that caffeine accelerates the regeneration and differentiation of these pluripotent cells into neural cells. Stem cell therapy for patients suffering from Parkinson's disease involves injecting stem cells directing into the basal ganglia, in hopes that these cells will differentiate into dopaminergic neurons. Caffeine may promote cell regeneration and neuronal differentiation in that context, as well, potentially improving therapeutic outcomes for patients with neurodegenerative disease.

### MATERIALS AND METHODS

Brown Planaria (*Dugesia tigrina*), a common freshwater representative of the phylum Platyhelminthes, was used in this experiment. The planaria were obtained from a commercial source, Carolina Biological Supplies (n=21). Kaffn8 liquid caffeine was used as the caffeine source. Three temporary holding containers were filled with spring water (Poland Spring Water) and seven planaria were assigned to each holding container. One gram of egg yolk was placed into each holding container as food. After 24 hours, residual egg yolk was removed.

The experiment began with creating two caffeine concentration solutions (30  $\mu\text{M}$  and 60  $\mu\text{M}$ ). Petri dishes (90 x 15 mm) were labeled to identify the treatment, and each petri dish was filled with 15 mL of the treatment per their label. Each planarian was transferred from the holding container to

a petri dish filled with spring water tracked with a unique identifier (labeled with numbers 1-21). A baseline photo was taken of each planarian using a digital camera on macro mode. After all photos were taken, each planarian was randomly assigned to a treatment group and placed into the associated treatment petri dish. The unique tracking number and treatment association was recorded. After 24 hours, each planarian was amputated by cutting the planarian slightly below its head with an X-Acto knife as it stretched to move. Post-amputation, each planarian was inspected to validate that the amputation was performed immediately below the head. The residual planarian (body without head) was placed back in the petri dish. The head section was placed in the surplus stock holding container (one for each environment).

To track the regeneration stages, a photo of the planarian in an elongated position was taken every 24 hours with a digital camera in macro mode (Figure 3). The photo was visually inspected, and the stage of development was recorded as follows: 1. Pre-wound closure - no visible regrowth to fill gap from wound closure, 2. Wound closure - cut has flattened and started to regrow, 3. Pattern formation - wound closed and round, formation visible, 4. Head translucent, but visible, 5. Head still mostly translucent, shape starting to peak, early photoreceptors now visible, 6. Head and photoreceptors visible, but still more translucent than rest of body, 7. Head fully peaked, photoreceptors visible, head color same as the remainder of body

The visual inspection was not blinded given the naming convention. However, the model reference stage pictures minimized potential bias.

The planaria were fed every seven days with one gram of cooked egg yolk. Any excess yolk was removed from each petri dish after two hours. At the conclusion of the experiment, the planaria had the opportunity to live out their natural lifespan.

The data was analyzed utilizing the statistical functions of Excel. The one-way ANOVA comparisons test was performed using GraphPad Prism (GraphPad Software, La Jolla California USA).

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