Scientific Research Publishing

Open Journal of Regenerative Medicine, 2023, 12, 125-132
https://www.scirp.org/journal/ojrm
ISSN Online: 2169-2521
ISSN Print: 2169-2513

# The Effect of Increasing mTOR Signaling on the Speed of Regeneration in *D. dorotocephala planaria*

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**How to cite this paper:** Danish, A. (2023) The Effect of Increasing mTOR Signaling on the Speed of Regeneration in *D. dorotocephala planaria*. *Open Journal of Regenerative Medicine*, **12**, 125-132.
https://doi.org/10.4236/ojrm.2023.123009

**Received:** June 5, 2023
**Accepted:** September 4, 2023
**Published:** September 7, 2023



## Abstract

Muscle strains are a common injury that can occur during physical activity or exercise, and they can range from mild to severe depending on the extent of the damage. The mTOR pathway is a highly conserved signaling pathway that has been implicated in various cellular processes, including tissue regeneration. Previous studies that have investigated protein synthesis in mice have concluded that the mTOR pathway can improve muscle regeneration. However, the specific effects of mTOR pathway activation on muscle regeneration in planarians have yet to be fully explored. Therefore, this study aimed to investigate the use of Arginine and Leucine to stimulate the mTOR pathway for muscle strains in planarians. During the experiment, planarians were amputated, and different dosages of Arginine and Leucine were used to stimulate the mTOR pathway in *Dugesia dorotocephala*. The speed of muscle regeneration was measured over 14 days in five groups, with thirty planarians in each group. The results showed that the rate of regeneration from 0.134 mM Leucine solution showed a significant increase compared to the control group, while the groups exposed to 0.1, 0.3 mM Arginine, and 0.0134 Leucine did not show significant changes in muscle regeneration. These findings suggest that mTOR pathway activation may enhance muscle regeneration in planarians and that the effects may be dose-dependent. These findings have important implications for developing new treatments for tissue damage. Further studies are needed to fully understand the mechanisms of mTOR pathway activation on muscle regeneration in planarians and its potential use in tissue engineering and regenerative medicine.

## Keywords

Muscle Regeneration, Muscle Strains, mTOR Pathway, Leucine, Arginine

## 1. Introduction

A muscle strain is a common type of injury that occurs when muscle fibers are torn, resulting in a range of symptoms such as swelling, bruising, weakness, and pain. This type of injury is particularly prevalent in sports, where sudden movements or excessive strain can cause damage to the muscles. Muscle strains can vary in severity, and severe cases may require extended periods of rest and rehabilitation before normal function is restored. Despite the prevalence of this injury, there are currently limited treatment options available, with the RICE method (Rest, Ice, Compression, Elevation) being the most commonly used approach. However, the slow and lengthy recovery process associated with muscle strains has prompted ongoing research to identify new and effective treatments that can accelerate healing and reduce the risk of recurrence.

Muscle strains can be caused as a result of over-exertion, sudden movements, or excessive force applied to the muscle. Naturally, when a muscle strain or tear occurs, the muscle fibers that make up the tissue must regenerate. Regeneration usually occurs 4 - 5 days post-injury. Muscle regeneration comprises five main stages, necrosis of the injured muscle cell, activation and proliferation of muscle stem cells, differentiation of the stem cells, maturation of the newly formed muscle fibers, and finally, the remodeling of muscle fibers [1].

A leading method in muscle strain regeneration is looking into many different signaling pathways attributed to the regulation of muscle regeneration, the most prominent is the mTOR (mammalian target of rapamycin) pathway. The mTOR pathway plays a crucial role in muscle regeneration as it is directly involved in stem-cell activation, cell proliferation, cell differentiation, maturation, and muscle fiber fusion to regulate protein synthesis and cell growth [2].

Previous studies involving mTOR on muscle regeneration used various methods to test for tetanic strength, muscle fiber size, and quality of regeneration.

One study was done on the mTOR pathway in mice, in which they deleted the mTOR gene has shown that the disruption of the mTOR pathway inhibits embryonic development and protein synthesis [2]. Thus, they concluded that the mTOR pathway is directly related to multiple stages of muscle regeneration. In another study, researchers investigated the effects of Leucine on damaged muscle in Wistar rats [3]. They used Cryo-lesion to damage soleus muscle tissue and measure Leucine's effects on the soleus's recovery. To measure the effects of Leucine on muscle regeneration, researchers completed tests for maximum tetanic strength pre- and post-workout.

In contrast to previous studies that tested the mTOR pathway for muscle function, size, and quality of regeneration, this particular study dives into the effects of the mTOR pathway on the speed of regeneration.

Additionally, the researchers measured muscle fiber size after Leucine supplementation. Based on the results of these tests, researchers were able to conclude that Leucine could increase cell proliferation and reduce protein ubiquitination, improving muscle size and function.

Several upstream signals, such as growth factors, nutrients, and amino acids, induce the mTOR pathway. In response to the upstream signal, the mTOR pathway is activated by a protein complex called mTOR Complex 1 (mTORC1) or mTOR Complex 2 (mTORC2). When active, the mTOR pathway triggers a signaling cascade leading to the stimulation of downstream targets such as ribosomal protein S6 kinase (S6K) and eukaryotic initiation factor 4E-binding protein (4E-BP). These compounds promote protein synthesis by facilitating the translation of mRNA into proteins.

In this particular experiment, two amino acids, L-leucine and L-arginine, were used to activate the mTOR pathway. Leucine is an essential amino acid that plays a critical role in protein synthesis and has been shown to be highly effective at stimulating the mTOR pathway [4]. When Leucine is present in sufficient amounts, it binds to a protein called Sestrin2, activating mTORC1.

Similarly, Arginine is a semi-essential amino acid that can also promote the mTOR pathway indirectly through the production of nitric oxide (NO), which activates mTORC1. Leucine and Arginine are commonly found in protein-rich foods, such as meat, fish, and dairy products, and their consumption can lead to increased mTOR activation.

To carry out this experiment, Leucine and Arginine were supplemented in the model organism *Dugesia dorotocephala*. Planarians are flatworms that are known for their remarkable regenerative capabilities. Despite a few key differences, a planarian's musculature consists of a complex organization of muscle fibers and an abundance of neoblasts (stem cells), similar to humans that enable them to regenerate their entire body in just 1 - 2 weeks. Many stages in planarian muscle regeneration are comparable to human muscle regeneration, such as stem cell activation, cell proliferation, and differentiation. Planarians are particularly relevant to this experiment because their first response to wounding results in the activation of the mTOR pathway, just like in any mammal species. The addition of Leucine and Arginine to the planarians' diet is expected to further stimulate the mTOR pathway, leading to increased cell growth and proliferation and potentially enhancing the regenerative capabilities of the planarians. Using a planarian as a model organism, researchers can study the effects of these amino acids on the mTOR pathway and the regenerative response in a living organism.

The rate of regeneration (growth in millimeters over time) over twenty-one days for each group was compared to one another to determine the most effective treatment. The results of this experiment provided a valuable perspective on whether further stimulating the mTOR pathway has a positive effect on the rate of muscle regeneration.

## 2. Hypotheses

$H_0$: There is no difference between the mean rate of regeneration of planaria when the mTOR pathway is accelerated and planaria when the mTOR pathway is not accelerated.

$H_a$: The rate of regeneration of planaria when the mTOR pathway is accelerated will be greater than that of planaria when the mTOR pathway is not accelerated.

## 3. Methods

The entire experiment lasted 34 days. Before the experiment, 2000 ml stock solutions of 0.4 mM L-arginine, 2.5 mM L-arginine, 0.134 mM L-leucine, and 0.0134 mM L-leucine were made in spring water as treatments. Upon arrival on Day 1, all planarians were placed into a bowl with spring water. Then, all planarians were fed bits of egg yolk, and the water was changed 1 - 2 hours later. To change the water, a separate bowl of spring water was created, and all planarians were transferred using tweezers or pipettes.

From Days 2 - 6, all planarians lived in their typical environment (spring water). The water was changed multiple times, and each group was fed once more. Planarians were starved from Day 8 to the end of the experiment to remove any interference with the treatments.

To ensure that each experimental treatment was safe for planarians to live in, each concentration was tested using extra planarians that were not a part of the experiment. New Arginine concentrations were altered and tested at 0.1- and 0.3-mM Arginine due to significant fatalities in the initial concentrations. Both 0.134 mM and 0.0134 mM solutions of L-leucine were deemed viable because all planarians survived. Additionally, the remaining planarians were used for practice amputating and measuring between Days 1 - 12.

On Day 13, the actual experiment began. One hundred fifty planarians were amputated and randomly divided into five groups. Planarians were placed in 5 mL of water. Then, using a scalpel, two cuts perpendicular to the A/P axis were made: the first cut was made between the eyes and the pharynx, and the second cut was between the pharynx and the tip of the tail. The head, trunk, and tail were obtained, and both the head and tail were discarded. Also, the blade was cleaned every 3 - 4 cuts to wipe planarian mucus off (Accorsi *et al.*, 2017).

There were four experimental groups; planarians were supplemented with either 0.134 m Leucine, 0.0134 m Leucine, 0.3 m Arginine, or 0.1 m Arginine. Environmental conditions were kept the same in all groups. Water changes and feedings happened on the same day to reduce the effects of possible confounding variables.

After amputations were made, 30 planarians were placed into each experimental group (10 planarians per standard-sized petri dish). They were kept in a cabinet to minimize light exposure. The water was changed 2 - 3 times weekly for three weeks post-amputation (Days 16, 19, 23, 25, 30, and 32).

Observation dates took place directly after amputation on Day 0 (post- amputation), halfway through the process on Day 7 (post-amputation), and the end of regeneration on Day 14 (post-amputation). Each planarian was placed onto the ice block to measure the regeneration rate, and measurements of the length in

millimeters were recorded three times using a metric ruler. The average length was then calculated for each separate group on each observation date (Days 14, 20, 27, and 34 of the total experiment).

An ANOVA test was completed directly after amputations to determine whether the mean lengths of planarians in each group differed. The null hypothesis was that there was no difference in the average lengths of planarians on Day 0 post amputation, where the p-value was 0.212. Even though there must have been slight differences in the lengths of planarians in each group post-amputation, because they were deemed statistically not different, the mean length of planarians at the end of the experiment could represent the growth over time and, therefore, the rate of regeneration.

## 4. Results

This experiment compared the regeneration of planarians with and without treatment. To support the hypothesis that stimulating the mTOR pathway with Leucine and Arginine enhances regeneration speed, a 2-sample t-test was conducted. It compared the mean lengths of the experimental groups to the control group on Day 14. The null hypothesis stated that there is no difference in mean length between planarians in the treatment and the control group. The alternate hypothesis stated that the treated planarians would have a greater mean length. A p-value less than 0.05 would reject the null hypothesis. The results were summarized in Figure 1. Only the 0.134 mM Leucine group showed a statistically significant difference compared to the control group.

Furthermore, a similar test compared Day 0 and Day 7, with the alternate hypothesis stating that the mean length of planarians in a specific group on Day 7 would be less than on Day 0. Figure 2 presented each group's p-values and statistical significance. Only the control group had a statistically significant difference, rejecting the null hypothesis. During the regeneration process from Day 0 to Day 7, planarians in the control group shrunk as they formed a blastema. However, the groups supplemented with Leucine or Arginine were able to retain their size and continue to grow.

To display the growth of planarians in each group over time, and scatterplot graph was created, Average Planarian Length vs. Time Post-Amputation (Figure 3).

Finally, observations were intended on Day 21, but insufficient planarians survived in the experimental groups beyond the 14-day regeneration period to obtain measurements.

Figure 1 shows the p-values of a 2-sample t-test comparing the mean length of planarians of the experimental groups with the control group on Day 14.

Figure 2 shows the p-values of a 2-sample t-test comparing the mean length of planarians on Day 0 versus Day 7.

Figure 3 is a scatterplot graph displaying the Average Planarian Length vs. Time Post-Amputation. By Day 14, there was a noticeable difference between

| Group | p-value | Statistically Significant |
|---|---|---|
| Arginine 0.1 mM | 0.8307 | No |
| Arginine 0.3 mM | 0.748 | No |
| Leucine 0.134 mM | 0.00031 | Yes |
| Leucine 0.0134 mM | 0.32 | No |

**Figure 1.** Mean length of planarians on Day 14 vs. control.

| Group | p-value | Statistically |
|---|---|---|
| Control | 0.0013 | Yes |
| Arginine 0.1 mM | 0.4631 | No |
| Arginine 0.3 mM | 0.3041 | No |
| Leucine 0.134 mM | 0.1638 | No |
| Leucine 0.0134 mM | 0.505 | No |

**Figure 2.** Mean length of planarians of each group on Day 7 vs. Day 0.

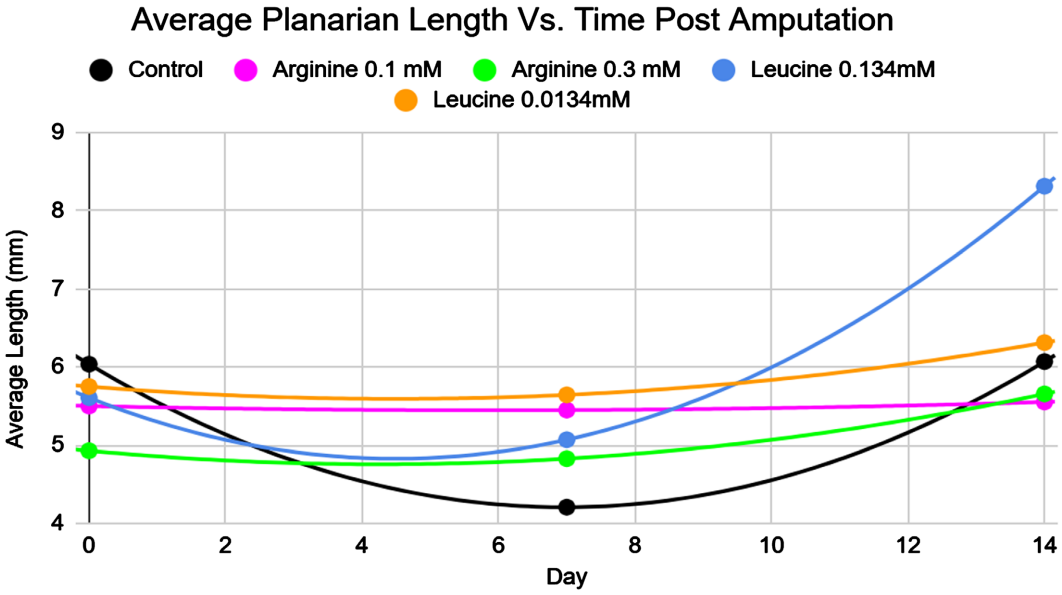


**Figure 3.** Average planarian length vs. time post-amputation.

the 0.134 mM Leucine group and the others.

## 5. Discussion

The present study aimed to investigate the effect of mTOR pathway activation on the speed of muscle regeneration in *Dugesia dorotocephala* planarians. The results demonstrated that the regeneration rate was significantly increased in planarians supplemented with a 0.134 mM Leucine solution compared to the control group. However, the groups exposed to 0.1 mM and 0.3 mM Arginine and 0.0134 mM Leucine did not show significant changes in muscle regeneration.

The results of this experiment pose a challenge as to why the other experimental groups do not significantly affect the speed of regeneration. Since the low concentration of Leucine (0.0134 mM) is ten times less than the high concentration, it is suspected that the dosage is too low to increase mTOR activation. On the other hand, as mentioned previously, Arginine is a known stimulant of the mTOR pathway, and higher concentrations of Arginine are deemed unsafe for planarians. While the reason for Arginine's ineffectiveness is unclear, a possible explanation is that Arginine and Leucine induce mTOR activity through two different pathways. Arginine activates the mTOR pathway through the PI3K/Akt signaling pathway and the Rag GTPase signaling pathway. In contrast, Leucine activates mTORC1 through the amino acid sensing pathway, which involves the intracellular amino acid sensor Sestrin2.

Even though it could not be concluded that three out of the four experimental groups affected the speed of regeneration, based on the results shown in **Figure 2**, in only the control group, the length of the planaria was significantly less on Day 7 than on Day 0. This is because planarians often shrink in size when forming blastemas in response to wounding. This study illustrates that planarians, when administered with an external activator such as Leucine or Arginine, can maintain their size rather than shrink as usual. Considering this, there may still be some beneficial effects of Arginine supplementation.

As stated earlier, the increased mortality rate from Days 14 - 21 (post-amputation raises an interesting concern about the long-term effects of the treatments). One plausible justification could be that planarians overdosed, meaning that further supplementation after regeneration could potentially be detrimental to planarian health.

The findings of this study support the hypothesis that stimulating the mTOR pathway can enhance the speed of muscle regeneration in planarians. These results are consistent with previous studies conducted in mice, which have shown that the mTOR pathway plays a crucial role in multiple stages of muscle regeneration [2]. Activation of the mTOR pathway has been associated with stem cell activation, cell proliferation, differentiation, and muscle fiber fusion, all of which contribute to protein synthesis and cell growth regulation.

In line with previous research, the current study used Leucine and Arginine to activate the mTOR pathway. Leucine, an essential amino acid, has been shown to be highly effective in stimulating the mTOR pathway and promoting protein synthesis [4]. Similarly, Arginine, a semi-essential amino acid, can indirectly promote the mTOR pathway by producing nitric oxide (NO) [4]. The supplementation of these amino acids in the planarians' diet aimed to further stimulate the mTOR pathway, leading to increased cell growth and proliferation and potentially enhancing the regenerative capabilities of the planarians.

This study has some limitations. The experiment only measured the regeneration rate up to 14 days post-amputation. Further, extended observation periods would provide a more comprehensive understanding of the sustained effects of

mTOR pathway activation on muscle regeneration.

Furthermore, while this study focused on the speed of muscle regeneration, it did not explore the underlying mechanisms of mTOR pathway activation and its specific effects on cellular processes involved in regeneration. Future research should aim to elucidate the molecular mechanisms by which the mTOR pathway influences muscle regeneration in planarians.

Since this experiment was conducted in a high school lab, factors like temperature fluctuations, contaminants from other experiments, and weekend care inconsistencies might have affected outcomes. Human error and bias during length measurements could have influenced the results. Future experiments should use advanced technology for precision planning and high-resolution histology. Limited resources prevented the utilization of such technology in this experiment.

## 6. Conclusion

In conclusion, this study demonstrates that activation of the mTOR pathway through Leucine supplementation can enhance the speed of muscle regeneration in *Dugesia dorotocephala* planarians. The findings suggest a potential role for mTOR pathway activation in developing new treatments for tissue damage and regenerative medicine. However, further studies are needed to fully understand the mechanisms underlying mTOR pathway activation on muscle regeneration in planarians and assess the long-term effects of such treatments.

## Conflicts of Interest

The author declares no conflicts of interest regarding the publication of this paper.

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