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Article

Determining the Effects of Fibroblast Growth Factor 2 on the Regenerative Abilities of *Echinometra lucunter* Sea Urchins

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SUMMARY

Advances in regeneration have the potential to benefit the healthcare field through contributions to wound healing, organ transplants, and many more related technologies. This experiment was performed to help contribute to further research in vertebral regeneration, as humans' capacity to regenerate is mostly limited to slower and less complex forms of regrowth. Due to their exceptional ability to regenerate entire bodily appendages, we used sea urchins of the species *Echinometra lucenter* as models for the study of regeneration. This experiment was constructed to examine the effects of fibroblast growth factor 2 (FGF2) on spinal regeneration time in the urchins. We hypothesized that the addition of this growth factor would cause urchins to regenerate a larger amount of their spinal tissue 14 days after severance. Although the mean percent regeneration of the experimental group was higher than that of the control, the results were not statistically significant, which reflects a possible lack of correlation between FGF2 and an increased regenerative ability. Further testing is required to discover the possible implications of the data and effect of FGF2 on humans.

INTRODUCTION

Almost all organisms possess a regenerative capacity to some extent, but for humans and most other mammals, this capacity is largely limited to smaller-scale regeneration processes such as wound healing and repair (1). Within humans, the exception to this is the liver, which has the ability to repair lost mass and grow to fit the size of the organism it inhabits (2). Each day, 20 people in the United States die while waiting for an organ transplant (3). Thus, the ability to stimulate regeneration in human organs other than the liver would have a profound impact on the scientific and medical community. It would reduce a recipient's need to rely on an organ donation, as organs could potentially be grown in laboratories (4).

Echinometra lucunter, commonly known as the rock boring sea urchin, has a unique ability to regenerate spines and tube feet and can potentially provide a model for regenerative growth. Echinoderms are ideal organisms due to their relatively quick ability to fully regenerate external appendages, most likely due to their abundance of multipotent cells. Furthermore, sea urchins are non-chordate deuterostomes and are related phylogenetically to humans, so they can also provide insight into mechanisms of regeneration in vertebrates (5). By examining a process that contributes to a greater efficiency of regeneration in echinoderms, we can identify potential factors that regulate regeneration in vertebrates (6).

A previous study's results indicated that the mechanism related to spine and tube feet regeneration in adult sea urchins required a functional Notch signaling pathway, which interacts with other signaling pathways to stimulate growth. This finding supported the hypothesis that the sea urchins that were given the mitotic inhibitor were unable to regrow their amputated appendages (5). Another study examined the effects of varying environmental conditions, specifically ocean acidification, upon adult sea urchins' ability to re-form body structures. The study discovered that the increase of atmospheric carbon dioxide greatly affected the seawater's chemistry and though the spines were able to regenerate, their structural integrity was greatly compromised (7).

Heparan sulfate proteoglycans (HSPGs) are glycoprotein components of the extracellular matrix of all animal cells (8). It is a suitable glycoprotein to focus on in our study of regeneration in sea urchins because it plays a large role in regulatory processes such as wound repair, coagulation, and cell migration (9). In addition, HSPGs interact with a variety of membrane receptors to promote extracellular matrix attachment and a variety of other extracellular interactions (9). For example, the liver has a high regenerative ability due to membrane HSPGs operating as endocytic receptors for the passage of ligands. In fact, studies have shown that HSPGs may assist in recovery from acute liver injury (9). Also, HSPGs can assist the process of growth factor dispersal (10).

In addition, HSPGs are critical in stem cell maintenance. In fact, when stem cells lack HSPGs due to an *Ext1* gene deficiency, they often lose their ability to differentiate and respond to growth factors (11). This highlights the vitality of HSPGs when it comes to the regeneration and differentiation of the cells that create animal tissue. Some of the growth factors capable of binding to HSPGs are fibroblast growth factors.

Fibroblast growth factors function to control the growth and differentiation of progenitor cells during embryonic development and organogenesis. They bind to heparan sulfate proteoglycans and through the use of signaling pathways, they regulate metabolic processes in mature tissues such as tissue repair and regeneration (12). Studies show that fibroblast growth factors (FGFs) likely play an important role in the successful regeneration of liver tissue because their inhibition diminished liver regeneration in rodents (12). In one study, mice that lacked *Fgf15* exhibited defects in regeneration due to an inability to properly regulate the cell cycle. Because researchers have found that echinoderms are capable of binding FGF2, we determined it to be the ideal growth factor for a study involving these animals (13). FGF2 increases the production of cells which stimulates healing. After two days of low dose FGF2 present in the skeletal system of the mice, the rate of cell growth increased by 10% (14).

FGF2 helps to promote angiogenesis, which can help individuals recover better and retain more blood flow (15). Discovering a method to promote regeneration in a variety of

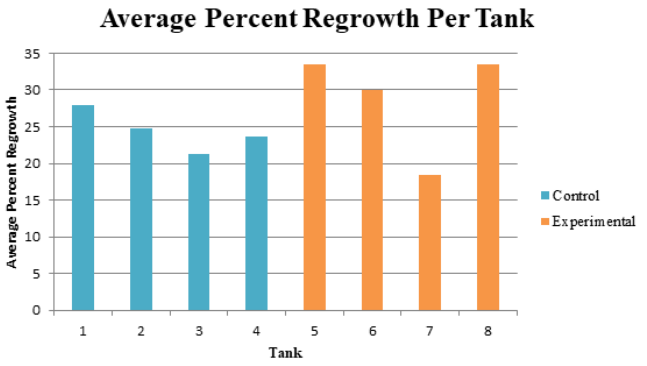

Figure 1. Average percent urchin regeneration by tank. Tanks 1, 4, 7, and 8 were each 10 gallons and contained 3–4 urchins per tank. Tanks 2, 3, 5, and 6 were 5-gallon tanks and contained 2–3 urchins per tank. Spine length after amputation was compared to spine length prior to amputation. Each bar represents the average percent regeneration per tank. Blue bars represent the control group of urchins and the orange bars represent the group of urchins that received FGF2.

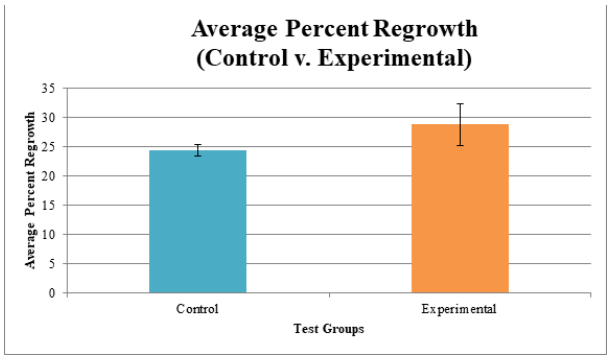

Figure 2. Average percent regrowth for both the control and experimental groups. The control group contained tanks 1–4 and held 11 urchins. The experimental group contained tanks 5–8 and held 13 urchins. The blue bar represents the average spine regeneration percentage of all the tanks in the control group and the orange bar represents the average spine regeneration percentage of all the tanks in the experimental group. The error bars indicate standard error. p=0.09 (one-tailed t-test).

human tissues and blood vessels would undoubtedly have a monumental impact on modern science and medicine. This study examined FGF2's effect on regeneration in the *E. lucunter* species. By studying the rate at which the urchins' appendages regenerate, the results can potentially support the possibility of growth factors increasing regenerative abilities in more organisms.

We hypothesized that the addition of FGF2 solution into the system of the *E. lucunter* would make the time of regeneration approximately 10% faster, as seen in previous studies (14). Therefore, we predicted that a single urchin spine with FGF2 would regenerate more of its original length in 14 days than the urchins of the control group (5). After experimentation, the mean percent regeneration of the experimental group was 28.86%, which was higher than that of the control which was 24.44%. The calculated p-value was 0.09.

RESULTS

Tanks 1-4 held the control group of urchins while tanks 5-8 held the experimental group of urchins. Discrepancies between the number of urchins at the start and end of the experiment signify that deaths occurred (**Table 1**). Two deaths occurred in Tank 1, one death occurred in Tanks 4, 6, 7, and 8, and no deaths occurred in Tanks 2, 3, and 5.

The control group (blue) had average spine regrowths of 27.99% (Tank 1), 24.79% (Tank 2), 21.35% (Tank 3), and 23.63% (Tank 4) (**Figure 1**). The experimental group (orange) has averages of 33.48% (Tank 5), 29.96% (Tank 6), 18.49% (Tank 7), and 33.49% (Tank 8). These percentages were found by cutting spines before and after FGF2 administration and comparing lengths.

The control group had a lower average percent of spine regrowth (24.44%) but also had a lower standard error (1.38), whereas the experimental group had a higher average percent of spine regrowth (28.86%) with a larger standard error (3.55) (**Figure 2**). The error range of the experimental group is 23.06-25.82 and the standard error of the control group is 25.31-32.41.

A test of normality was also conducted and the values were plotted. The plot was roughly linear, thus indicating that the data was approximately normally distributed. Once this was established, a one-tailed t-test was conducted. The p-value was 0.090521. A p-value of 0.09 indicates that there is a 9% chance that these results would occur if FGF2 had no effect on regeneration. This means that our hypothesis was not definitively supported as a 5% value would be required to suggest statistical significance.

DISCUSSION

The sea urchins in our study showed a greater average percent regrowth for the experimental group compared to the control group (**Figure 2**). However, since the standard error for the data is so large, the FGF2 may not have had an equal

Tank	Volume of Tank	Filter Type	Group	Number of Urchins at Start of Experiment	Number of Urchins at End of Experiment
1	10 gallon	Tetra Whisper Filter	Control	4	2
2	5 gallon	Tetra Whisper Filter	Control	3	3
3	5 gallon	Tetra Whisper Filter	Control	2	2
4	10 gallon	Tetra Whisper Filter	Control	2	1
5	5 gallon	Penn-Plrx Cascade Heat (Model: CH850)	Experimental	3	3
6	5 gallon	Tetra Whisper Filter	Experimental	3	2
7	10 gallon	Tetra Whisper Filter	Experimental	4	3
8	10 gallon	Tetra Whisper Filter	Experimental	3	2

Table 1. Tank parameters and number of urchins in each tank at the start and conclusion of the experiment.

effect in all experimental tanks. In particular, Tank 7 had a significantly lower percent regrowth than the rest of the experimental group (**Figure 1**). By the end of the regeneration period, the 10 urchins in the experimental tanks regenerated on average about 18% more spinal tissue than the 8 in the control group. This indicates that the FGF2 may have acted as an injury response agent and induced faster spinal regeneration. However, the *t*-test showed a *p*-value of 0.09, indicating that the data was not statistically significant.

One study showed an average spine regeneration of around 44% in 14 days without any growth factors added (5). Other researchers found that the addition of FGF2 causes spines to regenerate 48.4% of their original length in 14 days (14). The current study found that spines regenerated an average of 29% of their original length in 14 days.

Hence, our results showed a deficiency in the average percent regrowth compared to what was predicted. The reason for this may have been due to high stress levels in the sea urchins, indicated in the experiment when many spines fell off (16). This would have a significant impact on the data as there was no way to measure the lengths of prematurely detached spines. The stress could have been caused by inadequate filtration, mold in the bottom of the tanks from the live rock, and overhandling of the urchins. Because the water conditions varied between tanks, each tank was looked at separately. The varying conditions like mold and filter type likely impacted stress levels and therefore regeneration. Mold was present in Tank 7, which ended up having the lowest regrowth rate. Also, the results were compromised because some of the sea urchins died mid-experiment. The sea urchins that died were in tanks 1, 4, 6, 7, and 8. This could have been due to the aforementioned varying tank conditions. Therefore, there was a smaller sample size than what would have been optimal for this experiment, and this undoubtedly could have made it more difficult to draw conclusions because the statistical analysis is not as powerful.

In future experiments, steps could be taken to reduce variability. For example, a thinner pair of dissection scissors could be used to cut with a higher degree of precision. It was often difficult to cut close to the test (the skeleton of the urchin) using a large pair of scissors because of the surrounding spines. Additionally, a higher-quality waterproof adhesive would be advisable for tagging the urchins as the tape used in this experiment would occasionally detach from the specimen. There were also discrepancies between the filters in the different aquariums as some were different brands and therefore higher quality than others. This may have affected the health and stress levels of some of the urchins, as a low-quality filter possibly could have caused stress and contributed to their deaths. Accordingly, it is recommended that identical filters be utilized in each aquatic habitat in future experiments as it will reduce the likelihood of an additional variable having an impact on the results. Another possible factor that was linked to the variance in data was the amount of FGF2 administered per urchin. The amount of FGF2 given can be based on urchin size, so ideally all urchins would have been the same or similar sizes, allowing the administration of a standard dose of FGF2.

To further extend this experiment, rather than focusing solely on regeneration of appendages, morphallaxis in the *Hydra vulgaris* could be studied in order to gain an understanding of the effect of FGF2 on the process of full-body morphallactic regeneration after bisection. Additionally, the effect of FGF2 on tail regeneration in amphibians could be tested to examine if its effects transfer to more complex organisms that contain more highly differentiated tissues than those of *E. lucunter*. In the future, these studies could potentially be used to enhance understanding of regeneration in humans and could possibly be applied to methods of promoting faster rates of regeneration in human liver.

METHODS

Saltwater Tank and Habitat Set-up

Four ten-gallon tanks and four five-gallon tanks were set up for the sea urchins' habitat. Tanks 1, 4, 7, and 8 were the 10-gallon tanks and tanks 2, 3, 5, and 6 were the 5-gallon tanks. They were placed in conditions with 35 parts per thousand of salt, which is the salt content of their natural habitat, the Atlantic Ocean. Two pounds of live rock were kept in the ten-gallon tanks and one pound was kept in the five-gallon tanks to provide nutrition and shelter for the urchins. The temperature of the tank was maintained between 23.9–27.8 ˚C with constant filtration. The *E. lucunter* were fed eight cubic millimeters of algal-agar cube weekly. The algal-agar cubes were made by mixing 3.6 g of agar with 100 mL water and heating until boiling. Then 2 grams of green algae (crushed by mortar and pestle into fine powder) were added to 16 mL of water and were stirred to ensure uniform mixing. Next, the algae solution and the agar solution were mixed together. Then the algal-agar mixture was spread onto a microscope slide covered with wax paper, left to solidify, and cut into cubes of 2 mm^3.

FGF2 Administration

0.01 g (10 µL) of FGF2 obtained from Prospec Protein Specialist were mixed with 5,000 µl of distilled water and were then fed to the 13 experimental *E. lucunter* by inserting an insulin needle into their oral cavity to ensure that each urchin received the complete 501 µL of solution needed, which in turn gives them the full 5 ng of FGF2. The 13 experimental and 11 control urchins were then left for 2 days so that 10 % of FGF2 was released and was able to bind to the heparan sulfate located at the surface of the cells (14).

Identification of Urchins

If a tank contained two urchins of similar size, then one urchin was labeled using a strip of waterproof tape around a single spine. Tanks 1, 2, 5, and 8 all contained two urchins that were similarly sized, so one of each of those urchins was tagged to differentiate it from the other.

Spine Severance

Two days after the *E. lucunter* experimental group was fed the FGF2, four spines were cut off from one ambulacrum section of each sea urchin from both groups using dissection scissors. For future identification purposes, spines from each urchin were cut in a vertical line and numbered one to four (from top to bottom). The lengths of the severed appendages were measured using a caliper and recorded. After being amputated, sea urchins were left to recover overnight without disruption for 24 hours. The regrowth of the spines was then monitored over a course of 14 days (5).

Measuring Regeneration of the Spines

The lengths of the four regenerated spines of each urchin were measured at the end of the full two-week period. On the final day of the experiment, the sea urchins were removed from the tank, placed in a shallow container, and allowed to relax. Then, the regenerated spines were cut off with the dissection scissors. The electronic caliper was placed adjacent to the newly cut off spine, and the newly cut-off spine was then measured and compared to the length of the previously amputated spine and the percent regeneration was calculated. This growth showed how fast the regeneration of the spines occurred, which allowed the amount of regeneration of the experimental group to be compared to that of the control group (5). The average growth of each tank was taken and the average growth of the experimental group and the control group as a whole was calculated to determine whether the hypothesis was supported. A *t*-test was performed in Excel Spreadsheets to determine if the data was significant.

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