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Exploring the Effects of Varying Retinol Vitamin A Concentrations on Tissue Regeneration in the Species *Asterina coronata*

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ABSTRACT

The following study will look at the relationship between tissue and regeneration rate in the sea star species *Asterina* and retinol vitamin A. The overall purpose of the study is to evaluate whether concentrations of retinol vitamin A will produce a positive, negative, or null effect on the tissue regeneration in *Asterina coronata*. After amputation of the ray, 48 sea stars were subjected to four different retinol vitamin A concentration levels for sixteen hours and observed over a thirty-to-forty-day time period. The results show that the second highest concentration group produced the most consistent and noticeable growth. Moreover, the highest concentration group showed the second highest rate compared with the lowest concentration group and the control. Through these results, it can be concluded that retinol vitamin A does support tissue regeneration in *Asterina coronata* to a limit before it begins a negative feedback. The findings in this study add to an understanding of the mechanisms behind cell differentiation and whether stem cells involving tissue regeneration can be activated with the treatment of vitamin A. Hopefully the connections made in this paper can impact current research on human stem cell therapy itself, and the topic of whether sea stars can be made into viable stem cell therapy models.

Introduction

The initial observation by researchers Niazi and Saxena (1978) found that explicit vitamin A concentrations induce an excess of regenerative growth [1]. Findings in the study demonstrated that vitamin A paved the way for heteromorphic regeneration in amphibians, where limb regeneration was not simply a mere replication of the original pattern but resulted in the formation of abnormally long limb formation in tadpoles and frogs of Indian species [1,2]. Since this significant result, the scientific community has maintained a high interest in vitamin A's effect on regenerative capabilities.

Vitamin A is a family of organic compounds that consist of retinol, retinal, and provitamin A carotenoids [3]. For all vertebrate animal species, vitamin A is an essential nutrient. In the human body, it promotes cellular differentiation, benefits vision, and properly develops the embryo and fetus [4]. It also assists in the immune system, growth, and wound healing [4]. While vitamin A is a necessary nutrient, it may be toxic in higher doses. Acute toxicity in humans results in nausea, peeling skin, muscle weakness, and malaise, while chronic toxicity results in internal hemorrhage, spontaneous fractures, and skeletal malformations [5].

It is known that all living organisms have some ability to regenerate and resume stem cell activity to maintain tissue homeostasis and organs [6]. While several organisms have extensive regenerative abilities, such as the Hydra or Mexican salamander and amphibians in general, most organisms have limited regenerative abilities. These organisms are typically only able to regrow hair and skin, form thick scars, and heal bone fractures [6]. However, sea stars (also recognized informally as starfish) have fascinated researchers for decades with their exceptional ability to regenerate limbs and, in certain cases, their entire bodies at any given time. Sea stars can restore entire arms following

their loss by both autonomy and traumatic amputation [7]. This process can take time for some species—from a couple of months to a year. Their extensive regenerative abilities are mainly due to their stem cells that can differentiate into new limbs. When a limb becomes damaged, these stem cells receive a signal from the area and start regenerating. In other words, starfish have a central region of the body from which the limbs arise. If a limb is severed, a new one appears in the central region and extends outward [8]. Most sea stars need the central body intact to regenerate, but some species, such as the red and blue Linckia sea star, can regenerate a whole new sea star from a limb or ray only [9].

With such regenerative abilities, researchers are studying sea stars and their tissue growth. In particular, researchers often test to find a relationship between different factors and their effect on sea star tissue regeneration. Overall, sea stars are beneficial in studying wound healing and regeneration in humans, and it is imperative to further explore the workings behind the effect retinol vitamin A has on *Asterina coronata* sea stars that this study presents.

Literature Review

Following the foundational study by Niazi and Saxena (1978), researchers aim to understand the direct effects vitamin A may have on varying organisms and its possible implications in human tissue regeneration. In 1986, Mark L. Cecil and Roy A. Tassava of the Department of Zoology at Ohio State University attempted to determine if treatment of the wound surface with vitamin A palmitate in an untested frog species, *Rana pipiens*, would improve the regenerative response of forelimbs [10]. Previously, *Rana pipiens* normally exhibited a hypomorphic outgrowth at a low frequency. After amputation of the left forelimb, 30 frogs were immersed in a solution of vitamin A palmitate (25,000 IU, from Sigma Aldrich), where each treatment lasted 3 minutes. The amputated left forelimb of 20 control frogs received no treatment. Consequently, Cecil and Tassava found that vitamin A treatment did cause an enhancement in regeneration: the treated *Rana pipiens* frogs compared to the control were found to be larger and had improved hypomorphic growth, more complex structures and single-spike outgrowths [10]. Compared to the control, the treated forelimbs had a more intricate hand-like appearance [10]. These results coincided with a study conducted in 1984 by Niazi and Alam where young toad tadpoles of the species *Bufo melanostictus* were treated with a vitamin A palmitate solution. Niaza and Alam found that in more than 65% of cases, the resulting regenerating limbs were whole limbs that had the skeletal elements from the femur to the phalanges [11]. The results indicate that when properly administered, vitamin A, through the intensifying dedifferentiation of its cells, can cause limb regeneration blastema of amphibians to be the same as the original limb bud [11].

After multiple studies on a variety of frog species, researchers then went on to try to determine the effects of vitamin A on more complex organisms. In 2001, Shekhawat et al. examined the impact of vitamin A on lens regeneration in mice. Through intraperitoneal injection of vitamin A, vitamin A successfully induced lens regeneration in both young and adult mice. Results demonstrated that only 4 out of 20 young mice in the control group, compared to the 18 out of 20 young mice in the vitamin A treated group, led to full lens regeneration [12]. No adult mice of the control group led to lens regeneration, while 16 out of the 20 adult mice treated with vitamin A displayed lens regeneration [12]. The regenerated lens led to vacuolated fiber cells and small lentoids in tissue, but the process of developing lentoids was similar to lens formation. These results indicate that vitamin A greatly improves regeneration, even in adult mice where regeneration is not originally noticed, possibly due to the enhancement of the differentiation process [12]. An alternative complex organism studied is the lizard. In his 2019 study, Lorenzo Alibardi administered vitamin A in four adult wall lizards (*Podarcis muralis*) during tail regeneration. Regenerating epithelial tissues (apical epidermis and ependyma) simulated tail regeneration [13]. However, Alibardi decided to inject high doses of vitamin A to see whether high concentrations cause epithelial mucogenesis and muscle differentiation delay [13]. During the observational period, the epidermis did not form scales, and the differentiation of oberhautchen and β-layers was reduced [13]. Overall, the injection of high doses of vitamin A caused the degeneration of multiple tissues such as muscle and cartilage during tail regeneration, leading to soft tails rather than the stiffness in the original. Using these

results, Alibardi determined that the high dosage of vitamin A caused upregulation of a few genes that are involved in retinol metabolisms, such as retinoic acid-binding proteins and retinoic acid receptor gamma protein [13].

The revelation that vitamin A may instead hinder the developmental process and reverse tissue regeneration in high doses [5, 13, 17] is expanded upon in Zinder et al.'s paper. On a cellular level, retinol vitamin A binds to protein receptors such as RBP within the plasma membrane [14]. Since vitamin A is a fat-soluble property, there is a limited degree to how it can bind to intracellular proteins [14]. When retinol vitamin A does not bind to a protein in the plasma membrane, it can cause membrane lysis and extreme tissue damage as a result. For example, excess retinol vitamin A in the *gir* zebrafish mutant led to the absence of pectoral fins [15]. Fin development when subjected to retinol vitamin A, however, became normal when retinol vitamin A concentrations were below 7.5 × 10^-7 M to 1 × 10^−6 M in zebrafish tanks [15].

An increase in vitamin A concentrations can also cause an increase in serial reduplication or repetitions that go beyond the original sections removed by amputation. According to a study published by Maden, retinol vitamin A caused reduplication patterns in the proximodistal axis on axolotl larvae (*Ambystoma mexicanum*) during regeneration [16]. The degree of regenerative repetition in larvae treated with retinol scored around 3 times as much compared to the control [16]. During the period of vitamin A treatment itself, no regenerative rate took place in the larvae, and cell division was inhibited. Regeneration and serial reduplication resumed once the larvae were taken out of treatment. The same result occurred in larvae that were left in vitamin A for too long, but regeneration rate was permanently hindered, even after treatment. The reduplication patterns were unlikely to be the result of the delay in cell division since similar processes such as denervation do not lead to pattern abnormalities. Thus, the reduplication patterns were most likely due to mesodermal cell surface and membrane glycoprotein synthesis effects [16].

With modern advancements in molecular biology and the results of studies like the ones previously mentioned, researchers strive to understand how Vitamin A can regulate regeneration at a cellular and molecular level. Vitamin A, specifically retinol vitamin A, is involved in a complex cell signaling pathway that regulates gene expression [17]. According to Tafti and Ghyselinck, many clinical findings have indicated that vitamin A and retinoids play a significant role in embryogenesis, cell proliferation, differentiation, and apoptosis, while high doses can cause developmental abnormalities. [17]. Retinol vitamin A binds to small intracellular lipid-binding proteins to arrive at the nucleus. The resulting complex carries retinol vitamin A to specialized nuclear receptors that changes its signal in vivo as heterodimers with retinoid X receptors (RXR) [18]. Thus, retinol vitamin A is considered critical in the connection of vitamin, nutrition, homeostasis, and development through the regulation of gene expression [17, 18]. However, recent studies have shown that vitamin A also targets the activation of the mitogen-activated protein kinase (MAPK) signaling pathway [19]. Through the phosphorylation cascade, a protein-receptor complex is formed that can eventually stimulate the transcription of a gene. This type of signaling pathway activates genes that are not direct retinoic acid receptor targets, expanding the functions of retinol vitamin A.

Organisms such as frogs, mice, and lizards are known as typical subjects to test for regeneration. With their capable regenerative abilities, sea stars are also model subjects in tissue regeneration: hundreds of studies have been conducted to examine and observe the regeneration rate anatomical process in sea stars. One major study in this area aimed to investigate the possibility of a salinity level that maximizes arm regeneration in the starfish *Luidia clathrata.* As the study progressed, researchers found that arm regeneration can be up to five times slower in lower salinities compared to ocean salinity [20]. Additional studies also test the effects of temperature and pH on sea star regeneration. However, in literature and recent studies, there has yet to be a study that observes the relationship between vitamin A (retinol) and sea stars. This gap in research leads to the overall research question of this study: To what extent can varying concentrations of retinol vitamin A affect tissue regeneration in the sea star species *Asterina coronata*?

Thus, this study aims to investigate whether different concentrations of retinol vitamin A create a positive, negative, or null effect on regeneration rate after amputation for 48 *Asterina coronata* sea stars through a 30-40 day observation period. This study holds significance in creating a new understanding of how vitamin A may impact a large segment of invertebrates.

Combining evidence gathered from different studies that high concentrations of vitamin A cause the repression of cell division and tissue damage, along with the understanding that specific doses of vitamin A beneficially impact tissue regeneration, leads to the hypothesis that the middle concentrate (3.3 µg/mL) of retinol vitamin A would cause the most regenerative growth in *Asterina coronata* throughout the observation period.

Methodology

In order to effectively observe and analyze the relationship between tissue regeneration rate in *Asterina coronata* and retinol vitamin A, an experimental design framework was utilized. This methodology is generally preferred by multiple studies conducted in similar areas of research—as seen in Scadding and Maden (1986), where the authors used experimental design to successfully compare the effects of retinyl palmitate on limb development in *Xenopus laevis* tadpoles [21].

Altogether, experimental design serves to define a correlation between the independent and dependent variables. It provides control for the researcher towards the manipulation of the variables themselves and the subsequent environments. Additionally, the method has evolved from not just an explanation of the design of the experiment, but also an explanation of the statistical analysis [22]. As such, an experimental design optimization best fits the specific type of cause-and-effect research in this experiment. In this case, the independent variable was the varying retinol vitamin A concentrations, and the dependent variable was the overall tissue regeneration rate. The control group of the amputated *Asterina coronata* sea stars was not subjected to concentrations of retinol vitamin A, providing a baseline value for tissue regeneration without the influence of retinol vitamin A. To maintain organization, the method design was categorized into four major sections: (Section A) the establishment of materials and general setup, (Section B) the amputation and treatment process, (Section C) the observation period, and (Section D) data collection and analysis.

Anticipated limitations in the methodology included errors in the steps themselves, such as residue in the treatment wells, cross-contamination of retinol vitamin A through plate spillage, and handling effects.

A. Material Collection and Setup

A total of 48 *Asterina coronata* sea stars (mean body length 6.0 ± 0.4 mm from opposite arms) were used in the experiment, raised in a 125-gallon saltwater reef tank. *Asterina coronata* were selected due to availability, feasibility, and their close relation to *Asterina miniata*—a species featured in numerous cell and developmental studies [23]. Dimethyl sulfoxide (DMSO) and retinol vitamin A (R7632, synthetic, ≥95% HPLC, crystalline) were obtained through the biotechnology and chemical company Sigma-Aldrich. Eppendorf Research Plus 1000® and Socorex Acura 825® micropipettes were used to measure out quantities of DMSO and stock solution. Dissecting and handling equipment included a Miltex® micro-dissecting spring scissor and lab-grade tweezers. Four Costar® TC-treated cell culture 24-well plates held the sea stars: two plates for treatment and photography and two plates for observation. A saltwater reef tank housed the two 24-well plates during the observation period. Additional materials included a camera for photographic assemblage, baby brine shrimp as the source of nutrition for the sea stars, Adobe Photoshop as a software for quantitative data collection, a drill, and any laboratory safety equipment when handling the DMSO and retinol vitamin A (goggles, mask, and gloves). The setup before experimentation was as follows:

A-1: Retinol Vitamin A Stock Solution

Retinol vitamin A by itself is insoluble in liquids such as water or glycerol due to its fat-soluble property. As a result, researchers often use polar aprotic solvents such as dimethyl sulfoxide (DMSO) or ethanol that dissolve both polar and nonpolar compounds [25]. Hence, a retinol vitamin A stock solution in DMSO was necessary for this experiment. To prepare the stock solution, 20 mg (milligrams) of retinol vitamin A were dissolved in 1 mL (milliliter) of DMSO

based upon the manufacturer's suggested solution. Subsequently, this led to a basic 20 mg/mL stock solution. Additional stock solutions were created with the same criterion.

A-2: Preparation of 24-Well Plates

Two 24-well plates were used to hold the 48 sea stars in the tank after treatment. The plates were marked accordingly as Plate 1 or Plate 2 with a subsequent concentration indication for each level. The bottoms of the wells were drilled to create two 3-millimeter-wide holes to ensure water circulation once Plate 1 and Plate 2 were placed into the fish tank. The plates floated on the top while holding seawater in the wells, as seen in Figure 3.

A-3: Environmental Parameters

To effectively simulate the conditions of the sea stars' original habitat, environmental parameters were considered:
A-3-1: Tank temperature maintained around 23.5° C
A-3-2: Seawater salinity kept around 35 g/L
A-3-3: Phosphate and nitrite leveled 0 ppm
A-3-4: Sea stars exposed to full-spectrum LED light from 6 a.m. - 8 p.m. daily

B. Amputation and Treatment Process

For all 48 *Asterina coronata* sea stars, one of their arms was selected to be amputated with the micro-dissecting scissor. The amputation line was located at the middle of the arm which allowed for easier recognition and observation once tissue regeneration began. The amputation line was not extremely close to the central ring canal due to its key role in sea star regeneration, and the possibility of hindering the feeding area. With each sea star having a body diameter of around 5.6 - 6.4 mm (millimeters), an estimated 0.8 mm of the arm was amputated. Pictures of the sea stars were taken before amputation and promptly after the amputation.

Figure 1. (left) The oral surface of an *Asterina coronata* sea star with body diameter ranges and a general outline of the amputation line. (middle) Picture of an *Asterina coronata* sea star before amputation. (right) Picture of the same *Asterina coronata* sea star right after amputation.

Immediately after amputation, the sea stars were also subjected to their retrospective retinol vitamin A treatments. There were four primary concentration groups: Control (no retinol vitamin A), Concentration 1 (a 1 to 12000 dilution of stock solution: final working solution = 1.7 µg/mL of retinol vitamin A), Concentration 2 (a 1 to 6000 dilution of stock solution: final working solution = 3.3 µg/mL of retinol vitamin A), and Concentration 3 (a 1 to 3000 dilution of stock solution: final working solution = 6.7 µg/mL of retinol vitamin A). The sea stars were treated in their wells with a 2 mL working solution for 16 hours at room temperature. At the end of the treatment period, the sea stars were transferred to a seawater environment within the 24-well plates in the tank and kept therein for the remainder of the experiment.

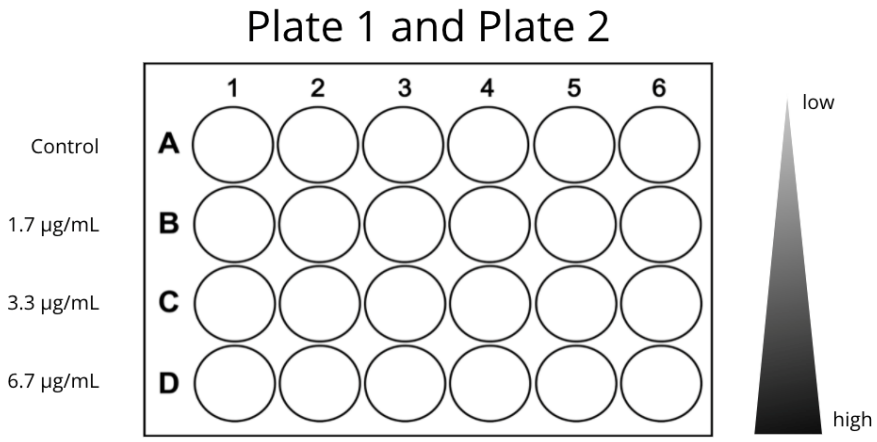

Figure 2. Treatment setup along with a concentration gradient.

C. Observation Period and Maintenance

Once the sea stars were adjusted to the 24-well plates in the tank, they were monitored for some time. Plate 1 was observed for 40 days from February 10, 2021, to March 22, 2021. Plate 2 was observed for 30 days (had to readjust space after 10 days of Plate 1 to fit Plate 2) from February 20, 2021, to March 22, 2021. During the observation period, the sea stars were fed once every two days on a diet of baby brine shrimp. The seawater in the 24-well plates was also replaced twice daily (typically once every 12 hours) to facilitate water circulation and maintain water quality in the wells. Once every ten days, the sea stars were transferred to two 24-well plates outside of the tank, and pictures were taken to keep track of the tissue regeneration rate.

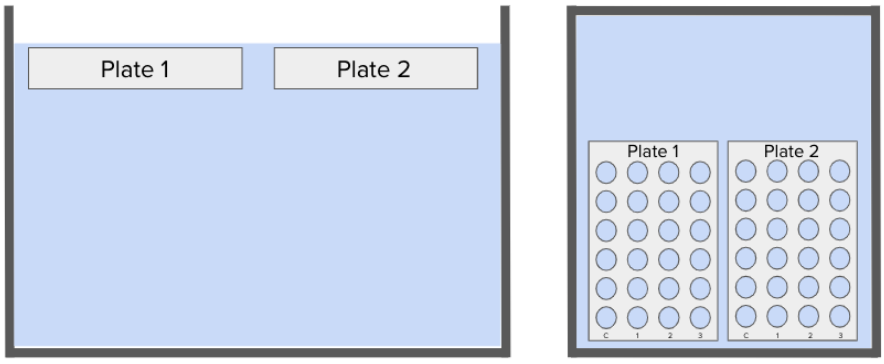

Figure 3. Diagrams of the tank and 24-well plate setup during the observation period. (left) Side view of the tank. (right) Downwards view from above the tank.

D. Data Collection and Data Analysis

After the observation period, quantitative data was obtained through the input of photos into Adobe Photoshop. With the "ruler" function that displays pixels by default, measurements were taken from the center of each sea star to the

edge of its amputated arm. Measurements from the center of each sea star to the adjacent arms of the amputated arm were also taken. With these values, a ratio was calculated using the formula below:

$$\text{Ratio} = \frac{Amputated\ Arm\ Length}{Left\ Adjacent\ Arm\ Length + Right\ Adjacent\ Arm\ Length}$$

A ratio ensured that factors such as the original size of the sea stars and photo angles would not create ambiguity among the results. The mean value of the ratios was calculated for each concentration group in every time frame.

Data analysis for the results consisted of standard deviation and T-tests to determine statistical significance. T-tests were performed with the conventional two-tailed distribution method to test for both positive and negative differences, along with two-sample unequal variance. P-values (calculated from the T-tests) of less than 0.05 were considered statistically significant. All values and calculations were stored and computed in Microsoft Excel.

Results

As stated previously, all quantitative values and data were stored and computed in Microsoft Excel. The mean ratio values for each concentration group at different time points in Plates 1 and 2 were stored in data tables (shown below). With such values, statistical analysis was conducted, and a bar graph was generated that includes the mean value points, standard deviation, and p-values, (also shown below). The horizontal axis for the bar graph represents the varying concentration groups of retinol vitamin A and the vertical axis represents the regeneration rate (in Adobe Photoshop's default pixel size). The results themselves will also be mentioned below.

When the original *Asterina coronata* sea stars were measured through Adobe Photoshop, the overall ratio was found to be around 0.53-0.57 for Plate 1 and 0.50-0.60 for Plate 2. After the sea stars were amputated, the ratio was lowered to around 0.39-0.41 (Plate 1) and 0.34-0.39 (Plate 2). As time passed, the mean ratio value also relatively increased for almost all groups. One such example can be seen in Concentration 2 of Plate 1. From the original mean value of 0.40 after amputation, sea stars in Concentration 2 had a final mean value ratio of 0.46. Similar trends were found in almost all other concentration groups. In Plate 1, Concentration 2 was the only group that was statistically significant with a p-value of 0.013. In Plate 2, Concentration 2 was the most statistically significant compared to the other values with a p-value of 0.00037. With this brief analysis of the results from the data table and bar graph, and through an understanding of the results themselves, it is clear that the Concentration 2 group had the highest and most consistent rate of tissue regeneration among the other groups.

Plate 1

Concentration Level:	2/9/2021 (before amputation) Mean Values	2/10/2021 (after amputation) Mean Values	2/20/2021 Mean Values	3/2/2021 Mean Values	3/12/2021 Mean Values	3/22/2021 Mean Values
Control	0.578039680	0.417013889	0.410009493	0.434878439	0.412869467	0.418906158

1	0.531120215	0.408989959	0.400714792	0.428898046	0.403589087	0.407046765
2	0.555636313	0.396294673	0.426813712	0.447943871	0.452556616	0.459383557
3	0.5516037349	0.4055601257	0.4323891147	0.4192686496	0.424428462	0.449069382

Figure 4. (Plate 1) mean ratio values for each concentration group (control, 1, 2, 3) in 24 sea stars (6 sea stars per group) over the time period from 2/9/2021 to 3/22/2021

Plate 2

Concentration Level:	2/19/2021 (before amputation) Mean Values	2/20/2021 (after amputation) Mean Values	3/2/2021 Mean Values	3/12/2021 Mean Values	3/22/2021 Mean Values
Control	0.5069987189	0.3442239135	0.4041819641	0.4035304823	0.3924030355
1	0.6001734585	0.3904918216	0.4058738977	0.4200954617	0.4237507344
2	0.5488179418	0.3711026972	0.4108512917	0.4361968849	0.4571515147
3	0.5488179418	0.37698357	0.3876644767	0.4399609559	0.4193577913

Figure 5. (Plate 2) mean ratio values for each concentration group (control, 1, 2, 3) in 24 sea stars (6 sea stars per group) over the time period from 2/19/2021 to 3/22/2021

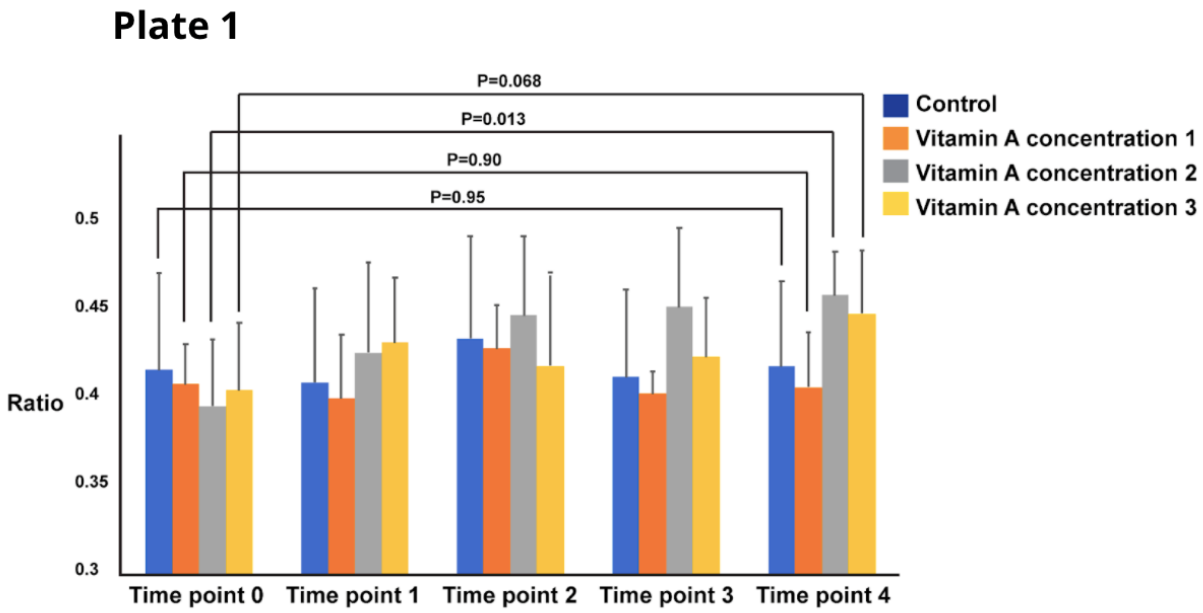

Figure 6. Bar graph containing mean value points, standard deviation and p-values for Plate 1

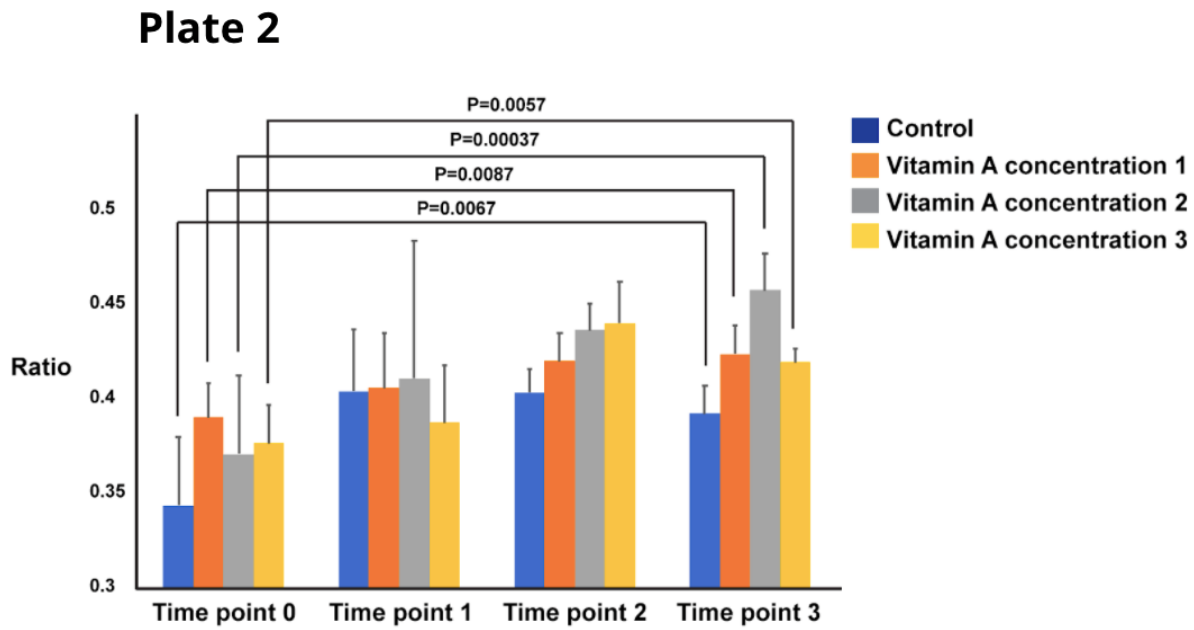

Figure 7. Bar graph containing mean value points, standard deviation and p-values for Plate 2.

Discussion

The results gathered from this study created multiple areas of discussion. As such, the discussion section is divided into three major sections.

A. Proper Concentrations of Vitamin A is Critical for Tissue Regeneration

Prior studies indicate that there is a vitamin A gradient during regeneration and extremely high or toxic concentrations of vitamin A arrest and inhibit overall tissue regeneration rate and growth. In this study, with Concentration 3 being the highest concentration, it eventually did not lead to the highest regeneration rate suggesting that Concentration 3 had already reached a level that will impact tissue regeneration negatively. This supports the idea that in regard to the general mechanisms of regeneration, vitamin A concentrations reach a certain point where a negative feedback mechanism or toxicity effect is employed and begins to inhibit cell proliferation and cell division. Interestingly, it is also noticed that Concentration 3 still led to a higher tissue regeneration rate compared to the control and Concentration 1 (In Plate 1 the control, Concentration 1, and Concentration 3 values were all statistically insignificant, but Concentration 3 had a lower p-value) indicating that the impact of vitamin A treatment in Concentration 3 had not regressed to the point where it would begin to greatly hinder regeneration. It also shows that even at higher levels, retinol vitamin A still played a greater role in tissue regeneration compared to no concentration at all in this experiment. Data suggests that a higher concentration of retinol vitamin A than Concentration 3 will be needed to display completely inhibitory effects. Overall, the results from this study confirm that proper concentrations of retinol vitamin A are required to stimulate tissue regeneration effectively.

B. Vitamin A is Crucial for Regeneration in Different Species

As previously mentioned, vitamin A has been suggested to be involved in tissue regeneration in multiple vertebrate species such as toads, frogs, lizards, salamanders, and mice [12]. No study has been performed on the relationship between vitamin A and invertebrates. Although invertebrate sea star species have been used as a well-established regeneration model for decades, there is no existing literature or research studying tissue regeneration in sea stars with vitamin A. The type of organism utilized in this experiment was the invertebrate sea star species *Asterina coronata.* For the first time, the results of this study indicated that vitamin A does have some positive effects on tissue regeneration rate in sea stars, signifying how the general studied impact of vitamin A covered both vertebrates and invertebrates.

It is well known that the components of a few important signaling pathways such as Wnt-signaling pathways are extremely conserved amongst different species and preserved through animal evolution. For example, the Wnt-signaling pathway which is critical for human development is initially identified in drosophila flies. With vitamin A to be involved in regeneration across a broad range of species as well, it is possible that components of the retinol vitamin A signaling pathway such as the membrane receptor and nuclear complex may also be extremely conserved among sea star species to mice.

C. Potential Cross-talking Between Vitamin A and Other Signaling Pathways

Signaling pathways such as Wnt and BMP signaling play a major role in tissue regeneration [26] in different species. The Vitamin A signaling pathway receptor RXR can form complexes with other receptors in the nucleus such as PPAR, vitamin D receptors, and hormone receptors indicating there could be a cross-talk (or interaction) between the vitamin A pathway and other signaling pathways during tissue regeneration. Furthermore, RXR can impact or interfere

with TGF-beta/SMAD signaling activity by interacting directly with SMAD 3 and 4, which is critical for tissue regeneration as well [27].

Conclusion

This study yielded some limitations that future research could address to enhance the understanding of retinol vitamin A and its effect on tissue regeneration rate in *Asterina coronata*. The principal limitation was the time dedicated to the observational period. Due to resources and time restrictions, Plate 1 was observed for 40 days, while Plate 2 was observed for 30 days. Compared to studies in a similar field—with observational periods of usually 60 days or more [10]—this study's observational period is somewhat shorter than preferred. In the end, the sea stars' amputated arms had still not fully developed, and coarse marks of the amputation line were still visible. These observations indicated that the regenerative process was still ongoing and could lead to different results over a longer period. This limitation could also explain why some results were statistically insignificant: there was not enough time for a noticeable change in tissue growth for certain groups. Another limitation included the number of testing samples. The use of 48 sea stars was decided due to feasibility, space in the tank, and maintenance, but a larger sample size is optimal for more reliable results. This would allow for less error to be accounted for in the data and situate for outliers. The last limitation concerns the number of concentration variations where only three levels of treatment were tested. More levels would provide more information on which specific vitamin A concentrate hits peak positive impact.

Future research should take all previously mentioned limitations into account, and researchers should attempt to have an observational period of at least 70-80 days or until regeneration, have a sample size of at least 100, and contain more retinol vitamin A concentrations for treatment. Besides improving the limitations for future research, researchers could also check for proliferation markers such as Ki67, a gene expression associated with cell proliferation, along with differentiation markers through a histology analysis to see whether they had a presence in the regeneration rate.

The results provide multiple implications in the field of stem cells and regeneration. It inspires an additional understanding on whether stem cells involving tissue regeneration have been activated with the treatment of vitamin A, and overall cell differentiation in the wounded area. With the combination of results in other papers regarding regeneration, scientists and researchers alike can apply the results into stem cell therapy in humans and create a rough correlation between the behavior of regeneration rate in *Asterina coronata* after retinol vitamin A treatment and regeneration rate in humans. With this correlation, more information on specialized stem cells can be used to apply its applicability in real life: using specialized stem cells to repair damaged cells in the body. Additionally, understanding the role of the vitamin A signaling pathway during regeneration using simple animals that are cost effective and easier to maintain could benefit biomedical research related to human clinical trials.

Overall, this study successfully accomplished its goal of testing to determine whether different concentrations of retinol vitamin A create a positive, negative, or null effect on regeneration rate in *Asterina coronata* and the hypothesis was fulfilled. While concentration 3 had the highest retinol vitamin A concentrate, it did not lead to the highest rate of tissue regeneration and as expected, the medium concentrate, Concentration 2 provided the most accurate, strong, and definite results. Despite some statistical insignificance in data, this study still provides definitive results in demonstrating the trend of vitamin A properties in regeneration. As the properties and mechanisms behind vitamin A and tissue regeneration continue to be explored, its findings can add on to the understanding of human stem cells and provide groundwork in utilizing stem cell research to benefit human health.

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