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Animalization of Sea Urchin Embryos Using Zinc Chloride and Theophylline

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I. Introduction

Sea urchins are exemplary models for developmental biology principles. In a broad sense, embryonic movements and intraflagellar transport can be studied in sea urchins by monitoring ciliogenesis (Burns, 1973). The study of ciliogenesis in developing sea urchin embryos involves microtubule assembly and localization of microtubule proteins (Burns, 1973). Structurally, a cilium is microtubule scaffolding, which rests upon a centriole (Stephens, 1995). The centriole serves as the basal body of the cilium, connecting the ciliary outgrowth to the cell interior (Stephens, 1995). The microtubules are assembled in a 9 + 2 axoneme and encased in the plasma membrane (Stephens, 1995). The cilium is a cellular structure that contributes to the motility of the entire cell.

Cilia formation occurs in the mid to late blastula stage of sea urchin development and a single cilium grows on each blastomere (Masuda, 1979). In normally developing sea urchin embryos, ciliogenesis is initiated by the ninth or tenth cleavage (Masuda, 1979). A method for studying ciliogenesis in development is to "animalize" developing sea urchin embryos. Normal embryos at this stage in development have long apical tuft cilia at the animal hemisphere (Stephens, 1995). Animalizing embryos involves treating them with a zinc chloride solution (Stephens, 1989), the result of which is unusual elongation of the apical tuft cilia and non-apical tuft cilia localized throughout the animal hemisphere of the embryo.

Animalization of embryos does not occur in a sea urchin's natural environment and is thus experimentally created. Zinc ions must be added at the time of fertilization. In previous experiments, the purpose of animalizing embryos has been to isolate the elongated cilia to examine the expression of a microtubular protein, beta-tubulin (Stephens, 1995). It was found that transcription of beta-tubulin is upregulated in animalized embryos, suggesting that there is a mechanism within the ciliary structure that responds to the zinc chloride solution (Stephens, 1995).

The objective of this experiment is to be able to create animalized embryos and to image them using phase microscopy. It is possible to animalize embryos using both a zinc chloride solution and a theophylline solution. Theophylline is a chemical related to caffeine. It is hypothesized that these two solutions will give different resulting ciliary lengths at the time of imaging, thus making it possible to conclude that one is a better "animalizing" agent.

II. Materials and Methods

Set Up

Before starting the experiment, the following items were obtained: 0.5 M KCl, syringe, live sea urchins of the species Lytechinus variegatus, container of fresh natural sea water (FNSW), a Petri dish, 3 transfer pipettes, a 200-1,000 uL pipette, 3 small Eppendorf tubes. Two solutions, 1.0 mM zinc chloride (ZnCl2) and 1.0 mM theophylline, were made up before conducting the experiment (see Appendix A for a detailed explanation of the procedure used to make up these solutions). Also, a Nikon 80i microscope was found in the ICUC which was employed in imaging the cilia.

Fertilization of Sea Urchin Embryos

The first step taken to animalize sea urchins embryos was to obtain embryos. This was performed by injecting sea urchins (of the species Lytechinus variegatus) with a 0.5 M KCl solution (Allgood, 2004). This salt solution initiated the urchin to shed its gametes because it a high salt environment signals to urchins that a storm or other environmental change is taking place (Allgood, 2004). Urchins were shed in this manner until eggs and sperm were obtained and put in separate containers (Allgood, 2004). Eggs were collected in a beaker filled with FNSW and sperm was collected dry and pipetted into an Eppendorf tube (Allgood, 2004). The eggs were then examined to determine their health and whether it would be wise to proceed with the experiment (Allgood, 2004). The sperm was activated by adding approximately ten milliliters of FNSW to dry sperm in a Petri dish (Allgood, 2004). Once the health of the eggs was determined to be good, the diluted, activated sperm was added to the egg Petri dish (Allgood, 2004). The dish was labeled with the time and date of fertilization and the researcher's initials (Allgood, 2004). After a few minutes time had elapsed, a sample was pipetted onto a slide and observed under the microscope for fertilized embryos (Allgood, 2004).

Animalization of Sea Urchin Embryos

If fertilization appeared normal in these embryos, then about four drops of fertilized embryos from a transfer pipette were placed into each of three separate small Eppendorf tubes. Solutions of Zinc Chloride and Theophylline were added to the developing embryos in the Eppendorf tubes and were made up to a final concentration of 0.5 uM. It should be noted here that the zinc chloride was extremely hydroscopic and had a great deal of water in the bottle. Also, the theophylline was extremely old and hadn't been used in many years. The Zinc Chloride came from Sigma Chemical Company (No. 1633). The Theophylline also came from Sigma Chemical Company (No. 3500). The embryos were kept at room temperature (about 25 degrees Centigrade). The time for ciliation had to be determined since this species was new to the researcher. After about ten hours, the ciliated blastulae were ready to be imaged.

Imaging of Animalized Embryos

The embryos were pipetted out of the Eppendorf tubes and observed under a microscope in brightfield under 40x magnification to determine their developmental state. If the embryos had ciliated at the point of observation, then the control and two experimentals were placed on separate slides using a transfer pipette and then had a cover slip placed over the top of them. The slide was then imaged using phase 1 microscopy on the Nikon 80i microscope. Once a healthy embryo was located on the 40x magnification, a picture was taken and a one-minute movie was shot. The image software used was Spot Software. Image J was the imaging program used to crop the images of the ciliated blastulae. The pictures and movies were saved to the hard drive of Zodiac and also saved to the ICUC server under 'Anabel and Erin'. The pictures taken were analyzed for ciliary length of the control, zinc experimental and theophylline experimental. Pixels were converted to microns with a 4.1 pixel per micron scale which was previously determined (Allgood, 2004). This scale was set in Image J by choosing the "Analyze" option from the top toolbar and from there choosing "Set Scale". In "Set Scale", 4.1 was entered into the "distance in pixels" box, 1.00 was entered into the "known distance" box and 1.0 was inserted into the "pixel aspect ratio" box. Microns (um) were set as the "unit of length". This set the scale for all lines that were drawn subsequently. In Image J, a line was drawn parallel to the cilium of interest and then the size was determined by choosing "Measure" from the "Analyze" menu. The measurement given was the length of that line in microns. The images were somewhat blurry, but a few cilia were able to be discerned. Ten of these cilia were chosen from each image (control, theophylline and zinc) and their lengths were measured. These ten lengths were then averaged to give an average length of cilia for each embryo and as a point of comparison between the embryos (Allgood, 2004). These averages were then put into a graph format (see Figure 4).

III. Results

This experiment was run three times on three different days. Originally, the eggs were fertilized in the test tube and then zinc chloride or theophylline was added. This yielded undesirable results because a good deal of the eggs were still unfertilized or lysed at the time of observation. An alternative method was developed where the eggs were fertilized in a Petri dish and then introduced to an Eppendorf tube containing the FNSW, zinc chloride or theophylline solution. The last run of the experiment yielded pictures of cilia on the embryos, as can be seen in Figures 1, 2 and 3.

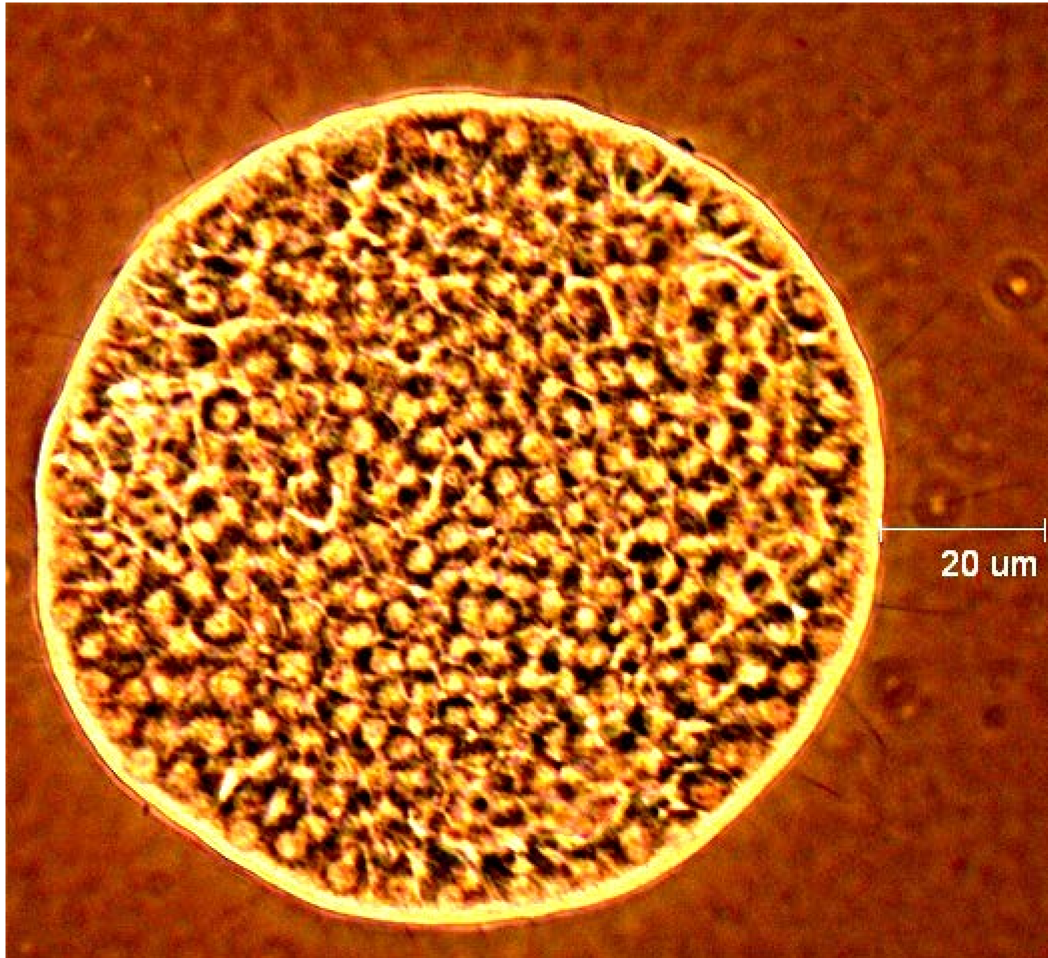

Figure 1: This is an image of a control embryo. This embryo was raised in FNSW at room temperature (25 degrees Celsius) for approximately ten hours. The cilia are slightly difficult to see, but are the darker lines projecting from the surface of the embryo. The scale bar shows 20 um which is roughly the lenght of the cilium above it. Note the varying lengths of all the cilia around the embryo.

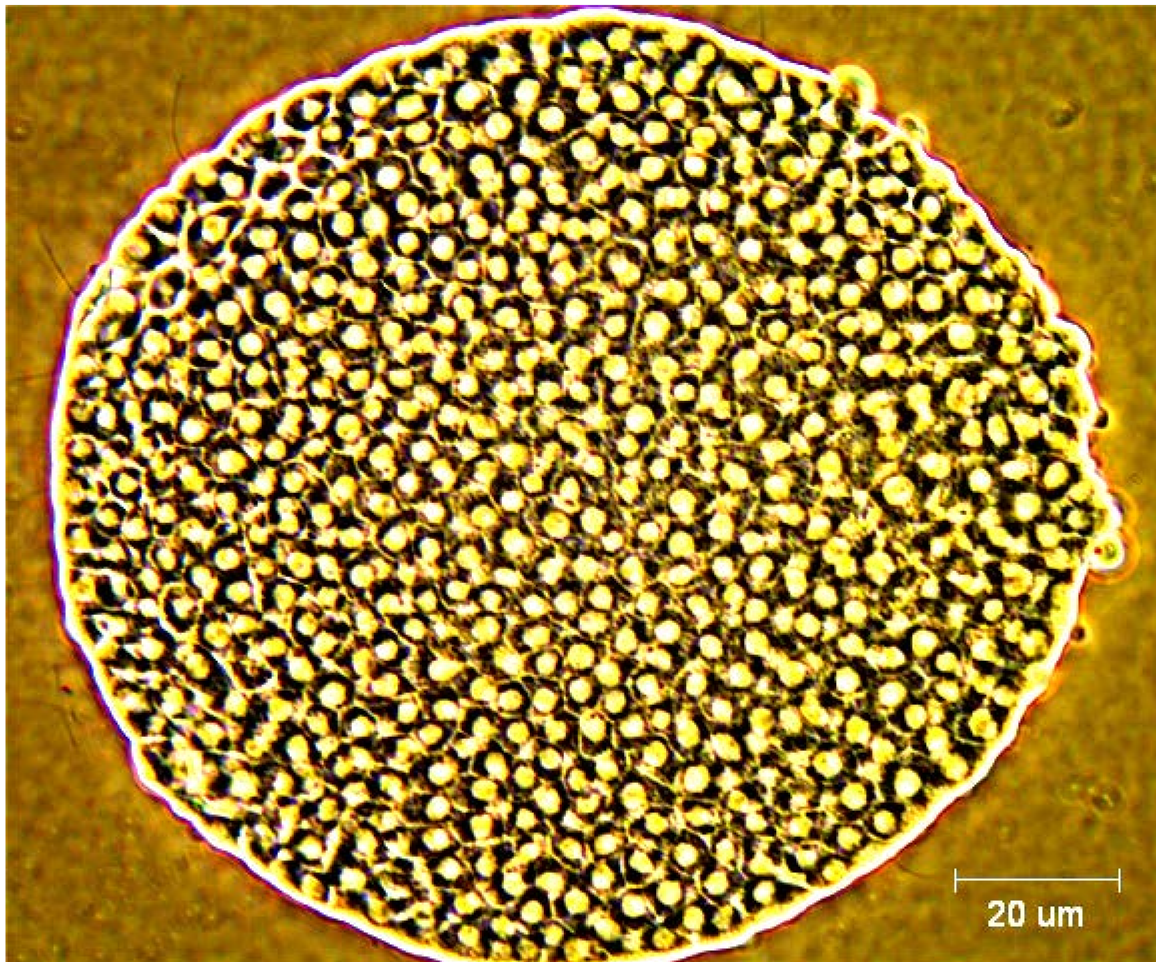

Figure 2: This is an image of a Theophylline treated embryo. This embryo was raised in a solution of 0.5 mM Theophylline at room temperature (25 degrees Celsius) for approximately ten hours. The cells at the right side of this figure are beginning to lyse. The cilia on this image are very faint, but there are a few present in the upper left hand corner. The scale bar in the lower right hand corner helps to place the actual size of the embryo.

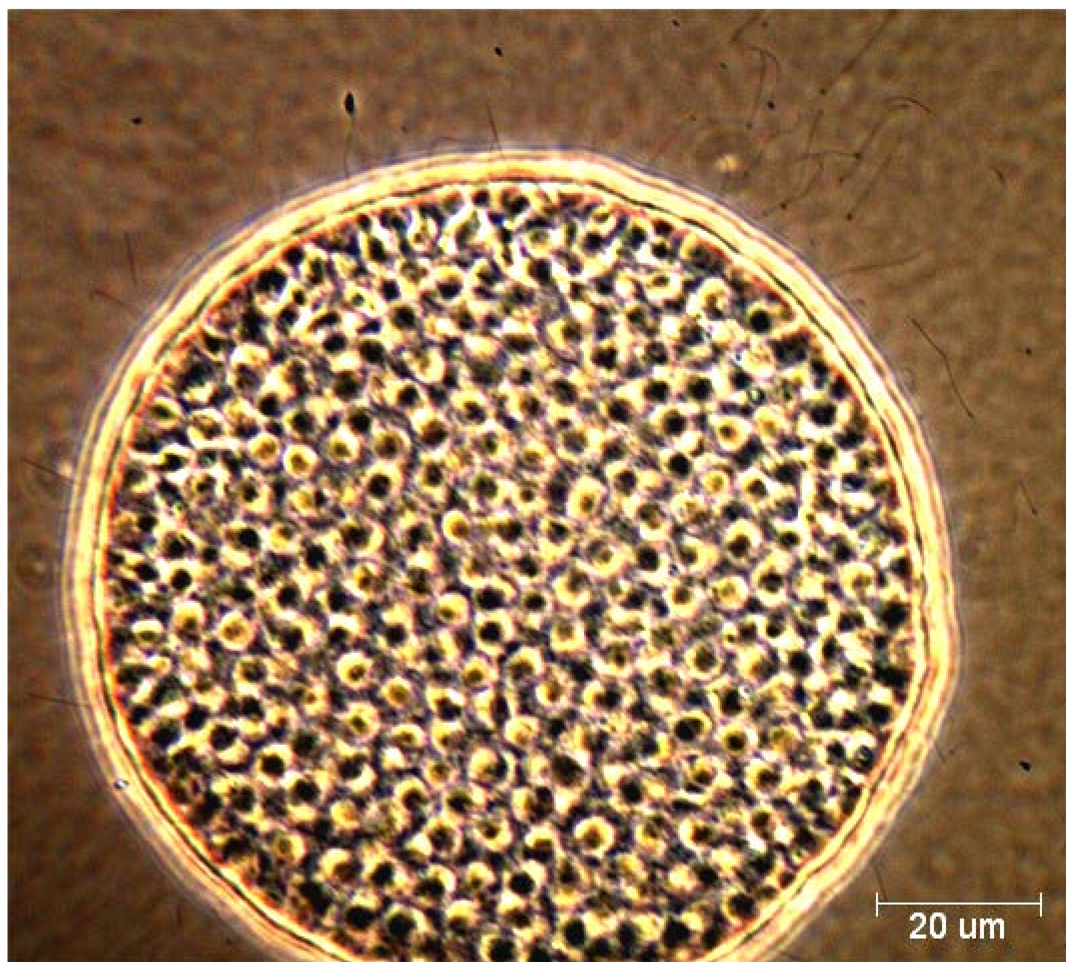

Figure 3: This is an image of a Zinc Chloride treated embryo. This embryo was raised in a solution of 0.5 mM Zinc Chloride at room temperature (25 degrees Celsius) for approximately ten hours. The cilia are more visible on this embryo. There appear to be some dismembered cilia diffusing away from the embryo in the right hand corner. The scale bar shows 20 micrometers and helps to place the actual size of the embryo.

Figures 1, 2 and 3 all show embryos at the mid to late blastula stage. Cilia are just starting to form on these embryos. The cilia can be seen as small, thin projections from the cell surface. There is a low quantity of cilia present on these embryos.

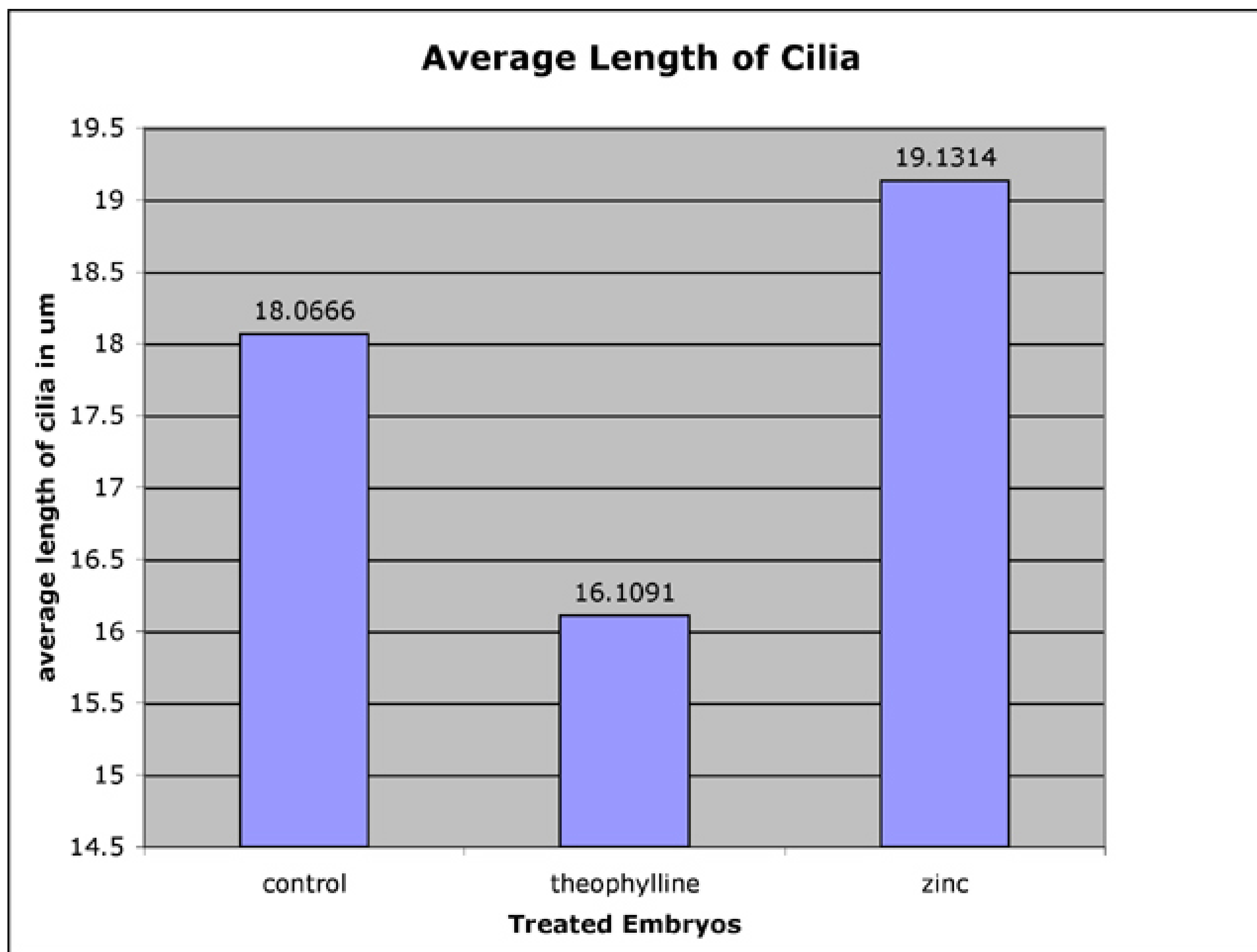

Figure 4: This graph shows the average lengths of cilia for each embryo. Ten cilia were chosen at random from each embryo and their lengths were measured and then averaged.

Figures 1, 2 and 3 all show embryos at the mid to late blastula stage. Cilia are just starting to form on these embryos. The cilia can be seen as small, thin projections from the cell surface. There is a low quantity of cilia present on these embryos. These pictures were taken with phase microscopy on the Nikon 80i microscope at a 40x magnification with a Spot camera and software.

IV. Discussion and Conclusions

Unfortunately, the data from the average ciliary lengths are inconclusive. The hypothesis that zinc chloride and theophylline treated embryos would yield longer, “animalized” cilia was not supported by the data. Animalized cilia are range from 27 to 75 um. The cilia observed in this experiment all showed an average length of less than 20 um. There are several possible reasons why this may have occurred.

The zinc chloride also was very hydroscopic and contained a great deal of water. The retention of water in the zinc chloride may have lowered the concentration of the zinc chloride, also making it less likely to produce a high enough concentration for animalized cilia to form.

A coverslip spacer was not used in observing the embryos under the microscope due to a mistake on the researcher’s part. It was believed that for such quick imaging a coverslip spacer would not be necessary. This could be a methods procedure change as the embryos were most likely crushed. Although this would not impact greatly the observable ciliary length, it is preferable to have intact, uncrushed embryos.

Also, enough time may not have elapsed to produce a properly animalized embryo. The embryos were spinning at the time of observation, but the time period was not worked out very well. It was estimated that the time to ciliation was roughly ten hours. In a future experiment, it would be necessary to wait eleven or twelve hours after fertilization to observe the Lytechinus variegatus embryos. This could account for the short cilia and lack of observable cilia covering the animal hemisphere. The embryo could have been caught at the time where it was just beginning ciliation and did

not have all of its cilia been forming at the time of observation. The time period for ciliation will vary and is dependent upon a preset number of cell divisions within a given species (Stephens, 1995) and that time period could only be approximated for Lytechinus variegatus due to a lack of available literature.

Once conclusive evidence is obtained concerning the different effects of theophylline and zinc chloride on sea urchin embryos, it would be possible to see if varying concentrations of theophylline and zinc chloride correlate to longer cilia or if there is a threshold concentration that embryos have before mortality sets in. It would also be interesting to investigate if the kinetics of ciliogenesis in theophylline and zinc chloride embryos change with concentration as well, showing that perhaps a better animalizing agents yields longer cilia.

On the whole, the data that was collected, although inconclusive, did suggest that the methods of growing the embryos up that were developed for this project did work by not killing the embryos. Unfortunately, the materials used (i.e. reagents and observation slides) and time period elapsed were two possible sources of error. Future experiments would entail getting brand new theophylline and zinc chloride, waiting longer than ten hours to observe the embryos and using a coverslip spacer.

V. Bibliography

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VI. Appendix A

How to mix up solutions of ZnCl2 and Theophylline

The molecular weight of ZnCl2= 136.28 g/mol. The molecular weight of Theo= 180.2 g/mol. A 1.0 millimolar solution of ZnCl2 and Theophylline is needed for a final concentration of 0.5 millimolar Zinc Chloride or Theophylline on the cells.

The final concentration needs to be 0.5mM, so a 1.0mM solution is suitable for the working solution. The total volume to use is 5ml of embryos in solution. So, if 2.5 ml of eggs in FNSW is used, then 2.5 ml of a 1.0 mM solution of ZnCl2 or Theo will end up at a concentration of 0.5 mM. If there are ten trials of the experiment, then only 25 ml of each solution is needed.

A 25 ml of 1mM solution of ZnCl2 and Theo for the working solution is needed.

Zinc Chloride:

136.28 g in 1000 ml = 1 M solution

For a 125 ml solution, divide 136.28 by 8: 136.28/8 = 17.035 g in 125 ml FNSW

For a 25 ml solution, divide 17.035 by 5: 17.035/5 = 3.407 g in 25 ml FNSW

For a 1.0 mM solution, divide 3.407 by 1,000: 3.407/1000 = 0.003407 g in 25 ml

Theophylline:

180.2 g in 1000 ml = 1 M solution
For a 125 ml solution, divide 180.2 by 8: 180.2/8 = 22.525 g in 125 ml FNSW
For a 25 ml solution, divide 22.525 by 5: 22.525/5 = 4.505 g in 25 ml FNSW
For a 1.0 mM solution, divide 4.505 by 1000: 4.505/1000= 0.004505 g in 25 ml

This is how the solutions were prepared to be at a concentration of 1.0 mM.