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Calcium's Effect on Cell Adhesion and Tight Junction Formation in Sea Urchin Blastulae

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I. Introduction

Sea Urchins are an ideal creature to study because they have an easy to observe and quick developmental process. After only two minutes following fertilization, the fertilization envelope can be seen, and after about an hour, the first round of mitosis occurs. Approximately 24 hours after fertilization, the blastula stage is reached (Vade Mecum 2 2003). A blastula is the name for an embryo once it has formed a hollow, fluid-filled cavity. Blastomeres, cells generated by cleavage, form a sheet and surround the blastocoel, the space in the middle of the embryo (Wilt and Hake 2004). Intercellular junctions hold these cells together and separate the outside seawater from the blastocoel. In the blastula stage, the cells are tightly adhered to each other with tight junctions and gap junctions (Wilt and Hake 2004).

Cells interact biochemically with other cells using receptors that bind to soluble signaling molecules on their cellular membranes. Cell adhesion molecules (CAMs) are a diverse set of proteins which are usually anchored to the cell membrane. CAMs hold cells together by having their receptors bound with another receptor on a neighboring cell. Binding of CAMs can be either homophilic (occurring between the same kind of molecules) or heterophilic (when two cells have different kinds of CAMs). Morphogenesis, the generation of shape, is driven by changes in cellular adhesion. Changing a cell's adhesion for the surrounding cells will have a profound effect on that cell's shape (Wilt and Hake 2004). Tightly adhered cells are squarer in shape, but cells that are not adhered to other cells or to the extra cellular matrix are "unzipped" and have a rounder shape.

The developmental process of cell adhesion and changing cell adhesion molecules is important for many reasons. First, the important epithelial to mesenchymal transformation occurs when the cell adhesion receptors for the basal lamina and for other cells are turned off. Epithelial cells adhere tightly to each other, but mesenchymal cells do not – they are surrounded by an extensive extracellular matrix. When you change the adhesion of a cell, you can change the location of that cell. Cell adhesion is also involved in the control of cell proliferation, and in addition to this, the penetration of a target cell by a virus is initiated by adhesive recognition (Bongrad 1988). Cell adhesion is such an important process to study because without morphogenesis, cells cannot change their shape, and advanced forms such as the blastula can not form.

Many CAMs, such as cadherins, are dependent upon calcium to function properly and bind homophilically. Cadherins are thought to be the most important molecules of adhesion in vertebrates, and they also have an important function in cell adhesion for many invertebrates. The expression and pattern of cadherin's during development plays a role in the morphogenesis of the tissues and organs. Once the tissues are formed, cadherin's are still important because

a loss of cadherin expression can cause some types of tumors (Boggon et al. 2002). There is little doubt today that calcium is important in cell adhesion, the question is just how. Many hypotheses have been made about the importance of calcium in cell adhesion, such as it having a binding function in tight junctions, that its depletion causes a separation of cells by its effect on the strength of the membrane, or that it provides stable cross links within the extracellular material of each cell (Trinkaus 1969). In any of these cases, the removal of calcium would cause the cells to dissociate, or come apart. It is not known which hypothesis is correct, but calcium's importance in cell adhesion could be due to a summation of its various activities.

I chose to study cell adhesion in sea urchin blastomeres because sea urchins are easily available, develop quickly, and produce beautiful images. I hypothesize that cell – cell adhesion and tight junction formation in the blastula stage of the sea urchin embryo, as measured by the average circumference of the embryo, the number of cells around the periphery of the embryo, and the average width of these cells, is dependent on extracellular calcium. In the blastula stage, the cells are adhered tightly together, so a lack in extracellular calcium will affect the CAMs. To carry out the experiment, sea urchin eggs and sperm will be fertilized in natural sea water. After reaching the blastula stage, the embryos will be placed in filtered natural sea water (the control) or calcium free sea water, and then imaged using an E200 light microscope. To quantify the data, image analysis using Adobe Photoshop will be used to determine the width of the cells around the embryo, and Image J will be used to determine the circumference of the embryo. The number of cells around the periphery of the embryo will be counted manually.

II. Materials and Methods

Materials:

Sea urchin eggs and sperm
Filtered natural sea water (FNSW)
Calcium free sea water (CFSW)
0.5 M KCl
Injection needle
Egg collection jar
Parafilm
Pipettes
Large test tubes
Glass slides
Calcium free seawater stock solution
1M EGTA
BTV Camera
Nikon Eclipse E200 microscope
Adobe Photoshop 7
Image J
Tweezers
Petri Dishes

Tests of PABA on development:

FNSW-PABA
FNSW with 0.5% p-aminobenzoic acid
Nitex mesh: 80 micrometer pore size
International Clinical Centrifuge
Pipette with rubber bulb
Pipetteman

Methods:

Shedding and Fertilization:

To induce the sea urchins to release their eggs or sperm, a female and a male urchin were injected with 0.5 M KCl into their mouth regions. The KCl caused the smooth muscles to retract, which induced the egg or sperm release. They were then shaken for 5 seconds. To collect the eggs, the female urchin was placed with its mouth over a jar full of FNSW – the eggs then settled at the bottom of the jar. Eggs from more than one female were used in this experiment. The sperm was collected by placing a male urchin over a piece of dry parafilm. Some of the sperm for this experiment was also collected by cutting open a male and removing one of its gonads with a pair of tweezers. The gonad was put into a small Petri dish and was squeezed with the tweezers a few times to release the sperm. The sperm was then activated in a 1:1,000 dilution of sperm in FNSW. As quickly as possible, one drop of the activated sperm was added to one milliliter of egg suspension. The fertilized eggs were then imaged under a light microscope on a slide to verify successful fertilization. Successful fertilization was determined by the presence of a fertilization envelope around the eggs.

Tests of PABA on development:

The original proposed experiment was to use fluorescent dextrans to measure the ratio of fluorescence of the inside of the blastula to the outside, but we could not get healthy cells after stripping the fertilization envelope (Procedure based off Tracewski 2003). To find out what was wrong with our procedure, we did one control and three other conditions with fertilized sea urchin eggs: a control of the eggs in FNSW, condition one with PABA in FNSW, condition two with PABA in FNSW and stripping with a nitrex mesh, and condition three in FNSW and stripping with a nitrex mesh.

To test the control and conditions, four large test tubes were obtained and a few ml of egg suspension was placed into each tube. Half of the volume of each test tube was then filled with FNSW. After this, a drop of sperm was added to each tube to fertilize the eggs. At this point, the control (eggs in FNSW) was covered and it was placed on its side for the embryos to develop. For conditions 1 (with PABA) and 2 (with PABA and net stripping), the formation of fertilization envelopes was observed by placing a drop of water from the tube on a slide on an E400 microscope at 10x. When most of the eggs had fertilized, a wash of PABA was added to the two test tubes. To make the PABA solution, .342 g of PABA was dissolved in 250 ml of FNSW.

In between the three washes with PABA for conditions 1 and 2, we used the International Clinical Centrifuge in the ICUC at speed 3 for one minute to make the eggs form a pellet at the bottom of the test tube. Most of the liquid was then sucked off the top of the test tube with a transfer pipette and then more PABA was added. This was repeated two more times to make a total of 3 washes with PABA. After this, FNSW was added to conditions one and two, and condition one was then put on its side for the embryos to develop.

Conditions 2 and 3 were then passed through a nitrex net two times to remove the fertilization envelopes. They were passed through an 80 micrometer Nitrex mesh using a transfer pipette to remove the fertilization envelopes off of the eggs. This length was used because it was slightly larger than the diameter of the sea urchin eggs (which is around 100 micrometers). After this, the eggs were washed in FNSW to wash away the removed envelopes. To confirm that the fertilization envelopes are removed, the eggs were viewed under a light microscope. At this point, conditions two and three were closed and put on their side for the embryos to develop.

Experiment:

A new batch of embryos was used for this experiment because it was performed the week following the tests of PABA on development. For this experiment, we put 3 ml of egg suspension in a large test tube, filled it three quarters of the way up with FNSW, and then added an ml of sperm. The experiment started once the embryos reached the blastula stage after 5 hours. Half of the eggs in the tube with the FNSW were taken out with a transfer pipette and put into the same size tube with CFSW in it. The other half was placed in a tube with just FNSW as a control. To make the CFSW, the following components were mixed together: 12.5 ml of the 4x stock solution, 37.5 ml FNSW, and .375 ml of 1M

EGTA (this was measured using a pipetteman). The two FNSW and CFSW tubes were closed and laid on their side for 15 minutes. After 15 minutes in the two solutions, three drops of the blastula suspension from each were placed on separate slides and the embryos were viewed and imaged under 10x on an E200 microscope.

Data Collection:

All of the data was collected after multiple images of the embryos had been taken from three drops of each of the FNSW and the CFSW tubes. First, the number of cells around the periphery of the embryo was counted. This was done by simply counting the cells manually. The outermost cells around the edge of the embryo were counted. The cells of seven different embryos were counted from the FNSW and the CFSW to get an average number of cells around the periphery for each.

Next, using Adobe Photoshop, the width of these cells was quantified. The rectangular marquee tool was used to draw a box that was the width of one of the cells. To get how many pixels wide this box was, it was copied and pasted into a new document. Then, going to “options” and “size” gave how many pixels wide the box was. Ten cells were measured for each the FNSW and the CFSW to get an average for each. No more than three cells were measured from any individual embryo.

The third set of data that was collected was the circumference of the embryo in FNSW and in CFSW. To do this, the multiple line tool in the program Image J was used to make a connected line all the way around the circumference of the embryo. Care was taken to measure any space in between cells. The circumferences of four embryos were measured this way in the FNSW, and the circumferences of five embryos were measured in the CFSW to get averages for each.

Measurement Conversion:

To change the measurements from pixels to micrometers in all of the quantifications (except the first one, the number of cells around the periphery of the embryo), a stage micrometer was used. A stage micrometer allowed us to determine the pixel dimensions of the images collected on a BTV camera on an E200 microscope at 10x. First, the image of the stage micrometer was taken on the same microscope also at 10x. This image was then opened in Adobe Photoshop and the rectangular marquee tool was used to highlight a region of known dimensions (600 microns in our case). Going to edit then copy and opening a new file allowed us to record the number of pixels wide the area was. To convert, I took this number and divided it by 600 micrometers to get a conversion factor of 1.62 micrometers/pixel.

To collect the results quantitatively for the tests of PABA on development, after 24 hours of development, from each control or condition tube, 3 drops of embryo containing solution were placed on a slide and viewed under an E200 microscope at 10x. The number of healthy and unhealthy embryos was counted from each of these drops. “Healthy” embryos were defined as normally dividing and developing embryos, and “Unhealthy” embryos were defined as embryos that did not divide or did not develop normally.

III. Results

Tests of PABA on development results:

The series of experiments on the effects of PABA on development showed a great deal about the effects of the PABA solution on sea urchin embryos. Between the control and the three conditions, there were large differences in the percentage of healthy embryos that developed after 24 hours. The differences in the development of the embryos could be clearly seen at 10x on an E200 microscope. The control group had the largest percentage of healthy cells (72%), but the two conditions with PABA (conditions one and two) had almost no healthy cells.

Percent of Healthy or Unhealthy cells in the tests of PABA on development

	Percent healthy cells	Percent unhealthy cells
Control 1	72%	28%
Condition 1	3%	97%
Condition 2	0%	100%
Condition 3	60%	40%

Table 1. This table shows the results from the tests of PABA on development. The overall percent of healthy and unhealthy cells, taken from all 3 drops from each test tube, is shown for the control and three conditions. For control 1, n = 43, for condition 1, n = 32, for condition 2, n = 16, and for condition 3, n = 72.

In condition one (with PABA in FNSW), the cells were either completely undeveloped, or severely deformed, as can be seen in figure 1. In condition two (with PABA and fertilization envelope stripping with the nitrex net), most cells had their fertilization envelopes removed successfully, but the cells did not develop normally; most simply never developed. In condition three, many of the healthy cells still had their fertilization envelopes around them, but quite a few were still healthy and had their fertilization envelopes removed.

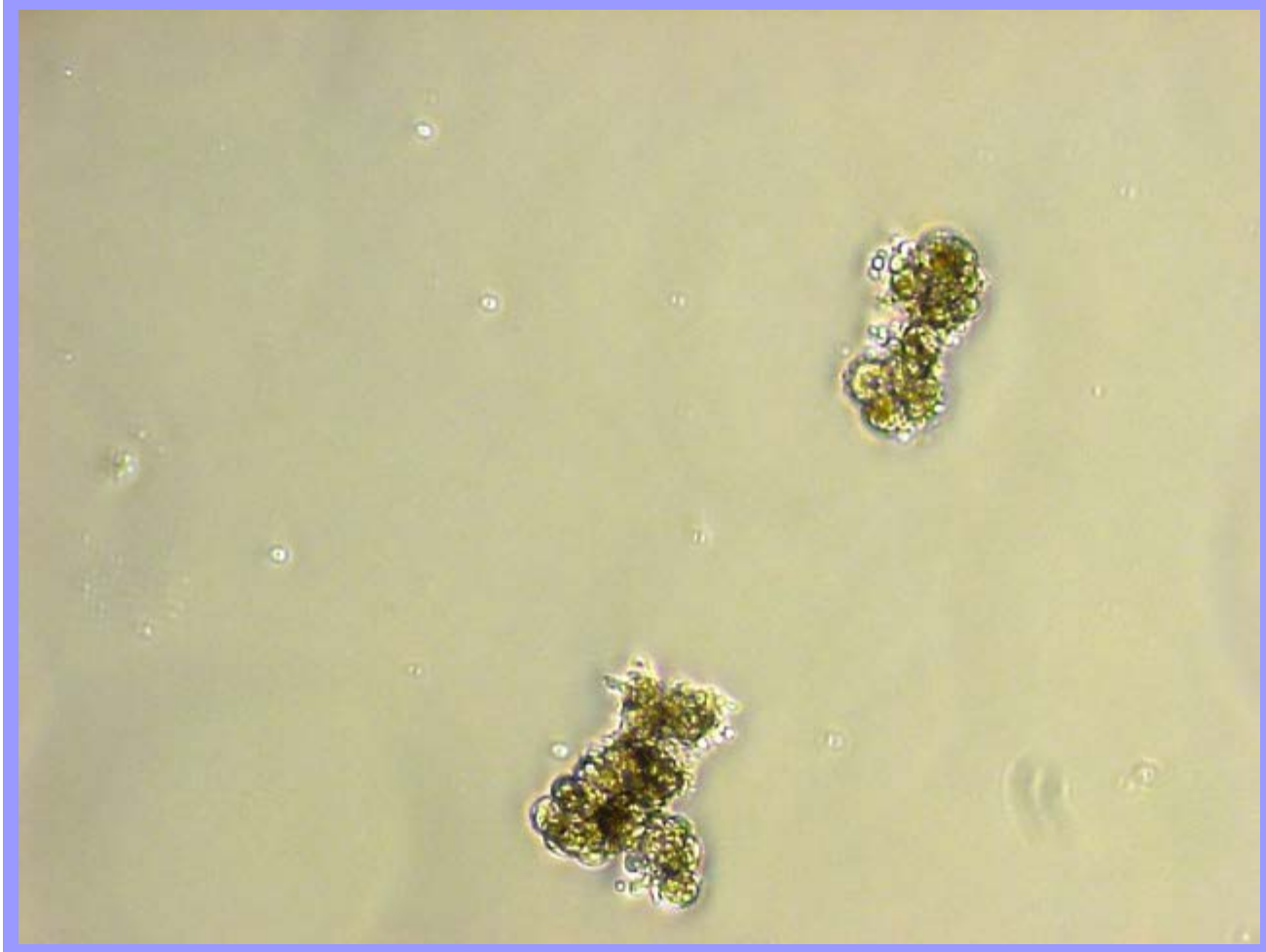

Figure 1. Severely deformed embryos from condition 1 (PABA in FNSW). This image was taken on an E200 microscope at 10x.

Experiment Results:

Putting sea urchin embryos in the blastula stage in either filtered natural sea water or calcium free sea water allowed differences in cell adhesion to be seen. Imaging the embryos at 10x using an E200 microscope made it easy to count the number of cells around the periphery of the embryo, measure the width of these cells, and measure the circumference of the embryo.

The embryos in FNSW looked considerably different from the embryos in CFSW after 15 minutes. Both of the embryos in FNSW and in CFSW looked healthy (neither were seriously deformed), but the embryos in FNSW had more cells around the periphery of the embryo and all of the cells were held tighter together. The cells from the embryos in CFSW looked a lot more spread out and were not held tightly together. In addition, these cells looked a lot rounder than the cells in the FNSW.

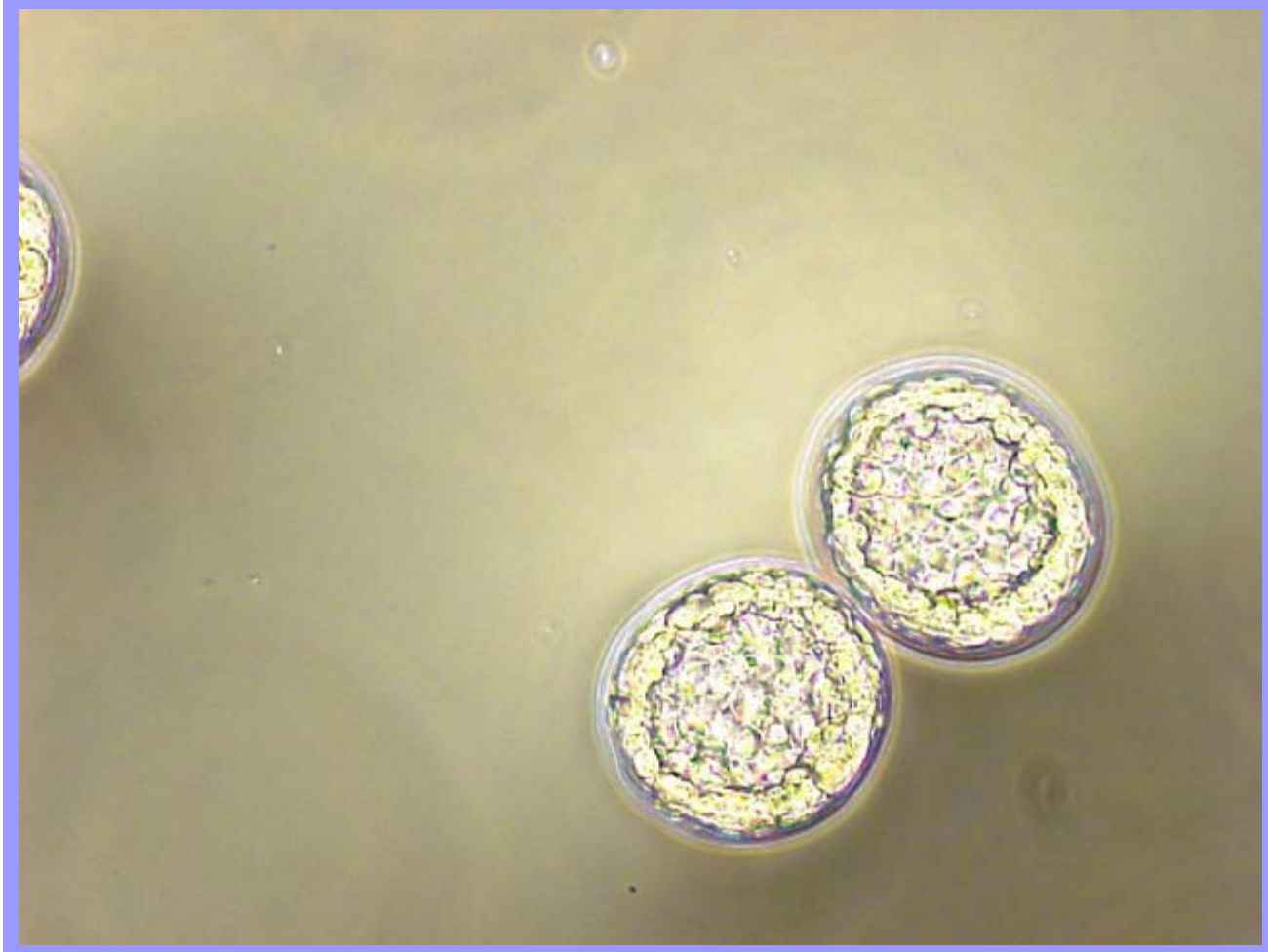

Figure 2. Two embryos in FNSW. The cells around the periphery of the embryo are held tightly together. This image was taken on an E200 microscope at 10x.

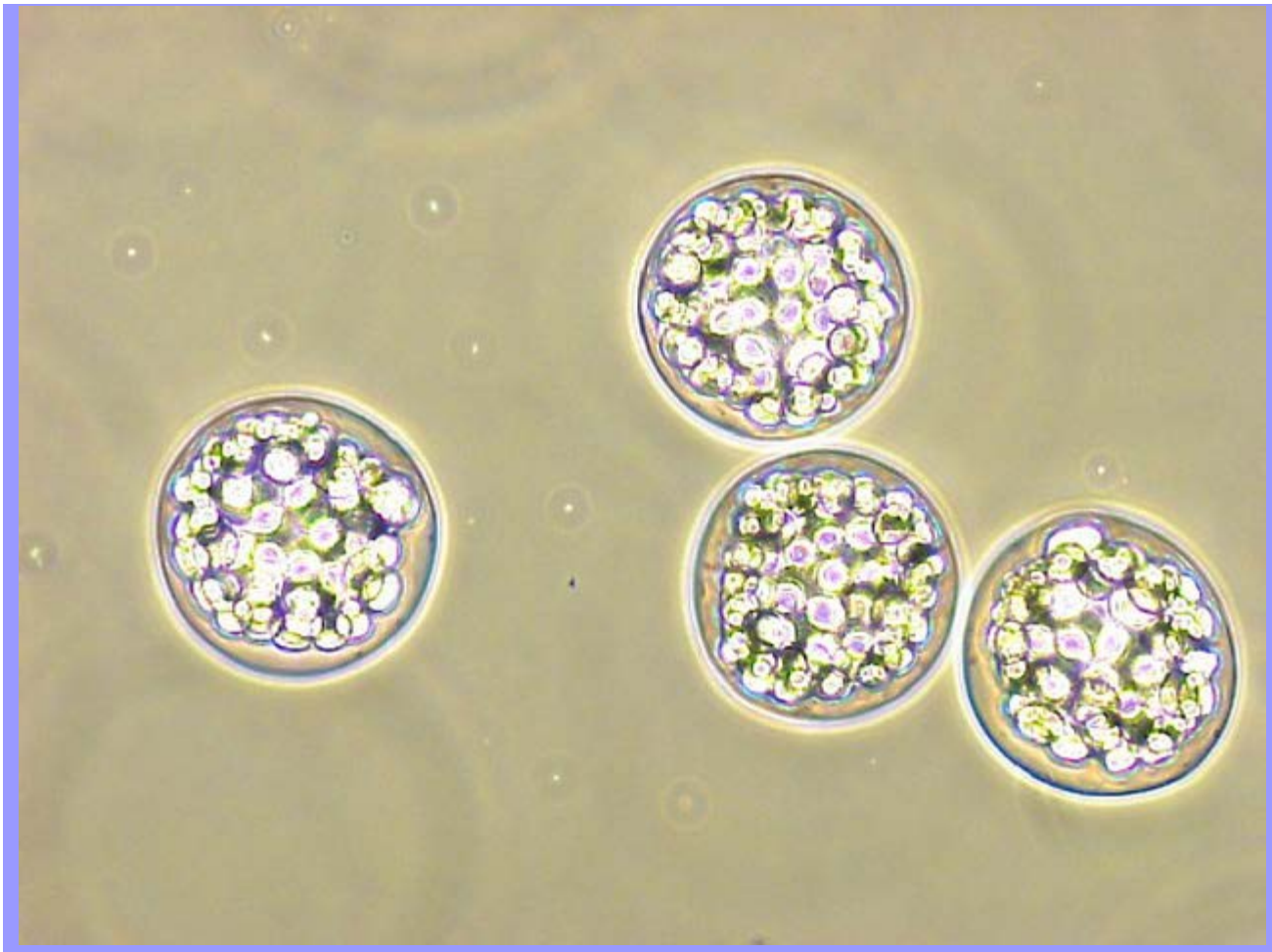

Figure 3. Four embryos in CFSW. The cells around the periphery and in the rest of the embryo are rounder and spaced apart, and the circumference of the embryo is dimpled, not straight. This image was taken with an E200 microscope at 10x.

Exposing embryos in the blastula stage to calcium free sea water for 15 minutes changed the number of cells that were around the periphery of the embryo. Calcium free seawater decreased the average number of cells around the periphery by an average of 10 cells.

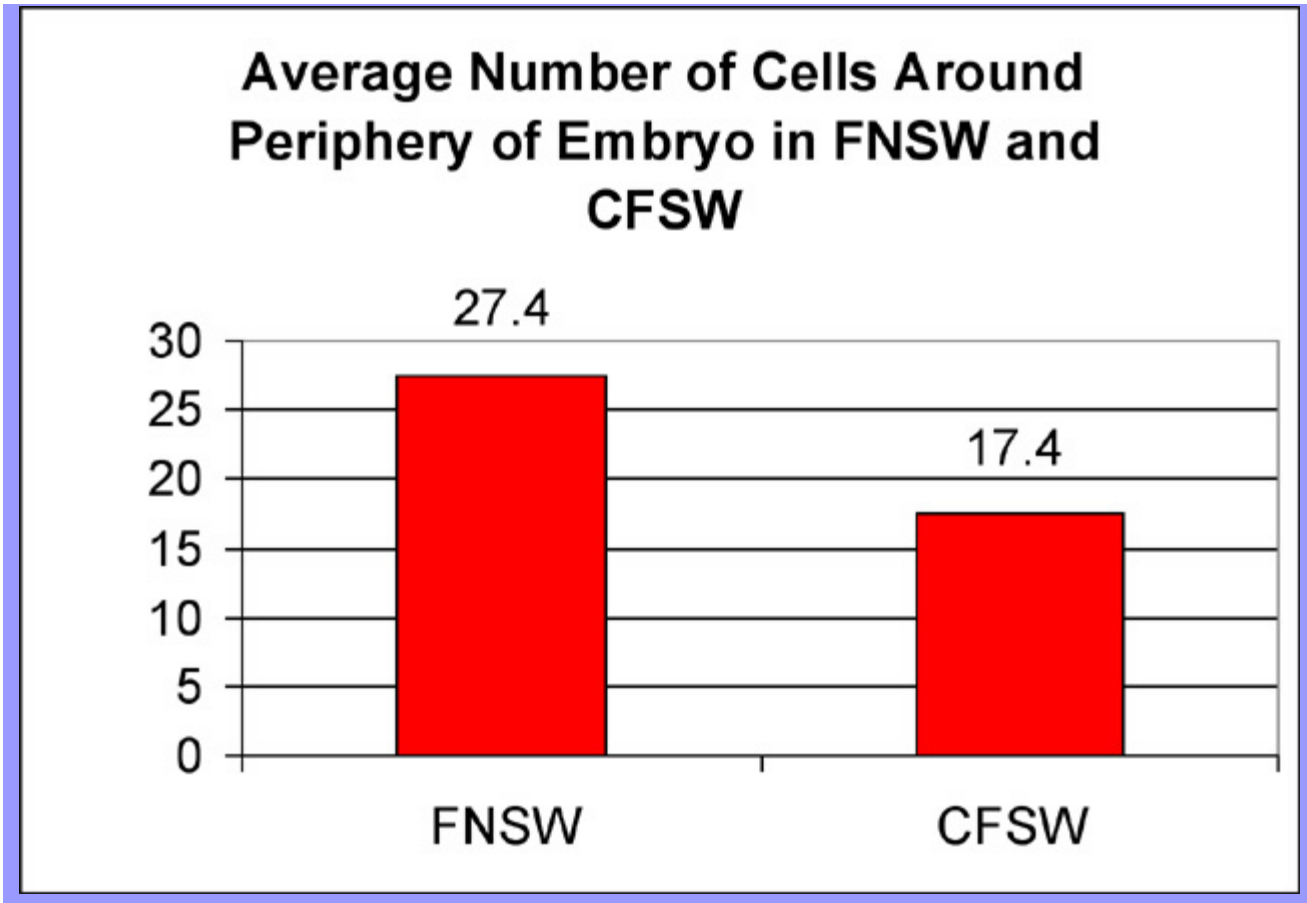

Figure 4. This graph shows the average number of counted cells around the periphery of the embryo in FNSW and in CFSW. Calcium free seawater decreased the number of cells around the periphery of the cell. For both FNSW and CFSW, n = 7.

In addition to decreasing the number of cells around the periphery of the cell, exposure to calcium free seawater for 15 minutes caused the average width of these periphery cells to increase by almost 20 micrometers.

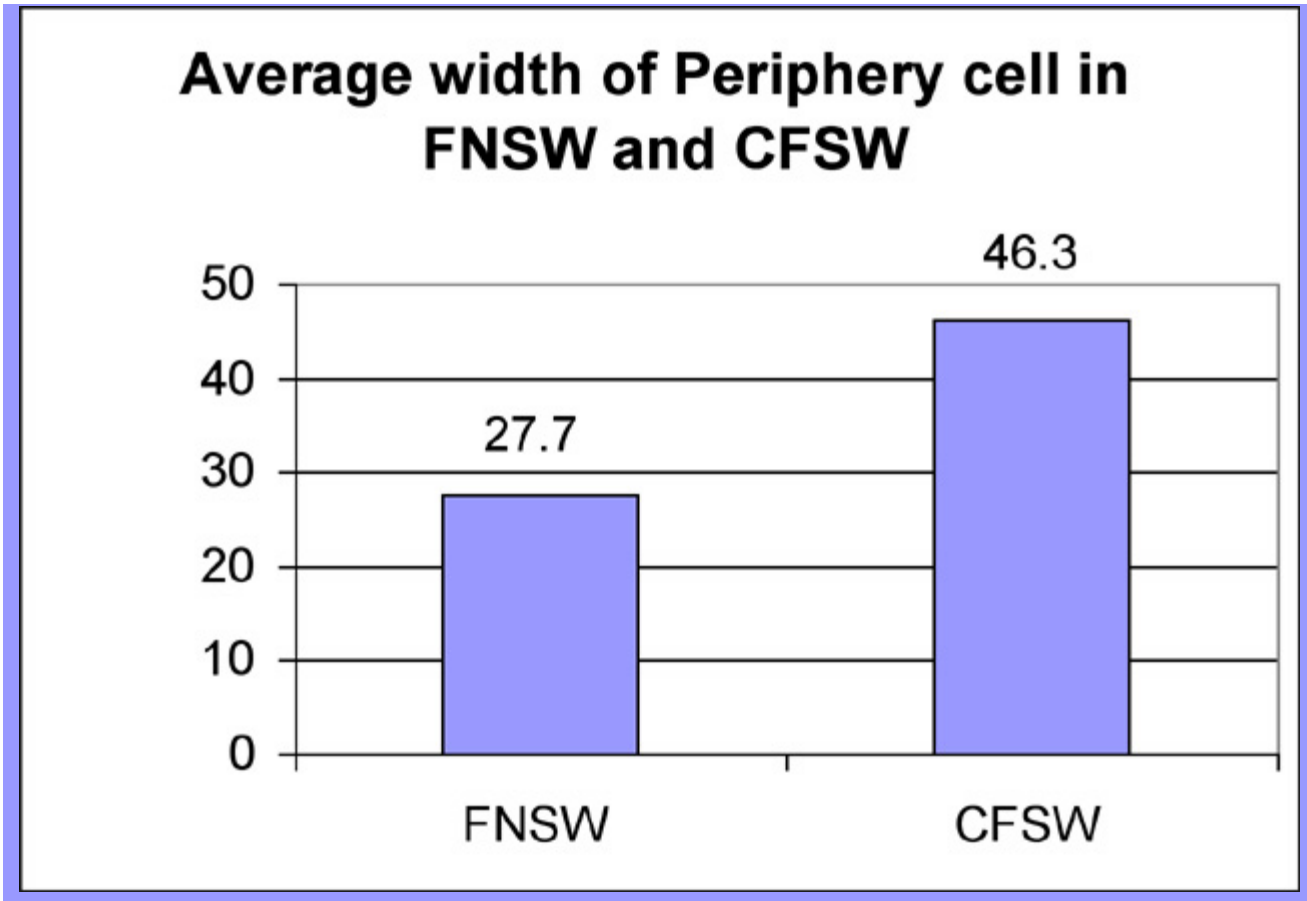

Figure 5. This graph shows that the average width of the periphery cells in increased CFSW. For both FNSW and CFSW, n = 10.

Exposure to calcium free seawater also increased the circumference of the embryo by almost 100 micrometers.

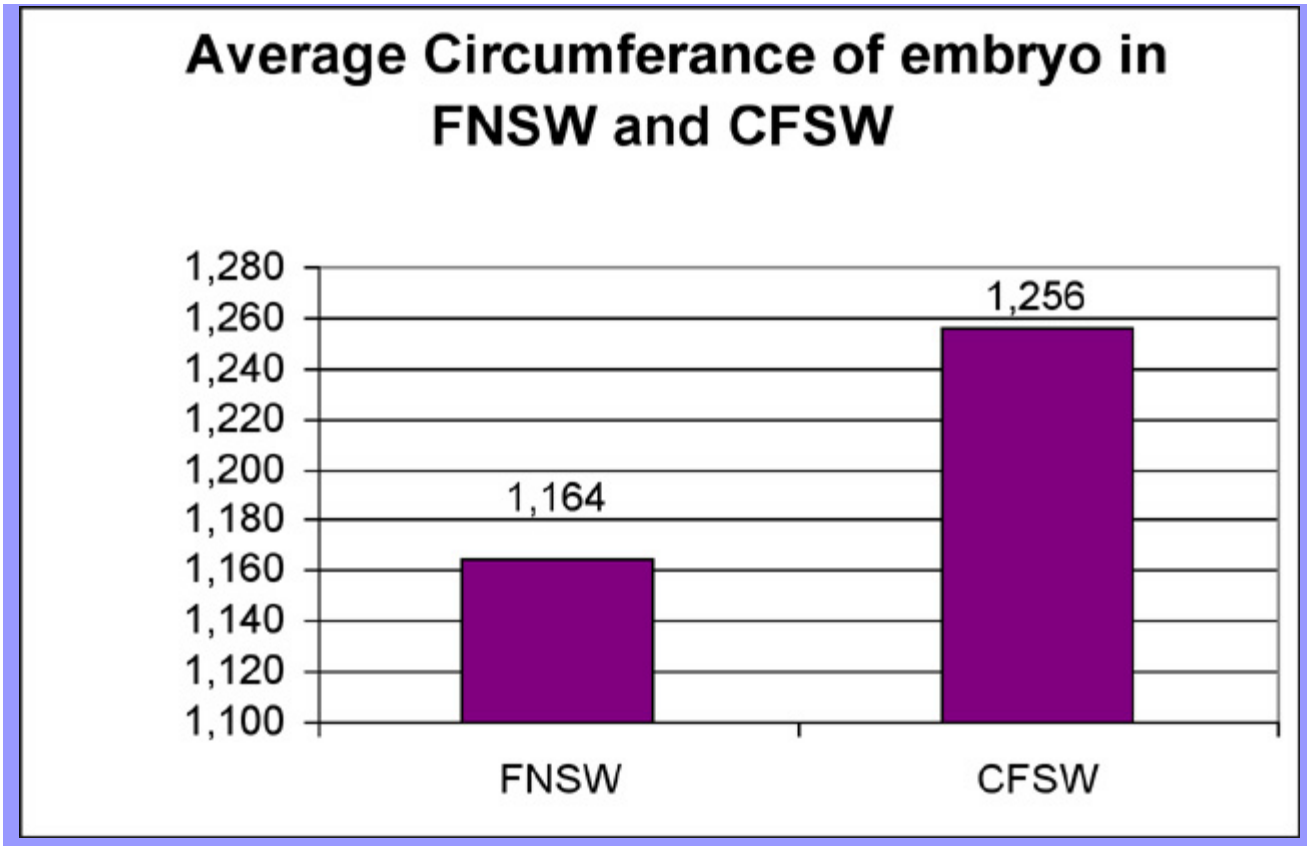

Figure 6. This graph shows that calcium free seawater increased the average circumference of the embryo. For FNSW, n = 4, and for CFSW, n = 5.

IV. Discussion and Conclusions

The first part of the experiment, the tests of PABA on development, was successful in evaluating what the factor was that caused all of the cells to die in our original experiment involving fluorescence. From the results of the PABA tests, it can be concluded that the PABA solution was the problem with the deformed cells. In the two conditions where PABA was added (conditions one and two), only 0% and 3% of the cells were healthy after 24 hours. The eggs and sperm themselves were not bad because the control in FNSW produced 72% normally dividing cells. In condition 3 where the cells were passed through a nitrex mesh, 60% of them looked healthy after 24 hours. This number is not as high as the control of 72% healthy cells, but it is clearly a lot higher than the controls involving PABA, so the net could not have been the factor that killed all of the cells. The net did decrease the number of healthy cells after 24 hours, but condition 3 still produced 60% healthy cells. Because the PABA solution was the factor that caused deformities in the embryos, we concluded that PABA should not be used in the experiment because something was wrong with it. Instead of stripping the fertilization envelopes with the PABA, it was decided the envelopes would be left on and two trials of the embryos in FNSW and CFSW would be conducted instead.

Results of our experiment indicate that cell - cell adhesion and tight junction formation in the blastula stage, measured by the average circumference of the embryo, the number of cells around the periphery of the embryo, and the average width of these cells, is dependent on extracellular calcium. By removing calcium and adding it, we have shown that calcium is both necessary and sufficient for cell adhesion. The process of changing cell adhesion molecules in the blastula stage is dependent on calcium because without it, the cells separate and lose their adhesion.

From the results of the experiment and the three quantifications, it is clear that the experiment worked. The embryos in the blastula stage being in the CFSW for only 15 minutes caused the cell adhesion and tight junctions to weaken. There were fewer cells around the periphery of the embryo in the CFSW because once cell adhesion is lost, the cells

move further apart and are not held tightly together. Some of the cells around the periphery of the embryo likely got pushed into the center of the blastula as cell adhesion was lost.

This same reasoning holds true as to why the cells in the CFSW had a larger width. When cells are held tightly together, their cell membranes are pushing against each other, and the cells are in more of a rectangular shape. When the cells lose their adhesion to each other because of a lack of extracellular calcium, the pressure from the neighboring cells is weakened, the cells become rounder, and thus become wider.

The average circumference of the cells in the CFSW embryos was larger because with lost cell adhesion in the calcium free sea water, little spaces formed between the round cells. When the cells were in FNSW, the periphery cells were held so tightly together that little space was able to form between them. Once there was not enough calcium to keep the cell adhesions and tight junctions intact, little dimples, or spaces, formed between the newly spaced apart cells. This extra periphery area caused the circumference of the embryo to become larger. These results confirm the hypothesis that cell – cell adhesion and tight junction formation in the blastula stage of the sea urchin embryo, as measured by the average circumference of the embryo, the number of cells around the edge of the embryo, and the average width of these cells, is dependent on extracellular calcium.

The main sources of error in this experiment had to do with our preliminary trials. We were not able to perfect a technique for stripping the fertilization envelopes off of the cells. One possible explanation is that we did wash enough of the PABA off of the cells once the envelopes were removed. Some extra PABA still surrounding the cells while they were developing could have caused some of the cells to die or develop abnormally.

To refine this experiment next time, I would do more trials of the embryos in the CFSW and the FNSW. The main part of the experiment could only be done once because the tests of PABA were done the week before. Because of this, all of the data came from only one day with only one set of eggs and sperm. In addition, I would find the exact time that the embryos reached the blastula stage to expose them to the CFSW. During this experiment, the embryos were roughly at the blastula stage, but it was not known if they had already formed most of their tight junctions or not. If the embryos had not formed many of their tight junctions yet, then taking away calcium would have less of an effect on the cells than if the tight junctions had been fully formed. To refine the preliminary experiment, I would use a less concentrated solution of PABA to try to remove the fertilization envelopes and I would make sure that I did more washes with FNSW to wash away the PABA.

For future experiments, I would try to find a way to remove the fertilization envelopes so the fluorescent r-dextran dye could be used. The r-dextran dye could be used to find a ratio of mean luminosity between the inside and outside of the embryo. The dye could tell us if there was lost cell adhesion between the cells; if there was space between the cells, the dye would leak out and the ratio of mean luminosity would be lower. A possible future experiment that could be based off of our experiment in this report is exposing the embryos to CFSW for different time intervals. The minimum amount of time that a blastula sea urchin embryo could be exposed to CFSW before cell adhesion is lost could be concluded from this experiment. The same quantifications used in this experiment could be used in the possible future experiment. As another experiment, it would also be interesting to see if cell adhesion in the blastulae was gained back after calcium was added back to the cells in the CFSW. The cells in the CFSW could be pipetted back into FNSW, and then the amount of time for cell adhesion to be regained could be concluded. An experiment where calcium is added back would provide further evidence that cell-cell adhesion and tight junction formation is dependent upon extracellular calcium.

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