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The Effects of Salinity on the Acrosomal Reaction in *Lytechinus Variegatus* Sea Urchin Embryos

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I. Introduction

In this lab we will be examining the effect that salinity has on the development of *Lytechinus Variegatus*, a warm water sea urchin species. We will use Rhodamine Phalloidin and Hoescht as means of highlighting actin filaments during the acrosomal reaction and during the first cleavage stage of mitosis by using the process of florescence microscopy. With an Epi-Fluorescence microscope and the process of Epi-illumination, which projects onto the sample from above thus causing it to glow, we will be able to highlight the actin filaments both during the acrosomal reaction and at early cleavage (Morris, 2004). Using the digital cameras that are attached to the microscope we will be able to photograph and observe the role of actin and how varying salinities affect the acrosomal reaction and the process of mitosis.

Actin is a globular protein with an ATP binding site in the center of the molecule. Monomers of actin polymerize to form long thin fibers that are approximately 8nm in diameter and are known as microfilaments. The actin filaments have multiple functions. Actin plays an important role in fertilization envelope lift off. The inner membrane of the egg is lined with actin filaments, which will be visible through the process of immunofluorescence microscopy. The acrosome in the sperm as well as the acrosomal reaction will be visible Rhodamine Phalloidin will highlight the actin (Wilt & Hake, 2004). Actin also forms a band beneath the plasma membrane that provides mechanical strength to the cell, links trans-membrane proteins to cytoplasmic proteins, anchors the centrosomes during mitosis and pinches the dividing cells apart during cytokinesis (Kreis, 1993). The Rhodamine Phalloidin will allow us to see this process by highlighting the actin filaments.

When sea urchin sperm are activated in proximity to unfertilized viable sea urchin eggs, the acrosomal filament, located in the foremost section of the head of the sperm, elongates and approaches the egg surface. Once the acrosome and the egg surface come into contact the structure of the egg cortex beings to change. The sperm head fuses with the eggs plasma membrane, pushing out the sperm pronucleus. At this point the cortical granules within the egg have exocytosed causing the formation of the fertilization envelope from the granules and the vitelline envelope. Once the sperm pronucleus completely enters the egg cytoplasm, fertilization envelope lift off occurs. The acrosomal filament of marine invertebrates contains long strands of polymerized actin (Wilt and Hake, 2004). The goal of these experiments is to highlight the actin during the acrosomal reaction and subsequently see how varying salinities affect the actin during the acrosomal reaction.

Aside from the acrosomal reaction, actin filaments carry out an important role during early stages of mitosis. Mitosis is a process of cellular division. After fertilization has occurred the sea urchin zygote begins a process of cell division to form the early embryo. The process of mitosis consists of six stages: interphase, prophase, metaphase, anaphase, telophase and cytokinesis. Interphase is the longest phase of mitosis during which DNA replicates, the centrioles

divide, and proteins are actively being produced. During prophase, the first mitotic stage, nucleolus fades, chromatin condenses into chromosomes and the microtubules within the cytoskeleton disassemble. The microtubules are used to form the mitotic spindle. Following prophase is metaphase. During metaphase the spindle fibers align the chromosomes along the metaphase plate. Throughout anaphase the spindle fibers shorten and the daughter chromosomes are pulled apart. Finally during telophase the daughter chromosomes arrive at separate poles and the spindle fibers begin to disappear. To end the cell division cytokineses occurs when the actin filaments pinch the cell membranes resulting in two identical daughter cells. The microtubules then reorganize and prepare for interphase again (Cells Alive, 2004). In this lab the cells will not be allowed to mitosis completely but will be fixed by the process of methalation immediately after the cleavage furrow forms. The first stages of mitosis will be observed under different salinities to see the effect that varying conditions have on the process of mitosis, most specifically the role of actin.

Salinity is an important variable to test in sea urchin embryos because sea urchins live in tide pools that have varying salinity concentrations depending on weather. For example, when tide pools are exposed to extreme sunlight evaporation occurs and the salinity concentration increases. In contrast when tide pools are exposed to excessive rains the salinity decreases. Sea urchin embryos must be able to adapt to these constantly and drastically changing conditions. The average salinity for northeastern seawater is approximately 35 parts per thousand (ppt) (US Hydrology Investigation, 2004). The expected results of these experiments are that the sea urchin eggs will be less likely to successfully undergo fertilization and mitosis in higher salinities than in lower salinities. Because of this the actin will be less visible because the acrosomal reaction and the beginning of mitosis will not have been as successful. We predict that the eggs will be more sensitive to a higher concentration of salt than to a lower concentration.

II. Materials and Methods

-Eggs and Sperm from *Lytechinus Variegatus* sea urchins
-Potassium Chloride (KCl)
-Filtered Natural Sea Water (FNSW)
-Distilled Water
-Rhodamine Phalloidin
-Hoescht
-Poly Lysine
-Phosphate Buffered Saline (PBS)
-3%BSA PBS buffer
-3%BSA PBS buffer with Rhodamine Phalloidin
-3%BSA PBS buffer with Hoescht
-Slides and cover slips
-Sterile Pipettes
-Varying concentrations of sea water (33ppt as the control and 39ppt as the high salinity)
-Sea Salt
-Salinity Measuring Device
-Microscope for Fluorscence Microscopy

Procedure:

Creating the Salinities:

To create the different concentrations of filtered natural seawater you will dilute the FNSW to create a salinity of 33ppt and add sea salt to create a salinity of 39ppt. To dilute the FNSW to 33ppt add distilled water and continuously check salinity with a salinity-measuring device. To create the high salinity add sea salt and mix it in with the FNSW. Again, continue to test the salinity until it reaches 39ppt.

Shedding the Urchins:

In this lab we were provided with sea urchin gametes and thus did not need to shed the urchins. However, if you needed to shed them yourself you would inject the male and female sea urchin with KCl into a single gonapore. Sea urchins shed their gametes when every they are shaken up, for example during a hurricane. To mimic that setting to make the urchin shed quickly, wrap the urchin in a paper towel and very gently “shake” the urchin. Unwrap the urchin

and turn the female urchin upside down and place her on top of small glass dish filled with FNSW. She will then shed her eggs into filtered natural seawater (approx 33ppt). The process for shedding the sperm is very similar except that you keep them "dry" as to not activate them until you are ready. After injecting the male urchins gonapore with KCl, wrap the urchin in a paper towel and gently "shake." Shed the sperm onto parafilm. This way the sperm will not be activated which allows them to survive for a longer period of time. You will only need to shed each urchin once to get more then enough eggs and sperm for these experiments.

Checking the Viability of Eggs at Different Salinities. To make sure that the salinities you will be using will not immediately kill the eggs or cause them to lice you much check their viability in the varying salinities. Observe a drop of the eggs in the 33ppt FNSW on a glass slide under the microscope to make sure that the eggs are viable. Be sure to record all results in lab notebook. All of your eggs will e in 33ppt FNSW and therefore to observe them in the high salinity you must wash the eggs in the 39ppt FNSW. To wash the eggs pipette them into a test tube and then pipette out as much of the FNSW as possible without removing the eggs. Then add the 39ppt FNSW with a pipette into the test tube. Repeat 2-3 times. The eggs are not ready to be observed. Put a drop of the 39ppt eggs on a slide and observe under microscope. Check the eggs to make sure they are viable under the high salinity. Record results in your lab notebook. In order to insure that the eggs will be viable under each salinity, do three trials for the 33ppt FNSW and three trials of the 39ppt FNSW. Like checking the eggs for viability you must also check the sperm for viability. To do this stick the tip of a pipette into the inactivated sperm and then activate them on a slide with a drop of 33ppt FNSW. Observe the sperm under the microscope. Healthy sperm will be in great number and will be rapidly swimming around. Do the exact same thing for the next set, but activate them with the 39ppt FNSW. Observe them under the microscope to make sure the sperm are healthy and viable at a higher salinity. Record all results in your lab notebook. Like you did for the eggs, do three trials with the 33ppt FNSW and 39ppt FNSW to insure the viability of the sperm.

Preparing for the Cold Methanol Fixation:

To prepare to do the fixation you will need to have certain solutions prepared and ready to use. To begin with you will need to create a block solution. In this lab you will be using a 3% BAS PBS block solution. Obtain the BSA and PBS and mass out 1.5g of BSA into a weigh boat. Add the 1.5g of BSA to 50ml of PBS and repeatedly invert to mix. This solution will be your block that you will use in the multiple washings of the cells. Next obtain an additional 50 ml of PBS. You will use this also in the washings of the slides. While you are preparing the other parts it is a good idea to keep these solutions refrigerated.

Next you will need to obtain 7 Poly Lysine treated cover slips in a Petri dish. If they a have just been made let them sit for 30 minutes and then rinse them with distilled water and let them dry. Once you have your cover slips ready to go you will obtain sea urchin eggs and wash them in 39ppt FNSW as explained above. Also have a test tube with eggs in 33ppt FNSW. Label the test tubes that they are in as to not confuse them later when you are ready to do the fixation (Cole, 2003).

Doing the Cold Methanol Fixation:

Obtain cold methanol (MeOH) and a 6 well culture cluster. Pour MeOH into each well until it is about 3/4 full. Place the culture cluster into a –80 degrees Celsius freezer.

In these slides you will be observing the acrosomal reaction.

Using a pipette add a big drop of 33ppf FNSW eggs onto one of the cover slip. On a slide active sperm with 33ppt FNSW and add them to the cover slip with the 33ppt FNSW eggs. Immediately pick up the cover slip using a pair of forceps and drop it into the culture cluster. Do another replica of this cover slip because one will be used as a control and one as an experimental. Be careful not too touch the freezer walls as it is extremely cold. Be sure to draw a diagram in your notebook showing which cover slip is in which slot. Next pipette 39ppt FNSW eggs onto a cover slip. Activate some of the sperm with 39ppt FNSW and fertilize the 39ppt eggs on a cover slip. Using forceps drop into the cold methanol and shut the freezer door (Cole, 2003).

In these slides you will be observing early mitosis,

Activate some of the sperm in 33ppt FNSW. Fertilize the eggs that are in 33ppt FNSW on a slide. Place the slide under a microscope and observe. You will want to make two slides again, as one will be for your control and one for your experimental. In this one you will be looking for early mitosis and thus you will have to wait approximately one hour before you can drop them into the methanol. You will know when mitosis is beginning because the cleavage furrow will appear. Activate some of the sperm with 39ppt FNSW. Fertilize the eggs that are in 39ppt FNSW with these sperm on a slide. Be sure to keep in eye on the slide as to prevent the slides from drying out. If need be add more FNSW of the corresponding salinity to keep wet.

Once the cleavage furrow forms immediately remove the slide and pipette the 33ppt fertilized eggs onto a treated cover

slip and drop into the methanol. Do this for two cover slips. Next pipette the 39ppt fertilized eggs onto a different cover slip and drop into methanol.
Let cover slips sit in the methanol for at least 2 hours to allow complete fixation to occur. If need be this cover slips can sit in the methanol over night.

Washing the Cells and Adding the Dyes:

Once you are ready to continue, remove the culture cluster from the –80 degree freezer. Wash the cover slip 3 times with PBS buffer by pipetting the methanol into a waste beaker and adding PBS buffer into the culture clusters. Then wash them with 3% BSA in PBS to block using the same procedure. Next, obtain 6 pedestals for your cover slips and place them in a large Petri dish. Remove cover slips from culture cluster and place on pedestals. You are now ready to add the dyes to the cover slips. Do this in as dark a room as possible as the dyes are photosensitive. Pipette 600 microliters of 3%BSA in PBS into a tube. Add 30 microliters of Rhodamine Phalloidin. Pipette 100 microliters of block plus Rhodamind Phalloidin onto the 39ppt cover slip and onto one of the 33ppt cover slip. BE SURE TO LEAVE ONE COVER SLIP WITH NO DYE. Place cover slips on pedestals in Petri dish (Cell Biology Education, 2003). Place in a dark place for 1 hour. After 1 hour you will stain the cover slips with Hoescht. Prepare 50mh/ml dilution by adding 900 microliters of block to 50 microliters of Hoescht. Pipette enough Hoescht onto each cover slip to cover it and let sit for 15 minutes in a dark place (Cooper lab. 1997).

Creating and Sealing Slides:

To create slides, pick up the cover slip and place if face down onto a clean slide. Paint around the edges of the cover slip with nail polish to seal. Do this to all slides. Label each slide. Place in refrigerator (Morris, 2004).

Doing the Fluorescence Microscopy:

To do the fluorescence microscopy you will need to use and Epi-Fluorescent microscope. To begin make sure the computer connected to the microscope you are using is turned off. Then turn on the mercury bulb. The bulb should click once and then ignite. Listen for this click so you know that the bulb lit properly. Let bulb run for 10 ready to begin observing the slides under the fluorescent lights. Rinse slides with distilled water and dry with a Kim wipe. There are multiple setting for the fluorescent lights and you will want to become familiar with the different settings for each dye.
It will be much easier to see the slides under the fluorescent lights if the overhead lights in the room are turned off. Begin by using your negative control for the acrosomal reaction in 33ppt FNSW. Observe slide under regular transmitted light at 4X. Jump magnification up to 40X. Look for cells. Place a cap over the transmitted light and observe under UV-2A filter. Look for visible fluorescence. Under this filter blue highlighting should be visible. This is the Hoescht stain. Switch the filter to the green light and look for red fluorescence. This is the Rhodamine Phalloidin stain. Observe any fluorescence you see and note its shape. You will want to make sure that what you are seeing is actually cells. Dirt is fluorescent under these lights so it may be confusing. However, eggs should be easily recognizable and sperm heads are very uniformly shaped.
Repeat this procedure for each slide in the following order: 33ppt acrosomal reaction, 39ppt acrosomal reaction, 33ppt mitosis negative control, 33ppt mitosis, 39ppt mitosis (Cell Biology, 2003). Record all data in notebook and take a picture of each slide, even if cells are not visible.
Print out the pictures and label them and place them in your notebook. Analyze your data in notebook.

III. Results

Although the procedure outlines the steps for both mitosis and the acrosomal reaction the results of this paper will focus specifically on the acrosomal reaction. On the 33 ppt negative control for AR cells were visible both under transmitted light and under fluorescent light. Many fertilize eggs were visible under transmitted light and when we switched to fluorescent light sperm heads and the egg surface glowed. These results meant that on the 33 ppt negative control slide for AR the poly lysine worked and that the fixation was successful. Also the multiple washings were not too vigorous as to wash all of the cells away. Next upon examination of the 33 ppt AR experimental slide under transmitted light there were no visible cells. There was no fluorescence highlighting under either of the fluorescent lights. These results mean that the washing of this slide may have been too vigorous and thus all of the cells were washed off. It is also possible that the poly lysine and methanol fixation were not successful on this particular slide. Lastly, upon examination of the 39 ppt AR experimental slide no cells were visible under transmitted light however upon switching to fluorescence that highlighted Rhodamine Phalloidin a red highlighted object appeared. It appeared

as an egg, however there were no visible sperm under any light, which makes is difficult to analyze. In conclusion it is possible that the fixation worked, and then the multiple washings were too vigorous for all of the cells to adhere but that not all of them were washed off.

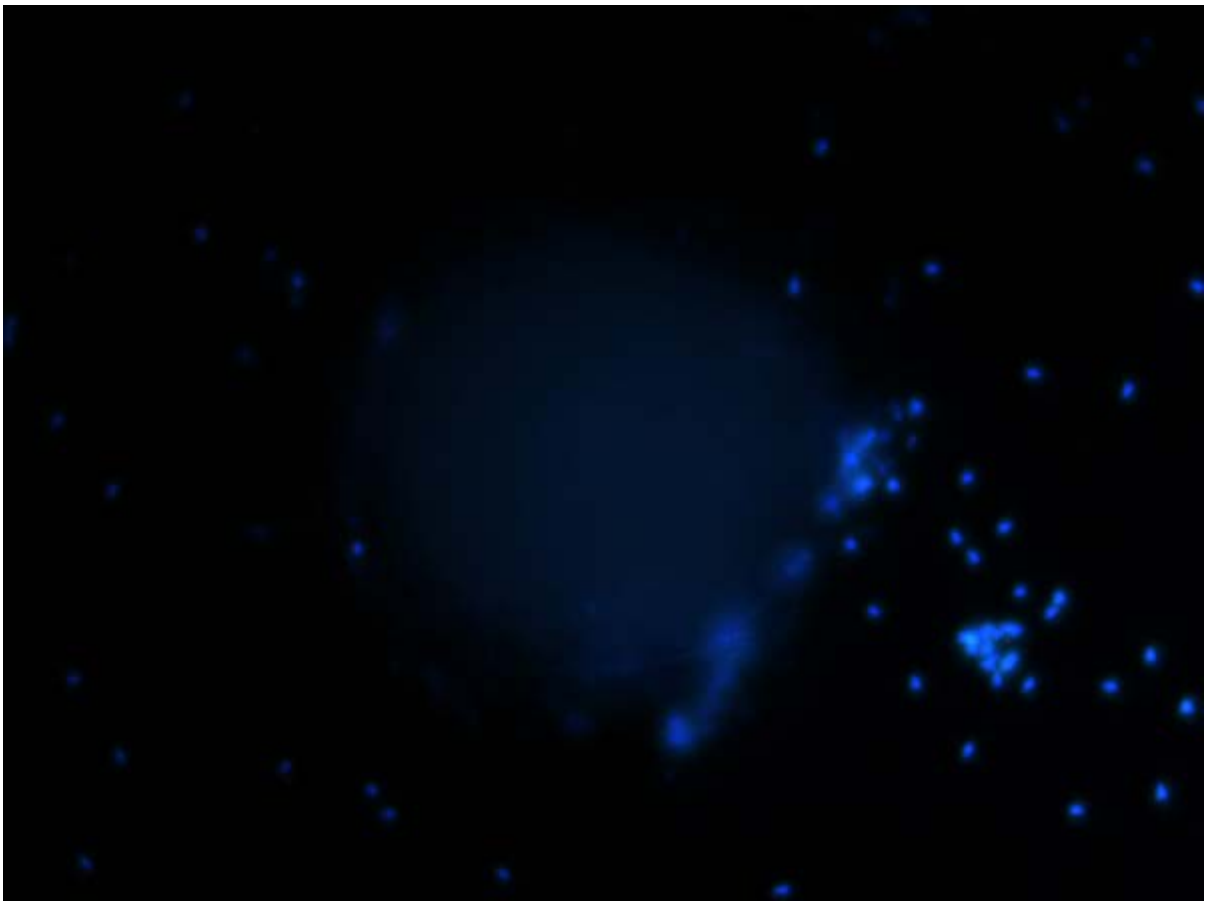

Figure 1: 33ppt Negative Control Acrosomal Reaction. There is lots of visible fluorescence of sperm heads and egg surface.

Figure 2: 33ppt Acrosomal Reaction. There were no visible cells on this slide.

Figure 3: 30ppt Acrosomal Reaction. The Rhodamin Phalloidin highlighted the surface of an egg, however no sperm were visible.

IV. Discussion and Conclusions

In this lab it was difficult to analyze whether the data would support the hypothesis because there was not sufficient data to analyze the effects the salinity would have on the AR. The data did not support the hypothesis however this was not due to unexpected results but due to the lack of result that would allow us to analyze salinity There was a large margin for error in this lab due to the complicated procedure. With each step there was a risk of losing the cells. From the results that we did obtain we can hypothesize as to what went wrong. To begin with, the poly lysine coated cover slips may not have made the cells adhere and thus the cells washed off into the cold methanol. Secondly the cold methanol fixation may not have fixated the cells. However the most likely place where error occurred was with the multiple washings of the cover slips with buffers and block. The washings appear to have been too vigorous and thus washed the cells off of the cover slips. Any of these errors would have led to the results that we obtained. In order to achieve better results in future experiments there are steps that should be changed. To begin with, we only had one trial for each different salinity. This left no way of comparing slides of the same salinity or reducing the chance of error by having more slides. It would have been more effective to have three trials for each different salinity. Also it would be important to be incredibly careful and gentle when washing each cover slip. This would help to prevent the cells washing off. Lastly, on each cover slip one should put as many eggs as possible. This will increase the chances of cells adhering to the cover slip. The procedure for this lab is an accurate procedure, however with a few changes the results of this lab would easier allow an analysis of ones hypothesis.

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