

Patterns of Apoptosis in the Feet of Chick Embryos at Day 7 and Day 9 of Development Using Nile Blue A Sulfate Staining

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I. Introduction

The formation of free digits in amniota vertebrates requires a massive amount of programmed cell death (apoptosis) in the interdigital mesoderm. Prior to the onset of apoptosis, the interdigits are covered by ectoderm and consist of a mesodermal core rich in blood vessels with complex extracellular matrix scaffolding. During apoptosis in chick embryo limbs, the degeneration of interdigital mesoderm cells, along with the subsequent disintegration and regression of the extracellular matrix and the blood vessels occurs (Zuzarte-Luis, et al., 2002). The detachment of ectodermal tissue, which is released into the amniotic fluid, is also experienced by the developing embryonic limb. The area in which apoptosis occurs in the vertebrate autopod is termed the interdigital necrotic zone (INZ), and is essential in sculpting the shape of the digits (Zuzarte-Luis, et al., 2002). In addition to the INZ, early amniotes contain anterior and posterior necrotic zones, which have been related to the reduced number of digits in chick embryos. A normal chick embryo contains three digits in each wing and four digits in each leg. Also, it is important to note that the term “necrotic,” which is used to define the different zones, is a holdover term from the time period when no distinction was made between necrotic cell death and programmed cell death. The cell death occurring between the interdigits in chick embryos does not occur by necrosis, but rather as a result of genetic programming. A dramatic decrease in a cell’s level of DNA, RNA, and protein synthesis is experienced by the cell during the time period between when a cell’s death is determined to time of the actual death (Gilbert, 2000). Specifically, the apoptosis of cells in the interdigital tissue is associated with the fragmentation of the DNA in the cells. The death of specific cells in a vertebrate limb is genetically programmed and has been selected for during evolution (Gilbert, 2000).

The triggering signal for apoptosis in the chick embryo autopod most likely consists of a combination of bone morphogenetic proteins (BMPs). BMP’s were originally described as proteins involved in the formation of bone and cartilage cell tissues. In the developing chick autopod, BMPs induce mesenchymal cells to either undergo apoptosis or to become cartilage-producing chondrocytes (Zuzarte-Luis, et al., 2002). The effects of BMPs are dependent on the stage of development and the age of the target cells, and are crucial in the formation of limbs. It is thought that the mechanisms controlling apoptosis in the INZ are fairly similar and related to the mechanisms regulating the proliferation and differentiation of the cells. BMPs are known to trigger apoptosis in limb mesodermal cells and in the ectoderm of the apical ectodermal ridge (AER). The AER is a ridge of tissue delimiting dorsal from ventral at the tip of

the developing limb bud, which becomes more prominent as the limb bud grows.

According to patterns of gene expression, three BMPs are thought to be responsible for triggering apoptosis in the limb bud, BMP 2, BMP 4, and BMP 7. Each of these three BMPs are each expressed in the interdigital mesenchyme, and blocking BMP signaling through the use of a BMP antagonist results in the inhibition of interdigital apoptosis. Since the expression of these three BMPs is present throughout the progress zone mesenchyme, it is speculated that without active BMP suppression, apoptosis occurs as a default state (Gilbert, 2000). Suppression of BMP may naturally occur from the Noggin protein, which is made in the developing digits, and the perichondrial cells surrounding them. Noggin is known to bind to BMP's in the extracellular space, consequently preventing the activation of BMP receptors. When Noggin is present throughout the limb bud, apoptosis does not occur (Gilbert, 2000).

In order to examine the patterns of apoptosis occurring in the developing limbs of chick embryos, Nile Blue A sulfate (NBS) staining was used. NBS uptake only occurs in dead or damaged cells, allowing the color of healthy cells to remain unaltered. This dye is extremely useful in observing patterns of apoptosis because of its selectivity, and also for the dark stains produced, allowing a clear distinction between stained and non-stained cells during microscopic analysis. In order to obtain developing limbs, chick embryos were explanted from their shells at various stages of development and were immediately stained and viewed under a microscope. The chick embryos were ideal organisms to use when examining apoptosis primarily because of the formation of digits arising from the limb buds. Chick embryos contain fairly defined limb structures, which allows for a more precise identification of the stain's location. The rapid rate of chick embryo development was also a benefit in this experiment. In this experiment, the hypothesis tested was that if apoptosis occurred during digit formation in chick embryo feet, then the exposure of the whole embryo to NBS during different stages in development would result in the staining of all apoptotic cells associated with the formation of the digits. The methods used in this experiment were heavily modified from those found in various lab manuals. Methods development was a crucial part of this experiment and many of the modifications were made throughout the course of the experiment.

II. Materials and Methods

This experiment on apoptosis was conducted over a period of nine days and involved the staining of chick embryos during two different stages of development. Eight eggs were incubated intact at 37 degrees C. During day 7 of development, five of the eggs were taken out for staining. The remaining three eggs stayed in the incubator until day 9 of development, which is when the second staining session took place. Each staining session lasted about 90 minutes while observations of staining results generally lasted close to 60 minutes. A Nikon E200 compound microscope was used in indentifying and observing stain patterns. Controls were not used in this experiment due to a shortage of eggs containing live and healthy embryos. There should have been four controls and four experimental embryos. For each staining session, two control embryos and two experimental embryos should have been used. The controls should have been explanted using the same method used for the experimental embryos. The control embryos also should have gone through the same steps as the experimental embryos from the beginning of incubation to viewing and analyzing, with the exception of one step. The controls should have been excluded from soaking in the stain solution occurring in Petri dish "2" of the staining procedure. The purpose of the controls would have been to compare rates of development, and also to show that the NBS was responsible for any staining patterns produced. Since the NBS was added to a prepared solution of HBSS, the experimental controls could have shown that the HBSS possessed no staining properties, and that all staining observed was derived solely from the NBS (Carlson, 2004).

Making the Nile Blue A Sulfate Stain Solution

In order to make a 1:1000 solution of Nile Blue A sulfate in HBSS, a saturated solution of NBS in distilled water was first made. A plastic 50 ml centrifuge tube with a screw cap was filled with 20 ml of distilled water, and the Nile Blue sulfate powder was added in very small quantities until the solution was dark blue. The cap was then screwed on the tube and was rigorously shaken for a few seconds. The tube was opened again and more of the powder was added until a very heavy dark blue color persisted. The solution was assumed to be saturated when small clumps of dark blue

powder appeared on the sides of the tube after being shaken. The collection of undissolved NBS powder that settled to the bottom of the tube was also used in order to determine that the solution was saturated. Once the saturated solution of NBS in distilled water was made, it was then used to make a 1:1000 solution of NBS in HBSS solution. HBSS was used in staining the chick embryos at day 9 of development and the Tyrode's solution was used in staining the chick embryos at day 7 of development. The staining solution was made several times over the period of this experiment, but the final dilutions all remained the same. In order to make 20 ml of the stain solution during day 9 of chick development, a plastic centrifuge tube with a screw cap was filled with 20 ml of HBSS. Using a pipetman, 20 microliters of the saturated NBS solution in distilled water was added to the 20 ml of HBSS. The tube was shaken for a few seconds and was immediately used. This solution was stored in the refrigerator when not immediately used (Carlson, 2004).

Staining the Chick Embryos

The same method for explanting chick embryos was used for all the eight embryos. The embryos were explanted using sterile technique on to large sterile weighboats. The embryos were explanted using sterile metal forceps and small Styrofoam bowls for placing the broken bits of egg shell. The wide ends of the eggs were gently cracked with the forceps, and holes were gradually made by removing small bits of the egg shell at a time. The holes were about the size of the air-spaces at the wide-ends of the eggs, and the edges of the holes were made as smooth as possible. Once the edges were smooth, each egg was then flipped over a weighboat so that the hole was pointing down. Another small hole was then made at the top of each egg, which allowed the contents of the eggs to slide into the weighboats. In order to reduce the amount of injury on the embryos during this procedure, each weighboat was placed gently over the large holes before the eggs were flipped over. They then gradually followed the hole as the eggs were gently flipped over. This reduced the chance of damage to embryos by reducing the impact between the embryos and the weighboats. Once the eggs were explanted onto the weighboats, Pasteur pipettes were used to remove as much albumin as possible. The yolks were then gently popped and all yolk was removed. All traces of surrounding membrane were removed using forceps and all attached blood vessels were disconnected and removed. The prepared NBS stain solution and a small portion (about 10 ml for each embryo) of HBSS were then taken out of the refrigerator and put into the incubator for fifteen minutes in order to heat the solutions to the temperature inside the incubator. Once the solutions were warm, three Petri dishes were obtained. The first Petri dish (Petri dish 1) was filled with HBSS, the second (Petri dish 2) was filled with the NBS in HBSS (1:1000) solution, and the third (Petri dish 3) was filled with HBSS. Using forceps, the embryos were gently transferred from the weighboats to the first Petri dish of HBSS. The embryos were left in this dish for 30 minutes and were periodically moved carefully from side to side with forceps in order to remove all excess yolk and blood. After being soaked for 30 minutes, the embryos were then placed in the second Petri dish. The embryos were left in this solution for 45 minutes, and more of the staining solution was added if parts of the body were not completely submerged. After soaking the embryos in the NBS stain for 45 minutes, the embryos were then gently placed in the third Petri dish containing HBSS in order to allow all excess dye to be washed away. The embryos were soaked for another 30 minutes in this solution and were then prepared for viewing under the microscope (Carlson, 2004).

Preparing the Specimens for Viewing and Analysis

As soon as the staining procedure was finished, the embryos were taken to a Nikon E200 compound microscope for observations. A glass microscope slide and coverslip was obtained for each embryo being examined. Freshly stained embryos were kept in HBSS during preparation and prior to viewing in order to prevent the embryo from drying out. In order to examine patterns in apoptosis occurring in the feet, the legs were cut around the thigh area using forceps. The severed limb was then placed on the glass slide, and a cover slip was pressed over the specimen. The limbs were then observed for any blue staining patterns under both 40x and 100x magnifications. When patterns were found, images were captured and saved using BTVpro (Carlson, 2004).

Data Collection and Quantifying Observations

Images of all observed staining patterns present on the limbs of the chick embryos were captured and saved on the Wheaton College ICUC server. The images were then opened in the ImageJ program for measurements. For each image, the length of the staining pattern was taken using a tool measuring the number of pixels present along the length of the stain. Since two different magnifications were used, a picture of a ruler was taken at the two different magnifications. The two images were opened in ImageJ and the number of pixels between a millimeter at both 40x and 100x was determined. Using the calculated number of pixels in one millimeter at each of the two magnifications

allowed for the conversion of pixels to millimeters for each of the staining images. The lengths were then graphed using Microsoft Excel in order to examine the trends of staining lengths. The images were modified using Adobe Photoshop through the addition of arrows showing the location of stained cells (Carlson, 2004).

III. Results

All of the eight embryos used in this experiment were stored in the same incubator, and were put in at the same time. During day 7 of development, a total of five eggs were removed from the incubator for staining. The explanting of the chick embryos took place and the only eggs containing embryos came from two eggs that had no signs of dirt marks or shell damage. The other three eggs contained either ruptured yolks or no embryo. The two living embryos obtained were not similar in size. One of the embryos was noticeably smaller by about 2mm in height, and was surrounded by a thick dark yellow substance attached to the yolk. The limbs of the smaller embryo could not be removed and were not viewed under the compound microscope. The feet of the larger embryo did not contain noticeable digits and no signs of staining were observed.

At day 9 of development, the remaining 3 eggs were removed from the incubator and were explanted to expose the embryos. After the contents of the three eggs were explanted, one of the eggs had no signs of containing an embryo, and had no blood vessel formation present on the yolk. The two remaining eggs contained live embryos with one of the embryos considerably smaller in size than the other. This embryo had very short stubs which resembled limb buds, but had no visible sign of digit formation. The other embryo, which was tan in color, possessed feet with clear digit formation. After the two embryos were stained, the limbs of the smaller embryo were not disconnected from the body due to the size. The larger embryo was observed using a compound microscope, and contained dark stain patterns between the digits in the feet. Three different locations of staining patterns were observed, and images of these three locations were taken at both 40x and 100x magnifications. Figure 1 is an image of the left foot of the embryo at 40x magnification. The stained cells appear to follow a crease between two separate digits. This image is shown at 100x magnification in Figure 2 for greater detail of the stained cells. Another image of the stained cells on the other side of the left foot is displayed in Figure 3, which shows the image under 40x magnification. The stained cells once again appear to follow a crease between two digits. This image is shown at 100x magnification in Figure 4, which provides a more accurate location of the stained cells. Stained cells are also present in the right foot of the embryo. Figures 5 and 6 are images of the stained cells present in the right foot. The line of stained cells in these images also follows along a crease between two defined digits. The lengths of the stains found in the feet of all embryos examined are represented in Figure 7.

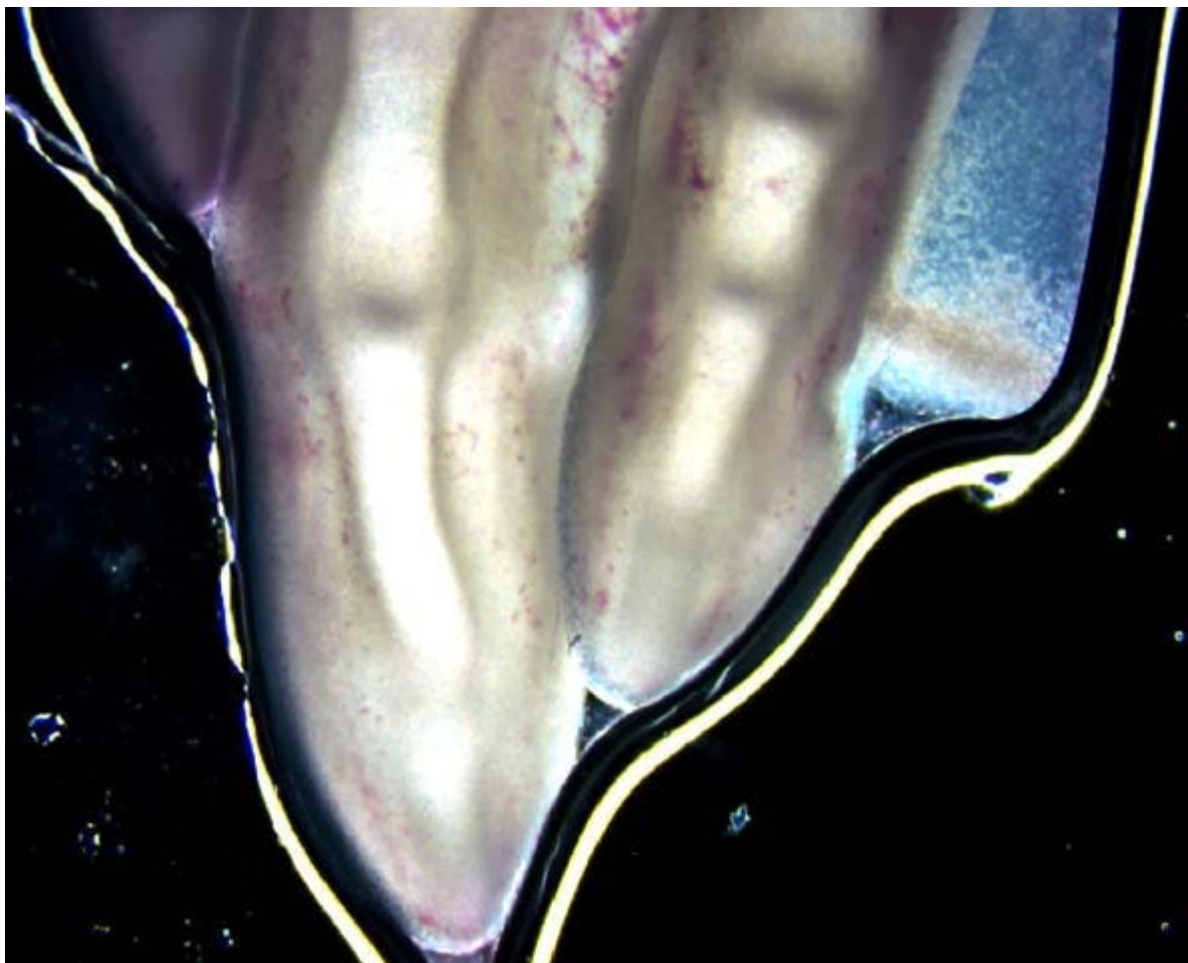


Figure 1 ? This is an image of a developing chick embryo foot at Day 9 of development. The left foot is shown at 40x magnification taken with a Nikon E200 compound microscope. This image shows shows the NBS stain pattern that occurred in the left foot of the embryo.

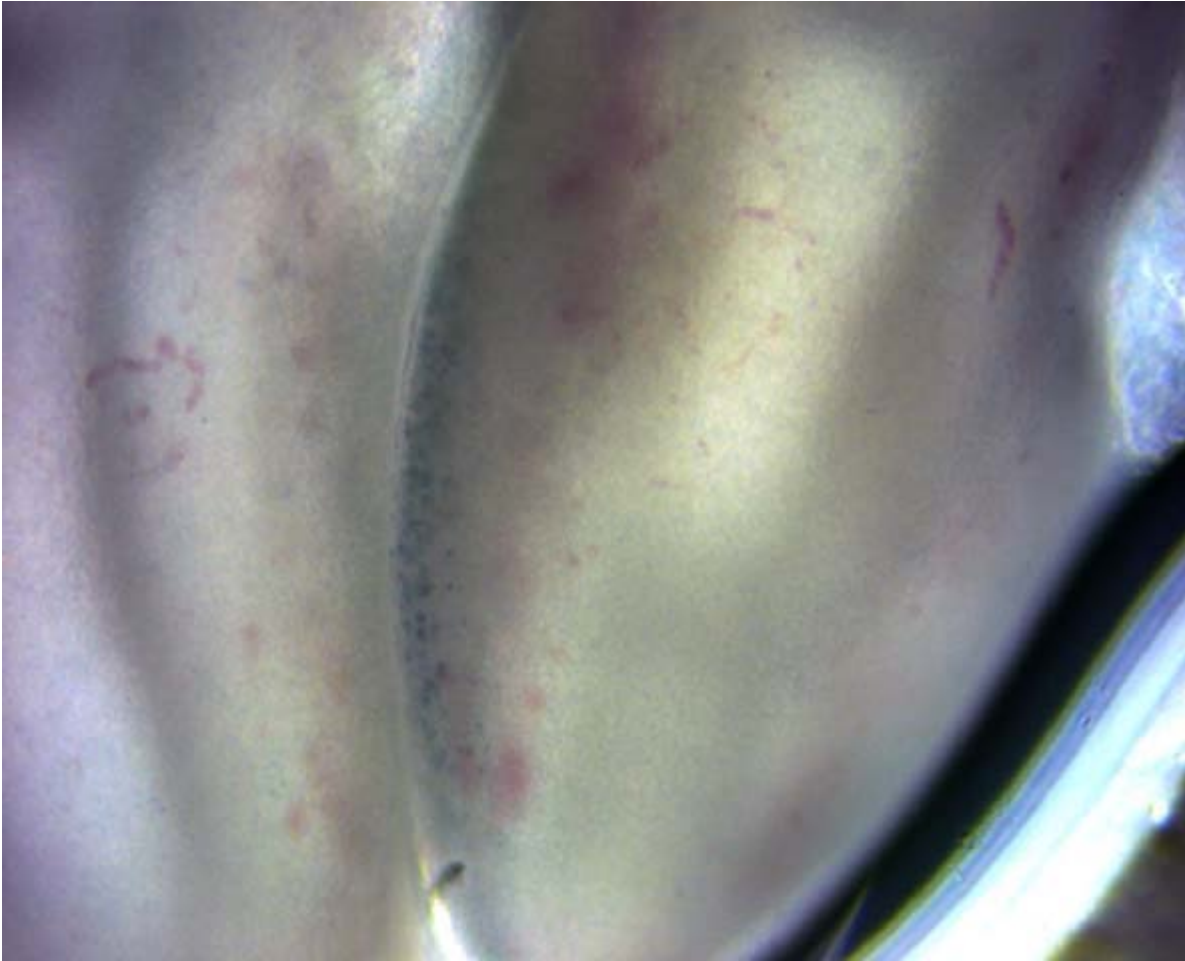


Figure 2 - This is an image of a developing chick embryo foot at Day 9 of development. The left foot is shown at 100x magnification taken with a Nikon E200 compound microscope. This image is a magnified version of the left foot depicted in Figure 1.

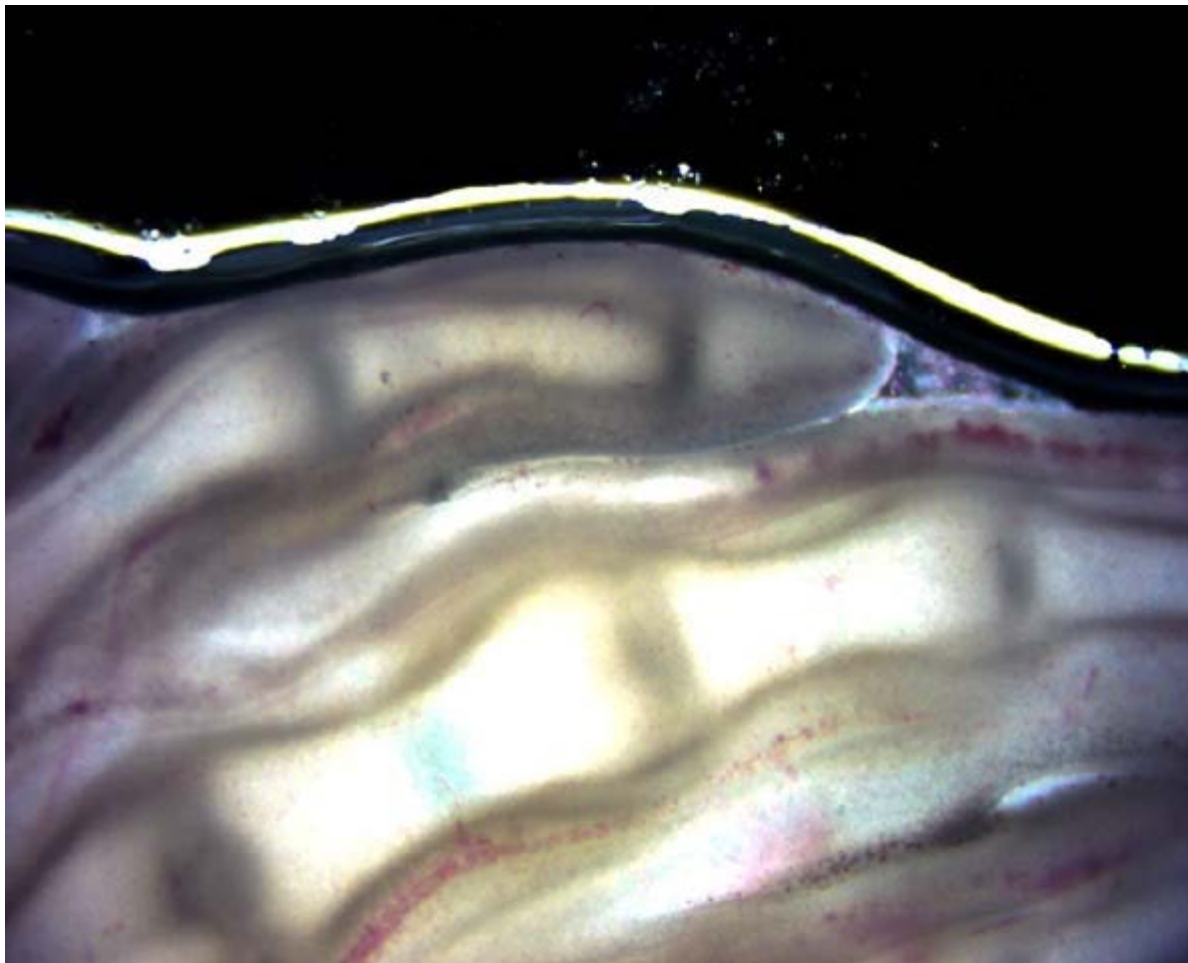


Figure 3 - This is an image of a developing chick embryo foot at Day 9 of development. The left foot (opposite side of Figure 1) is shown at 40x magnification taken with a Nikon E200 compound microscope. This image shows a NBS stain pattern found in a different location of the left foot.

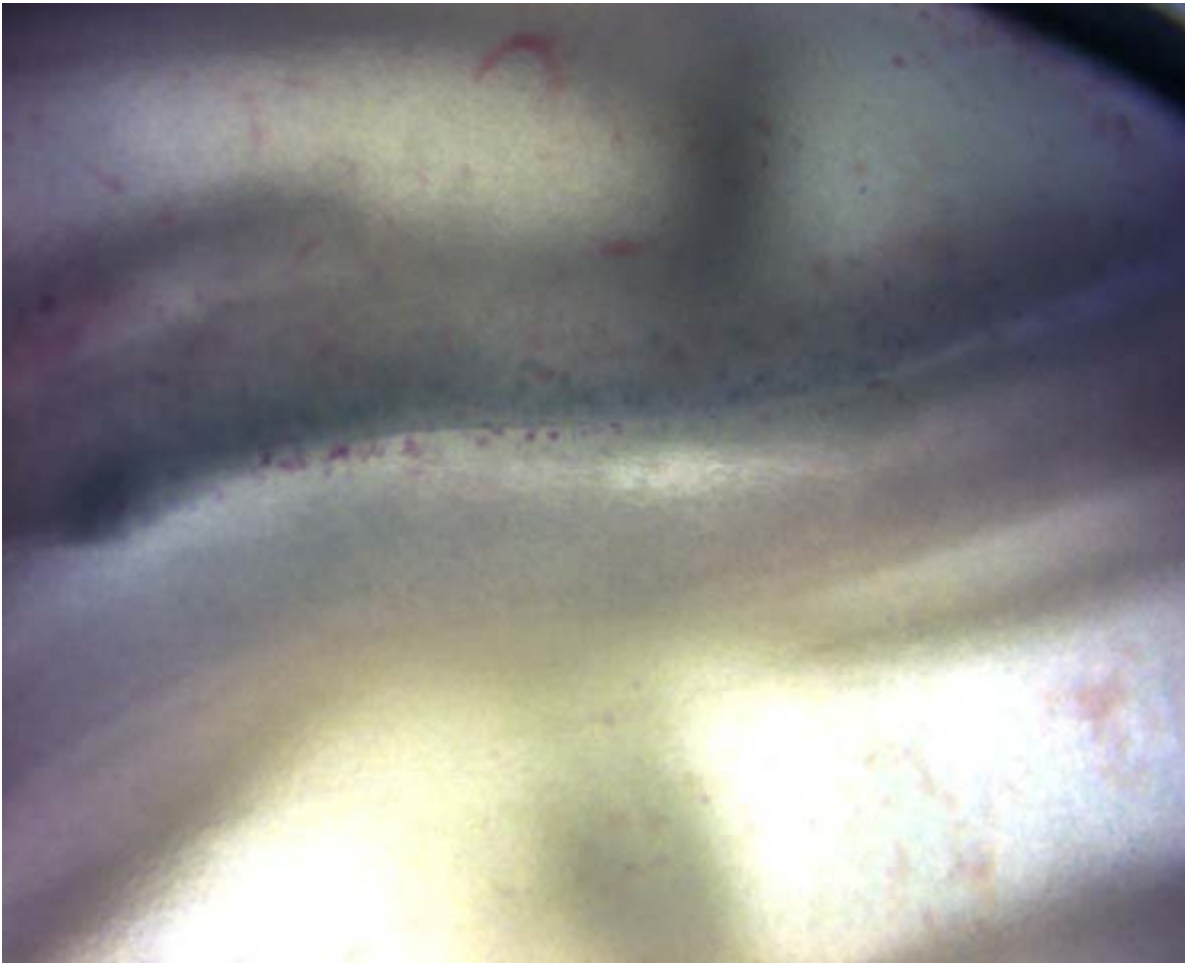


Figure 4 - This is an image of a developing chick embryo foot at Day 9 of development. The left foot (opposite side of Figure 1) is shown at 100x magnification taken with a compound microscope. This image is a magnified version of the foot shown in Figure 3.

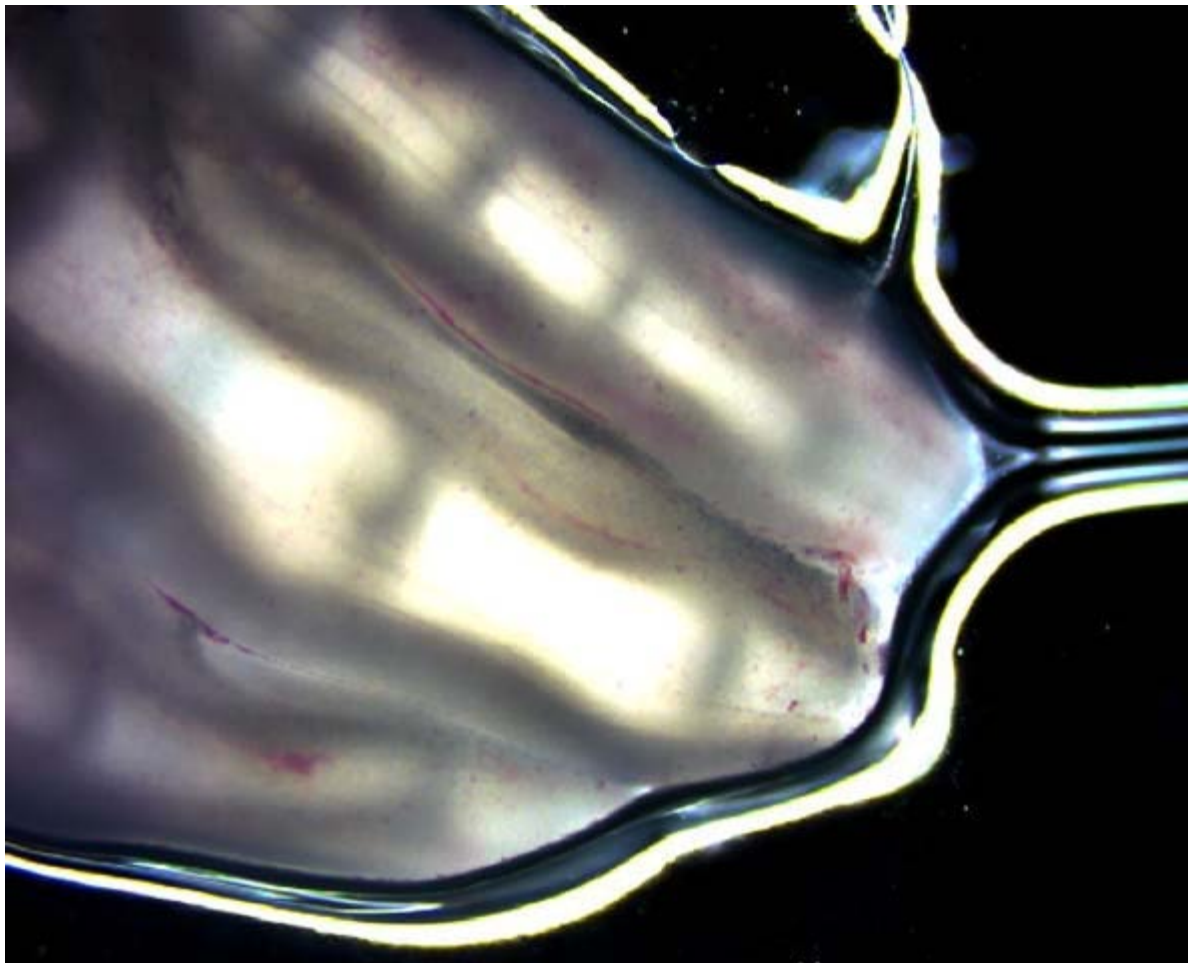


Figure 5 - This is an image of a developing chick embryo foot at Day 9 of development. The right foot is shown at 40x magnification taken with a Nikon E200 compound microscope. The NBS stain pattern is represented as the strip of blue-colored cells.

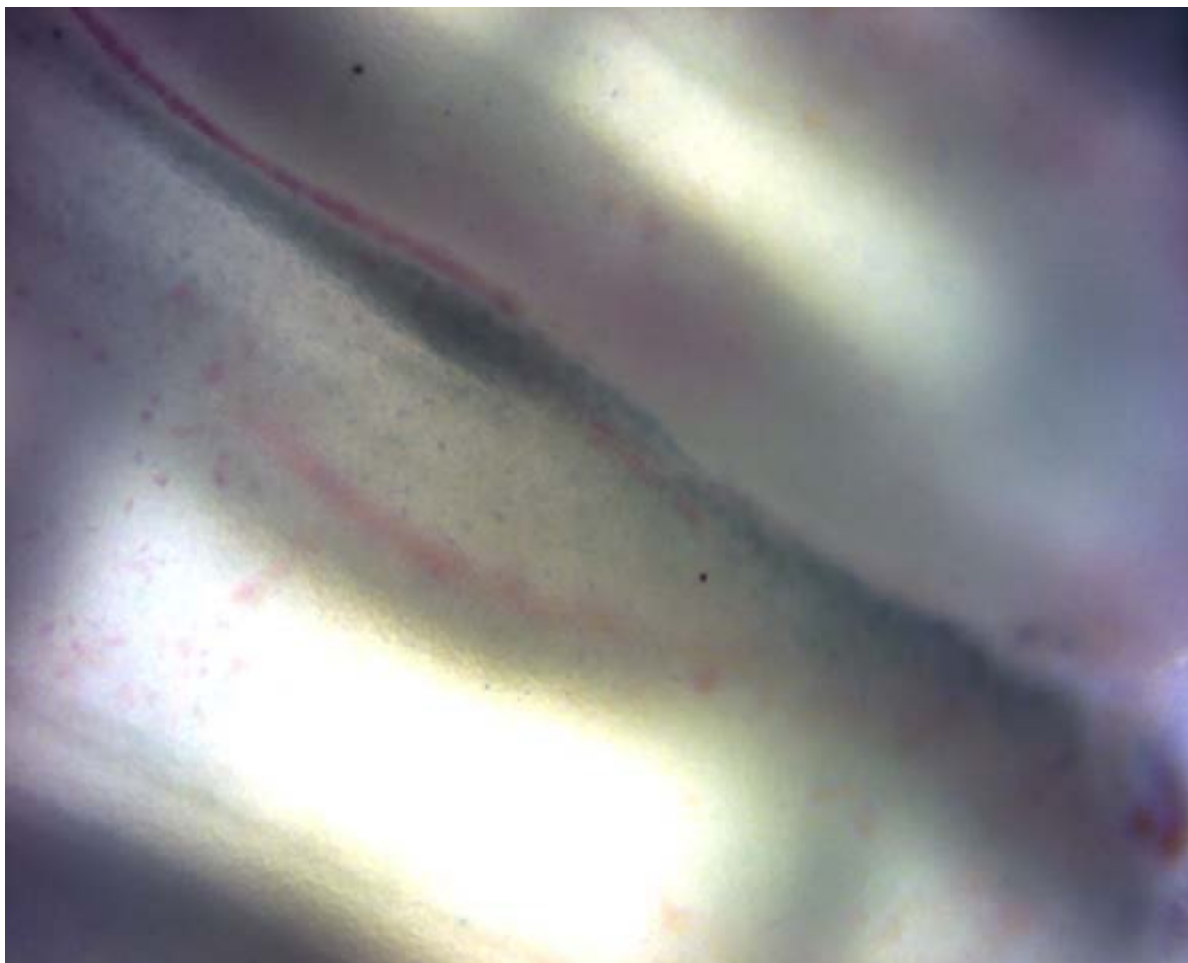


Figure 6 - This is an image of a developing chick embryo foot at Day 9 of development. The right foot is shown at 100x magnification taken with a Nikon E200 compound microscope. This image is a magnified version of the right foot found in Figure 5.

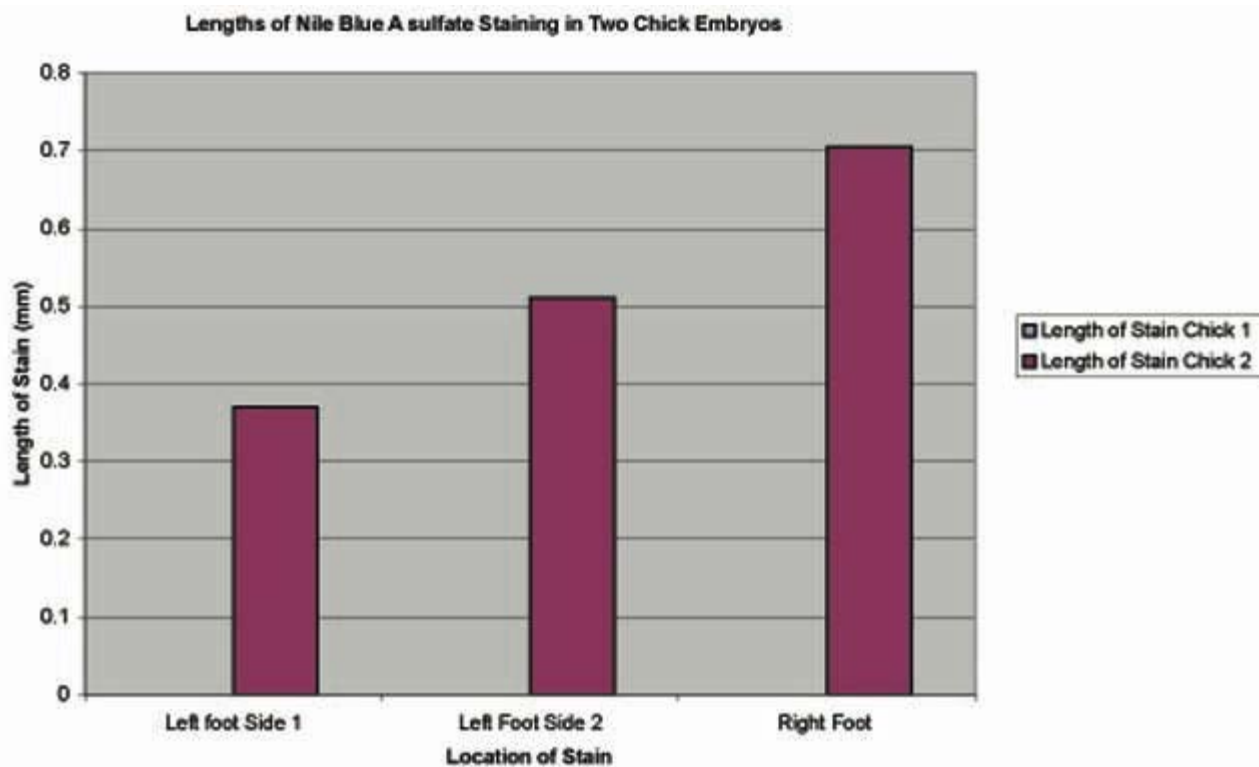


Figure 7 - This graph shows the different lengths of the stains found in the feet of two different embryos. The line for Chick 1 represents the measurements taken from a chick embryo at day 7 of development and the line for Chick 2 represents the measurements taken for a chick at day 9 of development.

IV. Discussion and Conclusions

The results of this experiment were extremely difficult to interpret due to the fact that six of the eight embryos were either dead or in extremely poor health. Although only one embryo displayed stained cells, it is also important to note that this was also the only embryo that possessed visible digit formation. The lengths of the stains in Figure 7 show that the stains were fairly long and variable in length. The most visible characteristic of all the stains present was the pattern. In Figures 1-6, all stains appear to closely follow the creases between the digits. These patterns reflect an extremely precise system of apoptosis present in the feet of the chick embryo. It can be concluded from these images that apoptosis occurs only along the creases of the developing embryo during day 9 of development.

The hypothesis for this experiment can not be fully supported through the data taken in this experiment, but also can't be fully rejected, due to the fact that apoptosis in the foot area may have actually only occurred in one embryo. Since digit formation occurs as a result of apoptosis, the staining of cells was not expected in the chick embryo at day 7 of development since no digit formation was present in the feet. The hypothesis tests that the NBS would stain apoptotic cells if apoptosis were to occur during digit formation. In that sense, the hypothesis was supported since both digit formation and staining occurred in both feet of the chick embryo at day 9 of development. The hypothesis can not be fully supported though because of the lack of controls, and the poor health of all but two embryos. There was a lack of controls in this experiment because the designated controls either contained dead or absent embryos. Consequently, any healthy living embryo obtained was immediately stained and examined.

In terms of stain dilution, it can be concluded from the data obtained that the dilution of the stain used is capable of staining apoptotic cells, and should undergo further testing to determine its staining capabilities over a large amount of specimens. It can also be concluded that the use of more eggs would have been beneficial toward obtaining more interpretable data. Since embryonic death or malformation occurred in the majority of the eggs, a larger number of

eggs would have increased the chance of obtaining healthy embryos. Also, the use of controls as explained in the methods and materials section should have been an essential part of this experiment. The use of controls in similar future experiments is essential, and serves a crucial component in interpreting the results. It was determined that the main source of error for this experiment was the use of unhealthy embryos. The eggs were left intact in the incubator until the staining was carried out, which should have increased their survival and health rate, but it appears a greater amount of care for the eggs was needed. A simple tool that could have been used to decrease the amount of damage to the shells is the use of an egg crate in the incubator. The eggs were stored on a wire mesh crate, which could have also contributed to the damage of the eggs. Other sources of error in this experiment were associated with explanting technique and yolk removal. In order for the embryonic limbs to be properly stained, all the cells present on the limb must be in direct contact with the staining solution. In several cases, the yolk sacs of the eggs ruptured as the contents were explanted onto the weighboats. This caused the embryos to come into direct contact with the yolk, which decreased the chances of successful staining. The yolk acts as a type of barrier decreasing the degree of limb cell exposure to the staining solution. The methods used in this experiment included a step in which all explanted embryos were washed in HBSS, but this step should have been more extensively carried out. Specifically, the embryos should have been allowed to soak in the HBSS for a greater amount of time immediately following removal from the egg. The HBSS in the Petri dish could have also been replenished several times, which would have more efficiently removed the yolk from the embryo. Improvements made to this step would further decrease the amount of yolk on the embryo, and would thus maximize the degree of exposure between the embryonic limb and the NBS stain solution.

The results of future experiments involving interdigital apoptosis in embryonic limb development could potentially contribute to the creation of a map extensively detailing the patterns of apoptosis. In addition to the increased chance of obtaining healthy embryos, the use of more eggs would allow to test for apoptosis over a wider array of stages in development. If more measurements were to be taken during more specific stages of development, averages could be made to develop a chart depicting the average length of apoptosis during each stage of limb development. The creation of a "standard" chart showing the length of apoptotic cells could act as a protocol for further research to use and build off of. Research involving apoptosis is extremely important in the field of developmental biology and also in medicine. The study of the molecular mechanisms involved in interdigital apoptosis could potentially provide information on how diversification is accomplished in the course of evolution. Studies in this area could also contribute to the explanation of the basis of soft tissue syndactyly, which is the most common human malformation (Ganan, et al., 1998). Apoptosis occurs throughout human bodies and is a crucial component in the removal of tumors. Research involving apoptosis is extremely important in developing clinical treatments for cancer and is one of the main focuses in the study of oncogenesis. There are many aspects of apoptosis that remain unknown to this day, making it the main focus of many types of research. Future research involving apoptosis holds a great deal of potential in explaining many biological processes and providing a base for the development of future clinical applications.

V. Bibliography

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