

The Effect of 2% Ethanol on a Developing Chicken Embryo's Vascular Area: A Study in Fetal Alcohol Syndrome and Blood Vessel Branching Patterns

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I. Introduction

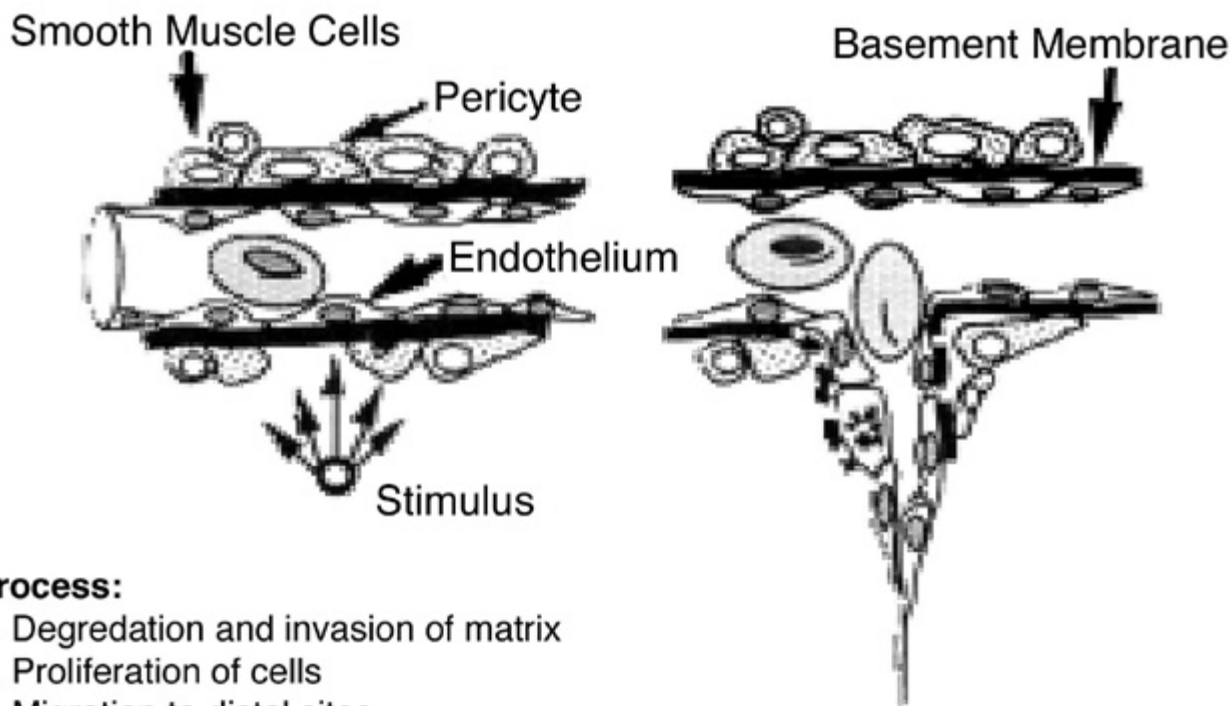
The developmental process that we will study is cardiovascular system generation in early chicken embryos. In particular, the number of branches or branching pattern within the developing embryos will be focused on. In this study we tested the hypothesis that the embryos exposed to ethanol would have more branching of blood vessels off the vitelline artery than the control embryos.

This is a significant study because it can show how the angiogenesis of vessels can be bad and can be a sign of depleted nutrients and oxygen. A larger vascular area and more branching suggest that the embryo has to compensate for what it cannot get easily. Therefore these studies can reinforce to mothers that drinking alcohol when pregnant is not a good idea if they want a healthy baby. We are testing our hypothesis with chicken embryos but the results are still significant because the observations from the chickens can be applied to other more complex vertebrates like humans. The chick embryo has been used as a developmental model in research for years within developmental biology. The chick embryo is a prime organism to study because it develops outside the mother and therefore is easy to observe. This external development also means that there are no maternal hormones present when testing the effects of external stresses on a particular part of the embryo, such as the vascular area or neural development (Ruijtenbeek et al. 2002). With the chicken embryos it is easy to watch their growth outside of the eggshell under specified conditions or also with a window in through the shell. The cardiovascular system of a chick embryo resembles the cardiovascular system of a human much more closely than many other organism models used in developmental biology. It is a lot easier to study development in chicken embryos and it is more ethical in present day society than using human embryos.

The development of the vascular area begins with the formation of blood islands by mesenchymal cells. These blood islands then create a network across the yolk of a developing embryo. Within the mesoderm of the embryo endothelial tubes develop to connect the blood islands (Wilt 2004). Most internal blood vessels are created by a process called vasculogenesis. This means that the blood vessels are produced by in situ formation of endothelial tubes (Wilt 2004). The blood vessel formation occurs via undifferentiated mesenchymal cells (Tertemez et al. 2004).

Angiogenesis is also another way of blood vessel formation although this type of formation happens by immigration of the tip of endothelium into the surrounding tissues (Figure 1)(Wilt 2004). It is the process of new vessel formation from preexisting vessels (Tertemez et al. 2004). Angiogenesis is important because it is crucial in the formation of new circulatory beds in embryo development as well as in the reproductive organs of adults and also after a trauma such as surgery. (Folkman & Shing 1992). With injuries and disease angiogenesis can be beneficial to providing healing nutrients and oxygen to the tissue. The presence of increased blood vessel growth can also be negative since it is a sign that something is wrong and the new vessels may feed tumors allowing them to proliferate and infect other tissues (Marsh 2002).

Angiogenesis In Vivo



Process:

1. Degredation and invasion of matrix
2. Proliferation of cells
3. Migration to distal sites
4. Realignment and vessel formation
5. Basement membrane formation

Figure 1: This depicts the process of angiogenesis in vivo. It shows the flat endothelial cells that migrate off to form the new vessels and the red blood cells circulating within the vessels that also aid in the branching process. The steps in the development of new blood vessels are numbered in the lower left of the image. The loosening of the basement membrane is due to the presence of vascular endothelial growth factor (VEGF) and a fibroblast growth factor (FGF-2) (Marsh 2002).

In all multicellular organisms the transport of oxygen, nutrients and waste are essential processes. No embryo can develop without a functional expansive vascular system. The development of such a region involves many signals from angiogenic factors and inhibitors, which are currently being explored and defined by research labs all over the world (Folkman & Shing 1992). The development of the vascular network begins with an angioblast that joins with similar cells to form plexuses (Gray 2000). Within the numerous solid plexuses masses, vacuoles form that contain plasma. This becomes the lumen of the blood vessel and the flat cells around the edge form the endothelium (Gray 2000). Blood cells develop from the masses of the original angioblast or from the endothelium. This accumulation of blood cells forms a mass that attaches to the wall of the forming vessel. This is what is known as a blood island and is the precursor to the blood vessels (Gray 2000). These blood islands then create a network across the yolk of a developing embryo (Figure 2). Within the mesoderm of the embryo endothelial tubes develop to connect the blood islands (Wilt 2004). As the cells and the hemoglobin accumulate the vessels appear and from those vessels new vessels can sprout off in the process called angiogenesis (Gray 2000)(Figure 1). The first large veins to develop are the primitive aortae and a connection between this and other vessels on the yolk sac. The vein that connects to the primary aortae is the vitelline vein which behaves as an umbilical artery (Gray 2000). The vitelline veins that initially enter the embryo through the anterior wall are later received by the posterior end after the two primitive aortae fuse to form the heart (Gray 2000). The vitelline veins drain the blood from the digestive tube until the liver forms and then that serves as the filter instead (Gray 2000). Therefore circulatory system and blood vessels have a great deal to do with organ development and the maintenance of tissue function including healing in adults (Marsh 2002).

There have been many studies done involving ethanol and fetal development for the purpose of understanding how

the habits of a pregnant mother can harm the growing child. By doing experimental studies the scientific community can provide direct data and observations that can tell the public that alcohol ingestion is fact detrimental to embryonic development. In other studies different concentrations of ethanol have been used.

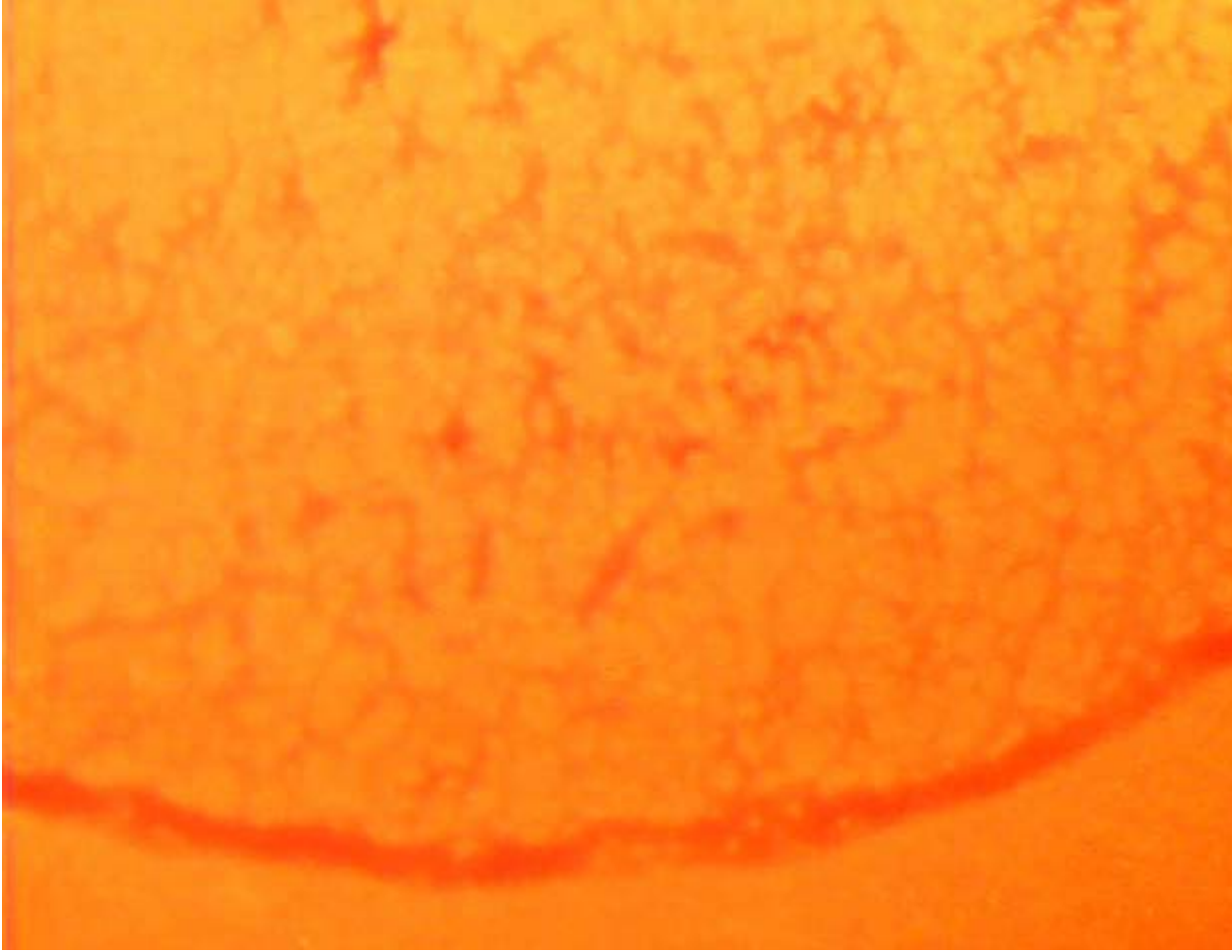


Figure 2: This image was taken on .8 magnification with the BTV Pro software in the Wheaton College ICUC. It clearly shows blood islands that are apparent before the development of distinct branching of blood vessels. This is a picture of the control Embryo on day 3.

A.C. Tufan and colleagues used ethanol concentrations of 10% ethanol in chick embryos and showed that that high a concentration severely disturbs the process of nutrient absorption that the embryo needs to develop properly and can lead to death. They also observed that the ethanol might also involve the establishment of reactive oxygen species that result in the initiation of oxidative stress which alters signaling and the expression of growth-regulatory vasculogenic factors and their receptors (Tufan 2003). A single low concentration of ethanol however may have an acute short-term positive effect or no effect at all compared to the controls (Tufan 2003).

We quantified the branching pattern which was defined as the number of branches off the vitelline artery to the right side of the chicken embryo (Figure 3). We believe that due to the increase of toxins in the 50ml environment of the weigh boat the embryo will compensate by increasing angiogenesis to get the nutrients and oxygen it needs to develop normally (Marsh 2002). We expect to see a higher number of branches in the ethanol exposed chicken embryos and a lower number in the controls. Because we are not using a high concentration we would not expect that the ethanol would disrupt the development of the vascular area enough to inhibit the growth of it and therefore the growth of the embryo. In this study we explanted the chicken embryos and exposed a portion of them to 2% ethanol (.02% in the weigh boat). We then counted up the numbers of branches every 24 hours in the experimental trials and in the controls.



Figure 3: This is an image of the entire embryo taken at 1 magnification using BTV Pro. The black box indicates the area in which the branches of the vessels were counted. The same area was used in all the embryos counted. It is the area to the right side off the vitelline artery.

II. Materials and Methods

Explantation

Before conducting the experiments we had to make sure the area was sterile. To do this the lab bench and hands were sprayed with 70% ethanol and wiped down with a paper towel. The materials needed were then collected and placed on the bench. A paper plate, sterile forceps, weigh boat and Petri dishes were collected. 8 eggs were explanted in each trial and there were two trials. Before explanting the eggs they were sprayed with 70% ethanol and let to air dry with the wide end of the egg facing upwards. By having the egg's wide end facing up the embryo orients itself at that end making explantation more effective (Armstrong et al. 1994). Forceps were used to chip away small parts of the shell away on the wide end of the eggs. This exposed an air space. The edges were made smooth as much as possible to avoid breaking the yolk with the eggshell (Armstrong et al. 1994). After a large enough hold was made the membrane over the yolk was pierced and the shell was flipped upside down. While upside down a small hole was pierced in the narrow end which made the egg contents fall into the weigh boat intact with the embryo ideally located on the top (Armstrong et al. 1994). This identical process was repeated for all the eggs. 1ml of penicillin/streptomycin (Pen/Strep) was added to each weigh boat (Calculation 1). The number eggs were split up into some that were used as controls and the others were used as experimentals. This numbers is dependant on how many of the initial 8 survive explantation (Armstrong et al. 1994). To the control no ethanol was added but .5ml of Tyrodes was added in place of the ethanol. To the experimentals .5ml of the .02% ethanol (Calculation 2) solution was added to the weigh boat (Armstrong et al. 1994). All of the weigh boats were placed in the 37 degree incubator in the tissue culture room in the Wheaton College science center. Daily observations were made of the embryos measuring the heart rate in beats per minute and the vascular area in millimeters. Pictures were also taken using BTV pro software and the stereoscopes in the Wheaton College ICUC of the embryo's body on 1 magnification and the vascular area on .8 magnification directly

below the embryo's tail.

Pen/Strep:

10,000 units penicillin (per ml)

10mg streptomycin

dilute it 100X: [] = 100 units, 100mg per ml Pen/strep

add .5ml pen/strep to embryo (1:50 dilution)

Final concentration Pen/Strep:

If 10,000 units/ 10mg per ml

Then 5000 units/5mg per .5ml (what we added)

$5000/50 = 100$ units $5\text{mg}/50 = .1$ mg = 100mg

[] = 100units/100ml per ml

Calculation 1: This calculation is for determining what the final concentration of the pen/strep was in the chicken embryo. .5ml of pen/strep solution was added to the weigh boat making a final concentration of 100units/100ml per ml.

2% ethanol:

If one needed a lot of the ethanol solution 2ml of ethanol would be added to 98 ml Tyrodes to make a final concentration of 2%. We don't need that much, we used about 5ml.

Need 5ml so divide by 20.

$2/20 = .1$ ml (100 ml)

$100/20 = 5$ ml ----> 2% (working concentration) ----> 5ml to 50 ml is a 1:100 ratio so final concentration of ethanol in the egg is .02%.

Calculation 2: This is a calculation showing the working concentration, and the final concentration of ethanol in the egg.

Data Collection and Analysis:

To ensure that the data collected is consistent across other embryos and to make sure that any conclusions reached are supported two different trials of chicken embryos were conducted. Each trial began with 8 possible embryos but with both trials only 5 were still alive with their yolk whole after explantation. This finding required us to drop the idea of doing two different concentrations of ethanol. 3 in each trial were used as controls. The controls were necessary because they contain no ethanol and only the Tyrodes salt solution. The purpose of the control is to allow the scientist to compare their experimental results to a result that is what would have occurred naturally if the subject has no outside manipulations. The purpose of adding the equivalent of Tyrodes to the controls is to ensure that it is not the Tyrodes in the ethanol solution that is causing any deformalities or abnormalities in the chick. This way it is monitoring what the ethanol, and only the ethanol does to the experimental chicken embryo. For these reasons the controls that were used in these trials were appropriately set up and provide the monitors with a good comparison to the experimental embryos.

The two trials went on for as many days as the embryos were alive. The embryos being alive was determined by a heart beat, red color of the embryo, and the condition of the vascular area. A dead embryo was characterized by no heartbeat, a white color in pallor and the breakdown of the vascular area. The first trial continued for 7 days while trial two continued for only 5 days. During these days the embryos were check on once a day. During this time each embryo was looked at under the stereoscopes and with the BTV pro software. A picture was taken of each embryo to show that it was alive, and pictures of the vascular area directly below the tail bud of the embryo were taken. These pictures were pictures of the vascular area coming off the right hand side of the vitelline artery in the same place. These pictures were taken using BTV pro on the 1034x779 image size and .8 magnification for the vascular area pictures and 1 magnification for the embryo pictures. Along with this the heart rate was taken every day and also the vascular area was measured in millimeters.

Once all the pictures had been taken for trial one and trial 2 the data had to be analyzed. The data collected was the number of veins coming off the vitelline artery to the right hand side of the embryo. To collect this data the pictures of the vascular area were used to count the number of smaller veins branching away off a branch of the vitelline artery extending away from the right side of the embryo in the images. To do this accurately the levels of the images was altered using Photoshop software. They were changed to 32:1.00: 217. This gave the image more contrast and made the smaller veins easier to count. The data was collected for each embryo for each day that pictures were taken. Some

days there were more than one experimental or one control alive, and those data numbers were averaged (Table 1). This data can be pooled because each embryo explantation was carried out in an identical manner and there should not be any variation between a control from trial 1 to the same from trial 2 and the same with the experimentals. For the rest of the days when just one embryo was alive the number of branches within that picture was used as the data for that day.

To analyze this data (Table 1) a graph was made to show the numbers of veins in the experimental conditions as opposed to the control embryos (Graph 1). Using this graph the values were weighed against each other and conclusions could be made from the comparisons of the numbers of veins branching in the experimental versus in the controls.

III. Results

Trial 1:

At the initial day 2 explantation 3 of the 8 eggs broke. This made it so we could no longer do two different concentrations. We decided to do only the 2% (.02% in the egg) ethanol in 2 of the 5 left and the other 3 to keep as controls. In the initial day 2 embryos had no development of the vascular area and the embryo was still transparent. On day 3 the vascular region had become visible but the diameter was still only around 15mm and slightly smaller for the ethanol-exposed chick. In the early day 3 embryos the veins have not clearly begun to form and the blood islands are still present. They were present throughout the area directly next to the growing embryo and also around the periphery of the vascular area. The heart was clearly visible with a strong heartbeat in all of the living embryos with the heart rate in the ethanol exposed embryo being considerably faster than the controls. On day 4 the vitelline artery had formed nicely and the branching of the veins were more pronounced. There were many less blood islands than before. Day 5 showed a vascular area on the experimental that was larger (29mm) than the control (26mm). The embryos are both beginning to show limb bud formation clearly now and the head is enlarging quickly during these few days.

Day 6 was incredible due to the first movements of the growing embryo. Both the experimental and the controls were moving slightly but it was very apparent under the dissecting scope. The heart rate in both was much greater now and the vascular was 45mm in the control and in the experimental. Now there seems to be a trend of more and more branching of the veins in the vascular area as the days go on. As the vascular area grows the number of branches also increases.

On day 7 the last control embryo died but the experimental was still alive with a 67mm vascular area and being about 2 cm in length. It continued to move and its heart rate was considerably faster at 140 beats per minute.

Trial 2:

Trial 2 started out with eggs that didn't appear to be in as good health as the eggs from trial 1. The eggs seemed narrower and the shells were very brittle. The yolks also seemed to be more fragile and explantation was much more challenging. Of the 8 eggs we were given we lost 3 again to rupturing of the yolk. So once again we decided to use 3 as controls and 2 as experimentals using the same concentration of 2% (.02% in the egg). The general growth pattern followed the same as trial one. There was no vascular area on day 2 when they were explanted and there was no clear branching and mostly blood islands on day 3. However, on day 4 all of the controls were dead except for one and it appeared as though there was no embryo on the one that was still alive. This could have been due to not being fertilized or it could have died. A transparent embryo is hard to see therefore is hard to declare dead. On the one experimental that survived the vascular area was larger than the trial one embryo at 22mm. We looked at Natalie Shelton's group control and experimental embryo to compare our data to something untouched by ethanol and to another experimental to make sure the data we were collecting was consistent with others data. Their control on day 4 looked like the controls we had on our first trial and the vascular area was 31mm as compared to the 22mm experimental vascular area. The vascular area on day 5 was 28mm in the experimental and the control was 39mm. The branching pattern of the embryos this time followed the same pattern as the first trial. The first few days were only blood islands, then as those continued to join together the visible veins began to form. Then as time went on and as the vascular area grew there were more and more branches within the vascular area off the main vitelline artery. This is shown in the data collected from the images taken by the BTV pro software on all days where the embryos were alive.

The general trends in the data are quite apparent once put into a bar graph (Graph 1). The control showed an abnormality on day 3. It showed a greater number of branches than the ethanol exposed embryos. And also it showed the highest number for the control throughout the entire experiment. Day 5, 6, and 7 showed numbers that increased as each day went on, which we expected due to the growth in the vascular area. Day 4 showed a different pattern with a higher number of branches in the control versus the experimental. Day 3 in both trials and in both the control and the experimental there were no branches due to the presence of blood islands and the primitive nature of the vascular area. It is apparent in graph 1 that these trends exist. The general trend is that the experimental over time showed more branching than the control (Figure 4). Day 3 is the only irregularity and this could be due to confusion between blood islands and branching or a slower initial development of the experimental.

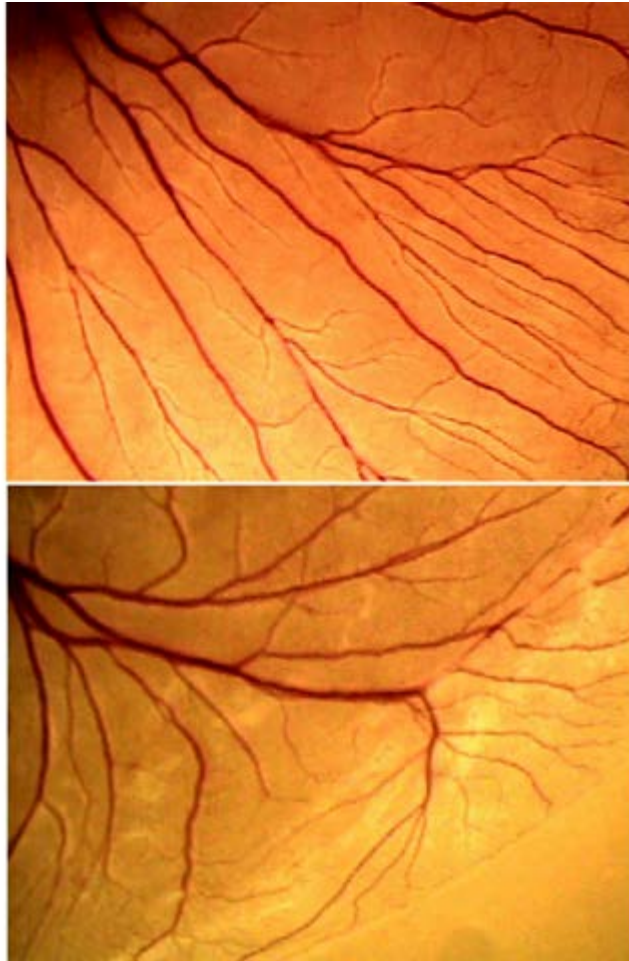
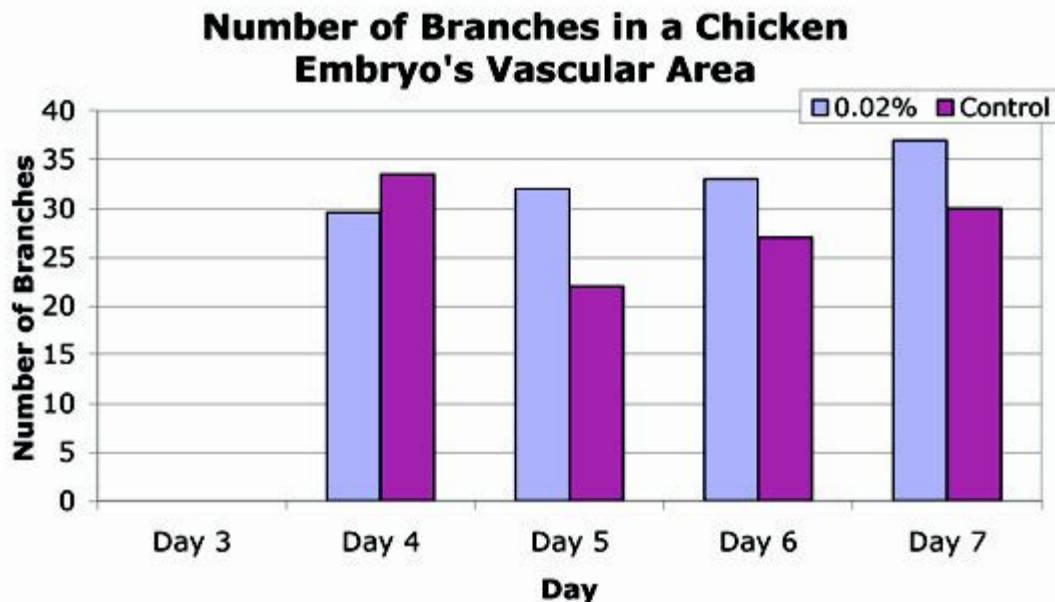


Figure 4: This shows the comparison between the ethanol exposed embryos and the controls. The ethanol experimental is on top in the figure and the control is on the bottom. Note how even without counting the ethanol appears to have more branching of the vascular network than the control does.



Graph 1. This graph displays the data collected from the ethanol exposed embryos and the control embryos. The graph shows that on day 3 there were no branching yet on day 3 due to the presence of blood islands. Day 4 is irregular data set having the control with a higher number of branches than the experimental. for day 5, 6 and 7 the data showed the opposite trend with the experimental having a higher number of branches than the control. Another trend is that over time the number of branches increases again with the exception of the day 4 control which started out as higher.

IV. Discussion and Conclusions

If the day 4 data is considered an outlier based on its irregularity as compared to the other data, the data directly supports the hypothesis that there would be an increased number of branches in the experimental versus the control. From the data it can be concluded that the number of branches over time increases and that the number of branches is greater in the experimental embryos than in the control embryos. This is consistent over both trials in all 10 eggs examined with the day 4 data considered an outlier and excluded. This conclusion supports the hypothesis and implies that the embryo may be creating more blood vessels as a way in which to battle the toxins within the yolk environment. The angiogenesis of blood vessels provide the growing embryo with a way to receive nutrients and oxygen when it becomes more difficult with the presence of the ethanol.

There were a few inconsistencies in the data such as on day 4 when the control's number of branches in the vascular area was observed as being higher than the experimental value. This could be due to different sources of error in the experimental process. One of these could have been in the counting method. Vessels could have possibly been counted more than once in the control on day 4 leading to the higher number of branches. This would have been an easy mistake to correct by recounting the veins in each picture. Also there could have been error in taking a picture where all the branches off the right side of the vitelline artery were not in the frame. If these techniques were to be refined there may have been a different result. Rather than counting the vessels on the screen in the BTV Pro software, a copy of the image should be printed out and then each branch checked off as they were counted. This could be a way to avoid counting the same veins twice. Also to ensure that the picture was taken including the entire section of veins better alignment of the section in the frame could have been done using the dissection scope and BTV Pro. A reason for the increased number of branches in the day 3 control may be due to contamination with ethanol or mixing of lids and dishes.

Some other ways in which this experiment could be done better exist in the methodology that went well in this initial experiment but could have been done better in future experiments. First and foremost it would be a lot better to use many more eggs in the study. This would lead to a larger data pool and therefore more accurate data and better

averages. I would suggest two trials of 30 or 40 eggs plus. Because of the few surviving day 2 embryos we were only able to do the single 2% experimental. With more eggs I would do the lower concentration of .2% ethanol and see if the number of branches in those embryo vascular systems were between the numbers of the control and the higher 2% concentration. Also having so few eggs that survived meant that we were unable to take averages of the data for all of the conditions and days therefore raw data was used for the interpretation. The conclusions made are therefore primary and more experiments would be needed to provide support for the observed results. As well as these changes to the experiment I would also perfect the explanation method to possibly cut down on the number of yolks broken on day 2 during the removal process. This would also be a way in which to increase the amount of data and useable eggs in the experiment.

There are many future experiments I would conduct in order to extend our results in new directions, and tell more about the effect of ethanol in different concentrations on the vascular branching of the chicken embryo. One future experiment would be to expose the chicken embryo to another type of toxin that a baby can be exposed to within the mother. An example of this would be mercury, which is considered to be very harmful to the heart and the nervous system of a growing fetus. It is especially a problem because of the amount of contaminated fish consumed by pregnant females. A study in chicken embryos is a way in which we can study the effects of such a toxin without harming humans in an unethical way and using a simple system to observe. Along with mercury there are also many drugs and other environmental contaminants like dioxin and fuel oil that would be used in a study such as this. Another future experiment would be to explant the chick embryo earlier and expose the embryo to the toxin earlier in development. This may have more of an effect on the developing embryo than exposing it at day 2. Rather than checking on the embryo once a day and taking a picture only at those times it may be interesting to check it more often to gather a more comprehensive data pool. This may allow us to see at what point in early growth the blood islands connect and form branches, and also the branching pattern and formation of the vascular area may be better imaged and observed when looked at every couple hours.

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