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The Effects of Ethanol on the Allometry of the Brain and Body of Chick Embryos

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I. Introduction

Fetal Alcohol Syndrome (FAS) is a set of birth defects which occur when developing embryos are exposed to alcohol. FAS encompasses a wide variety of defects, including brain damage, facial deformities, growth deficits, heart, liver, and kidney defects, as well as behavioral or learning disabilities (NOFAS, 2004). It is estimated that approximately 1 in every 1,000 human infants are severely affected by FAS, and approximately 3-4 in every 1,000 are mildly affected (Zagory et al., 2004).

It has been demonstrated that alcohol works by a number of different mechanisms to adversely affect the developing embryo. These include, but are not limited to, damage to cell mitochondria, effects on cell adhesion, changes in the regulation of gene activity, and changes in the transport or uptake of glucose (Goodlett & Horn, 2001). Most of these mechanisms ultimately result in the death of cells by either necrosis or apoptosis. Necrosis refers to the swelling and rupturing of cells and release of organelles into the intracellular fluid. Apoptosis refers to the breakdown of cells into small bodies, which are then destroyed by scavenging cells (Goodlett & Horn, 2001). These processes have extremely damaging effects on the development of the fetus, particularly that of the brain and nervous system.

Studies have shown that exposure to ethanol in the early stages of embryonic development results in a variety of adverse neurological and cranial effects. Dunty et al. (2002) showed that exposure to ethanol resulted in excessive cell death in the hindbrain and cranial nerve cells of mouse embryos. Similarly, Ye et al. (2001) tested the effects of ethanol on the sensitivity of glycine receptors in the brain of embryonic rats, finding that ethanol has a desensitizing effect. They concluded that this action of ethanol may contribute to neurobehavioral disturbances associated with fetal alcohol syndrome.

Non-human models are very useful to scientists for examining and understanding the effects of alcohol on a developing fetus. The chick is particularly useful because the stages of chick embryonic development are similar to those of a human embryo. Chick embryos can also be explanted to an artificial “shell,” allowing researchers to observe the same embryo over time. In this experiment, I explanted 2-day old chick embryos and exposed them to a 0.002% ethanol solution and a 0.02% ethanol solution, and observed the development of the brain. I compared their development to that of control embryos who had no exposure to ethanol. Brain development was quantified by measuring the length from the middle of the optic cup to the tip of the head. This measurement was chosen because the optic cup is one of the most prominent features of an embryo, making it easy to find. Measuring from this point to the tip of the head quantifies the sizes of both the diencephalon and the mesencephalon, which are both located above the optic cup. Measurements of the total body length were also taken to observe the allometry of the brain and body during embryonic development; that is, the growth of the brain relative to that of the body.

I hypothesized that the brains of the embryos who had been exposed to ethanol would develop at a slower rate than those of the control embryos, but that the rate of growth of the body would remain unaffected. Therefore, the allometric ratios of the brain and body of the experimental embryos should overall be smaller than those of the control

embryos. I also hypothesized that the brains of the embryos exposed to a greater concentration of ethanol would develop at a slower rate than those of the embryos exposed to a lesser concentration, therefore the allometric ratios of those exposed to the greatest concentration of alcohol should be the smallest of all, followed by those exposed to a lesser concentration of alcohol, followed by the controls. Finally, I hypothesized that the brain will increase at a faster rate than the body, therefore the allometric ratios should increase over time for all embryos, but that the ratios should increase at a slower rate for the experimental embryos than for the controls.

I chose to examine the development of the brain because it has been demonstrated that effects of FAS occur very commonly, although not exclusively, in the cranial region of the embryo. This experiment could be used to further understand the effects of FAS on the brain of an embryo and to develop future treatment techniques of this condition. Studying the allometry of the brain to the body could also be useful in observing changes in the growth and development of the body as the brain reacts to a teratogen during development. Finding a correlation between the reaction of the brain and the reaction of the body to a teratogen which is believed to act on just one system could help further understanding of the connections and signaling between the brain and the rest of the developing embryo.

II. Materials and Methods

Following the methods described in Armstrong et al. (1994), the experiment was performed first by sterilizing the materials with ethanol, including the lab bench, a pair of forceps, a paper plate, the plastic egg tray (also referred to as a "boat"), and the egg itself. Taking care to use sterile technique helped to ensure that the embryos did not become infected with bacteria during development. The corners of each boat were also folded up, to prevent the contact of the Petri dish lid with the boat itself and the formation of excessive condensation during incubation. Next, the yolk and embryo were explanted from the shell into the boat. This was done by cracking the egg at the marked end very gently with the forceps, then removing the shell bit by bit. When there was a sufficiently large hole at the top of the egg, the egg was flipped over the boat, and a very small hole was poked in the bottom of the egg with the forceps to allow air into the shell. This caused the release of the yolk and embryo from the eggshell into the boat. The same procedure was followed for the remaining five eggs.

Next, we added 0.5 mL of Tyrode's solution with penicillin to each boat in order to further protect the embryos from infection. This was done using a sterile syringe. The alcohol solutions were then added, also by a sterile syringe. Two of the six embryos received 0.5 mL of Tyrode's solution containing no ethanol. These embryos acted as the controls for the experiment and were labeled as "Control 1" and "Control 2." Two of the embryos received 0.5 mL of 0.2% ethanol in Tyrode's solution, and were labeled "Embryo 3" and "Embryo 4." The remaining two embryos received 0.5 mL of 2% ethanol in Tyrode's solution. These embryos were exposed to the greatest concentration of ethanol, and were labeled "Embryo 5" and "Embryo 6." Since 0.5 mL of each solution was added to approximately 50 mL of albumin, the final concentrations of ethanol to which the embryos were exposed were 0.002% and 0.02% ethanol.

All boats were placed inside and covered with Petri dishes immediately after explantation of the embryos. They were then placed inside the incubator for approximately 24 hours.

All embryos were observed at approximate 24-hour intervals for 7 days, or until death of the embryo. An embryo was considered dead if it had a visible heart, but not visible heart rate. It was also considered dead if no further development occurred after the date of explantation. During observation, each embryo was placed under the dissecting microscope SMZ 660, with a DC 200 camera attached, and an image was taken using the BTV Pro computer imaging software at magnification 0.8 x. If the embryo had a visible heartbeat, the heart rate was measured by counting the number of beats in 10 seconds. All measurements were taken from the image of the embryo, unless the entire embryo was too large to fit in the image. In this case, the measurements of body length were taken by hand, using a ruler, without the use of the microscope. When all embryos had died, this entire procedure was repeated with 6 new chick embryos, giving a total of 12 explants from which to record data.

At the end of the two weeks of experimentation, the images were sorted to extract only the images in which a clear embryo was present. All measurements of the length between the middle of the optic cup and the tip of the head, henceforth referred to as "brain size," were taken from these images, using the digital imaging programs Adobe Photoshop and ImageJ. First, the contrast of each image was altered in Adobe Photoshop, where each image was

darkened so as to see more clearly the defining lines and features of the embryo. The contrast of each image was changed to the same amount. Next, each image was uploaded to the program ImageJ, where the "line" tool in the tool selections bar was used to draw a line from the middle of the optic cup to the tallest point of the head. The program then gave the length of that line in pixels. This was repeated 3 times, and an average measurement was taken to ensure a greater degree of accuracy. This procedure was repeated for the body length of the embryo, if the entire body was visible in the image.

All measurements were recorded, and pixels were converted to millimeters using the image of a ruler taken at the same magnification as the images of the embryos. In ImageJ, a line was drawn between one millimeter of the image of the ruler, and the number of pixels in one millimeter was recorded. This number was used for the conversions from pixels to millimeters. Finally, the allometric ratios of the brain and the body were found by setting up mathematical equations, and multiplying the result by 100 yielded the percent of the total body size accounted for by the brain. This measurement allowed comparison of the size of the brain to the total body size, quantifying the growth of the brain in relation to the growth of the body. The control embryos yielded what would be considered a "typical" ratio of head size to body size, allowing for the comparison of this ratio to those of the experimental embryos.

III. Results

Quantifiable data were able to be gathered from just 3 of the 12 original explants. The remainder of the explants either did not develop after Day 3, or died too soon for data to be gathered. Three embryos lived long enough for data to be gathered: one control, one embryo exposed to 0.002% ethanol (referred to as "low ethanol embryo"), and one exposed to 0.02% ethanol (referred to as "high ethanol embryo"). The control embryo lived until Day 6, yielding a total of 4 images from which to take data. An image was used to gather data if the embryo was alive at the time the image was taken, and if the entire head of the embryo was clearly visible. With these criteria, just one image of the low ethanol embryo was able to yield quantifiable data, taken on Day 4. Two images of the high ethanol embryo were used, taken on Day 4 and Day 5. Therefore, data for all three embryos were gathered on just one day: Day 4 of Week 2, or November 12, 2004.

Figure 1 shows an image of the control on Day 3. This is an example of an image where both brain size and body length were able to measured on the image. The lines indicate the approximate points between which were measured to quantify brain size and body length. Figure 2 shows an image of the same embryo on Day 5. This is an example of an image in which the body length would not fit, and which was measured by hand.

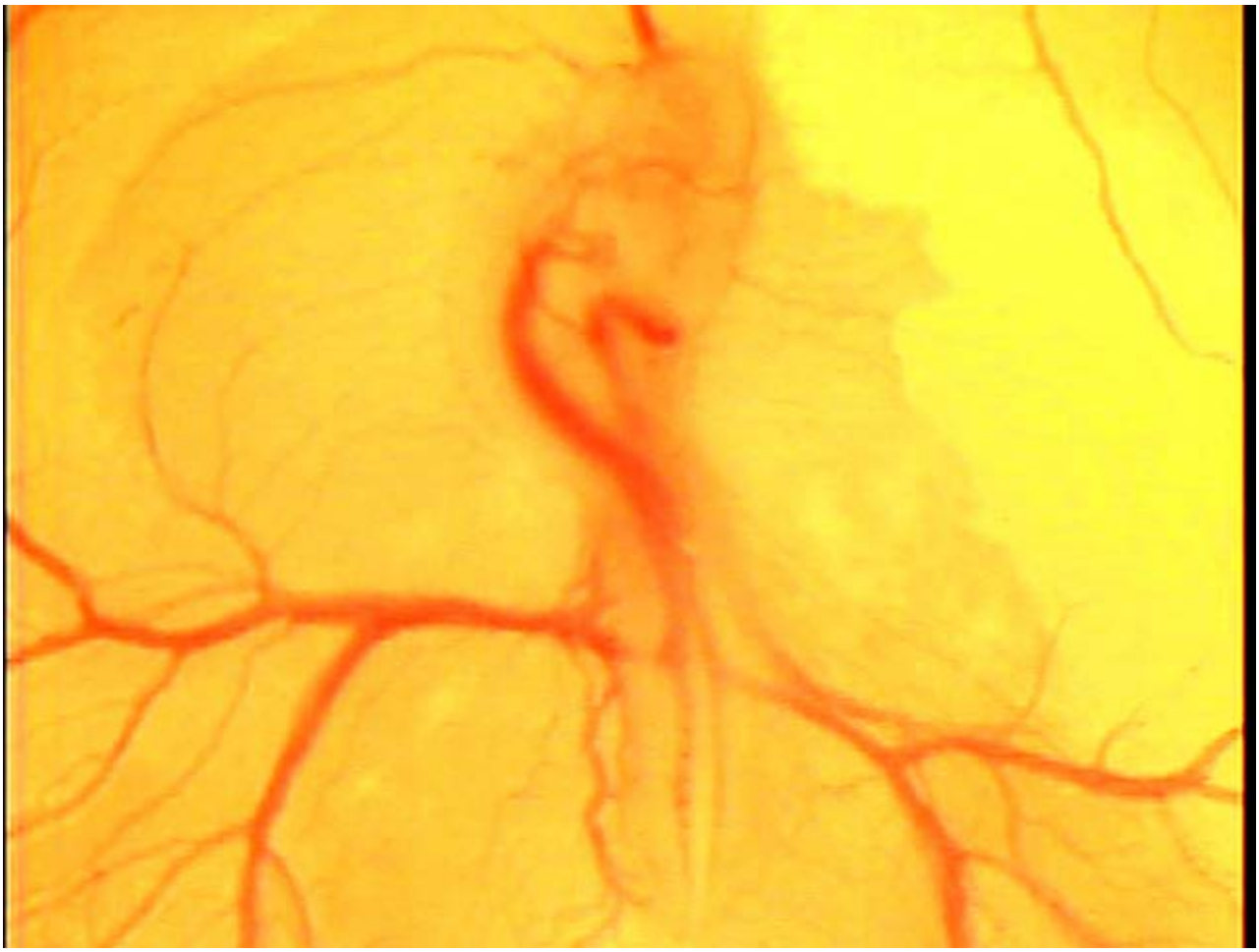


Figure 1. Image of Control Embryo 2 on Day 3. Image taken using a DC200 camera on an SMZ660 dissecting microscope, at an objective of 0.8 x. This image is an example of one in which both the brain size and the body length were measured using the digital imaging software ImageJ.

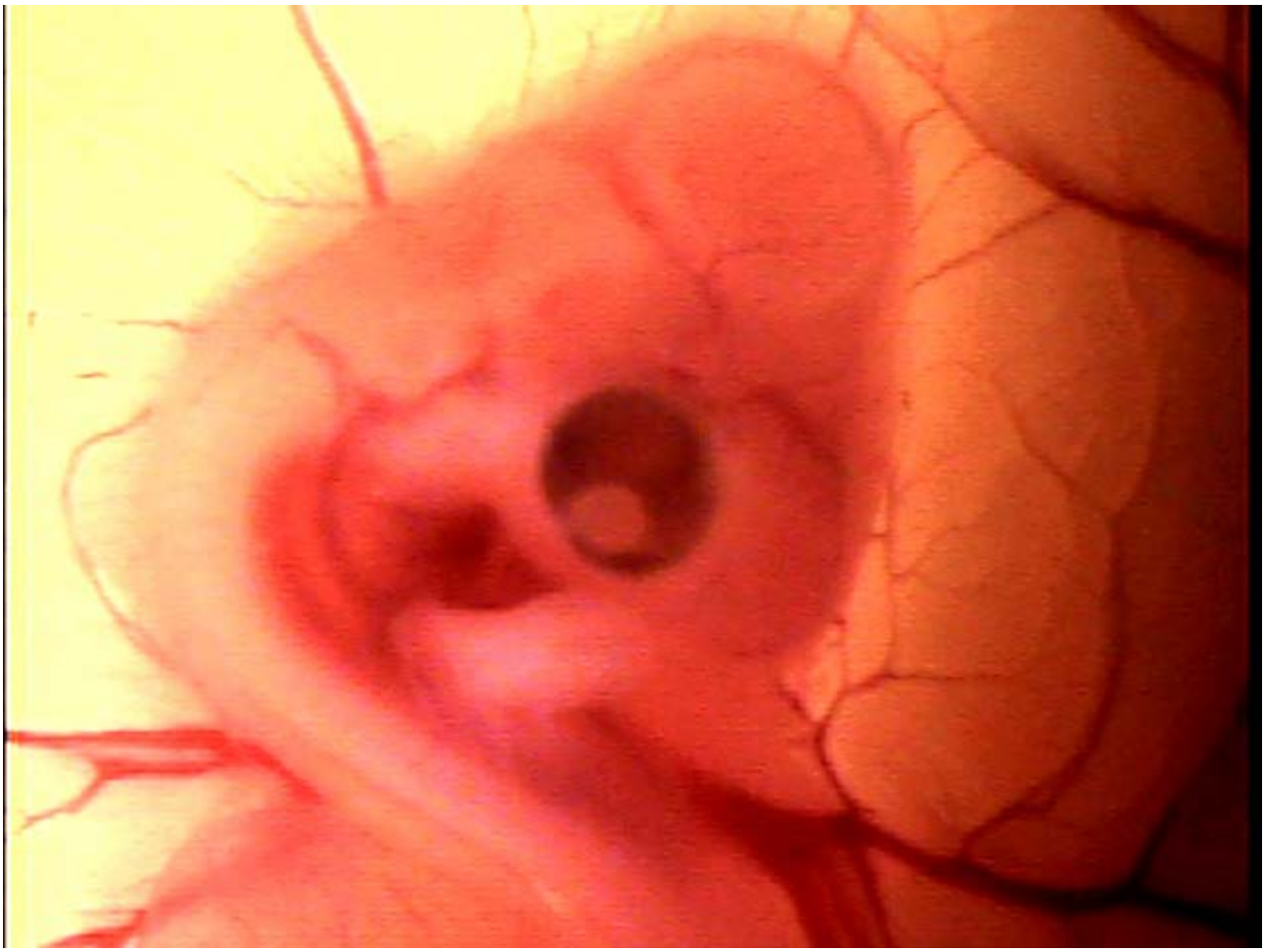
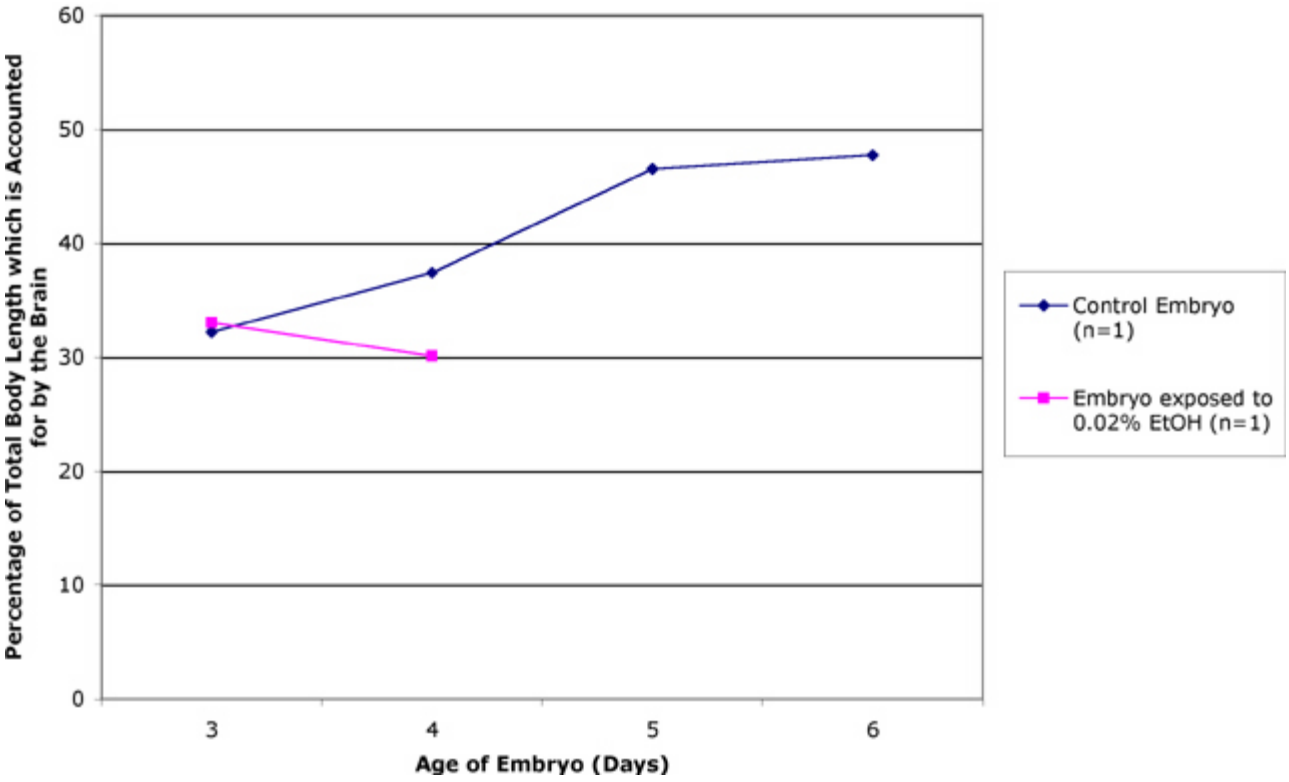


Figure 2. Image of Control Embryo 2 on Day 5. Image taken using a DC200 camera on an SMZ660 dissecting microscope, at an objective of 1 x. This image is an example of one in which the body length was too large to fit in the view frame, and which was consequently measured by hand.

In general, I observed marked increases in brain size and body size for all embryos over time, regardless of ethanol exposure. Calculations showed only slightly smaller brain sizes and body sizes for the experimental embryos as compared to the controls.

Figure 3 shows the comparison of the percentages of total body length accounted for by the brain for the control embryo and the high ethanol embryo from Day 3 until death of the embryo. The low ethanol embryo was not included in the figure, as only one image was used to gather data for this embryo. As can be seen from Figure 3, the brain size of the control embryo increased at a faster rate than the body size, as the percentage of the total body length accounted for by the brain increases over time. The brain of the high ethanol embryo did not increase as fast as the body, however, as this line slopes downward, showing a decrease in the percentage of total body length accounted for by the brain.

Figure 3: Comparison of the Allometric Ratios of the Brain and Body of Control Chick Embryo vs. Embryo Exposed to 0.02% Ethanol



IV. Discussion and Conclusions

My original hypothesis stated, first, that the allometric ratios of the brain and body of the experimental embryos would be smaller overall than those of the control embryos. As can be seen from the data shown in Table 1, this hypothesis was supported. My hypothesis also stated that the ratios would be smallest for those embryos exposed to the greatest concentration of ethanol. This point cannot be supported nor refuted, due to the small sample size of experimental embryos. Figure 3 shows that the brain of the high ethanol embryo was 33.0% of its total body length on Day 3, which was larger than that of the low ethanol embryo, at 32.1%. However, on Day 4, the ratio of the high ethanol embryo had decreased to 30.1%, and no comparable data is available for the low ethanol embryo for this day.

Finally, my hypothesis stated that the ratios would increase over time for all embryos, but would increase at a slower rate for the experimental embryos than for the controls. This portion of my hypothesis was refuted by the data, since the ratio for the high ethanol embryo actually decreased over time, as shown in Figure 3. It must be taken into account however, that only one experimental embryo was used to quantify data, and the rate of growth was able to be calculated over the course of just one day. A different trend might have been shown had more experimental embryos been used, over a greater amount of time.

Several conclusions can be drawn from the available data. First, the data shows that for embryos not exposed to ethanol, the brain appears to grow at a faster rate than the body, at least between Days 3-6 of embryonic development. This is illustrated by Figure 3, which shows a steady increase in the percentage of the total body length accounted for by the brain for the control embryos over time. It could be inferred from this data then, that the brain is one of the first organs to develop in an embryo, which would make sense considering the fact that the brain is the most complex of all organ systems, and is required to regulate almost all of the chick's physiological behaviors and functions, both prior to and after birth. The body might be slower to develop in the earlier stages of embryonic development because it is not quite as complex as the brain, and may "catch up" in size to the brain at a later stage.

The data also showed that the allometric ratios of the brain and body were overall smaller for the experimental embryos than for the control embryo of the same age, caused by smaller brain and body sizes. It could be inferred then that the ethanol affects the size of both the brain and the body between Days 3 and 4 of embryonic development, likely

through the causation of cell death through apoptosis or necrosis. The decrease in the percentage of body length accounted for by the brain of the high ethanol embryo between Days 3 and 4 shows that ethanol may affect the brain even more so than the body, as the brain decreased its growth rate relative to the body over this period of time, while the control embryo's increased. These conclusions, however, are only speculative at best, since there is so little data available for the experimental embryos.

One significant source of error may have affected these results. Measuring the body length by hand for those which grew too large to fit in the image likely yielded inaccurate measurements. The body length of an embryo looks quite small to the naked eye, and measuring this length using a ruler led to approximations of the actual measurement. This, in turn, would lead to inaccurate calculations of the allometric ratios of the brain and body.

In order to refine this experiment, it would be beneficial to take all measurements using a computer imaging program such as ImageJ. This could be done possibly by widening the frame of view to include the entire embryo, or by taking several pictures of the body of the embryo, so that the entire embryo could be viewed and quantified digitally. It would also be beneficial to use a greater number of explants, to raise the number of surviving embryos from which to gather data. It might also be useful to extend the experiment over a period of several more weeks for this same purpose of using a greater number of explants.

Further experiments could be performed on chick embryos by exposing them to various concentrations of ethanol, or other teratogens, and observing the allometry of different organs in relation to one another; not only the brain and the body, but also the heart, the optic cup, the wing bud, the leg bud, or somite growth. Examining the relationships between the growth rates of different organ systems could lead to the further understanding of the connections between the various parts of the body, and the signals they employ to communicate with one another during development. It may be found that a teratogen previously believed to have affected just one organ may indirectly affect others in more subtle ways.

V. References

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