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■ RESEARCH ARTICLE

An Analysis of the Effects of D-Glucose and L-Glucose on Chicken Embryos during HH18 and HH24

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ABSTRACT: This research examines glucose's effect on heart development and congenital heart defects. Chicken embryos and D-glucose and L-glucose were used together to model hyperglycemia. Each egg was windowed, glucose was applied, and data was collected in the form of images and videos using stereo microscopes. Each chicken embryo's heart length, rate, and outflow tract diameter were analyzed using two image/video segmentation software: MATLAB and ImageJ. In MATLAB, two different algorithms were developed to segment and analyze the chicken embryos. The delineation algorithm outputted the diameter of the heart outflow tract during heart diastole and the heart length in mm. The M-mode analysis algorithm outputted the contrast values of a cardiac cycle of a user-specified segment. In ImageJ, a linear measurement algorithm was used to quantify embryo characteristics. The results of our study show that adding glucose caused the heart rate of the embryo to increase, the length of the heart to decrease, and the outflow tract distance during heart diastole to increase, all-in-all demonstrating that adding any concentration/type of glucose induces hyperglycemia in chicken embryos and alters the development of their heart which can be projected to humans.

KEYWORDS: Biomedical Engineering; Cardiovascular System; Congenital Heart Defects; Hyperglycemia; Chicken Embryos; Heart Disease; D-Glucose; L-Glucose.

■ Introduction

Problem Statement and Research Aim:

In humans, by day 21, an embryo begins to absorb nutrients via the placenta, a temporary organ formed during pregnancy that aids the transportation of nutrients between the mother's and embryo's blood cells. The placenta has a collection of tissues that forms a blood barrier that restricts the passage of certain particles from mother to embryo while permitting others, such as oxygen and nutrients, to pass. As the mother eats, the nutrients from the food get broken down and absorbed into the mother's blood, which is then passed into the embryo's bloodstream. Due to the way the embryo receives its nutrients, if the mother has hyperglycemia, the embryo is likely to have high blood sugar as well.[1] Furthermore, previous studies have shown that this also likely causes heart defects.[2,3] To find how hyperglycemia affects the development of the human heart in its early stages, in this project, we applied two types of glucose, D-glucose and L-glucose, in two different molar concentrations, 0.03M and 0.05M, to *in vivo* chicken embryo models on Day 3 (HH18). These embryos were analyzed at the time of glucose deployment and subsequently on Day 4 (HH24). HH18 and HH24 chicken embryos (Days 3/4) were used as their heart's tubular structure greatly resembles human embryo hearts in their early developmental stages. The goal of this research was to segment, document, record, and analyze different features of HH18 and HH24 chicken embryo hearts, such as the ventricle and the outflow tract, to discover how glucose affects the development of an embryo heart and if it exacerbates any defects. This study will provide more insight into congenital heart defects and hyperglycemia-related diseases.

D-Glucose vs. L-Glucose:

This study aims to utilize in-vivo chicken embryo models to discover glucose's effect on heart development. Specifically, this study utilizes two different glucose molar concentrations and types: D-glucose (0.03M, 0.05M), L-glucose (0.03M, 0.05M). D-glucose and L-glucose can be differentiated by locating the OH- group of the penultimate carbon, which is positioned on the right side of the D isomer. In contrast, it is positioned on the left side of the L isomer.[4,5] D-glucose is abundant in nature and is found in foods like honey, fruits, and fruit juices. At the same time, L-glucose can only be synthesized and is not found naturally but is still present in several processed medications/food, such as laxatives and artificial sweeteners. L-glucose is indistinguishable taste-wise; however, it cannot be processed into energy like D-glucose during glycolysis (processing glucose). The Haworth Projection of D-glucose is shown in Figure 1, and the Haworth Projection of L-glucose is shown in Figure 2. The Fischer Projection of D-glucose is shown in Figure 3, and the Fischer Projection of L-glucose is shown in Figure 4.

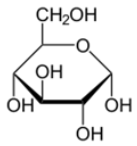

Figure 1: Haworth projection of D-glucose.[6]

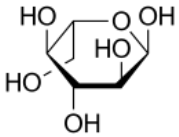

Figure 2: Haworth projection of L-glucose.[7]

DOI: 10.36838/v5i2.18

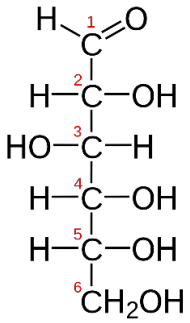

Figure 3: Fischer projection of D-glucose.[6]

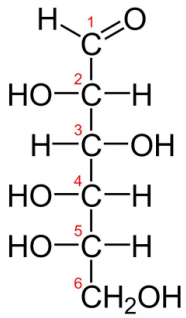

Figure 4: Fischer projection of L-glucose.[7]

Benefits of Using Chicken Embryos as a Model Organism:

The heart is the first functioning organ formed during embryonic development. A detailed understanding of its molecular and cellular mechanisms is imperative to providing necessary insights into congenital malformations and their effect on heart function and, eventually, the organism's survival. The chicken is a "classic" model organism for understanding embryonic development. The first meaningful information obtained using chicken embryos was found in the 17th century and showed that the embryos were not pre-formed but instead developed incrementally. Due to the advancements in scientific tools such as microscopes and advances in genome sequencing, the chicken egg became an ideal candidate for an animal model because the embryo develops *in ovo*, with an external pathway for oxygen already established. This allows the researcher to access the embryo at any developmental stage, which is a significant advantage. This ease of access means that the embryo can be manipulated and observed during all stages of development and tracked as the embryos mature. Another benefit to using chicken embryos as animal models is that a fully developed chicken heart has four chambers with in- and out-flow tracks. Despite some key differences, the chicken heart resembles the human heart more closely than other non-mammalian models.[3]

Hamburger-Hamilton Stages and Chicken Embryo Development:

Hamburger-Hamilton stages (HH stages) are the work of Viktor Hamburger and Howard Hamilton. In 1951 they compiled the photographs and drawings of other researchers to create an embryonic staging series. Hamburger and Hamilton used this data to develop 46 chronological stages of chicken embryos. The HH stages are still used today to accurately differentiate the time points of a chicken embryo's development.[8] An example chart of the HH stages can be viewed in Figure 5.[8]

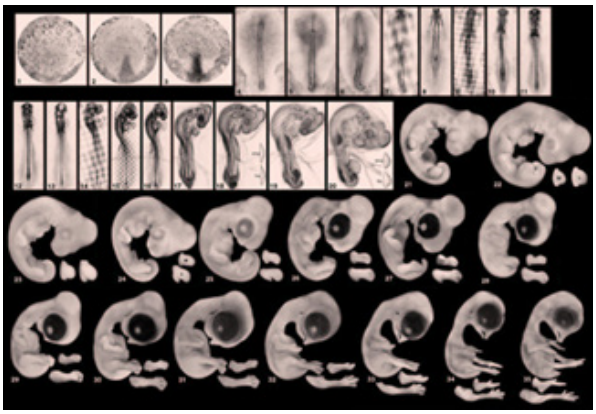

Figure 5: *Chicken embryo HH stages. Reproduced from*[8]

Another method of measuring the chicken embryo's development time is to use incubation days. The time of incubating a fertilized egg to the chicken hatching is approximately 21 days. During HH18, the limb buds enlarge, with the leg buds slightly larger than the wing buds. At this point, the amnion membrane is usually closed, but occasionally, a hole will occur over the back of the embryo. Around HH24, the limbs are now clearly longer than their own width, although the digits on the wings and legs are not yet clearly visible.[9]

Methods

**All chicken embryos used in this study received humane care, and the study protocols comply with Oregon Health and Science University's guidelines. The study conforms to the ethical guidelines of the 1975 Declaration of Helsinki.*

The experimentation was divided into multiple phases. In Phase 1, the incubated chicken embryos were windowed using curved forceps at HH18 (Day 3) of incubation. Once windowed, the outer embryo membrane was extracted using fine forceps so the embryo would be visible. Once visible, a strand of 0.41 millimeters diameter silver-plated modeling wire was cut and dropped near the tubular heart to provide a scale for image analysis conversions. Then, the embryo was transported to a Leica Stereomicroscope, and the embryo's heartbeat in beats per minute (BPM) was recorded. Fine-tuned images/videos of the embryo, its heart, and its heart cycle were acquired. After documentation, out of 29 viable fertilized eggs used in this study, four were labeled as the control group, six were labeled as D-glucose (0.03M) group, six were labeled as L-glucose (0.03M) group, six were labeled as D-glucose (0.05M) group, and seven were labeled as L-glucose (0.05M) group. For the control group, 50 microliters of Phosphate-Buffered Saline (PBS) solution were added to the embryo heart to simulate treatment. For each experimental group, 50 microliters of their respective glucose types and concentration solutions were added (glucose was dissolved into PBS to make the concentrations). After the embryos were treated, the wire was extracted using fine forceps and deposited into a plastic petri dish. Then using a glue stick, glue was spread around the windowed opening of the egg, where plastic wrap was used to seal the egg. The

sealed eggs were placed back in the incubator.

In Phase 2, after 24 hours of incubation, the plastic wrap was gently peeled off the eggs, and the wire was reinserted and dropped near the embryo's tubular hearts. Once again, the embryo's BPM was recorded, and fine-tuned images/videos of the embryo, its heart, and its heart cycle were documented. After data was recorded, the wire was extracted using fine forceps and deposited into a plastic petri dish. The egg was soon after discarded into a biohazard bin.

In Phase 3, previously documented images and videos were optimized and exported to two image analysis software: MATLAB and ImageJ.

In MATLAB, two different video/image segmentation algorithms were developed. The first delineation algorithm used a complete video of the embryo heartbeat cycle as an input, established a segmented region of interest of the heart through individual frame contrast values, and allowed the user to measure the distance in millimeters between two points on the heart. The conversion factor used can be seen in Equation 1 and is based upon calibration with the embryo wire. This algorithm measured the maximum diameter of the outflow tract during heart diastole for each treatment group at HH18 and HH24, as shown in Figure 6.

$$\frac{0.41\ mm}{x\ pixels} = y\ mm$$

Equation 1: Pixel to millimeter conversion factor.

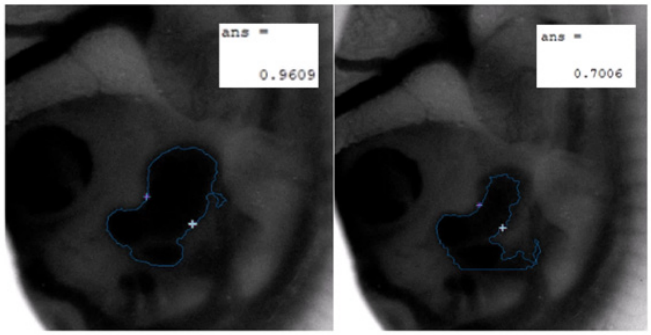

Figure 6: MATLAB image segmentation algorithm example images.

The second segmentation algorithm utilized was M-Mode Analysis. It first used a complete video/or specific frames of the embryo heartbeat cycle as an input, allowed the user to draw a line between two points, and displayed different contrast values of that line throughout the cardiac cycle by iterating through the video/frames. An example output of this M-Mode Analysis algorithm is shown in Figure 7.

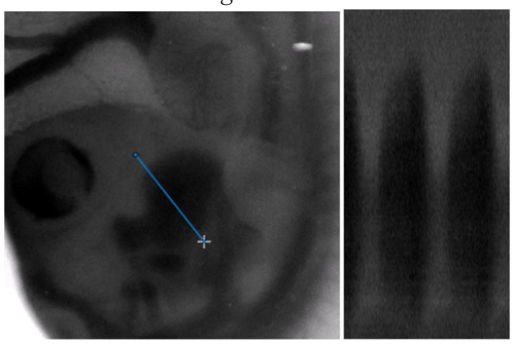

Figure 7: MATLAB M-Mode analysis algorithm example images.

In ImageJ, one frame from each embryo's video documentation was selected through random sampling to ensure minimal bias in results. Then using the linear measurement algorithm, the conversion of pixels to millimeters was calibrated using the equation shown in Equation 1. After calibration, the linear measurement algorithm was used to establish a line from the top to the bottom of the embryo heart, providing an embryo heart length measurement in millimeters. An example image is shown in Figure 8.

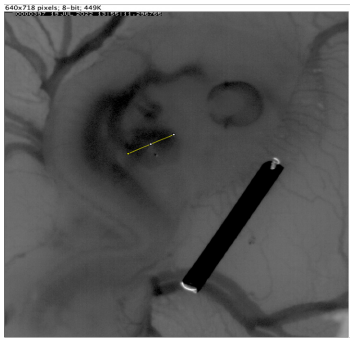

Figure 8: ImageJ linear measurement algorithm usage for calculation of heart length.

The same image analysis algorithm was used to establish a line from the top of the embryo heart to the top of the embryo head, providing a heart-to-head distance measurement in millimeters. An example image is shown in Figure 9.

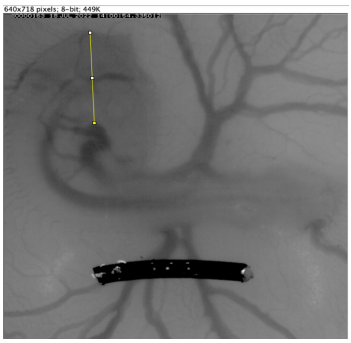

Figure 9: ImageJ linear measurement algorithm used for the top of the heart-to-head calculation.

Results and Discussion

Through thorough experimentation, our results show that embryos that had been treated with glucose had a higher mortality rate compared to our control embryos. On average, 44.4% of the incubated eggs were viable to use for the experiments as they contained an embryo on Day 3. 100% of the control embryos, which were treated with PBS, survived the entire experimentation duration (making it from Day 3 to Day 4). For the 0.05M D-glucose embryo group, 50% survived to Day 4. For the 0.05M L-glucose embryos, 28.5% survived the entire experimentation duration. For the 0.03M D-glucose embryos, 50% survived the entire experimentation duration. For the 0.03M L-glucose embryos, 66.6% survived the entire experimentation duration. Figure 10 shows the pie charts visually depicting this characteristic. The results show that chicken embryos with 0.05M glucose had a reduced number of surviving embryos compared to those with 0.03M glucose added. Thus, higher molar glucose concentrations added to the chicken

DOI: 10.36838/v5i2.18

embryos led to higher mortality rates. This can be viewed in Figure 10.

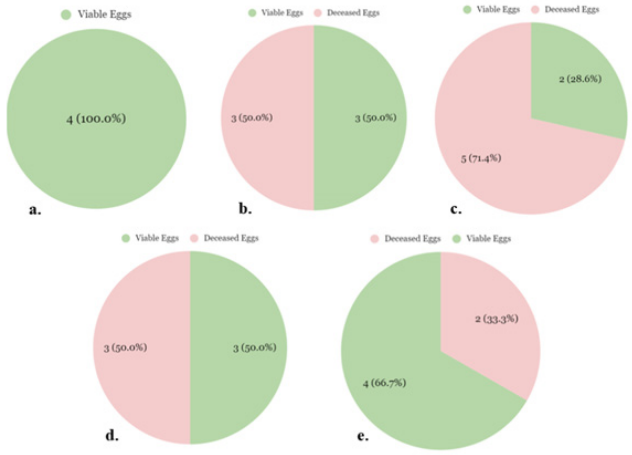

Figure 10: Mortality rate of (a) control embryos [n=4] (b) 0.05M D-glucose embryos [n=6] (c) 0.05M L-glucose embryos [n=7] (d) 0.03M D-glucose embryos [n=6] (e) 0.03M L-glucose embryos [n=6].

Additionally, we monitored the heart rate of each embryo for each treatment group during Day 3 and then also during Day 4. These heart rate values were calculated using visual reference points within a 1-minute time frame. They were validated using M-Mode Analysis in MATLAB, an example of which can be seen in Figure 7. Figure 11 and Table 1 depict this heart rate data. Each treatment group is defined using the following format: treatment group (control, D-glucose, L-glucose) - embryo number - molar concentration (0.03M, 0.05M). For example, D5-3 represents embryo number 5 of the 0.03M D-glucose treatment group.

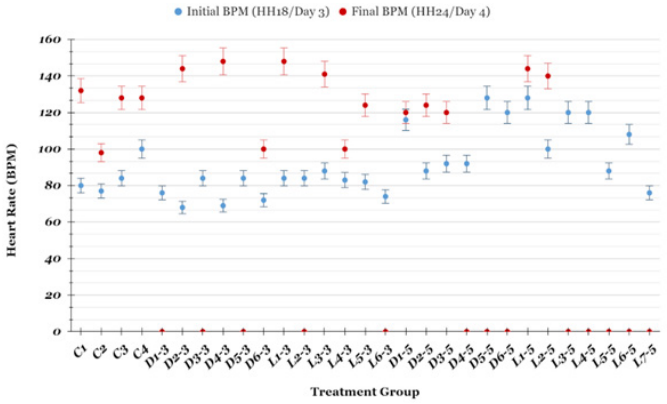

Figure 11: Measurements of the heart rate for each treatment group on Day 3 and Day 4 [n=29].

Table 1: Measurements of the heart rate for each treatment group on Day 3 and Day 4.

Treatment Group	Initial BPM (HH18/Day 3)	Final BPM (HH24/Day 4)	Net Change in BPM
Control (1)	80	132	52
Control (2)	77	98	21
Control (3)	84	128	44
Control (4)	100	128	28
Control (Mean)	85	122	36
D-Glucose 0.03M (1)	76	deceased	deceased
D-Glucose 0.03M (2)	68	144	76
D-Glucose 0.03M (3)	84	deceased	deceased
D-Glucose 0.03M (4)	69	148	79
D-Glucose 0.03M (5)	84	deceased	deceased
D-Glucose 0.03M (6)	72	100	28
D-Glucose 0.03M (Mean)	76	131	61
L-Glucose 0.03M (1)	84	148	64
L-Glucose 0.03M (2)	84	deceased	deceased
L-Glucose 0.03M (3)	88	141	53
L-Glucose 0.03M (4)	83	100	17
L-Glucose 0.03M (5)	82	124	42
L-Glucose 0.03M (6)	74	deceased	deceased
L-Glucose 0.03M (Mean)	83	128	44
D-Glucose 0.05M (1)	116	120	4
D-Glucose 0.05M (2)	88	124	36
D-Glucose 0.05M (3)	92	120	28
D-Glucose 0.05M (4)	92	deceased	deceased
D-Glucose 0.05M (5)	128	deceased	deceased
D-Glucose 0.05M (6)	120	deceased	deceased
D-Glucose 0.05M (Mean)	106	121	23
L-Glucose 0.05M (1)	128	144	16
L-Glucose 0.05M (2)	100	140	40
L-Glucose 0.05M (3)	120	deceased	deceased
L-Glucose 0.05M (4)	120	deceased	deceased
L-Glucose 0.05M (5)	88	deceased	deceased
L-Glucose 0.05M (6)	108	deceased	deceased
L-Glucose 0.05M (7)	76	deceased	deceased
L-Glucose 0.05M (Mean)	106	142	28

Our results also show that embryos treated with 0.03M of D and L-glucose had a reduction in heart length from Day 3 to Day 4, compared to the control embryos and embryos that were treated with 0.05M of D and L-glucose, which had an increase in heart length from Day 3 to Day 4. On average, the embryos treated with 0.03M of D-glucose had a net decrease in measured heart length of 11%, and those treated with 0.03M of L-glucose had a net decrease in heart length of 9%. The control embryos had a net increase in heart length by 16%, the embryos treated with 0.05M of D-glucose had a net increase in heart length by 44%, and 0.05M of L-glucose had a net increase in heart length by 9%. Figure 12 and Table 2 depict the measurements of the heart length for each treatment group from Day 3 to Day 4.

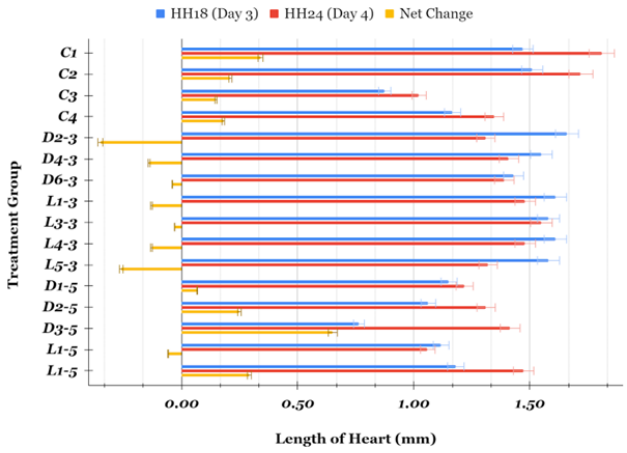

Figure 12: Measurements of the heart length for each treatment group on Day 3 and Day 4 [n=16].

Table 2: Measurements of the heart length for each treatment group on Day 3 and Day 4.

Treatment Group	Initial Length (mm)	Final Length (mm)	Net Change in Length (mm)	Percent Change (%)
Control (1)	1.47	1.81	0.34	23.13
Control (2)	1.51	1.72	0.21	13.91
Control (3)	0.88	1.02	0.15	16.91
Control (4)	1.17	1.35	0.18	15.42
Control (Mean)	1.26	1.48	0.22	17.34
D-Glucose 0.03M (2)	1.66	1.31	-0.35	-21.08
D-Glucose 0.03M (4)	1.55	1.41	-0.14	-9.03
D-Glucose 0.03M (6)	1.43	1.39	-0.04	-2.80
D-Glucose 0.03M (Mean)	1.55	1.37	-0.18	-10.97
D-Glucose 0.05M (1)	1.15	1.22	0.07	5.82
D-Glucose 0.05M (2)	1.06	1.31	0.25	23.42
D-Glucose 0.05M (3)	0.67	1.42	0.65	85.21
D-Glucose 0.05M (Mean)	0.96	1.32	0.32	38.15
L-Glucose 0.03M (1)	1.61	1.48	-0.13	-8.07
L-Glucose 0.03M (3)	1.58	1.55	-0.03	-1.90
L-Glucose 0.03M (4)	1.61	1.48	-0.13	-8.07
L-Glucose 0.03M (5)	1.58	1.32	-0.26	-16.46
L-Glucose 0.03M (Mean)	1.60	1.46	-0.14	-8.63
L-Glucose 0.05M (1)	1.12	1.06	-0.06	-5.19
L-Glucose 0.05M (2)	1.18	1.47	0.29	24.72
L-Glucose 0.05M (Mean)	1.15	1.27	0.12	9.77

Our results show that the outflow tract's diameter remained consistent and increased for all the chicken embryos used in the experiment. On average, control embryos had a net increase in outflow tract diameter by 45%. On average, the eggs treated with 0.03M of D-glucose had a net increase in outflow tract diameter by 23%. On average, the eggs treated with 0.03M of L-glucose had a net increase in outflow tract diameter by 19%. On average, the eggs treated with 0.05M of D-glucose had a net increase in outflow tract diameter by 32%. On average, the eggs treated with 0.05M of L-glucose had a net increase in outflow tract diameter by 18%. Figure 13 and Table 3 depict the measurements of the maximum diameter of the outflow tract during diastole for each treatment group on Day 3 and Day 4.

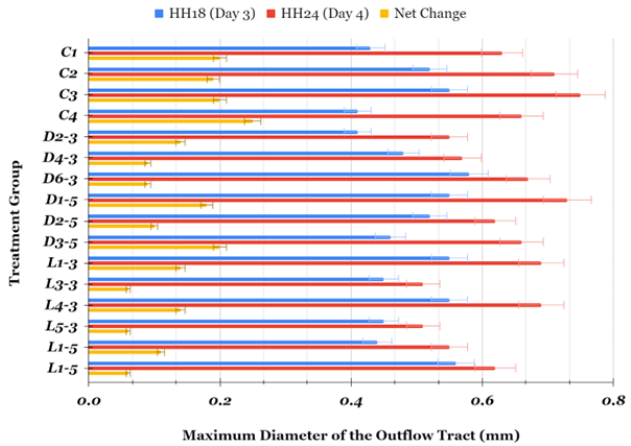

Figure 13: Measurements of the maximum diameter of the outflow tract during heart diastole for each treatment group on Day 3 and Day 4 [n=16].

Table 3: Measurements of the maximum diameter of the outflow tract during heart diastole for each treatment group on Day 3 and Day 4.

Treatment Group	Initial Length (mm)	Final Length (mm)	Net Change in Length (mm)	Percent Change (%)
Control (1)	0.43	0.63	0.20	46.51
Control (2)	0.52	0.71	0.19	36.54
Control (3)	0.55	0.75	0.20	36.36
Control (4)	0.41	0.66	0.25	60.98
Control (Mean)	0.48	0.69	0.21	45.10
D-Glucose 0.03M (2)	0.41	0.55	0.14	34.15
D-Glucose 0.03M (4)	0.48	0.57	0.09	18.75
D-Glucose 0.03M (6)	0.58	0.67	0.09	15.52
D-Glucose 0.03M (Mean)	0.49	0.60	0.11	22.81
D-Glucose 0.05M (1)	0.55	0.73	0.18	32.73
D-Glucose 0.05M (2)	0.52	0.62	0.10	19.23
D-Glucose 0.05M (3)	0.46	0.66	0.20	43.48
D-Glucose 0.05M (Mean)	0.51	0.67	0.16	31.81
L-Glucose 0.03M (1)	0.55	0.69	0.14	25.45
L-Glucose 0.03M (3)	0.45	0.51	0.06	13.33
L-Glucose 0.03M (4)	0.55	0.69	0.14	25.45
L-Glucose 0.03M (5)	0.45	0.51	0.06	13.33
L-Glucose 0.03M (Mean)	0.50	0.60	0.10	19.39
L-Glucose 0.05M (1)	0.44	0.55	0.11	25.00
L-Glucose 0.05M (2)	0.56	0.62	0.06	10.71
L-Glucose 0.05M (Mean)	0.50	0.59	0.09	17.86

The distance between the top of the embryo heart to the embryo head was also analyzed. For the control embryos, the distance between the top of the embryo heart to the embryo head decreased by 9% from Day 3 to Day 4. On average, the eggs treated with 0.03M of D-glucose had a net increase in the distance between the top of the embryo heart to the embryo head by 12%. On average, the eggs treated with 0.03M of L-glucose had a net increase in the distance between the top of the embryo heart to the embryo head by 9%. On average, the eggs treated with 0.05M of D-glucose showed a net increase in the distance between the top of the embryo heart and the embryo head by 24%. On average, the eggs treated with 0.05M of L-glucose showed a net increase in the distance between the top of the embryo heart and the embryo head by 34%. Figure 14 and Table 4 depict the measurements of the distance between the top of the heart to the head for each treatment group on Day 3 and Day 4.

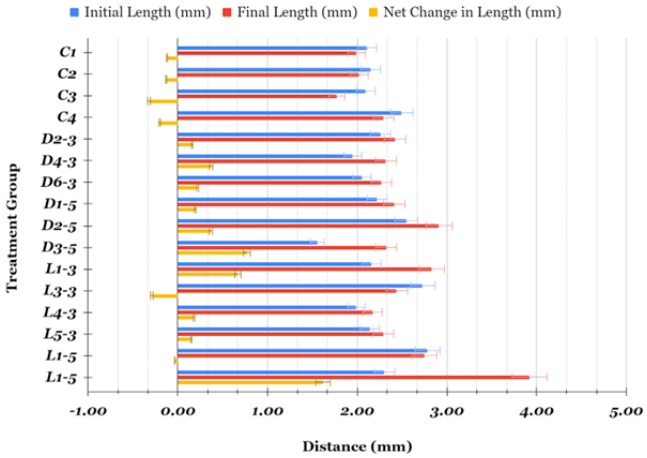

Figure 14: Measurements of the distance between the top of the heart to the head for each treatment group on Day 3 and Day 4 [n=16].

DOI: 10.36838/v5i2.18

Data Table 4: Measurements of the distance between the top of the heart to the head for each treatment group on Day 3 and Day 4.

Treatment Group	Initial Length (mm)	Final Length (mm)	Net Change in Length (mm)	Percent Change (%)
Control (1)	2.11	1.99	-0.12	-5.69
Control (2)	2.15	2.02	-0.13	-6.05
Control (3)	2.09	1.77	-0.32	-15.38
Control (4)	2.50	2.29	-0.20	-8.17
Control (Mean)	2.21	2.02	-0.19	-8.82
D-Glucose 0.03M (2)	2.26	2.42	0.16	7.08
D-Glucose 0.03M (4)	1.95	2.32	0.37	18.97
D-Glucose 0.03M (6)	2.05	2.27	0.22	10.73
D-Glucose 0.03M (Mean)	2.09	2.34	0.25	12.26
D-Glucose 0.05M (1)	2.22	2.41	0.19	8.70
D-Glucose 0.05M (2)	2.55	2.91	0.37	14.41
D-Glucose 0.05M (3)	1.55	2.32	0.77	49.58
D-Glucose 0.05M (Mean)	2.11	2.55	0.44	24.23
L-Glucose 0.03M (1)	2.16	2.83	0.67	31.02
L-Glucose 0.03M (3)	2.73	2.44	-0.29	-10.62
L-Glucose 0.03M (4)	1.99	2.17	0.18	9.05
L-Glucose 0.03M (5)	2.14	2.29	0.15	7.01
L-Glucose 0.03M (Mean)	2.26	2.43	0.18	9.12
L-Glucose 0.05M (1)	2.78	2.75	-0.03	-1.26
L-Glucose 0.05M (2)	2.30	3.92	1.62	70.18
L-Glucose 0.05M (Mean)	2.54	3.34	0.80	34.46

Conclusion

Around 3 to 6% of infants worldwide are affected by congenital disabilities, and about 1% are born with congenital heart disease, which causes them to have heart abnormalities in morphology.[11] Mothers with diabetes or high blood sugar are more likely to give birth to infants with heart defects. The placenta only manages to filter some molecules from entering the embryo's bloodstream, and the sugar or glucose content is not one of them. High glucose content in the embryo's blood can lead to hyperglycemia and malformations in the developing heart, causing CHD. People with CHD have a higher risk of heart failure and reduced life expectancies. The development of CHD occurs during the early stages of human embryonic development, and different variables, especially nutrition, play a prominent role in its formation.[10]

In this research, we use chicken embryo models to model heart development and study the effect of glucose during heart formation. Based on our experimental results, we learned that adding any concentration and type of glucose alters the development of the embryo's heart. The number of available chicken embryos limited this research. Further research could include the same experiment, comparing 0.03M D and L-glucose embryos with 0.05M D and L-glucose embryos to control embryos on a much larger scale (using 20-30 viable chicken embryos per treatment group), measuring more variables such as blood flow rate, as well as tracking the development of the embryos for longer than 24 hours, looking past HH24. This study can also be extended to other animal models, such as the embryos of quail, zebrafish, and mice, and see if similar results are produced, which can provide greater insight into early human heart development.

Acknowledgments

The Rugonyi lab supported this work at the Oregon Health and Science University (OHSU), the Kheradvar lab at the University of California-Irvine (UCI), and the National Science Foundation (NSF). It was part of the HEART (High school students Engaged in Applied science, Research, and Technology) internship at OHSU, sponsored by an NSF collaborative grant NSF/CBET 2109918 (to Dr. S. Rugonyi) and NSF/CBET 2109959 (to Dr. A. Kheradvar). The authors would like to acknowledge Dr. Sandra Rugonyi, Floyd Rubonal, and Katie Lee for their mentorship and assistance in performing the experiments and data analysis.

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DOI: 10.36838/v5i2.18