Addition of glucose solutions to aquatic environment of *Danio rerio* embryos in concentrations of 4 mM to 14 mM increases heart rate BPM for zebrafish exposed to elevated glucose levels

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Abstract

Background: Current scientific knowledge suggests that cells experience normal functional abilities when glucose levels are stable, as glucose represents the fuel implemented to generate energy for cellular functions. Previous literature cites cardiovascular complications as the leading cause of mortality in diabetic patients. A vital model in the biological field of study, zebrafish embryos can emulate the effects of glucose on the cardiovascular system for an insight to its impact on the human heart. Although studies have been conducted to address the implications resultant of high glucose consumption during human fetal development, the field of endocrinology lacks research explicating how high glucose intake during pregnancy leads to gestational diabetes.

Methodology/Principle Findings: The cardinal objective of the investigation wreaths identifying the correlation between the glucose consumption during pregnancy and fetal development of gestational diabetes. By measuring the heart rate and developmental abnormalities exhibited by zebrafish embryos exposed to divergent concentrations of glucose, we determined that in an elevated glucose environment-for a human, the surrounding amniotic fluid-the embryo seldom survives. Cardiovascular morphogenesis and embryonic development are highly conserved across species enabling zebrafish embryos to model human heart development and the human condition-gestational diabetes. The zebrafish heart has two chambers opposed to the human four. This difference in composition does not impede their similarities in cardiovascular phenotypes and morphogenesis. Through our observations, we noted embryonic stage delay and cardiac malformations in embryos exposed to 14 mM and 8 mM glucose concentrations as a result of hyperglycemia during organogenesis. Additionally, there appeared to be abnormal growth of these two experimental groups.

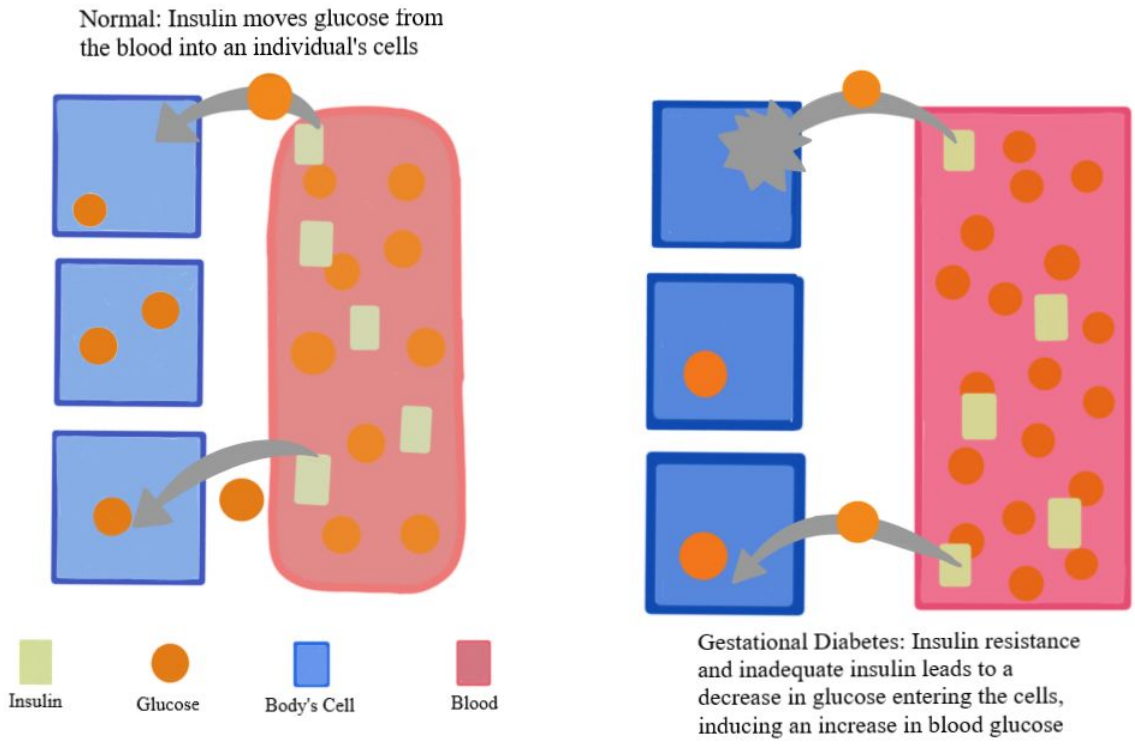
Conclusions: The statistically significant mortality rates of embryos exposed to 14 and 8 mM glucose environs as a result of cardiovascular complications emulates a possible consequence from heightened maternal glucose intake during pregnancy. An avenue for further research wreaths an assay to determine the genetic and molecular impact on elevated environmental glucose levels during embryological development. For future litigations, the medical field should endeavor to discern metabolic glucose adaptations on a molecular level to develop an exceedingly effective supplement for diabetic women and those who develop gestational diabetes during pregnancy. Neonatal and prenatal events at crucial developmental windows have been proven to result in deterred growth potential and organ complications. Exposure to above normal glucose concentrations during fetal development comprises the possibility of abnormal adaptations to the nutritional event and altered biological mechanisms. Such epigenetic phenomenons may affect the manner by which the individual regulates internal glucose levels later in life, dependent from the mother's nutritional sources. In summary, the present manuscript demonstrates that an increase in glucose exposure to the environment of a developing embryo leads to heart fatalities and physiological malformations.

Introduction

Amidst the United States, 29 million individuals endure diabetes, calculating to 9.3 percent of the entire American populace. In addition to this harrowing statistic, another 84 million personages are diagnosed with prediabetes, a condition that generally acts as a precursor to type two diabetes within the time frame of five years. Approximately 7 percent of United States pregnancies exhibit gestational diabetes resulting in 21% fetal mortality, (Madri et al., 2010). Contemporaneously, the slope of increasing diagnoses is slim. With that being said, diabetes delineates the seventh leading cause of mortality in the United States. Diabetes subsumes a mosaic etiology accrued from insulin production and signaling deficiencies, insulin sensitivity to target tissue modifications, and hepatic gluconeogenesis (Elo et al.,

2007). To extrapolate the predominant symptom of diabetes, individuals with a form of the disease experience augmented blood glucose, termed hyperglycemia which is characterized by insulin deficiency or insulin resistance. A disorder of one's metabolism, diabetes inhibits the functionality and performance of the digestive tract with regards to its ability to decompose carbohydrates, specifically the starches in foods, into glucose, the sugar that infiltrates into the bloodstream and provides energy for intrinsic bodily processes and mechanisms. One factor scientifically proven to cause diabetes is obesity, an expeditiously burgeoning heath impingement on modern society. In conjunction with the development of type two diabetes, obesity forbodes heart disease, elevated blood pressure, stroke, particular classes of cancer, and death. To provide an exemplar of a conventional product consumed by the general public, high fructose corn syrup is a substance incorporated in a plethora of processed foods and when uptaken by the body, the sugars are condensed to glucose and stored for later utilization. Copious quantities of glucose in the human body fabricates ample quantities of superfluous body fat, a herald to more inimical complications - cancer, type two diabetes, stroke, heart disease, and pregnancy complications. Each health burden enumerated results from an excessive accumulation of body fat from an overconsumption of copious amounts of unhealthy sugars. This trend is not limited to the nutritional choices executed by the individual. Consumption of a bounteous quantity of sugars during pregnancy may adversely affect the developing human embryo and child later in life. Gestational diabetes, the diabetes classification formidably stimulating implications upon the human embryo during pregnancy, developed in five to eight percent of women around week twenty-four to twenty-eight.

Figure 1. Glucose and insulin experience changes in gestational diabetes.



Delayed movement of glucose molecules into blood cells elicits the development of diabetes in the individual.

Knowledge in regards to the role of early nutritional stimuli as catalyzers of metabolism and overarching personal health is immensely sparse. Vascular complications depict the leading cause of mortality in diabetic patients and zebrafish embryos exemplify a quintessential model to test the effects of glucose on the cardiovascular systems of zebrafish to provide an insight to the impact on human beings. This study canvasses the effect of different concentrations of glucose solutions on the cardiovascular system and frequency of mortality experienced by *Danio rerio* embryos and through each stage of development. In this investigation, we strive to elucidate and clarify the following impelling question: do zebrafish embryos have a greater mortality when placed in wells with the highest dose of glucose 96 hours post fertilization as opposed to when placed in the well with the smallest doses of glucose over a span of 96 hours? In conjugation with this testable question, we speculate that glucose possesses adverse

effects on the cardiovascular systems of developing zebrafish embryos. Does the concentration of glucose exposure encompass a significant effect on the overarching heart health of the *Danio rerio* species?

Gestational diabetes, induced by elevated glucose exposure, elevates the implication of congenital heart defects in juvenile human beings experiencing hyperglycemia and adverse afflictions on endocrine cushion morphogenesis as well as the development of neural crest cells (Liang et al., 2010). There exists a considerable body of literature canvassing congenital anomalies in infants of diabetic mothers, including cardiovascular, genitourinary, and musculoskeletal malformations, (Mills, 2010). Emerging evidence propounds that vitamin D administration can ameliorate insulin sensitivity and glucose tolerance. Whether vitamin D supplementation can prevent gestational diabetes is contemporaneously beyond the scope of clinical knowledge (Burris et al., 2014). As an anti-diabetic drug, vitamin D has been approved by the Food and Drug Administration (FDA) for its qualification in insulin secretion amplification and insulin absorption reduction from the gastrointestinal tract. The importance of studying gestational diabetes mellitus lies in the manner by which hyperglycemia evolved during pregnancy increases the risk of maternal type 2 diabetes and predisposes the developing fetus to destitute metabolic function throughout life (Ruchat et al., 2013). A significant and illustrious question exists, canvassing the effects of hyperglycemia on heart organogenesis. Contemporary investigations explicate congruences in regulatory mechanisms of glucose metabolism in zebrafish and mammalian models. Such similarities include the production of insulin and the genes attributed to blood glucose level management.

We proposed the following hypothesis: if elevated glucose exposure causes adverse effects on the cardiovascular system of a developing embryo, and we compare the heart rate of zebrafish embryos, and we compare the heart rate of zebrafish embryos exposed to concentrations ranging from 4 to 14 mM for 96 hours post-fertilization (hpf), then there will be a statistically significant increase in heart rate leading to lethal heart complications in the group that is exposed to 14 mM glucose rather than in zebrafish embryos exposed to lower concentrations. Previous studies implementing zebrafish embryos have evinced

a broad spectrum of morphological and cognitive effects stimulated by glucose exposure, including developmental retardation, diverse cardiac malformations, cardiac looping defect, and hyperglycemia. Arising from an incapacity to regulate gluconeogenesis, hypoglycemia delineates a clinical hallmark of gestational and type II diabetes.

Materials and Methods

Table 1. Glucose chemical concentrations for larger stocks.

	Dissolve 180 grams glucose in 1 L of distilled water	Total	1000 mL of 1 Molar stock solution = 1000 mmol					
	Stock in Moles	Stock in mmol	L of stock glucose solution	mL of stock glucose solution	mL of Embryo Media	Final glucose in M	Final glucose in mM	Total volume
G1	0.1	100	0.04	40	960	0.004	4	1000
G2	0.1	100	0.08	80	920	0.008	8	1000
G3	0.1	100	0.14	140	860	0.014	14	1000

Moles of solute is equivalent to molarity multiplied by the liters of solution. Blood glucose levels exceeding 7.0 mmol/L (126 mg/dl) occurs when fasting and 11.0 mmol/L (200 mg/dl) two hours following. Normal blood glucose straddled between 4.0 to 6.0 mmol as represented by G1. G2 correlates to the blood glucose concentration possessed by an individual after fasting and G3 models a personage with a type of diabetes.

Table 2. Glucose chemical concentrations for reduced stock amounts.

Stock in reduced amounts	mL of glucose stock solution	mL of Embryo Media	Final in mL	Total volume
0.1	10	240	4	250
0.1	20	230	8	250
0.1	35	215	14	250

The values depicted above represent the quantities we formulated for the experiment to prevent a superfluity of solutions.

Table 3. Vitamin D3 chemical concentrations for stock solutions.

Number of capsules	Molecular weight	mL of distilled water	Molarity	mL of stock D3 solution	mL of Embryo Media	Final D3 in nmol	Final D3 in mM	Total volume in mL
8	385 g/mol	250	0.00001	75	175	3000	0.003 300 nM	250

Apply heat to 250 mL of distilled water and place eight vitamin D3 capsules into the heated solution. Utilize a glass stirring rod to evenly distribute the vitamin D and produce of homogeneous solution. After allowing the solution to reduce in temperature, add 75 mL of the vitamin D3 stock solution to 175 mL of embryo media. Distribute 1 mL of the solution to the six designated vitamin D3 wells.

Experimental Design

66 fertilized embryos at the same developmental stage 5 hpf were divided into 18 wells using plastic pipettes and exposed to six different environments. One milliliter of 4 mM, 8 mM, 14 mM glucose concentrations as well as vitamin D3 and vitamin D3 combined with 4 mM were pipetted into the wells. The toxant was changed twice everyday to maintain an unfluctuating environment. During non observational intervals, embryos and fish were maintained in an incubator set at 28℃ in a light and dark cycle, implementing a black, plastic cover to emulate a evening environment. Embryos and juvenile zebrafish were observed prior to solution exchange under either a stereomicroscope or a compound microscope, with the fish observed through a bridge slide. Mortality, hatching, morphological defects and characteristics, and heartbeat per minute was calculated and recorded on a data sheet. The magnification employed for the compound microscope was 4x and 10x. 1x and 2x magnifications were implemented during stereomicroscope usage. In addition, we used a compound microscope, formulating a bridge slide composed of six slides, to view the embryos and juvenile fishes.

Ethics Statement

Handled in conjugation with the protocols approved by the Animal Care and Use Committee (ACUC) of the University of Wisconsin-Milwaukee (UWM), zebrafish embryos were cared for and maintained in an ethically controlled environment.

Safety Implications

The glucose the we will be utilizing within our experiment must be handled with care and stored in a well sealed container that is separated from strong oxidants. It must be kept away from flames and other electrical equipment. If placed in a slightly ajar container during the course of an experiment the chemical must be ventilated consistently. Do not directly smell or inhale the substance as safety concerns and health issues will arise. Safety goggles must be worn at all times when handling the chemical and experimenting with it as a variable in a study.

Model Organism

Optically clear, silhouetting synchronous development within a clutch, rapid ex utero development, and produced in large numbers, *Danio rerio* emerge as an attractive model organism for investigating the effect of varying glucose levels on the developing embryo. Being a single clutch produces a sufficient quantity of eggs to orchestrate several litigations, the employment of zebrafish as the model organism in an experiment eliminates the conundrum of significant genetic variation between groups and individuals. The opthalmic distinctiveness sanctions detailed morphological analysis conducted through a stereo dissecting microscope. Due to its fecundity and genetic and physiological semblances to mammalian organisms-conserved developmental pathways between zebrafish and human-the zebrafish as evolved as an integral constituent in endocrinological investigations. In contemporary biological fields, the zebrafish exponentially emerges as an illustrious and informative

model system for studying vertebrate embryogenesis and organogenesis, implementing a standard for the analysis of nutrient exposure on the development zebrafish embryos in correlation to human disease simulations (Ali et al., 2011). In addition, gene functions and developmental pathways are conserved between zebrafish and human species, thus zebrafish delineate an impeccable model for human diseases and clinical investigations. Male and female zebrafish embryos were transported from the University of Milwaukee post-fertilization.

Table 4. Developmental phenotype and stage analysis.

Embryonic phenotype/stage	Criteria
1. Establishment of three body axes	From approximately 3 hpf to 10 hpf, anterior-posterior, left-right, and dorsal-ventral axes develop.
2. High dome stage	At 4 hpf, the embryos endure the process of epiboly, by which cells migrate down the barriers of the yolk whilst a layer of cells veil the entire yolk.
3. Gastrulation	5.25-10 hpf, inducing the formation of three distinct primary germ layers: endoderm, mesoderm, and ectoderm. These germ layers provoke tissue formation, akin to that found in the adult fish.
4. Segmentation period	Characterized by the formation of somites, a precursor to muscle and nervous system development. Chevron-shaped structures, somites are located in the posterior sector of the developing embryo.
5. Eye	By 13 hpf, the eye begins to develop and evident through a dissection microscope by 25 hpf.
6. Brain	By 25 hpf, the developing brain and optic vesicle of a forthcoming ear manifest, the heart begins to beat, and the yolk is wholly integrated in vivo of the body of the embryo.
7. Chorion degeneration	Between 48 hpf and 72 hpf, the zebrafish hatch from the embryo the the chorion dismantles. Movement becomes apparent.

Zebrafish embryonic development occurs in discrete steps and the abovementioned morphological markers aid in the identification and comparative analysis of a given stage observed in embryos exposed to divergent glucose concentrations.

Embryo Media

Synthesized from 0.42 grams of "Instant Ocean" salt in 2 Liters of distilled water.

Embryo Care

The *Danio rerio* embryos were maintained in an 28℃ incubator on a 14 hour light and 10 hour dark cycle. Handling and organism care adhered to local and national regulations. Sixty-six embryos were allotted amongst six rows of wells-three to four in each: high glucose, medium glucose, low glucose, vitamin D3, vitamin D3 and low glucose. To eliminate objectivity and bias, we henceforth refer to the glucose experimental groups as G1, G2, and G3. The three control group wells contained 1 mL of embryo media solution with three zebrafish embryos; G1 experimental group inhabited the second row constituting 1 mL of 4 mM glucose solution; row three housed G2 experimental group with 1 mL of 8 mM glucose solution; G3 experimental dwelled in row four exposed to 1 mL of 14 mM glucose solution. To test the efficiency of clinical supplements in reducing symptoms and signs of diabetes, two rows were implemented-one encapsulating 1 mL of G1 and 1 mL vitamin D solution and the second with solely 1 mL vitamin D solution. Devised from heating 200 mL of distilled water and eight capsules of vitamin D3, the vitamin D solution exemplified a clinical trial to substantiate the faculty of vitamin D as a predominant treatment of gestational diabetes in human beings. Solutions were exchanged twice a day to extract debris-an inhibiting factor of growth and development-whilst deceased embryos were removed instrumenting a 3 mL volume plastic pipette.

Toxicological and Pharmacological Treatments

Danio rerio embryos three hours post fertilization were arrayed in one and a half 12-well plates and maintained in embryo media in preparation for drug treatment. Glucose was dissolved in two hundred milliliters of distilled water through the implementation of heat to catalyze the process and guarantee the

even distribution of carbohydrate molecules in solution. The methodology for glucose solution concoction and synthesis is summarized in Tables 1 and 2, displaying the molecular weights required for 14 mM, 8 mM, and 4 mM solutions. For 96 hours, we applied two treatments for every 24 hour period to avoid dust contamination and pollution altering the results. Each treatment provided 1 mL of glucose solution to establish a constant for quantity.

Glucose Concentration Determination

Initially, the three experimental glucose test groups were referred to as high glucose (14 mM), medium glucose (8 mM) and low glucose (4 mM). After two days of exposure, to my stupefaction, the medium glucose and low glucose environments elicited the death of all embryos in the six wells housing a total of twenty-four embryos. Consequently, we presumed an entanglement when applying the initial treatments, thus referring to the experimental groups as G1, G2, and G3 to eradicate a biased opinion on the subject and proceed with experimental measures to determine the relative glucose concentration of each solution. The initial method utilized Benedict's solution, a water bath, and a test tube with the solution in litigation. After implementing the procedure and waiting an ample amount of time with no novel changes, we decided to progress in a divergent path: glucose test strips. The clinical reagent strips purchased from Flinn Scientific provide quantitative glucose determination, measuring in the range of 0 to 110 mmol/L. A color chart features six different pigments in correlation to a particular scope of glucose concentrations. In reference to the color chart photographed in Figure 2, the G1 solution, previously presumed the 14 mM high glucose solution, constituted low levels of glucose, between 1 mM and 5 mM, as disclosed by the light green pigmentation on the glucose test strip. By determining the concentration of G1, we confirmed these wells to contain 4 mM glucose solution, G2 to possess 8 mM glucose, and G3 constituting 14 mM glucose.

Figure 2.

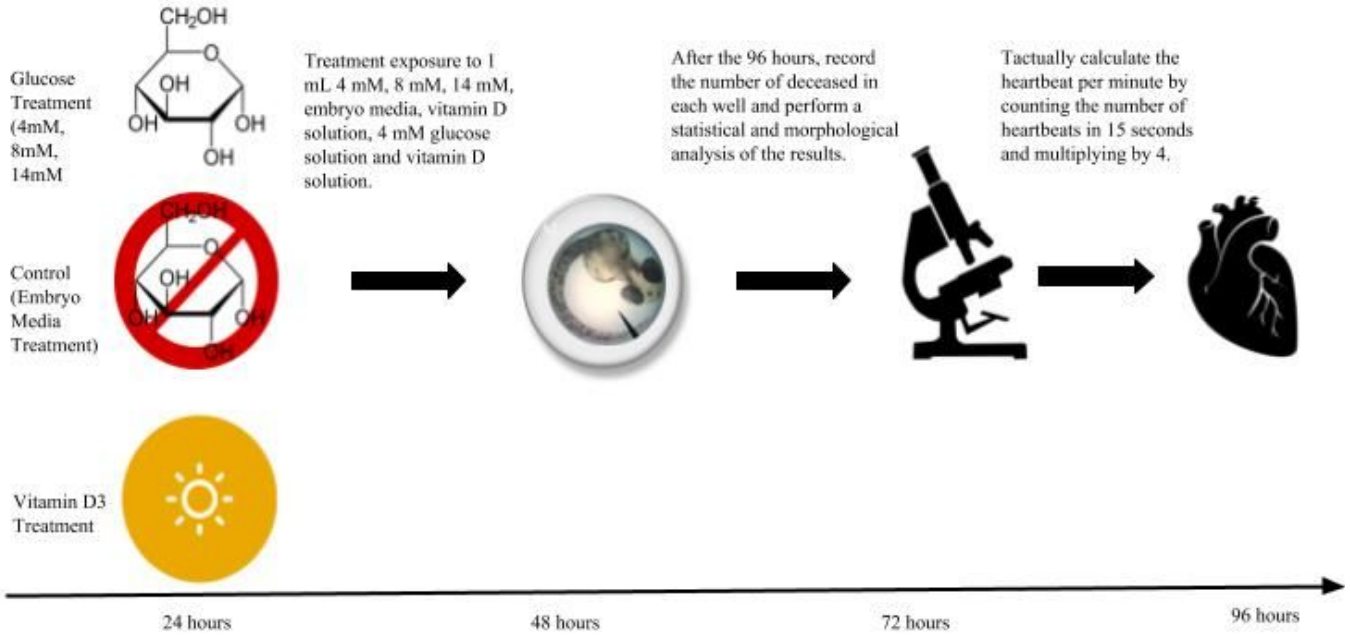
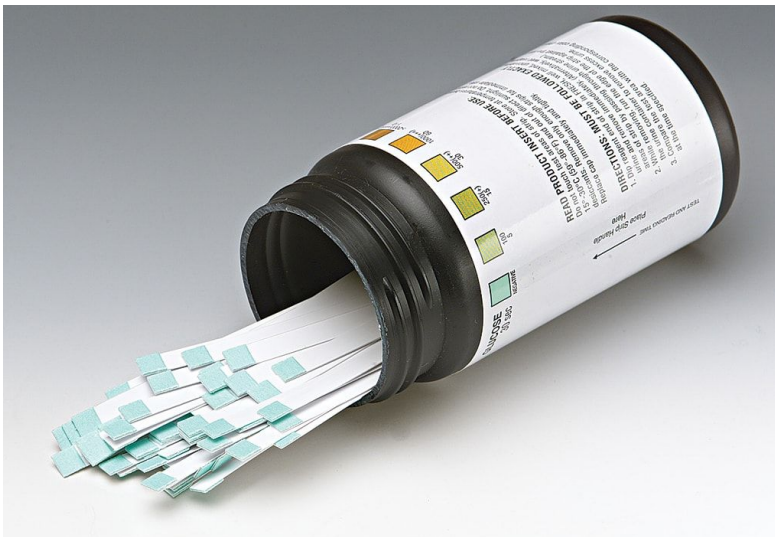


Figure 3. Glucose test strips and color chart.



A cyan, turquoise pigment represents no presence of glucose in solution; light green renders 5 mM glucose; moderate toned green delineates 15 mM glucose; honey yellow designates a 20 mM glucose concentration.

Data Collection

Data were collected and maintained through photography and videology. A majority of the photographs were obtained through a compound microscope at 1 X and 2 X resolution, constructing a bridge slide to house the embryos and permit visibility by means of light refraction. Capturing cardiovascular metamorphosis, adverse heart alterations, heartbeat, morphological changes, video and photo moulded a platform for further analysis and interpretation. Quantitative and qualitative data was tabulated in Google sheets. In an organizational scope, we labeled the wells as high glucose, medium glucose, low glucose, control, vitamin D, and vitamin D + glucose, indicating the chemicals and concentrations. To further explicate data management, we titled the observational notes and heartbeat calculations, catalogued the mortality rate, as well as signed and dated all documentation.

Heart Rate Measurement

Two different image-based stratagems were employed to qualitatively evaluate and analyze the cardiac rate and morphological metamorphosis in the embryonic zebrafish. On the basis and assessment of time-varying anatomy of the embryonic heart, the first method captured changes during vasculogenesis and the second analytical photography captured incremental stages of organogenesis. Orchestrating videography to document the beats per minute, we were able to calculate the average heart rate of the G1 experimental, control, and vitamin D3 test groups to determine whole heart arrhythmias. To quantify the heart rate, we documented the number of beats in 15 seconds and multiplied this number by four, to enumerate beats per minute.

Results

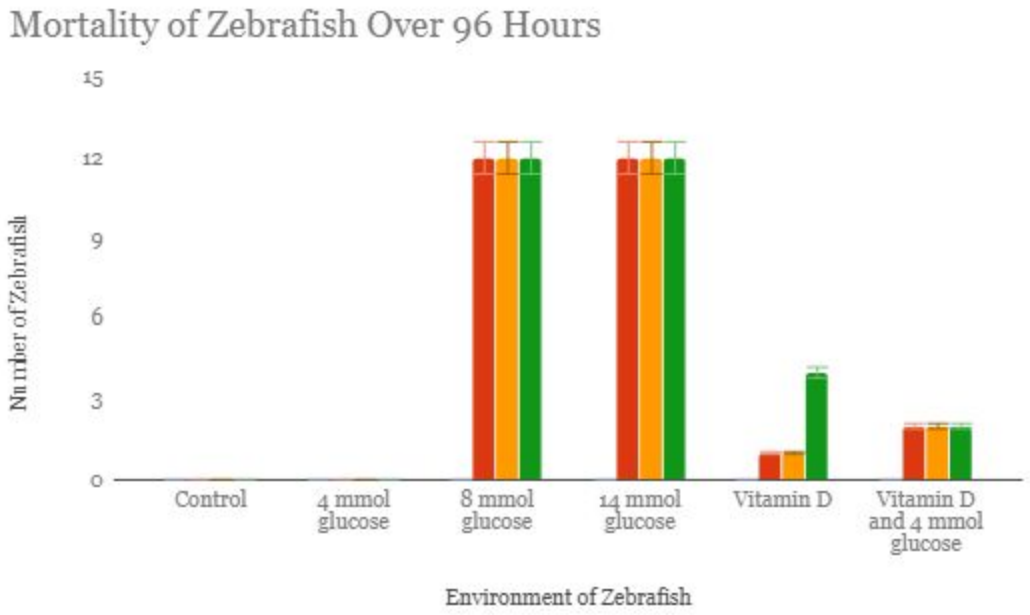
General Findings

To model zebrafish with high fasting blood glucose-hyperglycemia-we introduced 14 mM glucose concentrated solution to the aquatic environment, 8 mM ,mirrored blood glucose levels after fasting, and 4 mM contoured glucose ratios displayed by individuals at risk of diabetes development. Each solution was exchanged during two intervals each day. The experiment performed ranged from a 4 mM to 14 mM glucose exposure to zebrafish embryos as well as a trial interpolating the efficiency of a vitamin D3 supplement on embryos exposed to abnormal levels of glucose and thus mirrors the resulting regulation of blood glucose levels in the organism. In assessing the final mortality computations for the experimental groups, the zebrafish embryos exposed to 14 mM and 8 mM glucose experienced one hundred percent mortality. Accordingly, the physiological stages of these zebrafish embryos were not analyzed further. For the purpose of standardization, I administered 1 mL of a respective solution to each well.

Mortality and Hatching

Expediting growth, the vitamin D environments provoked early hatchure from the chorion 16 hours post fertilization (hpf). Initially, G3 did not experiences stunted growth, whilst G1, G2, and Vitamin D/G1 endured growth retardation, however, after 48 hours, this observation reversed. The mortality and survival of zebrafish embryos and larvae were observed and tabulated as delineated in Figure 2. Numbers for each category were amassed by counting alive and deceased embryos and larvae in each of the wells. No mortality was observed during the 24 hpf stage of development for all experimental groups. Past 36 hpf, embryos exposed to 8 mM and 14 mM experiences adverse cardiovascular implications and rapid heartbeat eliciting a heightened mortality rate. By 48 hpf, 14 mM and 8 mM succumbed to 100% mortality, whilst the control group and 4 mM faced no fatalities.

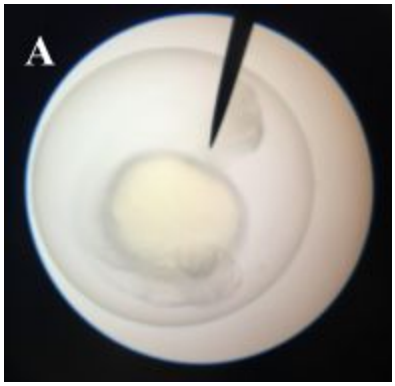
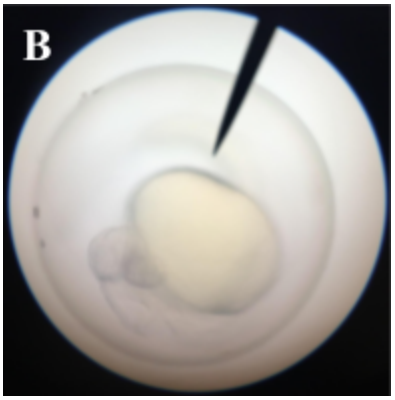
Figure 2. Zebrafish exposed to elevated glucose experience elevated mortality



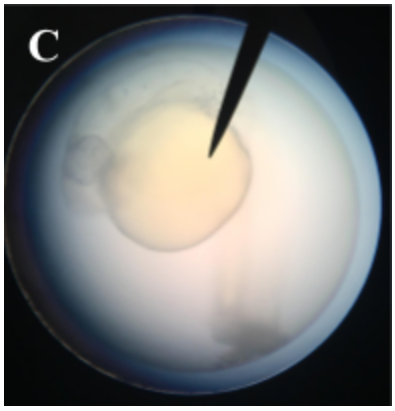
By the completion of the investigation, all of the zebrafish exposed to aquatic environments with 8 mM and 14 mM glucose concentration endured statistically significant rates of mortality, each still in their chorions at time of death. The 4 mM embryos all hatched and survived the duration of the experiment, vitamin D and vitamin D + 4 mM glucose experienced some death.

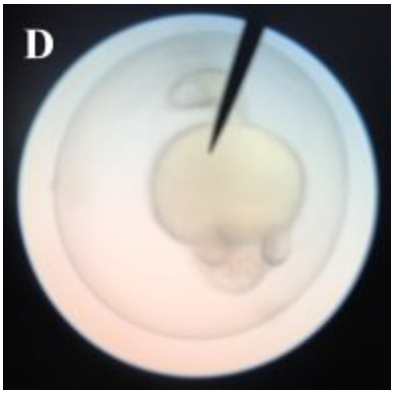
Developmental Abnormalities

Figure 3. Morphological analysis reveals growth retardation in embryos exposed to elevated glucose concentrations.

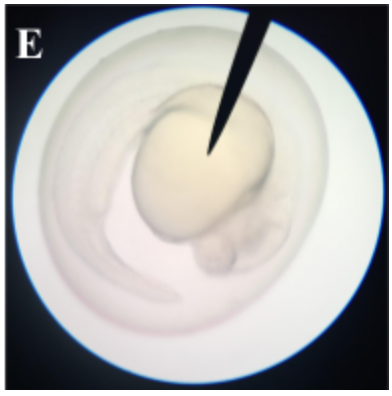
	17 hours post fertilization	
4 mM glucose		

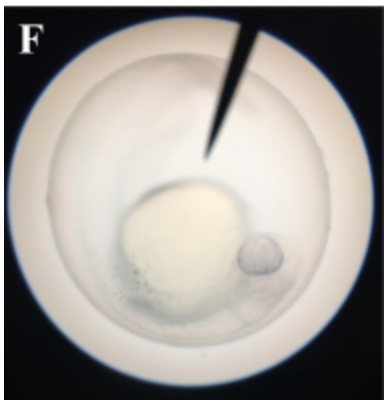
8 mM glucose



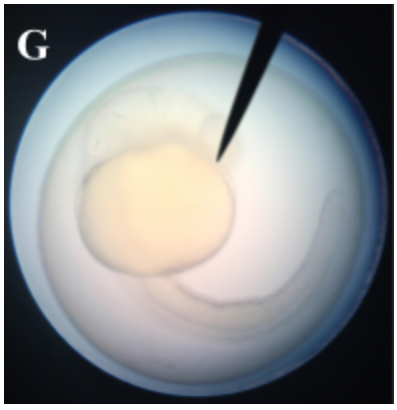


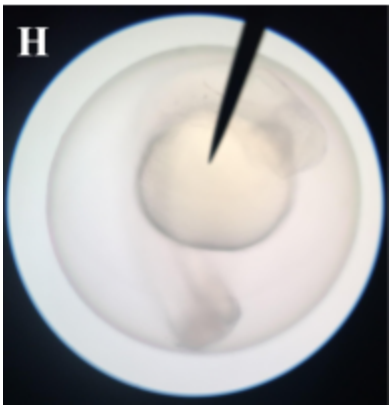
14 mM glucose



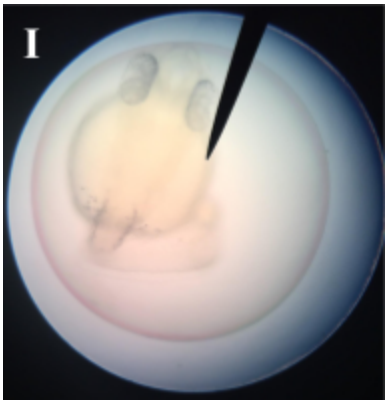


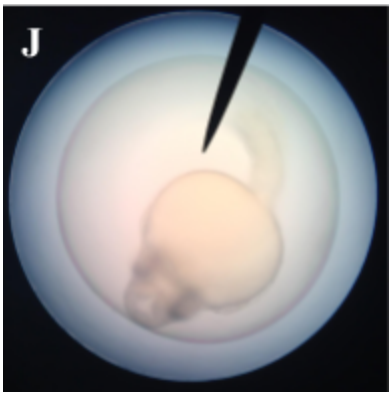
Control



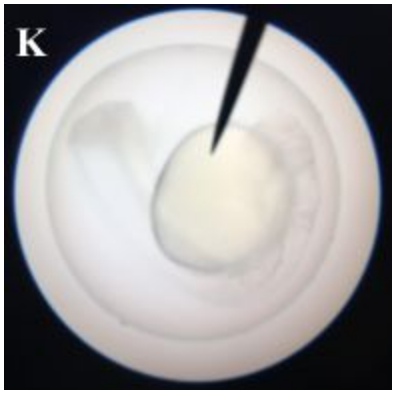


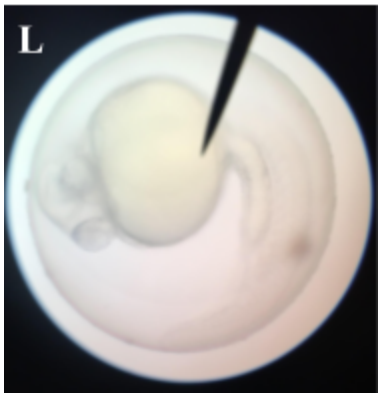
Vitamin D3 and 4 mM glucose





Vitamin D3

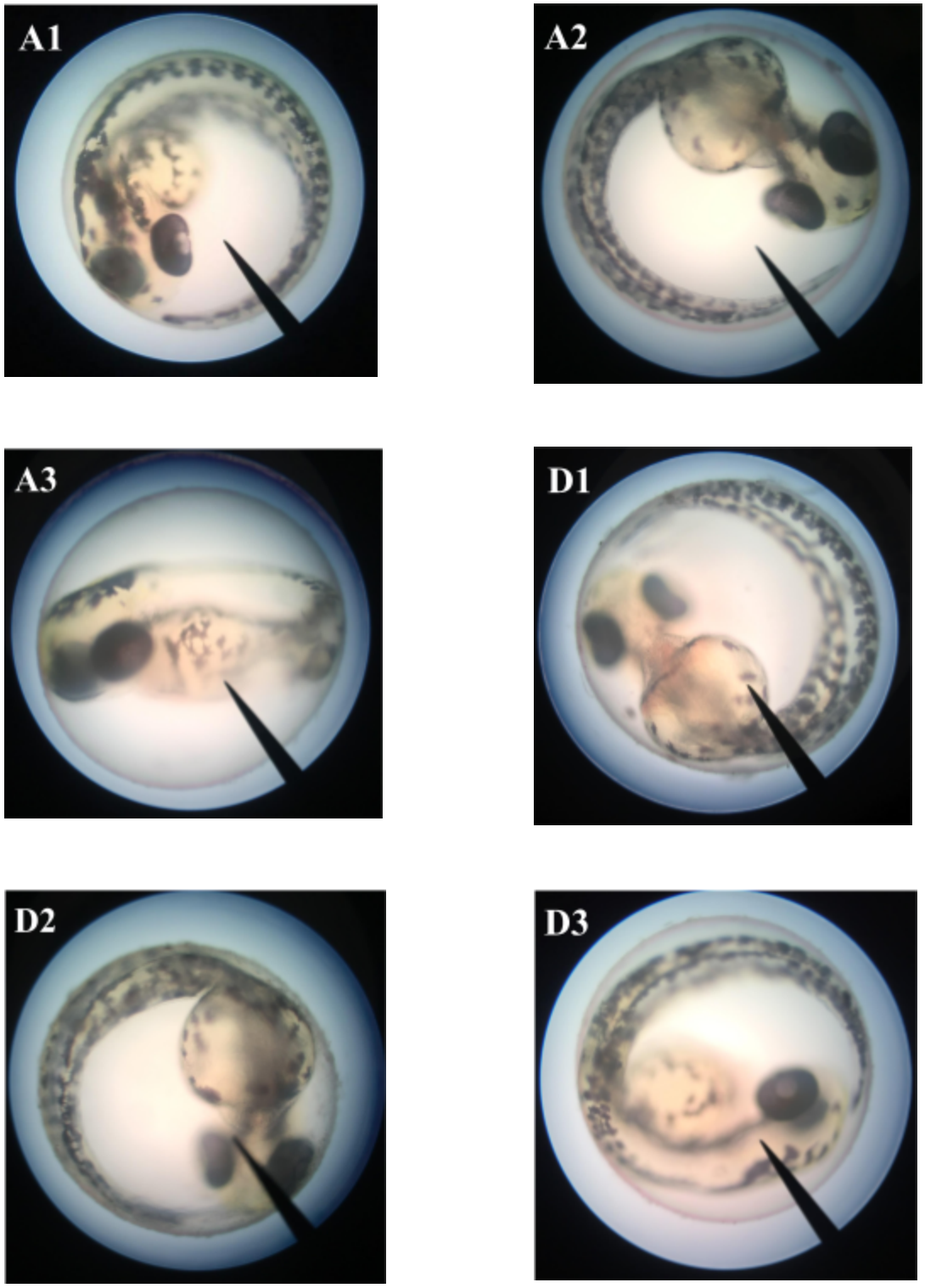


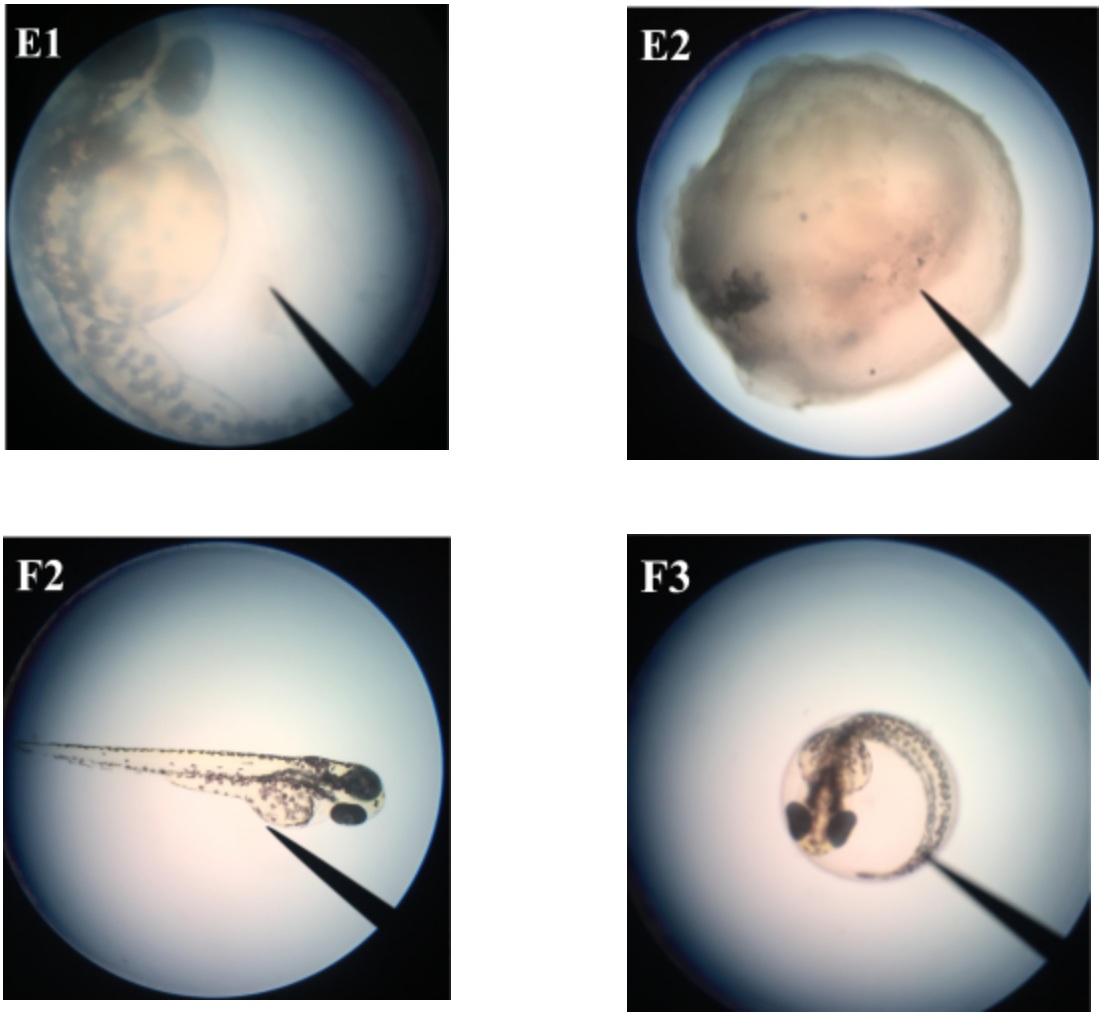


Danio rerio embryos 17 hours post fertilization are depicted above. **A** and **B** (4 mM glucose) externalize normal growth trajectory, whilst **C** and **D** (8 mM glucose), **E** and **F** (14 mM glucose), experienced developmental retardation silhouetting 12 hpf embryological development, as the notochord exhibits a decrease in length when comparatively analyzed with **G** and **H** (control group). Despite notochord shortening, 8 mM and 14 mM glucose embryos encompass a broadened yolk sac diameter. **I** and **J** delineate zebrafish embryos exposed to vitamin D3 solution and 4 mM glucose to simulate the effectiveness of an over-the-counter (OTC) clinical supplement in contesting gestational diabetes. The vitamin D3 evinces proficiency by inverting the growth deficiencies observed in 4 mM, 8mM, and 14 mM embryos. The eyes exemplify opaque black pigmentation and normal development of craniofacial featuring the typical dome configuration. Embryos **A-F** experiences delayed cranial skeletal ossification resulting in a flat head terminal. Embryos immersed in 4 mM, 8 mM, and 14 mM divulged acute embryonic movements, characteristic of a difficulty in acclimating to the elevated glucose concentrations.

Figure 4. Morphological comparison of *Danio rerio* 65 hours post fertilization

65 hours post fertilization





The embryos exposed to 4 mM glucose and vitamin D3 solution experienced an increase in ocular size and lateral pigmentation in comparison to those submerged in embryo media (control). The embryo heart is more patent in **D1** (control group), and the cardiovascular system in **A1-A3** (4 mM glucose) is moderately transparent-obscured through photographical data. **F2** and **F3** depict *Danio rerio* embryos rendered an environment infused with vitamin D3 and 4 mM. These juvenile fishes bared progressed hatching in lieu of the delayed development observed by the zebrafish in solely 4 mM glucose solution. **A1-A3** possess elevated pigmentation of the dorsal and ventral stripe. **E1** and **E2** illustrate embryos immersed in a vitamin D3 environment. A representation of a deceased embryo is delineated in **E2** with a light brown hue and clouded semblance. **A1-A3** endured delayed chorion degeneration-a morphological phenotype described in Table 4-resulting in delayed hatching. Upon hatching, juvenile fishes exposed to 4 mM exhibited delayed escape response when we extracted and resupplied solution dosages instrumenting a plastic pipette. Overall, 4 mM *Danio rerio* experiences a reduction in swimming activity, motor responses, and movement.

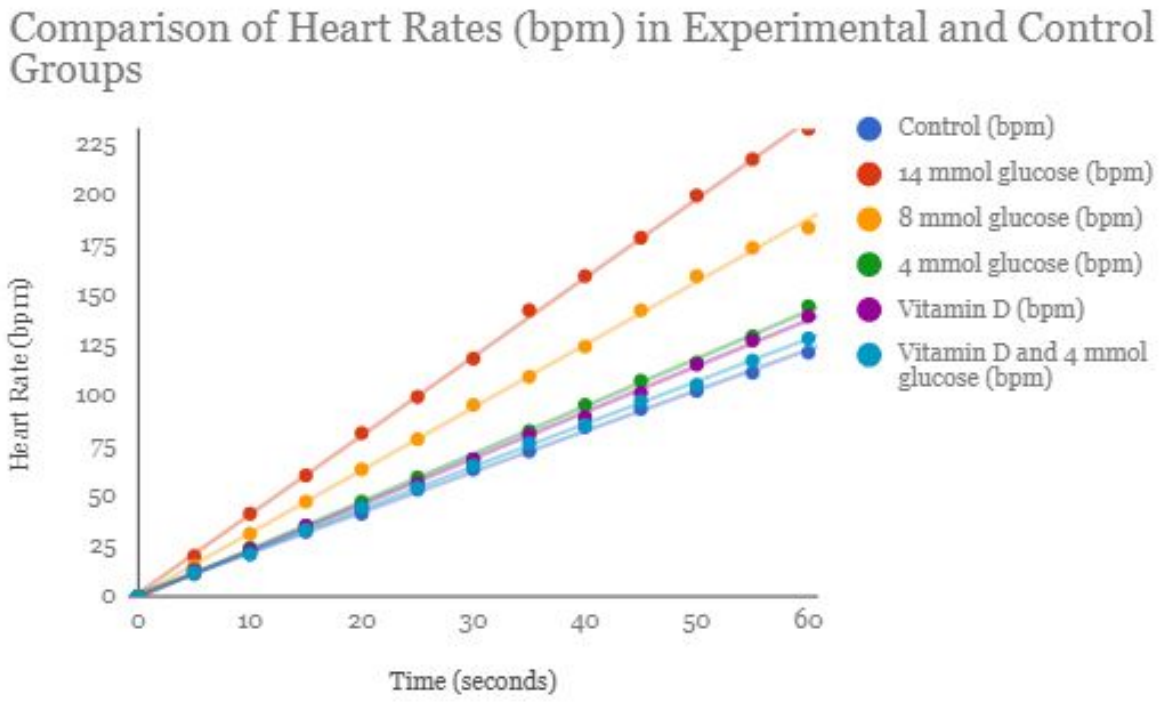
Vitamin D3 Supplementation

To validate the effectiveness of vitamin D3 as a diabetic treatment supplement, we administered 1 mL of vitamin D3 solution (eight capsules dissolved in 200 mL distilled water) to three wells in conjunction with 1 mL of 4 mM glucose solution. After 17 hours post fertilization, as shown in Figure 3, vitamin D hampered the growth retardation evinced in embryos exposed to glucose concentrations ranging from 4 mM to 14 mM. In addition, heart rate was reduced to normal levels, comparable to the control group. Consider Figure 5, in which the vitamin D3 and 4 mM glucose plots closely mimic that of the control group.

Heart Rate

The heart rates of zebrafish embryos exposed to 14 mM glucose concentrations were significantly faster than the heart rate of the control embryos with no glucose exposure. By implementing Fisher's Exact Probability Test to compare the statistical significance of the heart rate emanated by zebrafish embryos exposed to 14 mM glucose concentration to that of the control, a P value of less than 0.0001 was computed. By conventional criteria, this difference is accepted as extremely statistically significant. The fluctuations in heart rate between the control and experimental groups is represented in Figure 5. A decrease in glucose uptake from the environment elicited poor valve looping, inducing valve disgenesis and the observed increase in 8 mM and 14 mM embryonic heart rate

Figure 5. Increased heart rate in 14 mM and 8 mM embryos



Exposure of zebrafish embryos to 14, 8, and 4 mM glucose solution for 96 hours produced significant defective cardiac development in the 8 and 14 mM zebrafish embryos resulting in an elevation in heart rate for both experimental groups in comparison to the control group and 4 mM embryos. The line graph above illustrates the number of heart beats over the duration of 60 seconds, with an endpoint presenting the heart rate of each experimental group and control group.

Glucose Determination Conclusions

Upon investigation commencement, we attributed the terms high glucose, medium glucose, and low glucose to each row of wells in correlation to the concentrations of the solutions administered. Ensuing the first analysis session, the team remarked abnormal and fortuitous morphological features exhibited by the embryos coined “low glucose.” After purchasing glucose test strips from Flinn Scientific, we confirmed our entangled glucose hypothesis. What was initially identified as “high glucose” was verily the low glucose solution and vice versa.

Data Presentation

The null hypothesis reads: if elevated glucose exposure does not affect the cardiovascular system of a developing embryo, and we compare the heart rate of zebrafish embryos exposed to concentrations ranging from zero to 14 mM, for 96 hours post-fertilization (hpf), then there will not be a statistical significance in mortality in the group that is exposed to the greatest dosage of glucose in comparison to the zebrafish embryos exposed to lower concentrations. To determine the statistical significance of mortality in glucose exposure, we employed Fisher's Exact Probability Test (GraphPad Software Inc., San Diego, CA), chosen as the best fit for an expected group ≤ five. Two-tailed $p<0.05$ was considered to be statistically significant. This is summarized in Table 5.

Table 5. Fisher's Exact Probability Test

P	One-tailed	0.000000369801150525336
P	Two-tailed	0.00000073960230105067 2

To determine the statistical significance of mortality in glucose exposure, particularly 14 mmol and 8 mmol, we employed Fisher's Exact Probability Test. With an experimental value of 5 or less, this test fit with the population size of our test and control groups. By traditional criteria, computing a P-value less than 0.05 with the Fisher's Exact Probability test insinuates the data as extremely statistically significant, thus the null hypothesis can be rejected and the results are not due to environmental chance.

Discussion

Clinical features of gestational diabetes (GD) is broadly segregated into growth retardation, morphological malformations, and cardiovascular malformations, each manifested by the *Danio rerio* embryos administered 4 mM, 8 mM, and 14 mM concentrated glucose solutions. Macrophthalmia incarnated in embryos exposed to 8 mM and 14 mM glucose. Cataractous microphthalmia was analogically observed in mice with increased glucose deposits in the lens during embryogenesis (Gong et al., 2001). In conjunction with the aforementioned complications, the investigation explicated in this manuscript models the lethality of glycemic stress on congenital malformation development during the

critical period of embryo organogenesis. The molecular implications and phenotypes possessed by hyperglycemia is blueprinted in Figure 6. An elevation in osmotic stress and deficit of in vivo glucose regulatory transporters catapulted the development of hyperglycemia in 8 mM and 14 mM zebrafish embryos. We failed to accept the null hypothesis and our data supports the alternative hypothesis. Mortality and heart rate demonstrated a dose-dependent response. The decrease in adaptability to the divergent glucose environments can be ascribed to the osmoregulatory stress applied to the fish as the glucose diffuses through the permeable membrane of the zebrafish chorion. Whilst the embryos were not given diabetes, they were exposed to concentrations that are identical to the levels found in blood of women with gestational diabetes or hyperglycemia.

During vertebrate and zebrafish embryogenesis, the cardiovascular system represents the paramount unequivocally functional complex and developmental determinants are symbiotic to embryonic fatality. In neonates, abnormally high birth weight is conventionally pertinent with gestational diabetes and induced by unremittingly elevated glucose levels amidst the placental circulation, a theory corroborated by the observed tumescent yolk sacs evinced by embryos exposed to 14 mM and 8 mM glucose environments (Sermer, 1995).

Observation of mortality and heart rate has exhibited a concentration dependent increase. The decrease in adaptability to the divergent glucose environments can be ascribed to the osmoregulatory stress applied to the fish as the glucose diffuses through the permeable membrane of the zebrafish chorion. The embryonic stage delay observed in the embryos exposed to 14 mM and 8 mM glucose concentrations depicts a product of hyperglycemia. This outcome is in congruence with the increased embryo mortality and abnormal growth of these two experimental groups. Osmotic stresses effectuated on the 8 mM and 14 mM aquatic environments represent a possible explanation for the developmental retardation examined in these embryos. The increased concentration in environmental glucose and osmotic stress within the embryo-an accessory to gestational diabetes-provokes maturation hindrances

and impairment of organ structures, notably, the cardiovascular system. A decrease in proliferation of endocardial and myocardial cells was surveyed in the 14 mM and 8 mM models. Embryonic heart development is contingent upon the functionality of the endocardial, myocardial, and neural crest cells.

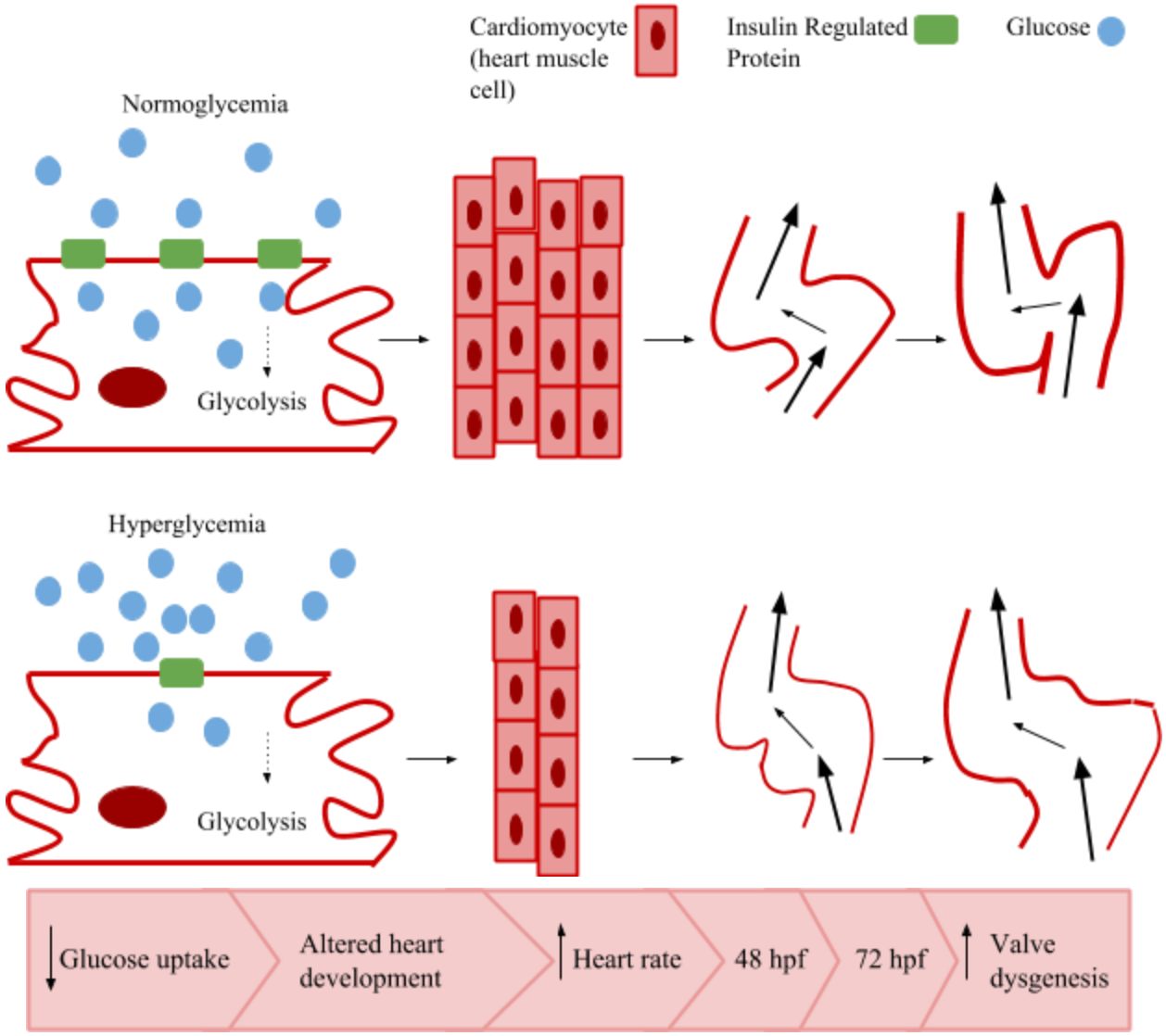
Hatching, a critical period in embryogenesis, has been employed as an endpoint in the early life zebrafish stages. Delayed hatching despite proliferated growth was observed in the vitamin D and G1 zebrafish exposure. Once spawned and fertilized, the zebrafish embryos perform with exorbitantly low chorion permeability. The period of accelerated embryonic development as the juvenile fish is en masse dependent upon yolk nutrients-antecedent to hatching-manifests as an immanent mechanism for testing nutritional programming and implications of glucose elevation on a developing embryo, applicable to terrestrial mammalian and aquatic craniate animals.

A critically sensitive period characterized by changes in environmental conditions alters the normal programme of embryogenesis (Gilbert, 2001). *Danio rerio* embryogenesis is sensitive to the introduction of glucose, as elucidated by a change in biological inveterate environmental factors. High genetic plasticity, cell proliferation, organs, tissues formation in early development, as well as immature regulatory mechanisms are deficient in the hatched zebrafish (Srinivasan and Patel, 2008). The morphological changes may have been induced by adaptive changes to the hypertonic environ during development inflicting gene expression pattern alterations on cellular and physiological phenotype, nutrient-sensitive signalling pathways, and cardiovascular abnormalities. Offspring of mothers diagnosed with type 1, type 2, or gestational diabetes possess an elevated risk of externalizing congenital anomalies, macrosomia, and neonatal childhood (Kalter, 2003). Such congenital malformations found in children conceived by diabetic mothers constitute neural tube defects-precursors to anencephaly, spina bifida aperta, meningocele, encephalocele, and septal defects (Kalter, 2003).

The embryonic stage delay observed in the embryos exposed to 14 mM and 8 mM glucose concentrations depicts a product of hyperglycemia. This outcome is in congruence with the increased

embryo mortality and abnormal growth of these two experimental groups. Osmotic stresses effectuated on the 8 mM and 14 mM aquatic environments represent a possible explanation for the developmental retardation examined in these embryos. The increased concentration in environmental glucose and osmotic stress within the embryo-an accessory to gestational diabetes-provokes maturation hindrances and impairment of organ structures, notably, the cardiovascular system. A decrease in proliferation of endocardial and myocardial cells was surveyed in the 14 mM and 8 mM models. Embryonic heart development is contingent upon the functionality of the endocardial, myocardial, and neural crest cells.

Figure 6. Hyperglycemia induces reduction in cardiac glucose uptake and vascular abnormalities



A decrease in glucose uptake from the environment elicits the condition hyperglycemia. The embryonic hearts of zebrafish exposed to 8 mM and 14 mM glucose environments exhibited a cardiovascular phenotype of poor valve looping, resulting in valve disgenesis.

For clinical drug response, the animal model, zebrafish embryos, were responsive to the anti-diabetic drug vitamin D3. Vitamin D3 is recommended to mothers at risk of developing gestational diabetes or possesses diabetes during pregnancy to prevent fetal morphogenesis abnormalities and cardiovascular complications. Vitamin D3 supplementation for embryos exposed to 4 mM glucose concentration experienced no detrimental cardiovascular defects and embraced normal growth, bolstering

vitamin D3 consumption during pregnancy as a prophylactic measure to hamper the development of fetal gestational diabetes. Recent studies concluded that vitamin D deficiency is affiliated with the development of gestational diabetes during early fetal development. Women bearing gestational diabetes possess an elevated chance of eventual type 2 diabetes ontogenesis and infants of diabetic mothers may contrive congenital anomalies and hyperglycemia.

A mammalian ortholog, *Danio rerio* encompass highly regulated metabolic machinery in the cardiovascular system to ensure optimal efficiency. Pathologies changing metabolic control possess consequences of cardiovascular deficiencies in embryological development, a precursor to heart failure and death. Studies have elucidated that proper cardiac structure development arises from a coalition of genetic, mechanical, and environmental factors. In an analogous perspective, valvulogenesis is sensitive to environmental disequilibrium-kin to that modeled by means of elevated glucose concentrations allotted to aqueous environs in which zebrafish embryos developed-arising defective valve formation. Whilst essential for normal fetal growth and metabolism, excess glucose concentrations exemplify a toxic aura for detrimental fetal malformations.

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