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Effects of Sodium Chloride on Zebrafish (*Danio rerio*) Embryonic Development and Analysis of the Zebrafish Embryo using ImageJ Software

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Abstract:- The zebrafish (*Danio rerio*) has emerged as an invaluable model organism for studying vertebrate development due to its transparency, rapid development, and genetic tractability. Sodium chloride (NaCl) is an essential component of the aquatic environment and plays a critical role in maintaining osmotic balance in fish. However, excessive salinity levels in aquatic ecosystems due to anthropogenic activities have raised concerns about their impact on aquatic organisms, particularly during early life stages. This experimental research was performed to investigate the effect of sodium chloride (common salt) on the embryonic development of Zebrafish (*Danio rerio*), and to examine the embryos before and after the treatment with Sodium Chloride (NaCl), and analyze the embryos using ImageJ tool. Zebrafishes were used in this experiment as they are considered as powerful model organisms, which means that the information obtained through them can help us better understand how Sodium affects the human embryos. Zebrafish embryos were placed in a tray, then the embryos were subjected to saline solutions at various concentrations for 24 hours during the experiment, including 0.002 g/mL, and 0.02 g/mL of NaCl. Furthermore, the dead embryos were separated out, data and observations were recorded. ImageJ software was used to analyze the early-hatched zebrafish embryo. The result of this experiment demonstrated that 0.002 g/ml of Sodium chloride is the ideal salt concentration for early hatching of zebrafish embryos and it has no side effects on Zebrafish embryos. The findings of this experiment suggested that Sodium chloride has minute or no side effects on the growth of human embryos, but further investigation needs to be performed.

Keywords:- *Zebrafish; sodium Chloride; Embryonic Development; Minimal Dose; Lethal Dose; imageJ Software.*

I. INTRODUCTION

Zebrafish (*Danio rerio*) is a tiny vertebrate tropical fish. It is a bony fish (teleost) that belongs to Cyprinidae family under the class of ray-finned fishes called Actinopterygii [1]. The zebrafish is a freshwater fish found in the tropical rivers of the South Asian Himalayan region, primarily in countries such as India, Nepal, Bhutan, Bangladesh, Pakistan, and Myanmar [2]. The Zebrafish has become one of the most powerful model organisms for understanding vertebrate development, and human diseases. As a matter of fact, zebrafish features high fecundity, also it is a good fit for genetic manipulation [3].

Recently, the zebrafish is utilized in behavioral neuroscience, and there are many neuroscience laboratories that have started employing zebrafish as their major model organism for genetics and behavioral neuroscience [4]. Moreover, it has developed into a reliable vertebrate model for examining in vivo metastatic events [5]. Zebrafish have been used since 1970s to study the development of vertebrates [1], and more recently, they have helped scientists to learn more about human diseases, and has developed various potential in genetic treatments. Most of the research has been performed using zebrafish as a model because of its innumerable benefits. Firstly, their genome is similar to the human genome, mainly because we share a common ancestor. They have over 26,000 protein-coding genes [6]. Their genome has been fully sequenced and is of high quality. About 70% of the genes in zebrafish are related to similar genes in humans. Around 84% of the disease-causing genes that have been identified in humans have at least 1 related gene in zebrafish [7].

They are small, and robust organisms. It is slightly expensive, much difficult to maintain and perform experiments on laboratory mice, whereas the zebrafishes are affordable and are very inexpensive to maintain when compared to other animal models such as mice [8,9]. Zebrafishes have higher reproduction rate, resulting in an abundance of embryos to work with and study them. Female zebrafishes can spawn around 200 to 300 eggs per day which makes for a lot of new test subjects [10]. They have a short lifespan of 3-4 years; hence they grow and develop quickly at a breakneck pace [11]. Zebrafish embryo are transparent, making it easier to observe their internal organ systems under the microscope. In 2008, Richard et al developed a genetically-engineered strain of zebrafish that was transparent through its whole life. That transparency allowed the scientists to see exactly what was going on inside the body of zebrafish, and watched the biological processes – like how cancer develops in the body [12]. Zebrafish can serve as a valuable model for studying the developmental origins of health and disease, as well as investigating the effects of toxicity across multiple generations [13]. Also, it has gained recent popularity as a research model for studying tissue regeneration due to its superior regenerative capacity, when compared to mice and

humans [14]. The phenomenon of regenerating tissues ceasing growth when the zebrafish reach the appropriate size is captivating but not well understood. There is still a lack of understanding regarding which specific cell types become active during regeneration and whether all cells possess equal regenerative capabilities [15]. Zebrafish are still not the perfect model for studying the human diseases as they do not have the lungs and mammary glands. Lot of their genome is made up of duplicate genes. Some of those gene copies might have mutated and developed functions that were not there in the gene of an ancestor – which would make them different from the human versions. As a vertebrate, zebrafish share similar tissues and organs with humans, sharing roughly 84% of disease-causing genes although the morphology of the embryo and larva has been well documented. Also, their genome assembly is of high quality, allowing for a more precise comprehension of important genomic characteristics. These include a distinct repeat composition, a low abundance of pseudogenes, a higher concentration of genes specific to zebrafish located on chromosome 4, and specific chromosomal regions that have an impact on determining sex [16]. In contrast to other animal models, zebrafish genes can be manipulated through the use of reverse genetic screening tools. These tools, such as morpholinos, can temporarily suppress specific genes of interest. Additionally, techniques like transposon-mediated insertional mutagenesis or CRISPR-Cas9 systems enable the modification of zebrafish genes [17].

II. EMBRYONIC DEVELOPMENT OF ZEBRAFISH

Zebrafish embryos are transparent and they develop from a Zygote, an externally fertilized egg. Within 2 to 3 days, the growth of a newly fertilized embryo from a single cell to a free-swimming larvae can be observed. There are various stages of Embryonic development of the zebrafish. It can be divided into eight stages, each marked by specific morphological and molecular changes. Here are the main stages of zebrafish embryonic development [18]:

A. Zygote Stage

This stage begins immediately after fertilization, when the male and female gametes (sperm and egg) fuse to form a diploid zygote. The zygote contains all the genetic information required for the development of a new organism.

B. Cleavage Stage

During this stage, the zygote undergoes rapid cell divisions called cleavage. The zebrafish embryo divides into smaller cells known as blastomeres, forming a solid ball of cells called blastula.

C. Blastula Stage

The blastula stage is characterized by the formation of a fluid-filled cavity called the blastocoel within the ball of cells, i.e., Blastula. The blastula consists of a single layer of cells, known as the blastoderm, surrounding the blastocoel.

D. Gastrula Stage

In this stage, the blastula undergoes gastrulation, a process in which the cells of the blastula rearrange and migrate resulting in the formation of three primary germ layers: ectoderm, mesoderm, and endoderm. The ectoderm forms the nervous system, epidermis, and other external tissues. The mesoderm develops into muscles, bones, blood cells, and other internal structures. The endoderm develops into the digestive system and other internal organs.

E. Segmentation Stage

During this stage, the zebrafish embryo undergoes segmentation, where the body becomes divided into distinct segments. The segments will later give rise to different regions of the body, such as the head, trunk, and tail. Segmental boundaries form along the anterior-posterior axis, and somites start to develop. Somites are transient blocks of mesodermal tissue that give rise to muscle, vertebrae, and other structures.

F. Pharyngula Stage

During the pharyngula stage, the zebrafish embryo begins to take on a more recognizable shape. The major organ systems, such as the heart, brain, nervous system, digestive system, and muscles, start to form and become more defined. The pharyngeal arches, which are critical for the development of gills, also begin to appear.

G. Hatching Stage

At this stage, the zebrafish embryo develops a specialized structure called the hatching gland. The hatching gland secretes enzymes that weaken the outer membrane, allowing the embryo to break free from the protective eggshell and hatch.

H. Early Larva Stage

After hatching, the zebrafish enters the early larva stage. The larva has a distinct shape with a prominent yolk sac that provides nourishment. It starts swimming and feeding, gradually developing more complex organ systems and undergoing further growth and development. These stages represent the major milestones in the embryonic development of zebrafish, but it is important to note that development is a continuous process, and different developmental events often overlap. While these stages provide a general framework for zebrafish embryonic development, the timing and specific events may vary between individual embryos. Additionally, there are further stages and sub-stages within these broad categories that involve more specific cellular and molecular processes. [19]

ImageJ is a popular image analysis program that is widely used in the biological sciences and other fields. ImageJ draws contributions from non-programmers, beginner programmers, and experienced developers equally due to its simple design, ease of use, recordable macro language, and flexible plug-in architecture. Allowing for such a diverse range of contributors has led to a big community spanning the biological as well as the physical sciences. ImageJ has long been the tool of choice for biologists who require both basic and advanced image

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analysis. During ImageJ's lifespan, a revolution in microscopy resulted in an order of magnitude rise in typical image sizes and multiple orders of magnitude improvement in image resolution [20].

III. MATERIALS AND METHODS

The materials used in this experiment included two test tubes containing 0.02 g/mL and 0.002 g/mL of saline solution, tray containing the zebrafish embryos in the sterile distilled water, one 50 mL beaker for dead embryos, transparent sterile disposable pipettes, one well plate with wells, one 28°C incubator, one DIY compound microscope, microscopic glass slides, and 10 zebrafish embryos. All the materials were provided by Bversity. The DIY microscope was constructed as per the instructions by the instructor. The Zebrafish eggs were obtained from the instructor and were collected from the tray. The live eggs were separated out from the dead ones by observing them carefully under the compound microscope. The saline solutions of 0.002 g/mL NaCl, and 0.02 g/mL NaCl were prepared in two separate test tubes.

The concentrations on each well were labeled appropriately. One well was kept as control, which was filled with 1 mL of water without the NaCl solution. The second well was filled with minimal dose of saline solution containing 0.02 g/mL NaCl, and the last well was filled with 0.002 g/mL NaCl solution, and was kept as lethal dose. Gloves were worn whenever work was done with sodium solutions. The embryos were divided so that 2 embryos were placed in each well. Zebrafish embryos were exposed to Sodium chloride (NaCl) at two concentrations, minimal dose (0.002 g/mL), and lethal dose (0.02 g/mL). Finally, all the embryos were placed in the 28°C incubator for 24 hours.

The plate was removed from the incubator after 24 hours. The dead embryos were removed and separated out from the well plate using a transparent disposable pipette. The dead embryos were then squirted into the waste beaker. Then the remaining embryos were checked for their growth and development. After this, the microscopic glass slides were washed with distilled water and using 70% ethanol to eliminate the pathogens present on the glass slide. The embryos were placed on the glass slide using the transparent disposable pipette. The slides containing the embryo were placed under a microscope and each embryo was carefully examined for the changes in the embryo development. The observations were recorded. Furthermore, Fiji, the ImageJ software was used to analyze the embryos before and after the development.

Fiji, a new version of ImageJ (ImageJ2) was used to analyse the zebrafish embryos. Throughput has increased by a factor of ten in Fiji. The Fiji project gives biologists effective tools for creating advanced image processing pipelines, including as scripting languages and feature-rich libraries for processing huge quantities of large images, while improving on the easy usage of ImageJ. Fiji retains ImageJ compatibility while adding more essential features. Fiji maintains a balance between usability, performance, and adaptability, and as such, it has an exceptional spot in the landscape of biological image analysis, complementing other tools while also providing new and innovative attributes [20].

Fiji (ImageJ2) was downloaded using the link: https://imagej.net/software/fiji/. The ImageJ software was opened and the microscopic images of the Zebrafish embryos were opened in ImageJ. The scanned color image of the pre-matured Zebrafish embryo was converted into 8-bit greyscale image. The height of the pre-matured embryo of Zebrafish was measured by selecting line tool, straight line. The line was dragged for the length measurement. Furthermore, it was measured by choosing Analyze and measure. The area of eye of the Zebrafish embryo was measured. The circle was adjusted for the area measurement. Spinal cord of the Zebrafish embryo was measured by selecting line tool, segmented line. The segmented lines were dragged along the curve for spinal cord measurement. Furthermore, the obtained results were interpreted.

IV. RESULTS AND DISCUSSIONS

Many people in India consume significantly more sodium than it is suggested. A high salt diet can have adverse effects on health among humans. With so many health risks surrounding a high sodium diet, one could speculate what would happen when a high sodium diet of a pregnant woman impacts the embryo that grows inside her. The purpose of this experiment was to see if the sodium concentration creates difficulties in developing zebrafish embryos so that toxic effects of sodium chloride on developing human embryos could be predicted. From this experimental research, it was found that the sodium chloride did not result in any of the toxicological effects, but it was found that the optimum amount of NaCl (0.002 g/ml in this case) is required to accelerate the early-hatching process in the Zebrafish embryos.

Table 1: Observations of all the three samples (Control, minimal dose, and lethal dose)

Well no.	Samples	Saline Solution (g/mL of NaCl)	Observations
1.	Control	-	slightly changed from normal egg to the bud stage embryo
2.	Minimal dose	0.002	showed premature embryo developed at prim-16 stage
3.	Lethal dose	0.02	reached to 21-somite stage

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Sodium was considered as the independent determining factor of this study. The dependent factors were the mortality rate, and the hatch rate. The results of the experiment did not support the hypothesis and showed that sodium had a negligible or no side effects on zebrafish embryonic development. But this study also showed little, but significant result. The hatching rate of Zebrafish embryo incubated in a well containing the minimal dose of Sodium chloride (0.002 g/mL of NaCl) had increased when compared to the control sample and embryo with the lethal dose of Sodium chloride. Therefore, according to this experimental study, the optimal amount of Sodium chloride required to improve the hatch rate of zebrafish embryo is 0.002 g/mL of NaCl solution. But it is not possible to assume that this is the perfect result. The results may vary according to different equipment, and procedures used, nature of the common salt (sodium chloride) may vary from different regions of the country, and changes in the environment. If the embryos of zebrafish are exposed to excessive amounts of sodium chloride, the embryos would be damaged substantially. Out of three samples, all the samples were in the initial stages, and none of them had hatched into embryo, before adding sodium chloride (NaCl) solution. After 24 hours of incubation, the three samples (Control, minimal dose, and lethal dose) revealed some growth and development after the addition of NaCl solution.

Sample 1: Control	Sample 2: Minimal dose	Sample 3: Lethal dose
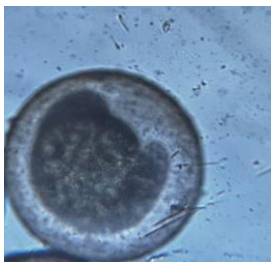	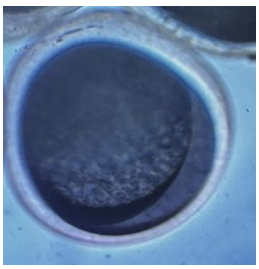	
Figure 1(a)	Figure 1(b)	Figure 1(c)

Fig 1: Zebrafish embryos before adding sodium chloride (NaCl) solution

Sample 1: Control	Sample 2: Minimal dose	Sample 3: Lethal dose
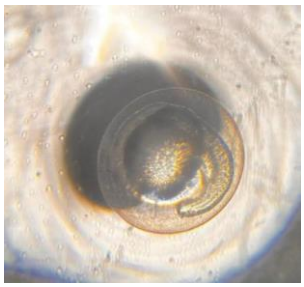		
Figure 2(a)	Figure 2(b)	Figure 2(c)

Fig 2:- Zebrafish embryos after adding sodium chloride (NaCl) solution

➢ *ImageJ Analysis:*

The following results of the early-hatched zebrafish embryo were obtained by using Fiji ImageJ software. The first image depicts the area of unhatched embryo before adding sodium chloride. The other three images represent the measurement of its spine, eye, and length of the matured embryo after adding NaCl.

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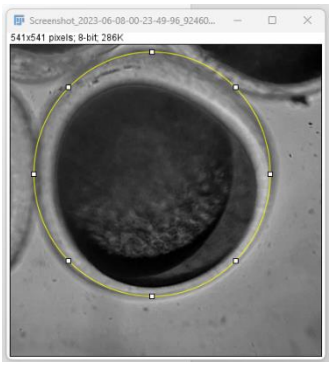
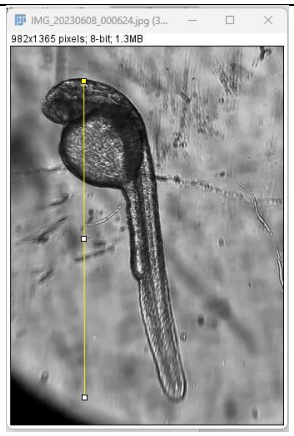
Table 2: Measurement of the pre-matured Zebrafish in ImageJ tool

S.no.	ImageJ measurements	Area	Mean	Min	Max	Angle	Length
1.		1144	164.301	84.853	211.003	-89.85	1143.004
2.		8532	106.398	81	164	0	0
3.		1165	144.363	76.668	247.267	0	1164.748
4.		137207	98.918	29	226	0	0

V. CONCLUSION

Zebrafish was used in this study as they can recapitulate many human diseases at a molecular level. The effects of sodium chloride on the embryos of zebrafish can vary based on factors such as concentration, exposure duration, developmental stages, and individual susceptibility. It is also essential to control the environmental conditions and salt levels while working with the zebrafish embryos to maintain their health and minimize the potential adverse effects. In this experimental study, 0.1 ml of NaCl (Minimal dose) is considered as the optimal amount of NaCl required for the premature hatching of the Zebrafish embryos. This may be same for the development of human embryos as they are surrounded by the amniotic fluid.

The amniotic fluid contains a mixture of water, electrolytes, proteins, hormones, various salts, including sodium chloride (NaCl) and other fetal waste products in humans. These components assist in the maintaining an appropriate environment for embryo development, facilitate proper growth, exchange of nutrients and protection against bodily impacts. For the maintenance of adequate osmotic balance and support for tissue growth and organ development, it is essential to contain salts such as sodium chloride (NaCl) in amniotic fluid. However, the results may slightly vary in humans as human beings are viviparous, whereas the zebrafishes are oviparous animals. In humans, it is essential to maintain the concentrations and precise composition of salts in the amniotic fluid. It can vary and are tightly regulated by the maternal and fetal systems to ensure optimal conditions for embryonic development.

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