The Alterations of Zebrafish Development, Neurological, and Cardiovascular Function Caused By Dextromethorphan

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Abstract

In this study, 40 zebrafish embryos were raised in various Robitussin solutions (0.02 mg/mL, 0.002mg/mL, 0.0002mg/mL, instant ocean control) to determine the effects the common ingredient dextromethorphan (found in many over-the-counter medicines) had on the development of fish. Previous studies on the effects dextromethorphan on humans have been conducted, and results have found that the common ingredient can block neurological receptors, altering brain functions. Experiments conducted on rodents found similar results to that of humans, meaning neurological functions were changed, causing unusual responses to movement and differences in development. The study took place over the course of a week, and offered significant results on how dextromethorphan changed zebrafish development. Embryos raised in higher concentrations of dextromethorphan showed slower signs of development, deformities, and alterations in neurological functions when compared to controlled zebrafish. The results from the conducted experiment can give better insight as to how common cold medicines containing this ingredient can affect human development when taken at high doses.

Introduction

Dextromethorphan, an addictive ingredient found in most over the counter drugs, has been proven to have dissociative effects on humans' neurological and cardiovascular function when taken in large doses. Misuse of the drug often times leads to hallucinations, differences in psychological responses, increased organ function, impaired memory, changes in motor function, and alterations in perceptual awareness. These effects are produced when dextromethorphan blocks a common neurotransmitter called glutamate. When glutamate interacts with NMDA receptors (N-methyl-D-aspartate) in the brain, responses related to cognitive function, memory, and the perception of pain are released. Few studies have been conducted to determine the effects of dextromethorphan on other organisms, but those that have found that the responses are similar to humans in that they alter neurological functions. A study performed by Rijen et al. (1991) observed rats’ responses to dextromethorphan when affected by ischemia, a disease that causes an inadequate blood supply to the body's organs. The results proved that dextromethorphan decreased the function of NMDA receptors, slowing metabolic functions and lactate formation; lactate is created during glycolysis, the breaking down of sugars to create energy, and is used to continue the glycolysis process. Another study conducted by Olney et al. (1989) found similar results in rodents with no abnormalities. The study showed that dextromethorphan-treated rodents developed Olney's lesions, a type of neurological disease that damages or kills brain cells. This occurs when certain NMDA antagonists, such as dextromethorphan, are taken at high doses, causing the brain cells to over-excite and damage themselves. Limited information exists about the exact causes of Olney's lesions, and scientists have failed to duplicate the results among other organisms such as monkeys.
The studies above can give insights as to how other vertebrates, such as zebrafish, respond to dextromethorphan. The objective of the following experiment is to determine the relationship between zebrafish and human development. The experiment will also allow better comprehension as to how common cold medicines taken by many expectant mothers can alter developmental rates and functions of the fetus. It can be hypothesized from this experiment that if zebrafish are raised from embryos in solutions containing dextromethorphan, then neurological function will be altered, and deformities will result from low lactate formation and changes in cardiovascular functions.

**Methods and Materials**
- Stock solutions of Robitussin (dextromethorphan)
  - 1% (0.02mg/1mL) Robitussin Solution
  - 0.1% (0.002mg/mL) Robitussin Solution
  - 0.01% (0.0002mg/mL) Robitussin Solution
- Instant Ocean/Embryo Media Solution (provided by a teacher)
- One bottle of sugar-free Robitussin
- Four 100 mL beakers (necessary for the creation of stock solutions containing robitussin)
- One 50 mL beaker (necessary for the disposal of liquid and dead embryos)
- One 10 mL graduated cylinder
- One 100 mL graduated cylinder
- Four disposable pipettes, minimum bore, 1.5 mm
- Four disposable pipettes, 1 mL (necessary for transportation of embryos)
- One plate with wells
- One 28.5 degree Celsius Incubator
- One Dissecting Microscope
- One stopwatch or timer

**Day 1:**

Stock solutions were prepared through the use of the 10 and 100 mL graduated cylinders, four 100 mL beakers, a 1 mL pipette, and one bottle of sugar-free Robitussin. The 1% (0.02mg/mL) solution was created by measuring out 99 mL of instant ocean using a 100 mL graduated cylinder. Then, 1 mL of Robitussin was added to the 99 mL using a 1 mL pipette and placed in a 100 mL beaker labeled 1% solution. The 0.1% (0.002mg/mL) solution was created by first measuring out 90 mL of instant ocean, then adding 10 mL of the 1% solution using the 10 mL graduated cylinder. The solution was then moved to a beaker and labeled 0.1% solution. The final Robitussin solution was created by first measuring 90 mL of instant ocean. 10 mL of the 0.1% solution was then added to the 90 mL and transferred to a beaker labeled 0.01% (0.0002mg/mL) solution. The control solution was created by pouring 100 mL of instant ocean into a beaker. All beakers were then covered with tinfoil and placed in the fridge overnight.

**Day 2:**

Ten freshly hatched zebrafish embryos were transported into each well through the use of a 1 mL pipette, creating a total of 40 embryos and four wells. The liquid that contained the embryos was disposed of using a 1.5 mm pipette and was replaced by a milliliter of the correct solution. The control solution (instant ocean) was placed in the first well. The second well contained the 0.01% (0.0002mg/mL) solution, and the third used the 0.1% (0.02mg/mL) solution. The final well was comprised of the 1% (0.02mg/mL) solution. Tape was placed on the well cover and stated the locations of each solution. Observations about the initial amount of embryos and the stage of development were noted, then the plate was placed in an incubator at 28.5 degrees Celsius overnight.
Day 3:
The plate was removed from the incubator and placed under the dissecting microscope for observations. The developmental stages of each well, the amount of living and dead, as well as the amount of hatched and unhatched embryos were recorded. Then, the liquid waste and embryos were removed using a 1mL pipette and placed in a 50 mL beaker for disposal. After, each well received new solutions of 1 mL (as seen on Day 2). The plate was then placed in the incubator overnight.

Day 4:
The plate was taken from the incubator and observed under the dissecting microscope. Observations about the development of the zebrafish, such as color, formation, and deformities were noted. The amount of living and nonliving embryos, as well as the amount of hatched and unhatched embryos was recorded. After, a stopwatch was used to record the number of heartbeats per minute of one zebrafish in each well. This was measured to assist in determining the effects of dextromethorphan on the cardiovascular functions. The liquid and dead embryos in each well were then disposed of using a 1 mL pipette and a 50 mL beaker. Each well was filled with 1mL of the correct solution (as seen on Day 2 and 3), and then placed in the incubator overnight.

Day 5:
The plate of wells was removed from the incubator and transported to a dissecting microscope for final observations on deformations, color, hatch rate, and the amount of alive and nonliving embryos or fish. The heartbeat of one zebrafish in each well was recorded for a minute to later compare with the findings from day 4. The liquid in each well was also moved around using a 1mL pipette to observe the zebrafish responses to movement. Then, the dead embryos and liquid were discarded using a 1 mL pipette and a 50mL beaker. Each well was replaced with control solution (instant ocean) to flush out the tested solutions (Robitussin). The remaining embryos and fish were then transported to a larger tank using a 1 mL pipette, where they would continue developing in a normal environment.

Note: Careful precautions should be taken while changing out solutions, insuring that no live embryos are harmed or discarded in the process. Gloves should be worn while conducting the experiment and hands should be washed after to ensure no chemicals are left on the skin.

Results

It was previously hypothesized that if zebrafish were raised from embryos in solutions containing dextromethorphan, neurological function would be altered and deformities would occur from changes in cardiovascular and neurological activity. The results of the experiment on the effects of dextromethorphan on zebrafish produced significant results relating to the stated hypothesis. Visual and statistical observations helped in determining the effects dextromethorphan had on the developmental rate, cardiovascular, and neurological functions of zebrafish.

Throughout the conducted experiment, temperature and the solutions the embryos were raised in were kept consistent in each well. The procedures and observations of embryos were conducted between 7:30am and 9:30am and kept the same each day. The results acquired from the concentrations of dextromethorphan were compared to the controlled embryos (instant ocean) to produce conclusions about alterations in zebrafish development. By keeping the experimental controls consistent through the duration of the experiment, the results of the effects of the various concentrations of dextromethorphan (independent variable), on the development, neurological, and cardiovascular functions of zebrafish (dependent variable) ensured better accuracy.
Visual observations of the four wells every 24 hours showed substantial differences in the developmental stages of the zebrafish. The initial observations before the zebrafish were placed in the dextromethorphan solutions stated that all embryos were clear in color and were showing early signs of development, forming tails and eyes. After being placed in the correct solutions for 24 hours, changes in development were beginning to become apparent. Those with a higher concentration of dextromethorphan were lighter in color, almost opaque, while those developing in the controlled solution began to develop spots and were darker in color. The second day of observation (48 hours), showed larger signs of the variation in development between solutions. Eyes, tails, and heartbeats were clearly visible in the zebrafish contained in the control solution. The hatched zebrafish were responding to movement, and fins were visible on some. The hatched zebrafish in the controlled solution were also staying upright, and their figures were straight, whereas those hatched in the dextromethorphan-containing solutions were slightly curved figures with hardly visible organs, such as the eyes and heartbeat. Responses to movement in the dextromethorphan-containing solutions were delayed or not evident. These visual observations demonstrated that as the concentration of dextromethorphan increased, the zebrafish developmental rate became slower, and deformities began to develop.

Through statistical observation, it was discovered that the ten zebrafish raised in the greatest solution including dextromethorphan (0.02mg/mL), were all deceased after 72 hours. Observations made under the compound microscope found that the three embryos alive after 48 hours were decomposed by Protozoa, single celled organisms that live off dead organisms, in the 24 hours following the observation. The second-highest concentration (0.002mg/mL), contained three living zebrafish after 72 hours of observation, and the solution containing the lowest percentage of dextromethorphan(0.0002mg/mL), held 8 live zebrafish following 72 hours. The control solution (instant ocean), contained 9 living zebrafish after the conducted experiment. When put into a chi square statistical analysis chart, the data collected on the amount of living and nonliving embryos after 72 hours had a degree of freedom of 3, with a statistical probability of 10.8, greater than the critical value of 7.82, proving that the null hypothesis was rejected (explained further in the conclusion).

The experiment also statistically measured the heartbeat of a zebrafish per minute in each well after 48 and 72 hours, as well as the hatch rate of zebrafish. The data gathered from the statistical observation of heartbeat is displayed in Figure 3. It was found that as the concentration of dextromethorphan increased, the cardiovascular function, measure in beats per minute, decreased. The hatch rate, displayed in Figure 2, changed slightly from each concentration, but did not show significant variations. When computed in the chi square statistical analysis chart, the hatch rate data had a probability of 4.58, lesser than the critical value of 7.82, showing that the null hypothesis was accepted (explained further in the conclusion).
Figure 1: The line graph depicts the relationship between the time spent in solutions of dextromethorphan and the amount of alive zebrafish. As the time and dextromethorphan solutions increase, the amount of live zebrafish decrease.

Figure 2: The line graph depicts the amount of hatched zebrafish overtime when immersed in different solutions of dextromethorphan. As the solutions of dextromethorphan increase, hatch rate decreases slightly.
Figure 3: The bar graph shows the relationship between heartbeats per minute and dextromethorphan solutions. The heart rate decreases slightly as the dextromethorphan solutions increased, it not enough to conclude that dextromethorphan causes a slower cardiovascular response.

The pictures depict the stages of development after 48 hours immersed in different solutions. The 0.01% solution is showing similar development signs as the controlled solution.
Discussion

The study conducted to determine the effects of dextromethorphan on zebrafish provided substantial insight as to how Robitussin and other over the counter medications can affect the cardiovascular and neurological functions of vertebrates. Zebrafish that were raised in the solutions containing dextromethorphan developed at a slower rate, showed deformities related to organ development, heart rate decreases, delayed responses to movement, and a lower life expectancy.

The chi square statistical analysis chart was used to determine whether dextromethorphan had an effect on the life expectancy of zebrafish, or the number of living zebrafish over time. It was previously determined that the probability computed was greater than the critical value of 7.82, proving that the null hypothesis was rejected. This means the correlation between zebrafish death and dextromethorphan exists, and the results were not caused by chance. It was proved that dextromethorphan decreases the life expectancy, or the number of live zebrafish, as the concentrations of dextromethorphan increased. The hatch rate was also tested using the chi square statistical analysis chart, but the null hypothesis was accepted. This means the results occurred by chance, and the hatch rate of zebrafish was not altered by the dextromethorphan concentrations.

Deformities observed in the zebrafish included opaqueness in color and alterations in organ development such as the eyes. It was discovered that dextromethorphan had the ability to create Olney's lesions, a disease that damages or kills brain cells. The deformities may have been caused by Olney's lesions, or another related disorder. Neurons in the brain are responsible for sending neurotransmitters that contain chemical signals relating to body function. According to the National Institute of Neurological Disorders and Stroke (2015), neurons create passageways called neural circuits by connecting to other neurons to relay information when the brain is developing. During development, the neurons transmitting signals are determined by other molecular cells, as well as which neurons they will connect to to form neural circuits. Once the brain is done developing, the neural circuits are permanent and new neurons must develop in a way to fit in with the neural circuit. Dextromethorphan may have damaged these forming neural circuits through Olney's lesions or another disease, altering neuron passageways and changing the developmental pattern of the zebrafish.

The delayed responses to movement of zebrafish may have been caused by low lactate formation when dextromethorphan blocks NMDA receptors in the brain. Lactate is a byproduct of glycolysis, the process of making energy from sugars, but can be used to continue the creation of ATP, or energy. By blocking the NMDA receptors, lactate formation is slowed, and energy levels are reduced. A lack of ATP can prohibit the use of energy through physical activities, providing an explanation as to why zebrafish raised in higher concentrations of dextromethorphan had delayed or no physical response to movement.

The correlation between dextromethorphan and cardiovascular function is not clear. It was found with humans that dextromethorphan increased cardiovascular function, whereas the cardiovascular functions of zebrafish decreased. The variations between the organism's responses may have resulted from the differences in the organism's body structure. Although both humans and zebrafish are vertebrates, the human body is much more complex, possibly explaining the differences in cardiovascular function.
Based on the results discovered through this experiment, it can be determined that the previously stated hypothesis "if zebrafish are raised from embryos in solutions containing dextromethorphan, then neurological function will be altered, and deformities will result from low lactate formation and changes in cardiovascular functions" is partially correct. Neurological functions were observed and proven to have been altered by dextromethorphan. Deformations were also discovered, although the cause was not because of low lactate formation, but most-likely because of a neurological change. Changes in cardiovascular function were not thoroughly proven, as the heart rate changes were not significant enough to determine if dextromethorphan was the cause. The hypothesis was correct about neurological changes, but incorrect about the deformities and cardiovascular functions of zebrafish affected by dextromethorphan.

To further investigate the effects of dextromethorphan on the neurological and cardiovascular, the study could have been conducted over a larger time period, and movements per minute could have been recorded to extend research on the alterations in neurological function. A point of error when conducting this experiment was not recording the heart rate of the zebrafish from the initial start. Observing the heart rate for only a short portion of the experiment did not allow for an accurate representation of the effects of dextromethorphan on cardiovascular functions, whereas recording heart rate for the whole duration of the study would have. Robitussin, the medicine used to conduct the experiment, is not purely dextromethorphan. It consists of one other active ingredient, guaifenesin, which may have altered results. It cannot be determined that dextromethorphan was the sole cause of the developmental alterations, but can be inferred to have caused some neurological and developmental changes based on past research.

This experiment conducted over the course of week allowed a closer look as to how dextromethorphan can affect the internal and external development of zebrafish. The results gathered can be related to the effects of dextromethorphan in humans and help to build a better understanding of how over-the-counter medicines can affect the neurological functions of the patients, as well as the development of fetuses in pregnant women.

References


