The Effects of Ibuprofen on the Embryological Development of Zebrafish

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Abstract:

Ibuprofen is one of the most common and frequently used non-prescribed NSAIDs (nonsteroidal anti-inflammatory drug), which may cause a vast amount of overlooked side effects. These effects include: gastronomical, cardiovascular, and circulatory system issues. The experimental species, zebrafish, are beneficial for scientific research due to the impressive similarities to human genes and human-disease causing genes. The purpose of the experiment was to test the effects of the NSAID ibuprofen on the hatching and developmental rates of zebrafish embryos. For a total of four days, zebrafish embryos were placed within wells containing varying amounts of the ibuprofen solution or nontoxic embryo media solution for the controls. Each day the solutions were replaced and all dead embryos were removed. All observations were recorded and the embryos were studied underneath a microscope. The final data concluded lower doses had a small overall impact of delayed development and few abnormalities, while higher doses resulted in an obvious delay in hatching and developmental rates, along with causing other severe abnormalities.

Introduction:

Ibuprofen is a nonsteroidal anti-inflammatory drug (NSAID) which treats mild to severe pain by decreasing hormone levels which cause inflammation and pain throughout the body. The NSAID is most commonly used to treat muscle aches, menstrual cramps, and headaches (WebMD, 2016). Nonetheless, ibuprofen has a vast amount overlooked side effects. These effects include, gastronomical, cardiovascular, and circulatory system issues. New research has revealed that NSAIDs can in fact be deadly. Additionally, a recent article released by the FDA concluded the risk of heart attacks and strokes can occur within the first weeks of using the NSAID. A further result being heighten chances of having blood clots by a jaw-dropping 80%. Individuals who have pre-existing cardiovascular conditions are advised to avoid use of ibuprofen, due to the overwhelming increase in chances of heart attacks. Moreover, the FDA is adding information to the drug label warning those who have already had a heart attack to avoid usage, as the NSAID will increase risk of additional heart attacks (FDA, 2015). Judy Racoosin, M.D., M.P.H., deputy director of FDA’s Division of Anesthesia, Analgesia, and Addiction Products stated, “Everyone may be at risk – even people without an underlying risk for cardiovascular disease” (2015).

The experimental species, zebrafish, are one of the most beneficial species for scientific research due to the impressive similarities to human embryos. A recently published paper in Nature, concluded humans and zebrafish have approximately 12,719 genes in common. In a percentage, 70% of human genes are found in the zebrafish. Zebrafish possess a backbone, brain
and spinal cord as well as several other organs, including a heart, liver and pancreas, kidneys, bones and cartilage. This makes them much more similar to humans than otherwise thought. Furthermore, 84% of human-disease causing genes are found in the zebrafish (Heath, 2013). Aiding these overwhelming statistics, zebrafish are easy to maintain and are inexpensive. The fish produce substantial amounts of offspring as well. These are just a few of the remarkable factors which make zebrafish beneficial for scientific research.

In this toxicological investigation, a total of two trials were run. During the first, the wells included a control, low, medium, and a high. The high concentration level was quickly ruled as too high of a dosage for the embryos, after all died in the solution overnight. As a result, the following experiment included only a control, low, and medium. New solutions were added each day, after cleaning out the previous day’s. The purpose of the experiment was to test the direct effects of ibuprofen on the hatching and developmental rates of zebrafish embryos. If the ibuprofen solution was added to the wells of developing zebrafish, then development would be delayed and abnormalities would occur because ibuprofen has a negative effect on developing embryos.

**Materials & Methods :**

The following materials were used in the experiment: 0.428 µg/mL and 4.28 µg/mL of ibuprofen solutions, 100 mL waste beaker, 250 mL embryo beaker, liquid disposal, sharpie, instant ocean/Embryo Media Solution, 1.5 mm disposable pipette, 1 mL disposable pipette, a well plate, 28.5°C incubator, and a dissecting and compound microscope.

Prior to the experiment, a spawning tank was set up, and the brine shrimp were fed for spawning preparation. After obtaining embryos, the plate was labeled in sharpie with the concentration of the ibuprofen. Using the pipettes, 1 mL of Instant Ocean/Embryo Media Solution was added to one well of the plate. The remaining wells were filled with the correct amount of the ibuprofen solutions. The embryos were then divided to ensure there were 10 in each well. The exact number of live embryos in each well was recorded on a data sheet and observations were made.

The next day, the well plate was removed from the incubator. All dead embryos were removed and placed into the waste beaker. The remaining embryos, hatched numbers of fish, and observations were recorded. Once all the observations had been recorded, the ibuprofen solution was then removed and fresh solution was added. The well plate was then placed underneath a microscope and any developmental markers and abnormalities were then recorded in the observation table. Lastly, the plate was returned to the incubator.

The following day, the same steps were repeated as the previous day. All observations were recorded and the solution was exchanged. These steps were repeated then for another consecutive day as well. On the last day, the teacher then properly disposed the embryos. All of the materials were provided by the Wisconsin Inquiry-based Scientist-Teacher Education Partnership (WInSTEP) Program, which is part of the NIH Science Education Partnership Award.
(SEPA) Program administered by the University of Wisconsin–Milwaukee and the Children's Environmental Health Sciences Core Center.

Using an online statistical t-test, on the GraphPad software website, the significance of the data was calculated.

**Results:**

Ibuprofen solution was added to wells of developing zebrafish to test the effects of the solution on the developing fish. The independent variable was the ibuprofen solution, while the dependant variable was the developing embryos. The control contained nontoxic embryo media solution. The hypothesis was: If the ibuprofen solution was added to the wells of developing zebrafish then development would be delayed and abnormalities would occur because ibuprofen has a negative effect on developing embryos.

Two total trials were run due to experimental complications with the concentration levels. During the first trial, the embryos in the solution wells were deceased within a day from the high levels of concentration. Within the first trial as well, the control were the only wells which survived. As a result, the concentration levels were decreased for the second trial. Within the second trial, the mortality rate decreased among all wells after the first day, one or two embryos were deceased within each individual well (Table 2 and Figure 2). The only differing well was within the medium, which had three deceased after the first day. However, this was the only mortality decrease within all the wells throughout the experiment. Additionally, the low and medium concentrations’ hatching rates were delayed overall, resulting in a total of five hatched within the low and no hatchings in the medium concentration (Table 1 and Figure 1). The control had a total of 14 hatch.

Furthermore, the heart rates of the embryos were recorded. A drastic difference appeared between the low and medium concentrations (Table 3 and Figure 3). The coloration of the hearts differed as well. The control and lows’ embryos hearts had the normal pace and expected shade of red. On the other hand, the medium’s were clear and had a much slower pace. The t test determined these results for heart rate were very statistically significant.
Table 1: Final Number of Zebrafish Hatched. This table displays the average number of hatched zebrafish after Day 4 of the experiment, the standard deviation, probability, and the result of the t-test.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Well 1</th>
<th>Well 2</th>
<th>Well 3</th>
<th>Well 4</th>
<th>Average</th>
<th>Standard Deviation</th>
<th>Probability</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5</td>
<td>3</td>
<td>4</td>
<td>2</td>
<td>3.5</td>
<td>1.290994449</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.428 µg/mL</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1.25</td>
<td>1.089724736</td>
<td>p = 0.0468</td>
<td>statistically significant</td>
</tr>
<tr>
<td>4.28 µg/mL</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>p = 0.0016</td>
<td>very statistically significant</td>
</tr>
</tbody>
</table>

p < 0.05 (result is not significant)
p > 0.05 (result is significant)

Figure 1: Final Number of Zebrafish Hatched. The graph shows the drastic differences in hatching rates from the control to the low and the low to the medium concentration. The bars represent standard deviation.

Averages for Ibuprofen Exposure and Final Number of Hatched Fry
Table 2: Final Number of Live Fry Exposed to Ibuprofen on Day Four. The table below displays the mortality rates of the zebrafish on the final day of the experiment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Well 1</th>
<th>Well 2</th>
<th>Well 3</th>
<th>Well 4</th>
<th>Average</th>
<th>Standard Deviation</th>
<th>Probability</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>9</td>
<td>10</td>
<td>8</td>
<td>8.75</td>
<td>0.829156197</td>
<td>6</td>
<td>Not Significant</td>
</tr>
<tr>
<td>0.428 µg/mL</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>0.1682</td>
<td>0.1609</td>
<td>Not Significant</td>
</tr>
<tr>
<td>4.28 µg/mL</td>
<td>7</td>
<td>4</td>
<td>10</td>
<td>5</td>
<td>6.5</td>
<td>2.291287847</td>
<td>0.1609</td>
<td>Not Significant</td>
</tr>
</tbody>
</table>

p < 0.05 (result is not significant)
p > 0.05 (result is significant)

Figure 2: Final Number of Live Fry Exposed to Ibuprofen on Day Four. As the solution amount was increased, a decrease was presented within live fry, resulting in a negative effect on the mortality of the developing embryos.
Table 3: Heart beats per minute of Zebrafish Exposed to ibuprofen. The decrease in heart rate of embryos exposed to a higher concentration is shown in the table below.

<table>
<thead>
<tr>
<th>Treatment Level</th>
<th>Trial 1 (bpm)</th>
<th>Trial 2 (bpm)</th>
<th>Average (bpm)</th>
<th>Standard Deviation</th>
<th>Probability</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>152</td>
<td>137</td>
<td>145</td>
<td>23.25</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.428 µg/mL</td>
<td>152</td>
<td>133</td>
<td>143</td>
<td>24.89</td>
<td>p = 0.7891</td>
<td>not statistically significant</td>
</tr>
<tr>
<td>4.28 µg/mL</td>
<td>92</td>
<td>107</td>
<td>99</td>
<td>7.33</td>
<td>p = 0.0034</td>
<td>very statistically significant</td>
</tr>
</tbody>
</table>

p < 0.05 (result is not significant)  
p > 0.05 (result is significant)

Figure 3: Heart beats per minute of Zebrafish Exposed to ibuprofen. Two time trials were conducted, first two 10 seconds trials, then two additional 15 second trials. The trials were conducted twice to ensure the most accurate outcomes. The averages of the two trials are included as well. A decrease in heart rates of embryos exposed to higher concentrations of the solution is shown in the table.
Figure 4: The control, low (0.428 µg/mL), and medium (4.28 µg/mL), concentrations of ibuprofen are shown below. Within the low concentration, delayed development was a result from the solution. Furthermore, in the medium concentration delayed development and additional abnormalities occurred as well.

A) Control  B) 0.428 µg/mL  C) 4.28 µg/mL

Discussion:
The concluded data supports the hypothesis by the developmental abnormalities which occurred within each well. The low concentration (0.428 µg/mL) of the solution caused a notable decrease in the hatching rates, allowing only five embryos to hatch, which the t-test determined was statistically significant to the control. However, despite the delayed development, the mortality rates were determined to not be significant. In each well, two embryos were deceased, resulting in an average of eight surviving per well. The heart rate average was very similar to the control as well. In the first trial, the numbers were close to exact and only a few off in the second trial. Continuing, within figure 4, few abnormalities are shown. A recorded observation is the embryos developed at a slower pace. The ibuprofen in low dose
resulted in delayed development with the tails particularly taking more time to develop. A conclusion that can be made is ibuprofen in low doses results in developmental delay and few abnormalities, but it does not have a significant impact on the developing embryos.

The low concentrations had a small overall effect on the developmental, hatching, mortality, and heart rates. However, the medium concentration had a much greater effect on the embryos. The embryos within the medium concentration (4.28 µg/mL) wells showed the direct and harmful effects of the drug, resulting in delayed and abnormal cardiovascular development as well as decreased hatching and growth rates. The medium concentration had no embryos hatch, with the test determining these statistics to be very statistically significant. However, despite the 14 deceased embryos in medium concentration wells, resulting in an average of 6.75 surviving per well, the test determined these statistics to not be significant. Moreover, the heart rate had a drastic decrease from the control and low concentration. The average of the control was 145 BPM (beats per minute) and the low concentration was 143 BPM. In contrast, the medium concentration had an average of 99 BPM. From figure 4, observations which were recorded included discoloration of the heart and deformed yolk sacks. The heart is a usual shade of red, while the embryos exposed to the medium concentration, had clear hearts. In addition, the yolk sacs were cloudy and the location differed on embryos. The embryos exposed to a the medium concentration level of (4.28 µg/mL) were directly harmed from the drug, ibuprofen. Hatching and developmental rates, along with severe abnormalities occurred within the wells.

For conducting future studies, one benefit would be continuing the experiment for a longer period of time. The experiment ran for a total of 4 days, not allowing the knowledge of what may occur if the embryos had been continually released to the ibuprofen to be known. Additionally, using a larger amount embryos would provide more in depth results, along with increased accuracy of what was occurring within the embryos exposed to the different amounts of the solution. On top of that, exposing the embryos to a variety of levels of the solution, would be beneficial for determining where the abnormalities and developmental issues begin to occur.

The two experimental errors included, a miscount within one of the wells, the total was 9 rather than the expected 10. The only other error may have occurred while adding fresh solution, one or two embryos may have been placed in an adjacent well of the same concentration.

The NSAID ibuprofen, used to treat pain and inflammation can be obtained at most local drug and home remedy stores, increasing use throughout the U.S.. The overlooked side effects of gastronomical, cardiovascular, and circulatory system issues, must be taken into consideration when pregnant users use the drug. As shown, increased doses of the NSAID have an outstanding impact on the developmental process of zebrafish embryos, resulting in severe abnormalities. Pregnant users are advised not to take ibuprofen within the last 3 months of their pregnancy, however, with the pre-existing risks and the conclusions made, it would be safest to avoid use throughout the entire pregnancy. In addition, taking the NSAID for a longer period of time increases the possibilities of risks with heart attacks. An inference which can be made is, if the pain-reliever is taken multiple times before the third trimester, the abnormalities still may occur.
due to the continual exposure. The nonsteroidal anti-inflammatory (NSAID), ibuprofen, used for treating pain and inflammation, should be avoided during pregnancy to eliminate all possible risks of delayed embryological development and critical abnormalities.

References:


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