Effects of Sucralose, Saccharin, Rebaudioside (in Stevia) and Aspartame on Development in *Xenopus laevis* (Clawed Frog)

By

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Dedicated to:
My friends and family
Abstract

Artificial sweeteners are a relatively recent addition to the human diet. These substances, developed to fight rising obesity and diabetes rates by providing a low calorie sugar substitute, have become very popular among consumers. The result has been increased consumption of chemicals whose effects on the human body are not fully understood. The purpose of these experiments is to determine the effects of the artificial sweeteners sucralose, saccharin, rebaudioside (in Stevia) and aspartame on development of Xenopus laevis embryos. Trials consisted of four experimental groups and one control group. Each experimental group consisted of one artificial sweetener dissolved in aged tap water at a concentration of 10 µg/ml. The control group was exposed only to aged tap water. The rate of subsequent development was measured and photos were taken of specimens to record any morphological changes. Results, with the exception of a single saccharin trial, indicated that the rate of development was unaffected by the artificial sweeteners. However, in several trials individuals in the aspartame group presented with tail defects in which the tail appeared underdeveloped and curved.
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Introduction

Background

Artificial sweeteners were developed to fight rising obesity and diabetes. They allow consumers to enjoy sweet flavored foods with fewer calories than sugar. As a result these substances have become extremely popular. However, the long term effects of continued consumption of these artificial sweeteners are not yet understood and potentially hazardous. Despite the potential dangers, the average American consumes about 125 pounds of artificial sweeteners (22). If aspartame, saccharin, sucralose or rebaudioside do possess the potential to alter development it would have a profound impact on public health. This study seeks to uncover any such causal relationship.

*Xenopus laevis* was selected as a model organism for several reasons. Even at very early developmental stages, the embryos of this species are easily observed and manipulated. The larval stages are transparent allowing for easy observation of any internal changes. Additionally, embryos are readily available and easy to obtain. Normal development of these embryos has been well documented making it easier to identify any changes in their development induced by the artificial sweeteners. This study used the Nieuwkoop and Faber development series (1994) as a reference for determining the stages of the specimens being observed (15).

Aspartame

Aspartame is an artificial, non-saccharide sweetener, used as a sugar substitute and found in the popular products Nutrasweet and Equal. Aspartame is a methyl ester of the aspartic aspartic acid/phenylalanine dipeptide with an IUPAC name of N-(L-α-Aspartyl)-L-phenylalanine, 1-methyl ester (10). It was approved by the US FDA in 1980 and has since been used in over 6,000 products including food, soda, and candy. Aspartame is common in sweet
Aspartame is a common cause of food-related health issues. It is responsible for over 75% of harmful reactions to food additives as reported by the FDA (1). Since 1982 there have been over 10,000 complaints filed by 7,000 people, listing 92 different symptoms due to aspartame consumption (3). Some of the reported reactions even include seizures and death. In fact, aspartame has been shown to be a direct cause of seizures in lab mice (17). Although it has been reported that aspartame has no effect on the brain by some researchers, other researchers have even gone so far as to refer to aspartame as a potent neurotoxin. In fact, recent studies have shown that the artificial sweetener contains the excitotoxin aspartic acid which elevates the excitotoxin levels in the blood when consumed. Excitotoxins such as this play a large part in the destruction of the myelin sheath on nerve fibers. This destruction of myelin-producing cells results in a condition very similar to multiple sclerosis (4). Additionally, aspartame is thought to be a possible cause of memory loss as it is degrades into aspartic acid and phenylalanine which, in the absence of other amino acids, act as neurotoxins (10).

Aspartic acid isn’t the only danger present in aspartame; when consumed, aspartame is broken down into phenylalanine and methanol in addition to aspartic acid. Phenylalanine plays an important role in neurotransmitter regulation, and aspartic acid is also thought to play a role as an excitatory neurotransmitter in the central nervous system. Methanol is converted to formate, which can either be excreted or can give rise to formaldehyde, diketopiperazine (a carcinogen) and a number of other highly toxic derivatives. It has also been reported that consumption of aspartame may cause neurological and behavioral problems. Headaches, insomnia, and depression are only a few of the neurological effects that have been reported as a result of
aspartame consumption (5). Another study suggests a possible link between the growing brain cancer rates in adults and children and increased consumption of aspartame and other artificial sweeteners (12). Yet another study indicates that aspartame may be a cause of birth defects such as low birth weight and brain damage (6). This is a debated claim as aspartame was described as an unlikely cause of birth defects in a separate study (20). However, it has been confirmed that mothers with a condition known as phenylketonuria are unable to consume aspartame without risking miscarriage or serious birth defects such as developmental delays, neurological disorders, behavioral disorders, reduced bone density and microcephaly (4).

The effects of aspartame have been studied on various species including humans, rats, mice and rabbits, but most studies have taken a macroscopic approach. In some of these studies, if no adverse effects were visible after a single large administered dose of aspartame, it was assumed that there were no negative side effects at all (2). This study looked at the effects of aspartame during a time period in which critical developmental phases took place in *Xenopus laevis* embryos.

**Rebaudioside (Stevia)**

Rebaudioside is an artificial sweetener extracted from the leaves of the plant *Stevia rebaudiana*. It is commonly sold under the name Truvia and Sweet Leaf. The active compounds of *Stevia* are steviol glycosides (stevioside and rebaudioside) which have up to 250-300 times the sweetness of sugar (19). *Stevia* does not have any major effects on blood glucose which makes it attractive to people who struggle with high blood sugar such as diabetics. *Stevia* was originally banned in the US in the 1990s as a result of studies which indicated *Stevia* may cause cancer. In 2008 the FDA approved some specific glycoside extracts. Since then, studies have shown that
Stevia based artificial sweeteners help reduce blood glucose levels in diabetic rats by increasing insulin sensitivity (5). While Stevia has been shown to be nonmutagenic, research on the effects of Stevia on development has not been done (11). A goal of this study was to record rebaudioside’s effect on the development of Xenopus laevis embryos and compares these effects to those of aspartame, sucralose and saccharin.

Sucralose

Sucralose, or trichlorogalactosucrose, is an artificial sweetener sold in several products but is most notably the main ingredient in Splenda which is used for the same purposes as aspartame and rebaudioside (17). Despite a lack of comprehensive research into side effects, it remains an attractive product because, “First, sucralose does not affect blood glucose levels. Second, sucralose has not been linked to the severe side effects associated with sweeteners made with other chemicals” (17). The fact that sucralose does not affect blood glucose makes it a particularly popular choice for diabetics (8). While sucralose does not have as many potential dangers as aspartame and has had more research performed on it than Stevia, there are still many unanswered questions regarding its effect on health. For instance, one study found a “causal relationship between sucralose and migraines” (17). However, no mechanism explaining how sucralose causes these migraines has been discovered. Additionally, a recent study found that there is an increase in malignant cancer in rats who consume Splenda which contains sucralose. However, a separate study concluded that sucralose had no carcinogenic affect. It is unclear what, if any role, sucralose played in the increase in cancer among rats. More research is clearly needed in addition to the undertakings of this study.
Saccharin

Saccharin is an artificial sweetener 300 times sweeter than sugar (7). It is also the first artificial sweetener, developed in 1879 (8). It is used in similar ways as the sweeteners mentioned above. Additionally, there is research which indicates there is a link between saccharin consumption and bladder cancer in male rats. When research was performed to try to discover such a possible link in humans, “Subjects who reported ever having used artificial sweeteners or artificially sweetened foods or beverages showed no elevation in risk. However, positive associations between various measures of use of artificial sweeteners and risk of bladder cancer were seen in several subgroups” (9). Still, the increased risk of bladder cancer in humans was deemed insignificant by the FDA and saccharin remains in food products available for human consumption. The primary side effects of saccharin consumption include acute diarrhea and vomiting. Chronic side effects include low birth weight and cancer in breast-fed animal offspring (19). These chronic side effects may indicate that saccharin causes harm during development.
Materials

- Human chorionic gonadotropin
- Three adult *Xenopus laevis* mating pairs
- Approximately 200 eggs
- Aspartame
- Rebaudioside A
- Sucralose
- Saccharin
- Aged tap water
- Aquarium
- Dissecting microscope
- Clear glass culture dishes
- Watchmaker’s stainless steel number 5 forceps
- Unitron, 12V, 400mA digital camera
- Micro adaptor for camera
Methods

Animal Maintenance:

Frogs were kept in thirty gallon aquariums with one mating pair per tank. Each tank was equipped with one Whisper Biobag Filter System, one hinged lid, one LED light on a twelve hour light/dark cycle, marbles (for substrate) and was filled about 75% with aged tap or reverse osmosis water. Frogs were fed two small pieces of liver each, three times a week. Water quality checks, water changes and filter changes were performed weekly.

Inducing Mating:

Male and female *Xenopus laevis* were placed in an aquarium in a quiet, isolated room. Using 2,500 IU of gonadotropin and 5 milliliters of sterilized distilled water, a solution of 500 IU/ml was made. Each frog was taken out of the aquarium individually and held using a paper towel. They need to be held firmly but gently enough not to damage them. The paper towel was placed over their eyes to calm them during the injection. Each frog received an injection of one ml of the gonadotropin solution in the dorsal lymph sac. The frogs were to be returned to their aquarium and covered to block out any light. This was typically done around 3:00PM.

Obtaining the Embryos

By 9:00AM the morning after injection the frogs typically produced a large number of eggs. After eggs were obtained a wide mouth transfer pipet was used to transfer the eggs into clear holding dishes. While looking through a dissecting microscope, watchmaker’s forceps were used to dejelly the eggs. The dejellying process is critical as the jelly layer would have prevented the solutions from being absorbed into the embryos. Additionally, improper jelly removal
technique results in the damage or destruction of the embryos. Once the embryos were dejellied, they were collected and divided up into the appropriate solutions.

After several injections the chorionic gonadotropin supply was depleted and embryos were acquired from a different source. In this study, embryos were ordered from xenopus1.com. Xenopus1 performed a procedure similar to what is described above and shipped the embryos overnight. Upon arrival, the same procedure was followed as with embryos which were obtained via injection of a mating pair.

**Preparing Solutions:**

Each artificial sweetener was dissolved in aged tap water at a concentration of 10µg/ml. Once the solute was dissolved in the aged tap water the solutions were stored at 2° C. After the eggs are dejellied, 200 ml of each solution at room temperature was poured in a glass culture dish in which the embryos were later placed.

**Incubation:**

Each culture dish was given a number which corresponded to the solution which it held, as shown in the chart below.

**Figure 1**

<table>
<thead>
<tr>
<th>Group Number</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solution</td>
<td>Control</td>
<td>Sucralose</td>
<td>Saccharin</td>
<td>Rebaudioside A</td>
<td>Aspartame</td>
</tr>
</tbody>
</table>

Each group consisted of five embryos which were placed in each dish at either stage 9 (blastula) or 19 (neurula) of development.
Photography:

The dissecting microscope was fitted with a Unitron, 12V, 400mA digital camera and pictures were taken every 8-12 hours to record morphology of the specimens. The photographs were taken throughout stages 9-42. The photographs taken were used to detect any irregularities in the morphology of the specimens.

Measuring Development:

Each time photos were taken, the average stage of each group was determined based upon the Nieuwkoop and Faber development series. The stage and morphology of the specimens in each experimental group were compared to the control group.
Results

Trial 1

The data presented in table 1 did not show a consistent pattern of altered growth rate in any experimental group. The most notable finding of this trial was a single specimen in the aspartame group which developed a tail deformity unlike any of the other test subjects. The tail was shrunken, curved to the side and functioned less effectively than the others. Aside from this single individual, no other abnormalities were observed during this trial.

Table 1

<table>
<thead>
<tr>
<th>Dish Number</th>
<th>Solution Type</th>
<th>AS* at 0 hours (start)</th>
<th>AS* at 8.5 hours</th>
<th>AS* at 20 hours</th>
<th>AS* at 48 hours (end)</th>
<th>Deceased Embryos</th>
<th>Underdeveloped Embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>18</td>
<td>24.25</td>
<td>30.75</td>
<td>39.75</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>Sucralose</td>
<td>19.75</td>
<td>23.75</td>
<td>31.75</td>
<td>40.2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>Saccharin</td>
<td>19</td>
<td>24.8</td>
<td>32</td>
<td>40.8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>Rebaudioside</td>
<td>19</td>
<td>25</td>
<td>31.75</td>
<td>39</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>Aspartame</td>
<td>19</td>
<td>24.5</td>
<td>32.5</td>
<td>37(^1)</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

\(^1\)This group contained a single outlier with the stage breakdown of the group being 29, 39, 40, 40

* AS = Average Stage

Trial 2

Trial two has three measurements instead of the four that were taken in trial 1 and 2. However, it does contain results which mirror the results of trial one. Once again, the rate of development did not appear to be affected in any groups. A single subject in the aspartame group
had a curved tail. However, the tail bud imperfection which occurred in this trial was far less severe than it was in the first trial.

**Table 2**

<table>
<thead>
<tr>
<th>Dish Number</th>
<th>Solution Type</th>
<th>AS* at 0 hours (start)</th>
<th>AS* at 20 hours</th>
<th>AS* at 40 hours</th>
<th>Deceased Embryos</th>
<th>Underdeveloped Embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>9</td>
<td>27</td>
<td>42.63</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>Sucralose</td>
<td>9</td>
<td>26.56</td>
<td>42</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>Saccharin</td>
<td>9</td>
<td>26</td>
<td>39</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>Rebaudioside</td>
<td>9</td>
<td>27</td>
<td>40</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>Aspartame</td>
<td>9</td>
<td>24.57</td>
<td>41.71</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

*AS = Average Stage

**Trial 3**

In this final trial, there were several noteworthy observations. The first was that embryos which initially appeared to be fertilized and healthy in the saccharin did not develop. Not a single embryo entered neurulation and, unlike the unfertilized embryos, they appeared to be suspended in development mid-stage 9. It is unclear why this happened.

The second noteworthy finding was that four of the five subjects exposed to aspartame had severe tail defects. The subjects were unable to swim in a straight line and only moved in a circular path. It was clear that they had not developed properly and were unhealthy. Both the saccharin and aspartame groups showed a marked difference from the control group.
Table 3

<table>
<thead>
<tr>
<th>Dish Number</th>
<th>Solution Type</th>
<th>AS* at 0 hours (start)</th>
<th>AS* at 7.5 hours</th>
<th>AS* at 14 hours</th>
<th>AS* at 33 hours</th>
<th>Deceased embryos</th>
<th>Underdeveloped embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>9.2</td>
<td>19.2</td>
<td>28.25</td>
<td>36.25</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>Sucralose</td>
<td>10.5</td>
<td>21.8</td>
<td>29.75</td>
<td>38</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>Saccharin</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>Rebaudioside</td>
<td>9.8</td>
<td>21.25</td>
<td>31.75</td>
<td>35.8</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>Aspartame</td>
<td>10.5</td>
<td>24.57</td>
<td>27</td>
<td>36.5</td>
<td>1</td>
<td>4</td>
</tr>
</tbody>
</table>

*AS = Average Stage
Discussion and Conclusion

Two hypotheses were tested in this experiment. The first, that one of the four artificial sweeteners tested would cause a change in the rate at which the embryos developed, seemed unlikely in aspartame, sucralose and rebaudioside. While there were some small differences in the average stage between these experimental and control groups, the differences were far from significant. The trial 1 and 2 saccharin groups exhibited similar rates of development as the control. However, all five embryos in the trial 3 saccharin group remained undeveloped. Thus, the results of this study are inconclusive in regards to saccharin’s effect on embryos rate of development. There is no evidence that aspartame, sucralose or rebaudioside cause acceleration or deceleration of the development process.

The second hypothesis, that any of the four artificial sweeteners being tested would cause morphological changes or even mutations in the embryos, cannot be rejected. In all three trials aspartame produced at least one subject who appeared to be altered relative to the other subjects involved. However, the third trial was the only trial which showed the tail defect with any level of consistency. In the second trial, the curve of the tail bud was very minor and possibly insignificant. The claim that aspartame caused these tail bud mutations cannot be substantiated by these data as the defect only appeared consistently in a single trial.

The lack of development in the entire saccharin group during the third trial remains unexplained and non-reproduced in any other trials. It seems unlikely the lack of development was a result of the saccharin; however it is possible. Still, it seems more likely that a contaminant was somehow introduced to that specific dish which was not present in any other group. However, this remains speculative as the embryos were not observed continuously and other
people were performing research in the adjacent area. It is possible someone could have accidentally introduced a contaminant while working but there is no way to be sure.

Further Research

The tail bud mutation discovery in the aspartame groups was particularly interesting. However, this study only focused on the earliest stages of development. A long term study of these frogs would be the best way to observe the impact of this mutation and determine whether there was a causal relationship between aspartame and the defect. Additionally, a study done over a longer time would be useful to observe whether tadpoles with tail bud defects develop into healthy frogs as the tail will eventually disappear.

The lack of development in the saccharin group in the third trial was worth further investigation. This result was only observed in the third trial but a study with larger sample sizes would be able to expose any causal relationship between the lack of development and saccharin exposure. Further research on this topic is critical as such a drastic effect on development would have serious real world implications for the way saccharin is handled.

A study similar to this one with a focus on neurulation would also be very useful. Many previous studies indicate that these substances impact nervous tissue more than others. It seems likely that, if there is an impact on development, it is most likely to occur during this stage. If there is an impact on neurulation as a result of consuming any of these sweeteners, it would be a major public health issue.
References


(14) Mann S.W., Yuschak M.M., Amyes S.J.G., Aughton P., Finn J.P. (2000). A combined chronic toxicity/carcinogenicity study of sucralose in Sprague–Dawley rats. Food and Chemical Toxicology, **38** (2) 71-89

(15) Nieuwkoop and Faber (1994) Normal Table of *Xenopus laevis* (Daudin). Garland Publishing Inc, **23** (2) 20-23


Appendix A

The developmental stages investigated in this study. Image is adapted from Nieuwkoop and Faber 1994 development series.
Appendix B

Trial 1:

Figure 1

Figure 1 shows the development of embryos in the control group during trial 1. One embryo was unfertilized and did not develop. The remaining four embryos developed normally.
Figure 2 shows the development of embryos in the experimental group exposed to sucralose during trial 1. One embryo was unfertilized and did not develop. The remaining four embryos developed normally.
Figure 3 shows the development of embryos in the experimental group exposed to saccharin during trial 1. All five embryos developed normally.
Figure 4 shows the development of embryos in the experimental group exposed to rebaudioside during trial 1. One embryo was unfertilized and did not develop. The remaining four embryos developed normally.
Figure 5 shows the development of embryos in the experimental group exposed to aspartame during trial 1. One embryo was unfertilized and did not develop. One embryo developed a tail defect in the later larval stages, as can be seen in the image taken at 7:26 p.m. on day 2. The remaining three embryos developed normally.
Trial 2:

Figure 6

Figure 6 shows the development of embryos in the control group during trial 2. Two embryos were unfertilized and did not develop. The remaining three embryos developed normally.
Figure 7 shows the development of embryos in the experimental group exposed to sucralose during trial 2. All five embryos developed normally.
Figure 8 shows the development of embryos in the experimental group exposed to saccharin during trial 2. Two embryos were unfertilized and did not develop. The remaining three embryos developed normally.
Figure 9 shows the development of embryos in the experimental group exposed to rebaudioside during trial 2. One embryo was unfertilized and did not develop. The remaining four embryos developed normally.
Figure 10 shows the development of embryos in the experimental group exposed to aspartame during trial 2. One embryo was unfertilized and did not develop. One embryo began to develop a minor tail defect in the larval stages, as can be seen in the image taken at 5:25 p.m. The remaining three embryos developed normally.
Trial 3:

Figure 11

Figure 11 shows the development of embryos in the control group during trial 3. One embryo was unfertilized and did not develop. The remaining four embryos developed normally.
Figure 12 shows the development of embryos in the experimental group exposed to sucralose during trial 3. One embryo was damaged while being transferred during the third set of observations at 3:29 p.m. The embryo had been developing normally up to this point. The remaining four embryos developed normally.
Figure 13 shows the development of embryos in the experimental group exposed to saccharin during trial 3. The embryos appeared to be developing normally during the first observation. However, no further development occurred.
Figure 14 shows the development of embryos in the experimental group exposed to rebaudioside during trial 3. One embryo was unfertilized and did not develop. The remaining four embryos developed normally.
Figure 15 shows the development of embryos in the experimental group exposed to aspartame during trial 3. One embryo was unfertilized and did not develop. The remaining four embryos developed tail defects. The defects can be seen in the early stages in the photo taken on day 1 at 3:29 p.m. The defects are more noticeable in the photo taken on day 2 at 10:48 a.m.