ISSN: 2167-7956
Journal of Biomolecular Research & Therapeutics

OPEN ACCESS Freely available online

Research Article

Effect of Bismuth Subsalicylate on the Survival Rate of Hyperglycemic *Danio Rerio*

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ABSTRACT

More than 37 million Americans have diabetes, and approximately 90%-95% of them have Type 2 Diabetes Mellitus (T2DM). T2DM usually begins with insulin resistance, a condition in which muscle, liver, and fat cells do not use insulin well. Certain genes can also increase the likelihood of getting T2DM. Currently, metformin is one of the most common medicines used to treat this chronic condition. However, companies are recalling metformin due to the possibility the medicines could contain Nitrosodimethylamine (NDMA) above the acceptable intake limit. Not only that, but metformin is not widely accessible due to the fact that it is a prescription medicine. Evidence shows salicylates, especially salsalate, appear to be a promising alternative for prevention or treatment of diabetes by lowering glucose levels. In this study, *Danio rerio* were hyperglycaemically induced to model T2DM in Phase 1 Phase 2 investigated the effect of bismuth subsalicylate on decreasing blood sugar levels, and thus increasing survival rate. After statistical analysis, T2DM zebrafish with 1 mg of bismuth subsalicylate were seen to have the highest survival rate, suggesting lowest blood glucose levels, than the control group of zebrafish and other experimental groups. This study provides insight on the effectiveness of bismuth subsalicylate as a treatment for T2DM. Further studies may investigate the direct correlation between T2DM and bismuth subsalicylate using blood collection procedures with humans and more concentrations.

Keywords: Diabetes mellitus; Bismuth subsalicylate; T2DM

INTRODUCTION

Patients with T2DM usually suffer from insulin resistance or inadequate insulin production. This chronic condition results in too much sugar circulating in the bloodstream, which can lead to a variety of health problems, such as heart disease and stroke. Metformin is generally the first medication prescribed to diabetic patients. It works by lowering glucose production in the liver and improving body sensitivity to insulin so that the body uses insulin more effectively, which in turn helps lower the amount of sugar in the body's blood [1]. However, researchers have recently found the high presence of Nitrosodimethylamine (NDMA). Low levels of NDMA aren't dangerous. But if at least 96 nanograms are ingested daily, it becomes a health concern. Over time, it could increase a person's cancer risk. Overexposure can also lead to jaundice, fever, vomiting, abdominal cramps, and dizziness. Long-term exposure may even lead to lung damage [2]. Salicylates, specifically salsate, have been proven to have potential in replacing metformin as a treatment for T2DM, without the severe side effects [3]. Bismuth subsalicylate is an antacid elixir medication commonly used to treat temporary discomforts of the stomach and gastrointestinal tract, such as nausea, heartburn, indigestion, upset stomach, and diarrhea. It works by decreasing the flow of fluids and electrolytes into the bowel, reduces inflammation within the intestine, and may kill the organisms that can cause diarrhea [4].

Some studies have investigated the relationship between various salicylates, not including bismuth subsalicylate, and glucose control, but present conflicting data. This study aims to end the debate on the efficacy of salicylates as a treatment for T2DM, instead of medications such as metformin and berberine. To determine if there is a direct relationship between bismuth subsalicylate and sugar levels, this study will use the model organism *Danio rerio* , also known as zebrafish. Zebrafish are commonly used in studies because they possess key organs that are important for regulation of energy homeostasis and metabolism in mammals, including digestive organs, adipose tissues, and skeletal muscle [5]. Additionally, zebrafish share 70% of genes with us. In fact, 84% of genes known to be associated with human disease have a zebrafish counterpart [6]. Not only do they have an exclusive advantage of their genome similarity to the human genome, but they allow for easy imaging of transparent embryos, well-characterized developmental stages, high fecundity, and low-cost husbandry and housing [7]. Care and maintenance in the lab setting is also not as difficult as other

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Received: 09-Jul-2022, Manuscript No. BOM-22-17399; **Editor assigned:** 12-Jul-2022, Pre QC No. BOM-22-17399(PQ); **Reviewed:** 26-Jul-2022, QC No. BOM-22-17399; **Revised:** 02-Aug-2022, Manuscript No. BOM-22-17399(R); **Published:** 09-Aug-2022, DOI: 10.35248/2167-7956.22.11.221

Citation: Sahni P (2022) Effect of Bismuth Subsalicylate on the Survival Rate of Hyperglycemic *Danio Rerio*. J Biol Res Ther. 11:221

model organisms.

Initially, bismuth subsalicylate would be tested as a potential treatment for T2DM by measuring blood glucose levels before and after exposure to the treatment. A decrease in blood sugar levels would indicate that bismuth subsalicylate does have an effect on the development of T2DM in zebrafish. Since zebrafish and humans have similar endocrine systems, the results of this study could be broadened to humans as an effective treatment for this chronic condition. The original hypothesis was that as exposure to bismuth subsalicylate increases, average blood glucose levels decrease in zebrafish. However, this method would prove to not be achievable in the pre-experiment stage.

During the pre-experiment stage, two main things were tested using extra zebrafish concentration toxicity and blood collection. The concentration 20 mg of bismuth subsalicylate was tested by immersing three fish in a glass fish bowl filled with distilled water. A thermostat was placed inside to keep the temperature constant. After one day, all three fish had died. A concentration of 10 mg was then used to test if it would also be toxic. However, this time, none of the fish had died. Therefore, it was concluded that 10 mg is a safe concentration to test on zebrafish for this study. Blood collection was also attempted using the spare zebrafish. Zebrafish were taken out of the 20 gallon tank after the acclimation period, and placed in ice-cold water using a small fish net, which was measured at 30 degrees Fahrenheit by a thermometer. Upon sedation, seen by a total loss of equilibrium and muscle tone, decreased respiratory rate and no response to stimuli, zebrafish were dried with a paper towel to prevent movement and placed on petri dishes prepared with agar [8]. A pin was then inserted on the tail of the fish to keep it in place. A 5 µL removable needle and syringe was inserted at a 30°-45° angle at the blood collection site Dorsal Aorta (DA) or Posterior Cardinal Vein (PCV) [9]. After several trials with a number of fish, a small amount of blood (approximately 0.4 µL) could be collected. However, this was not enough for the Precision Xtra glucometer to give results, which needs a sample size of 0.6 µL of blood. Therefore, it was no longer practical to directly collect blood glucose levels to assess the effectiveness of bismuth subsalicylate as a diabetic treatment.

As a result, survival rates of zebrafish were measured instead. In other words, zebrafish were still induced with hyperglycemia, but the number of fish dying was monitored instead. The new hypothesis is that if bismuth subsalicylate has an effect on development of T2DM, then hyperglycemic zebrafish will have lower blood glucose levels on average, and ultimately increased survival rate. This relationship would give insight on how the capability of bismuth subsalicylate to help treat T2DM.

Hypotheses

H_0: Bismuth subsalicylate has no effect on the survival rates and development of T2DM in hyperglycemic zebrafish.

H_a: If bismuth subsalicylate has an effect on development of T2DM, then hyperglycemic zebrafish will have an increased survival rate on average.

METHODOLOGY

While research has been conducted on the correlation between some salicylates, such as salsalate and glucose control, none have tested bismuth subsalicylate specifically. The concentration of bismuth subsalicylate (mg) is the independent variable, while the survival rate (%) is the dependent variable. To test the relationship between these two variables, a wild type strain of normal zebrafish was used. Prior to experimentation, four groups of 25 randomly assigned zebrafish were each placed into four 20 gallon tanks of distilled water for acclimation for one day. All tanks were kept at approximately 26°C-28.5°C and the pH of approximately 6.8 to 7.5 were checked daily. Filters were checked and changed regularly, and thermostats were checked regularly to keep temperature constant [10]. The 50 zebrafish in each tank were fed a third of a tablespoon of fish flake food every other day, so each fish would be fed a couple flakes [11].

Before experimentation began, four 100 mL beakers of varying bismuth subsalicylate concentrations were prepared. For each concentration, the beakers with bismuth subsalicylate powder were weighed on a weigh boat, after standardizing the scale, and covered with parafilm to prevent contamination. They were then left aside at room temperature until further use.

To prepare for experimentation, the two 20 gallon tanks that contained no zebrafish at the time were set up and prepared with a 2% solution of pure glucose powder. This was done by approximating the volume of each tank to be about 18 gallons, and this amount converted to mL was approximately 68,137 mL. To calculate a 2% glucose solution per tank, 68,137 mL was then multiplied by 0.02 to equal approximately 1,363 g of glucose per tank. Two samples of 1,363 g of glucose powder were weighed on a weigh boat, after standardizing the scale, and then mixed into each of the two 20 gallon tanks without fish to make a 2% glucose concentration solution per tank [12]. Two groups of 25 zebrafish each were then transferred into the remaining two tanks to create a total of two tanks of 50 zebrafish. As evidence from past studies has proven that chronic exposure to a 2% glucose solution for 14 days induced diabetic phenotypes, the fish were similarly kept in these hyperglycemic solutions for 2 weeks [13].

In phase 1, which included the two weeks of diabetes induction, the zebrafish were monitored and fed every other day. The tanks became visibly partially cloudy, as the glucose powder dissolved in the water. By the end of the two weeks, many fish died for a variety of reasons that could include collision with the filters, overconsumption, under consumption, and any experimental conditions. However, the exact or main reason for death cannot be pinpointed.

To address the concern that zebrafish were getting stuck in the filter, a filter sponge was placed on each of the filters to ensure that the filters would not cause a problem with the health of the zebrafish. Additionally, tanks were monitored and assessed to perfect the appropriate amount of flake food for the zebrafish.

In phase 2, survival rates of zebrafish were measured in each of the tanks. Each tank had a filter and thermometer to keep the water clean and at a constant temperature of about 27 degrees Celsius. Zebrafish were first split into four tanks of 15 zebrafish each. They were fed fish flake food every other day as in phase 1. The number of fish alive after 7 days was measured. Therefore, this gives an understanding of the effectiveness of bismuth subsalicylate as a treatment for T2DM.

RESULTS

Statistical analysis

For this study, a Fisher's Exact test was used, which is commonly used to determine if there are nonrandom associations between two categorical variables. It is practical for small sample sizes, like the ones in this study.

A Fisher's Exact Test calculator (https://www.socscistatistics.com/tests/fisher/default2.as px) was used to come up with observed P-value. In order to test for statistical significance, a 0.05 p-value level of significance was used, where $p>0.05$ is the probability that the null hypothesis is true and $p<0.05$ is the probability that the alternative hypothesis is true (Tables 1-3 and Figure 1).

Table 1: Data Comparison Between Control and Experimental Groups.

	Tank 1 Control	Tank 2	Tank 3	Tank 4
Treatment Dosage	0 mg	1 mg	5 mg	10 mg
Survived (fish)	3	13	1	0
Died (fish)	12	2	14	15

Note: The Fisher exact test statistic is 0.0007. The result is significant at $p<0.05$.

Table 2: Data comparison between the fisher exact test statistic values.

	Results		
	0 mg	1 mg	Marginal row totals
Survived (of fish)	3	13	16
Died (of fish)	12	2	14
Marginal column Totals	15	15	30 (Grand total)

Note: The Fisher exact test statistic value is 0.2241. The result is not significant at $p<0.05$.

Table 3: Data comparison between the survived and dead fish test statistic values.

	Results		
	0 mg	5 mg	Marginal row totals
Survived (of fish)	3	1	4
Died (of fish)	12	14	26
Marginal column Totals	15	15	30 (Grand total)

Note: The Fisher exact test statistic value is 0.5977. The result is not significant at $p<0.05$.

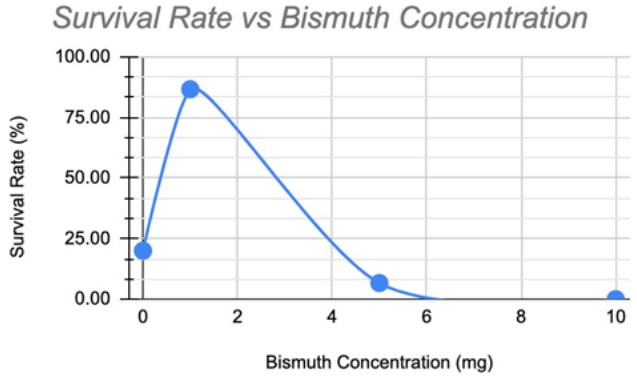

Figure 1: 2 × 2 Contingency Tables of the Correlation between Bismuth Subsalicylate Concentration and Survival Rate.

The statistical tests reveal significance between 1 mg vs. 0 mg in affecting the survival rate. Since $0.0007<0.05$, the null hypothesis was rejected in favor of the alternate hypothesis. This shows statistical significance in that hyperglycemic fish receiving 1 mg of bismuth subsalicylate will have a higher survival rate than the control group. However, there is no significance in the 5 mg and 10 mg concentrations since these p-values were greater than 0.05.

DISCUSSION

This study examined the relationship between bismuth subsalicylate and T2DM through observation of zebrafish survival rates. The results of this experiment proved that immersing 1 mg of bismuth subsalicylate is the best treatment for increasing survival rates of hyperglycemic zebrafish. The statistical significance in this relationship showed that the T2DM fish can use drugs, specifically bismuth subsalicylate to decrease their blood sugar levels.

On the other hand, the fish exposed to 5 mg and 10 mg of bismuth subsalicylate did not show a significant increase in survival rate. Perhaps, these higher concentrations were toxic to the fish, causing them to die. The pre experiment lasted only one day for each trial, but it should've been longer had time permitted [14]. This would have ensured that the 5 mg and 10 mg concentrations were safe for a week as used in the experiment, rather than just one day.

There were other areas in which study could have been improved. After realizing that blood collection was not feasible for the circumstances of this experiment, measuring survival rates still provides information for the effect of bismuth subsalicylate on T2DM. However, the survival rates should have been measured every day for the week in phase 2, instead of just at the end of the seven days [15]. This would give more insight on how bismuth subsalicylate works to improve survival over a period of time. Additionally, there was an assumption made that the zebrafish immersed in 2% glucose solutions for fourteen days were models for T2DM. However, since this is such a crucial part of the experiment, the assumption should have been tested somehow. In fact, initially, it would have been tested if blood was being collected, since the glucose levels would indicate if the zebrafish reached the threshold for T2DM. But, after switching to survival rates, this assumption was not tested [16].

Regardless, if zebrafish are an accurate model to mammals for these processes, then the experiment conducted reveals significance between the fish receiving 1 mg of bismuth subsalicylate and the control group.

The precautions to avoid cross contaminations of control and experimental groups by using a different fish net for each tank could be considered strength in this study.

Limitations included budget, method, and time constraints such as conducting the pre-experiment for a longer period of time. It would be difficult to manage this study if blood collection was conducted due to the fact that blood collection from all sample zebrafish would need to occur on the same day to keep the results accurate. This would be a time-consuming and tiring task. If blood was collected from the first fish a day before another fish, results may not have been accurate.

CONCLUSION

The way of administering anesthesia in organisms like zebrafish is heavily debatable to many researchers, however. Some claim that directly placing fish against ice while completing death by hypoxia

makes the fish feel pain, which is unethical, while others believe it is okay to have direct contact with the ice since it is believed that fish feel no pain. Additionally, MS-222 (tricaine), a known ion channel blocker, is often used to numb fish, however, MS-222 has been reported to increase blood glucose and potentially interfere with normal ion channel function in the β-cells of teleost fish. Therefore, hypothermia using some three to four ice cubes in water was chosen, after considering ethical and accuracy concerns. Overall, the study was ethically sound by following regular care and maintenance protocols of the fish.

These results offer an alternative treatment for T2DM than metformin, which contains dangerous compounds. Bismuth subsalicylate (Pepto-Bismol) is also more accessible than metformin due to the fact that it is an over-the-counter medication, unlike metformin which requires a prescription. An understanding of the relationship between this medication and treating T2DM is crucial in fighting against the diabetes crisis worldwide. Future studies may benefit from exploring if bismuth subsalicylate could be even more effective, with the combination of other medications. As a whole, this study provides insight on new ways to treat T2DM effectively, without the costs.

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