JOURNAL OF
EMERGING INVESTIGATORS

Article

The non-nutritive sweeteners acesulfame potassium and neotame slow the regeneration rate of planaria

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SUMMARY

The consumption of sugar substitute non-nutritive sweeteners (NNS) has dramatically increased in recent years. Despite being advertised as a healthy alternative, NNS have been linked to adverse effects on the body, such as neurodegenerative diseases (NDs). In NDs, neural stem cell function is impaired, which inhibits neuron regeneration. The purpose of this study was to determine if the NNS acesulfame potassium (Ace-K) and neotame affect planaria neuron regeneration rates. Since human neurons may regenerate, planaria, organisms with extensive regenerative capabilities due to stem cells called neoblasts, were used as the model organism. The heads of planaria exposed to either a control or non-toxic concentrations of NNS were amputated. The posterior regions of the planaria were observed every 24 hours to see the following regeneration stages: (1) wound healing, (2) blastema development, (3) growth, and (4) differentiation. We hypothesized that exposure to the NNS would slow planaria regeneration rates. The time it took for the planaria in the Ace-K group and the neotame group to reach the second, third, and fourth regeneration stage was significantly greater than that of the control. The results of this study indicated that exposure to the NNS significantly slowed regeneration rates in planaria. This suggests that the NNS may adversely impact neoblast proliferation rates in planaria, implying that it could impair neural stem cell proliferation in humans, which plays a role in NDs. This study may provide insight into the connection between NNS, human neuron regeneration, and NDs.

INTRODUCTION

Non-nutritive sweeteners (NNS), also known as sugar substitutes and artificial sweeteners, are defined by the FDA as low-calorie or zero-calorie alternatives to regular sweeteners that provide minimal or no carbohydrates or energy (1). NNS may be derived from plants or herbs, or even sugar itself (1). They occur naturally in foods, and they may be added in during food processing or by consumers before consumption (1). The use of NNS has risen significantly in the last few decades. Sylvetsky *et al*. found that there was a 200% increase in NNS consumption for children and a 54% jump for adults from 1999 to 2012. The researchers extrapolated that the numbers have likely only increased since (2). The dramatic increase in the consumption of NNS stems from their advertisement as a healthier alternative to regular sweeteners. In fact, it is advertised that using NNS instead of regular sweeteners can help consumers lower their daily caloric intake, lose weight, stabilize blood sugar levels, and sustain a healthy diet (3). However, NNS, including acesulfame potassium and neotame, have been linked to disorders of the central nervous system (CNS) suggesting they could impair neural stem cell function and neurogenesis (4-11).

Acesulfame potassium (Ace-K) is one of the major FDA-approved NNS in the modern diet. Many studies have linked Ace-K to adverse impacts on the CNS. Cong *et al*. determined that long-term Ace-K exposure causes many dysregulations in the CNS, including inhibition of glycolysis, decrease in intracellular ATP production, and inhibition of neuroprotective activity and cellular viability (4). The study also concluded that Ace-K exposure obstructed several essential signaling pathways in the CNS (4). Bian *et al*. concluded that Ace-K exposure altered the bacterial composition of the gut microbiome (5). Changes in the gut microbiome are linked to diseases of the CNS (6). Park *et al*. found that Ace-K exposure causes elevated endoplasmic reticulum stress levels on brain cells, blunts axon outgrowth, and that Ace-K is cytotoxic (7). The fact that Ace-K causes several severe disruptions in the CNS strongly indicates that it will negatively impact several other CNS processes, including neural stem cell function and neurogenesis.

Neotame is another common NNS approved by the FDA that has been linked to adverse responses in the CNS. Chi *et al*. found that neotame exposure altered the diversity of the gut microbiome in mice (8). Alterations in the gut microbiome have the potential to severely and adversely impact the CNS (6). The study also concluded that neotame exposure also negatively altered metabolic patterns and inhibited the function of some key genes involved in metabolism (8). Metabolic disorders can play a role in neural inflammation and neuronal death (9).

Neotame is an analog of the NNS aspartame. This is concerning because aspartame has been shown in many studies to cause neurodegeneration. Humphries, Pretorius, and Naude found that aspartame exposure causes the degeneration of neurons in the Meynert nucleus, a region of the brain (10). Loss of neurons in this region has been found in Alzheimer's patients. Ashok and Sheeladevi found that the FDA-approved daily acceptable intake of aspartame (40 mg/kg) caused neurobehavioral changes and the activation of neurodegenerative neuron cell death in rats (11). The fact that neotame causes several severe disruptions in the CNS, and is an analog of a substance linked to neurodegeneration, indicates that it may negatively impact several other CNS processes, including neural stem cell function and neurogenesis.

The ability to perform neurogenesis, the process where

new neurons are generated from neural stem cells (NSCs), is a primary concern in the study of neurodegeneration and NDs. The process of neurogenesis relies on the complete function of NSCs. If the function of NSCs is impaired, then neurogenesis would be unable to occur (12). Demars *et al*. found that the inhibition of neurogenesis is linked to the development and progression of neurodegeneration and NDs (13).

NDs are a common and growing cause of morbidity and mortality worldwide. NDs occur when neurons in the brain and spinal cord begin to deteriorate, which causes disruptions in emotional, cognitive, and locomotor functions (14). NDs are a building concern due to their increasing prevalence and increasing health and socioeconomic implications. NDs strike primarily in the elderly because the ability to perform neurogenesis diminishes most considerably in the human brain as it matures (15). Because NDs strike primarily in mid- to late-life, the incidence is expected to soar as the population ages. By 2030, as many as one in five Americans will be over the age of 65 (16). If left unchecked, 30 years from now it is predicted that more than 12 million Americans will suffer from NDs (16).

This experiment uses planaria (*Dugesia dorotocephala*) to evaluate the effects of NNS on neuron regeneration. Planaria are small, aquatic organisms that have the ability to regenerate due to pluripotent stem cells, called neoblasts, distributed throughout the body, which differentiate into all cell types. After planaria undergo amputation, neoblasts migrate to the wound site and increase their proliferation rate. Their progeny forms an unpigmented mass of new tissue called the blastema, in which cellular differentiation is coordinated to restore the missing body parts (17). The planarian nervous system is remarkably like that of humans (18). In fact, planaria are considered the most primitive form of cephalized animal with a CNS like that of vertebrates. Like vertebrates, planaria have a CNS with a brain (18). Planaria have many of the same neurotransmitters as humans, including serotonin (18). Serotonin controls axon and neuronal regeneration in both the vertebrate and planarian nervous system (19, 20). Furthermore, planaria share with vertebrates all the major developmental signaling pathways of cells (17). This includes the Wnt/β-catenin signal transduction pathway, which plays a critical role in vertebrate development and regeneration (21). These similarities present an opportunity to research stem cell function in planaria to better understand mammalian disease and development and to better analyze relevant molecular processes in humans.

The objective of this study was to determine how exposure to the NNS, Ace-K and neotame, affected the regeneration rate of planaria. Due to their negative effects on CNS processes, we hypothesized that Ace-K and neotame would slow the regeneration rate of planaria. Data from our experiments showed that Ace-K and neotame significantly slowed the regeneration rate of planaria. This indicates the Ace-K and neotame slow neoblast function in planaria (3-7,9).

RESULTS

Determining the working concentration for Ace-K and neotame

Since the effects of Ace-K and neotame on planaria were unknown prior to this experiment, we had to perform a preliminary experiment to determine the appropriate working concentrations of each NNS for the main experiment. To identify the appropriate (non-toxic) concentrations of Ace-K and neotame in this species of planaria, we analyzed the mortality incidence of planaria incubated in varying concentrations of each NNS over the course of three days. Both Ace-K and neotame solutions of 1M, 1dM, 1cM, 1mM, 100μM, 10μM, 1μM were prepared and tested in this experiment. The findings of the preliminary experiment indicated that the 100μM concentration of Ace-K would be the working concentration of Ace-K (**Table 1**). The results also indicated that 1mM was the ideal working concentration of neotame for the main experiment (**Table 2**). These concentrations visibly slowed the planaria but had a 0% mortality incidence.

Determining how NNS impact the regeneration rate of planaria

To determine if Ace-K and neotame affected the rate of regeneration of planaria, we tracked the time it took for the planaria in each group to reach each regeneration stage after head amputation (**Figure 1**). We amputated each planaria below the head and visually inspected each to determine that the amputation was completed properly. Every 24 hours, we took a photo of each planaria, visually inspected the photo, and recorded the regeneration stage (**Figure 2**). We assessed the regeneration stage based on the presence

	Number of Planaria Alive			
Solution	**Day 0**	**Day 1**	**Day 2**	**Day 3**
Control	5	5	5	5
1M	5	0	0	0
1dM	5	0	0	0
1cM	5	2	2	0
1mM	5	5	5	4
100μM	5	5	5	5
10μM	5	5	5	5
1μM	5	5	5	5

Table 1: Ace-K Preliminary Experiment Results. The table shows the number of planaria alive throughout the 3 days after exposure to solutions with Ace-K concentrations of 1M, 1dM, 1cM, 1mM, 100μM, 10μM, 1μM, or a Poland Spring Water control (n=5).

	Number of Planaria Alive			
Solution	**Day 0**	**Day 1**	**Day 2**	**Day 3**
Control	5	5	5	5
1M	5	0	0	0
1dM	5	1	0	0
1cM	5	2	1	0
1mM	5	5	5	5
100μM	5	5	5	5
10μM	5	5	5	5
1μM	5	5	5	5

Table 2: Neotame Preliminary Experiment Results. The table shows the number of planaria alive throughout the 3 days after exposure to solutions with neotame concentrations of 1M, 1dM, 1cM, 1mM, 100μM, 10μM, 1μM, or a Poland Spring Water control (n=5).

Figure 1: Planarian Amputation. Picture of a planaria pre-amputation. The red line is indicative of where the amputation was performed.

of developmental milestones for each of the four stages of regeneration, as specified in the material and methods section. We recorded the elapsed time (in days) required for each planaria to reach each stage of regeneration. We calculated the average elapsed time for each stage for each experimental group (**Figure 3**). On average, both the Ace-K and neotame groups took considerably longer to reach each regeneration stage than the control group. We analyzed all results using a one-way ANOVA test with a post-hoc Tukey test. From this, we concluded that both Ace-K and neotame significantly delay the regenerative response of planaria. We found that the planaria exposed to Ace-K took significantly longer to reach the second, third, and fourth stages of regeneration than the planaria exposed to the control ($p < 0.05$). However, there was no significant difference in the time it took planaria in the Ace-K group and planaria in the control group to reach stage one ($p > 0.05$). The planaria exposed to neotame also took significantly longer to reach the second, third, and fourth stages of regeneration than the planaria exposed to the control ($p < 0.05$). There was also no significant difference in the time it took planaria in the neotame group and planaria in the control group to reach stage one ($p > 0.05$). From this, we concluded that both Ace-K and neotame significantly delay the regenerative response of planaria.

DISCUSSION

The results indicate that an Ace-K or neotame treatment hinders planaria regeneration by demonstrating that the NNS increased the time required for planaria to regenerate following amputation below the head. Based on the average time it took each planarian to reach each stage of regeneration, planaria in the Ace-K group ($p < 0.05$) and the neotame group ($p < 0.05$) regenerated significantly slower than the control group. Thus, because the regeneration time of the planaria treated with the NNS was significantly longer than that of the control, the hypothesis is supported.

Planaria cultivate a large population of neoblasts. This study suggests that the NNS Ace-K and neotame decelerate the regeneration and differentiation of these neoblasts into

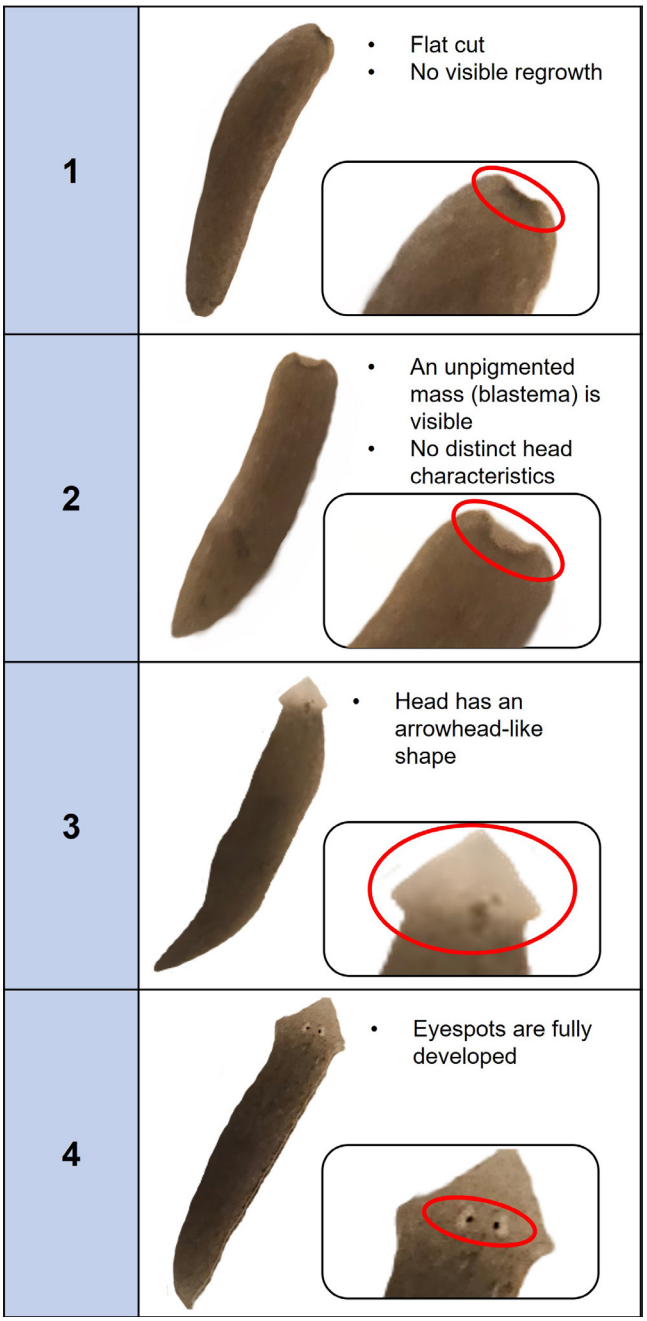

Figure 2: Planaria Regeneration Stages. The regeneration rate of each planarian was determined by recording the time (in days) it took to reach each regeneration stage. The stages of regeneration are: (1) wound healing, (2) blastema development, (3) growth, (4) differentiation.

neural cells. These findings imply that Ace-K and neotame may compromise the function of NSCs in the human body, thus impairing neurogenesis. These findings add to the studies by Lohner *et al.* which concluded that NNS have negative implications on human health (3). The findings concur with the studies of Cong *et al.*, Bian *et al.*, Park *et al.*, and Chi *et al.* that Ace-K and neotame may negatively impact CNS processes (4-8). The findings also warrant additional investigations into whether NNS really are safe for human consumption. However, it must be noted that the impact demonstrated in a model

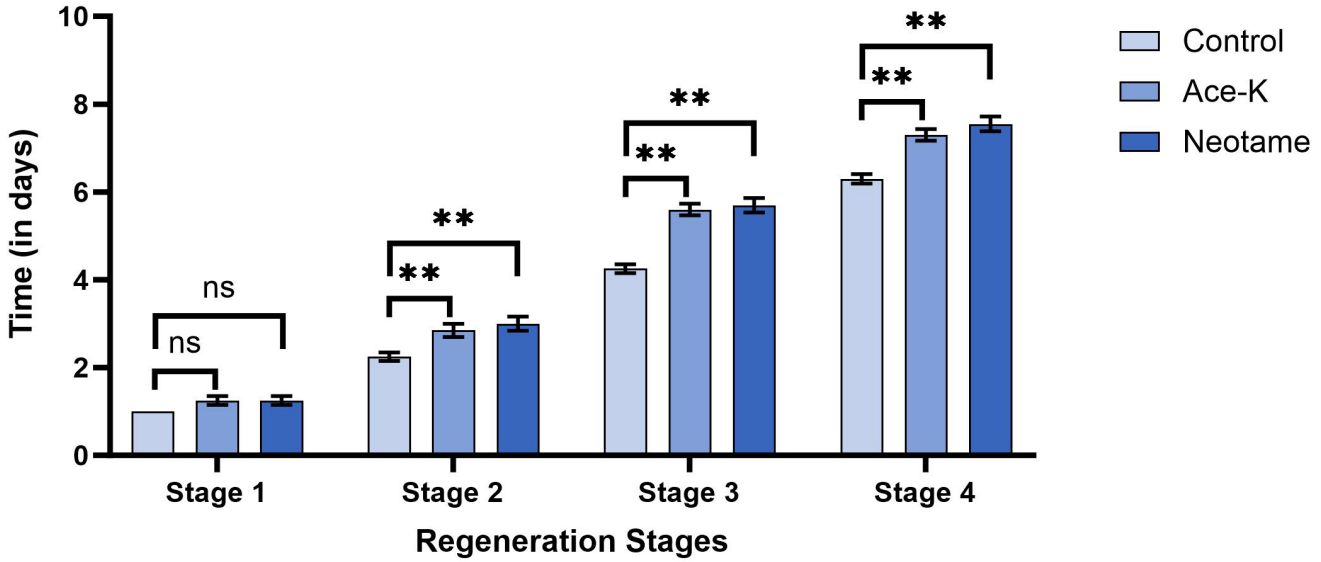

Figure 3: Time to Reach the Regeneration Stages. Bars represent the mean time in days the planaria of each group took to reach each stage of regeneration (n=20). Planaria were amputated below the head and then photographs were taken every 24 hours and visually inspected to determine the stage of regeneration. Error bars present the standard deviation. One-way ANOVA **p < 0.05 when compared to the control.

organism does not necessarily always translate to humans, and that the potential of NNS to play a role in disease does not indicate that they definitively do. Also, this study does not take into account whether the dosage used in the planaria is anywhere in the realistic range for what humans might be exposed through from consumption.

Impaired neurogenesis can lead to failure to adequately respond to neurodegeneration and NDs. Impaired neurogenesis can also compromise the effectiveness of treatment for NDs. Stem cell therapy for patients suffering from NDs such as Multiple Sclerosis and Parkinson's disease involves injecting stem cells directly into the basal ganglia, in hopes that these cells will differentiate into dopaminergic neurons (14). Ace-K and neotame may prevent cell regeneration and neuronal differentiation in that context as well, which could potentially worsen therapeutic outcomes for patients with neurodegenerative disease.

It also must be noted that the amputation of planaria in this experiment removes more than just CNS tissue. The regeneration requires the proliferation of many different cell types. Due to the location of the amputation in this experiment, planaria must also regenerate muscle, epidermis, and eyespots (18). The findings, specifically the results relating to stage four, suggest that that the NNS may slow the specialization of neoblasts into muscle, skin, and eye cells in planaria. This could indicate that these NNS would also slow human stem cell specialization. This would prevent humans from renewing and repairing many body tissues, including skin and muscles.

There were some limitations to this study that future research could aim to address to form a better understanding of the impact of the exposure to Ace-K and neotame on the regeneration rate of planaria, and by implication, neural stem cell function. Several of these limitations originated from material constraints that stemmed from the COVID-19 pandemic. There was a limited amount of planaria available for this experiment. Thus, only 5 planaria were used per solution concentration in the preliminary experiment. If a larger sample size were used, it may have revealed that a lower molarity concentration of the NNS was the more suitable working concentration. Furthermore, due to the COVID-19 pandemic, the student researcher was the only person present during the experimentation. Using a third-party blind observer who did not know what groups each planaria were assigned to and therefore wouldn't be biased in identifying the regeneration stage would have increased the validity of this research.

The results of our study indicate that Ace-K and neotame adversely impact the regeneration of planaria. Future research could look into how Ace-K and neotame impact other CNS processes such as learning, memory, or addiction. This also inspires further inquiry into how other unresearched NNS, such as monk fruit extract and Advantame, impact planaria regeneration and other CNS processes.

MATERIALS AND METHODS

Care and Maintenance of Planaria

The planaria were purchased from Carolina Biological Supply Company and placed in Poland Spring water. Poland Spring water was used throughout experimentation to avoid the toxicity that may be caused by chlorine in tap water. Chlorine has been found to induce mortality in planaria (22). The planaria were maintained at 70-73°F and had their water changed twice weekly, including once after their feeding period, to cultivate a suitable living environment and prevent mortality. Planaria were fed beef liver once a week with the feeding period lasting for 2 hours. The planaria were fed 24 hours prior to experimentation. The planaria were not fed for the duration of the regeneration experiment.

Non-Nutritive Sweeteners

We used Prescribed for Life Acesulfame Potassium (Ace-

K) ® as the Ace-K source. We used MarkNature® Neotame Powder Sweetener as the neotame source. Solutions were prepared as 1L solution to allow for each petri dish to have its designated solution changed bi-weekly. We made the NNS solutions by dissolving the chosen NNS in Poland Spring water.

Preliminary Experiment

For the preliminary experiment, we used 75 planaria, 5 for each concentration of each NNS. Both Ace-K and neotame solutions of 1M, 1dM, 1cM, 1mM, 100μM, 10μM, 1μM were prepared and tested in this experiment. These solutions helped to determine appropriate concentrations of Ace-K and neotame to be used in the main experiment. We put 5 planaria in each of the 7 solutions of Ace-K and 7 solutions of neotame; we also put 5 planaria in water alone, which served as the nontreated control for this experiment. We observed the planaria for 3 days and recorded the number of surviving planaria. We chose the concentrations that caused slight stress characteristics in the planaria but did not kill them. We compared the effects of the Ace-K and neotame treatments to the control to determine which was the most suitable for the main experiment.

Main Experiment

For the main experiment, we used 60 planaria. Prior to experimentation, we rinsed all petri dishes (90x15mm) with Poland Spring water. We used 20 petri dishes in each experimental group, one for each planaria. For the control groups, we filled the petri dishes with 20mL of Poland Spring water. For the treatment groups, we filled petri dishes with 20mL of the NNS solution. We prepared all solutions as a 1L solution to allow for each petri dish to have its designated solution changed bi-weekly. The concentration of the Ace-K solution that we used in this experiment was 100μM. The concentration of the neotame solution that we used in this experiment was 1mM. We labeled each petri dish with an identifier number 1-20 and a color to indicate what treatment group it was in for easier data retrieval and analysis.

We randomly allocated each planaria to a petri dish containing the assigned treatment immediately following their feeding period. 24 hours after random allocation, we bisected each planaria horizontally, slightly below the head with a scalpel as it stretched to move. Post-amputation, we inspected each planaria to validate that the amputation was performed the appropriate distance below the head (**Figure 1**). We placed the residual planarian (body without a head) back in the petri dish. We placed the head section in the surplus stock holding container (one for each condition).

We determined the rate of regeneration of each planarian by measuring the length of time in days it took for the planarian to reach the 4 stages of regeneration. The 4 stages of planaria regeneration are: (1) wound healing - the cut has flattened but no regeneration is visible, (2) blastema development - the wound has closed and a rounded blastema forms at the site of the cut, but distinct head characteristics are not apparent, (3) growth - the blastema has proliferated, and the distinct arrowhead shape of the head is visible and, (4) differentiation - the cells are becoming specialized, which is demonstrated by the complete development of eyespots (**Figure 2**).

To track the time it took the planaria to reach each of the regeneration stages, we took a photo of the planarian in an elongated position every 24 hours with an iPhone with a macro lens attachment. We visually inspected each photo and recorded the stage of development. The visual inspection was not blind given the naming convention. However, the model reference stage pictures minimized potential bias.

Data Analysis

We analyzed the data sets to determine how regeneration was affected by each solution. We compared the Ace-K and neotame solutions to the control solution containing only Poland Spring water. We then determined the differences between the rate of regeneration of each NNS solution and the control. We performed a one-way ANOVA test and a post-hoc Tukey test using Excel to determine if the results were statistically significant.

ACKNOWLEDGEMENTS

We would like to thank Mr. Richard Kurtz for his support throughout our experiment. We would also like to thank Dr. Scott Rawls for his guidance pertaining to planaria care and experimentation.

Received: June 15, 2022
Accepted: December 19, 2022
Published: November 29, 2023

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