The Effect of Artificial Sweeteners on Reactive Oxygen Species in the Growth of Yeast

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Background

Artificial sweeteners have become a comparable option to sugar, especially for those who may struggle with obesity or cannot tolerate sugar in their diets, such as diabetics. Although artificial sweeteners may have the same sweet taste as sugar, there have been many rumored side effects and controversial arguments surrounding artificial sweeteners. The Food and Drug Administration is responsible for the regulation of artificial sweeteners and have currently approved eight high-intensity sweeteners including saccharin, aspartame, acesulfame potassium (Ace-K), sucralose, neotame, advantame, Steviol glycosides, and Luo Han Guo fruit extracts**.** These sweeteners can be divided into nutritive and non-nutritive groups depending on if they provide calories. Artificial sweeteners are currently on the market as food additives in prepared food or as table-top sweetener.[9]

The process of approving artificial sweeteners includes animal models and possibly human trials. The focus of the FDA when considering the safety of a compound is the chronic toxicity to fertility, reproduction, fetal development, teratogenicity, carcinogenicity, and mutagenicity. However, there may be milder effects on the human body not included in these studies.[10] This provides an area for research to provide reasoning to the rumored side effects and the underlying mechanisms by which they occur.

Approved Artificial Sweeteners

Saccharin was the first artificial sweetener to be discovered in 1879. It is non-nutritive and is marketed as Sweet'N 'Low. It is a cyclic imide that is not absorbed or metabolized in our bodies and is ultimately excreted by the kidneys.

Acesulfame-K, a potassium salt 6-methyl-1,2,3-oxathiazine-4(3H)-one-2,2,dioxide, was discovered in 1967 and was approved by the FDA in 1998. Like Saccharin, Acesulfame-K is not metabolized by the body and is excreted unchanged.

Sucralose was discovered in 1976 and was approved for general-purpose sweetening by the FDA in 1999. Sucralose is a sucrose molecule with three chlorine atoms in place of the hydroxyl groups. It is non-nutritive and is not digested in the body.

Aspartame was discovered in 1965 by a chemist studying treatments for gastric ulcers and was approved by the FDA in 1981. Aspartame is a dipeptide, L-aspartyl-L-phenylalanine methyl ester and is hydrolyzed in the intestinal lumen into aspartic acid, phenylalanine, and methanol. These components then enter the blood and are metabolized.

Neotame is a derivative of aspartame and is the "newest" artificial sweetener. It is non-nutritive, removed completely from the body within 72 hours, and was approved by the FDA in 2002. Neotame consists of a t-butyl group added to the amine group of aspartic acid. Due to the chemistry of the 3,3-dimethylbutyl group, peptidases are unable to break the peptide bond between aspartic acid and phenylalanine and this results in a reduction in the availability of phenylalanine.[10]

Advantame is a non-nutritive sweetener that is approximately 20,000 times sweeter than sucrose. It was approved by the FDA in 2014 and is generally used for baked goods since it remains sweet even at high temperatures. The FDA examined data from 37 human and animal trials looking for possible toxic effects.

Steviol glycosides are natural components of *Stevia rebaudiana* Bertoni plant leaves. It is non-nutritive and is approximately 200-400 times sweeter than sucrose. Luo Han Guo fruit

extracts are also non-nutritive components of the fruit *Siraitia grosvenorii* Swingle. These extracts contain different levels of mogrosides and are considered to be 100-250 times sweeter than sucrose. [9]

Although there are controversial side effects mentioned from each of these artificial sweeteners, they have been considered safe, especially since most are not metabolized by the body. However, some scientists disagree about safety because even if they are not metabolized, some of the metabolites have been shown to cause deleterious effects contributing to the acute side effects not specifically of interest to the FDA when considering approval.

Aspartame

It is noted by Whitehouse, Boullata, and McCaulley that "By far, aspartame has been the most controversial artificial sweetener because of its potential toxicity." As mentioned, aspartame is one of the artificial sweeteners that is metabolized in the body. It is hydrolyzed into aspartic acid, phenylalanine, and methanol. Aspartame is particularly toxic to those with phenylketonuria who lack or have insufficient phenylalanine hydroxylase required to breakdown phenylalanine. The resulting accumulation of phenylalanine can alter brain function in humans. [10]

Phenylalanine is a neurotransmitter regulator and aspartic acid is an excitatory neurotransmitter. state that if neurotransmitter regulation is altered, neurobehavioral disturbances may result. A study by Lindseth, Coolahan, Petros, and Lindseth indicated that increased levels of phenylalanine and aspartic acid led to decreased dopamine and serotonin

production. Aspartame is also known to cross the blood-brain barrier allowing an increase in the permeability to catecholamines, like dopamine. [6]

Other potential negative effects result from the metabolization of methanol into formaldehyde and formic acid, which can induce acidosis or methanol toxicity. An article by Ashok and Sheeladevi, focused on the brain's antioxidant status and apoptotic changes in the brain resulting from long-term aspartame exposure. Methanol is metabolized to formaldehyde and then to formate, which is considered to be the cause of methanol toxicity. Formate inhibits cytochrome oxidase, leading to the generation of superoxide, peroxyl, and hydroxyl radicals. Another cellular outcome of methanol toxicity is damage to the mitochondria resulting in an increase of oxygen radical production. Overproduction of free radicals in these ways is directly related to death of immature cortical neurons and DNA damage.[1]

Many studies have concluding evidence of other negative effects of consuming aspartame even at the dosage that is considered "safe" by the FDA. In the study by Lindseth, Coolahan, Petros, and Lindseth, high-aspartame diets led to more irritable mood, more depression, and performing worse on spatial orientation tests. The level of aspartame that produced these results is below 40-50 mg/kg of body weight/day, which is the maximum acceptable daily intake.[6]

Reactive Oxygen Species

Reactive Oxygen Species are the result of the reactive nature of molecular Oxygen, which produce products that may be oxidants or reductants, and thus, have the ability to affect the redox homeostasis within a cell. Primary ROS are produced from the metabolism of molecular oxygen, but other reactive species may be produced from the reactions of the

primary ROS with other compounds. The superoxide anion is the main ROS generated from Oxygen and primarily forms from electrons leaking from the mitochondrial respiratory chain. Hydrogen peroxide is produced during detoxification of the superoxide anion, which is catalyzed by superoxide dismutases. It is relatively unreactive, but may crosses biological membranes and have deleterious effects when it is converted to the reactive hydroxyl radical.

ROS are particularly known to cause damage to cells resulting from radicals and molecules that are products of primary ROS reactions. ROS may damage nucleic acids, proteins, and lipids. Thiols are particularly susceptible to oxidation from ROS and can be oxidized to disulfides and sulphenic or sulphinic acids. In addition, ROS may cause damage to DNA through altering the amino acids on protein backbones, causing fragmentation, cross-linking, and unfolding of DNA. Damage to nucleic acids plays a role in mutagenesis and carcinogenesis. DNA damage or replication stress by mutations has been shown to lead to apoptosis.

Since a variety of forms of reactive oxygen species exist, the cell responds to excess ROS in a complex manner. The cell's goal is either detoxification, repair of damage, or maintenance of metal ion homeostasis. The cell's reaction is often to produce metabolites and enzymes with antioxidant functions. However, if ROS reach a level as to where they exceed the cell's detoxifying ability, this leads to oxidative stress, which may trigger apoptosis in the cell. [2]

The mitochondria is a major source of ROS generation under normal oxygen conditions. Damage to the macromolecules in the mitochondria from ROS leads to even further mitochondrial damage and consequently, an increase of ROS production. The mitochondria is also associated with apoptosis in mammalian cells and in yeast cells. In mammalian cells the proteins Bcl-2, Bcl-xl and Bax are known to influence the apoptotic pathway. Yeast cells (S.

cerevisiae) do not have homologues to these genes, however, research has shown that the mechanism through which programmed cell death acts is conserved in yeast.[3]

Yeast

Yeast are considered to be model organisms because they are some of the simplest eukaryotic organisms with many of the same essential cellular processes as humans. The species Saccharomyces cerevisiae (S. *cerevisiae*) is commonly used. Yeast can be genetically manipulated easily in labs and have short-life cycles, making them convenient to study. Programmed cell death is among the cellular processes that functions nearly the same in yeast as it does in humans. This is significant because oxidative stress and its role in programmed cell death can be studied in yeast, which has implications in many pathologies such as cancer, neurodegenerative diseases, and aging. [3]

Materials and Methods

An HAO strain of Saccharomyces cerevisiae was grown in YEPD liquid media comprised of the following: 2% glucose, 1% yeast extract, and 2% bacto-peptone. The yeast was incubated at 37° C in 24 well plates on a plate shaker at 130 rpm. A stock of the yeast was grown on YPD solid media (YEPD media recipe with 2% agar and stored in a 4° C refrigerator. To obtain cell counts, OD600 using a UV VIS spectrophotometer was utilized to determine approximate population size of each well.

The well plates contained three of each of the following concentrations of glucose, stevia leaf extract (Truvia), aspartame (Equal), and sucralose (Splenda): 0.025, 0.05, 0.1, 0.3, and 0.5 M. OD600 readings were taken at 24, 48, and 72 hours. The concentration of 0.3 M was

used for each sugar to perform lysis of harvested cell cultures and for indication of reduced glutathione levels.

Lysis of harvested cells was performed by centrifuging 1000 µL of the samples from the well plate at 5000 x g for 3 minutes, washing in cold deionized water, and resuspending in the lysis cocktail (100 mM Tris-HCl [pH 7.4], 20 µg/mL leupeptin, 10 µg/mL pepstatin A, 0.264 mg/mL apropinin, 1 mM PMSF, and glycerol 10% [v/v]). Two hundred microliters of acid-washed glass beads were added to the solution and centrifuged on high speed for 30 seconds. After the cells were taken out of the centrifuge, they were cooled on ice. This process was repeated four times.

Fifty microliters of the lysis supernatant was added to a reaction buffer (0.1 M phosphate buffer [7 pH], 0.5 mM EDTA, and 3.0 mM 5-5'-dithro-bis-(2-nitrobenzoic acid) and incubated at 30° C for 5 minutes. 0.4 mM NADPH was added to each centrifuge tube and incubated at 30° C for 20 minutes.[4] Absorbance at 412 nm was measured using the UV VIS spectrophotomer and compared with the molar absorptivity of TNB to get a concentration of TNB in moles.[8] Figure 1 shows the reaction of $DTNB^{2-}$ with free sulfhydryl groups and the production of a mixed disulfide and the TNB^{2-} ion.

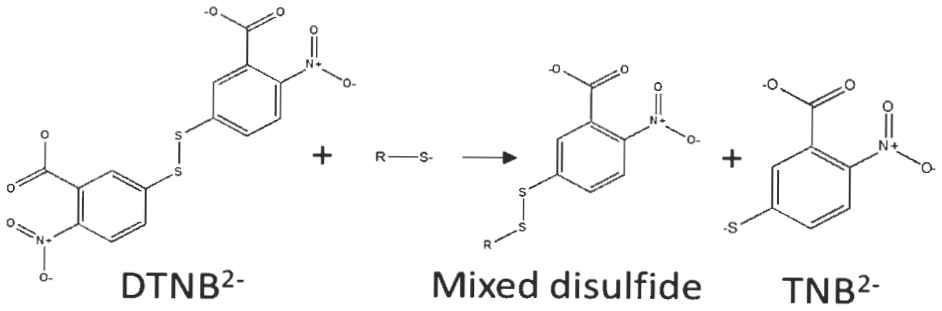

Figure 1. The reaction of DTNB with a free sulfhydryl group.

Results

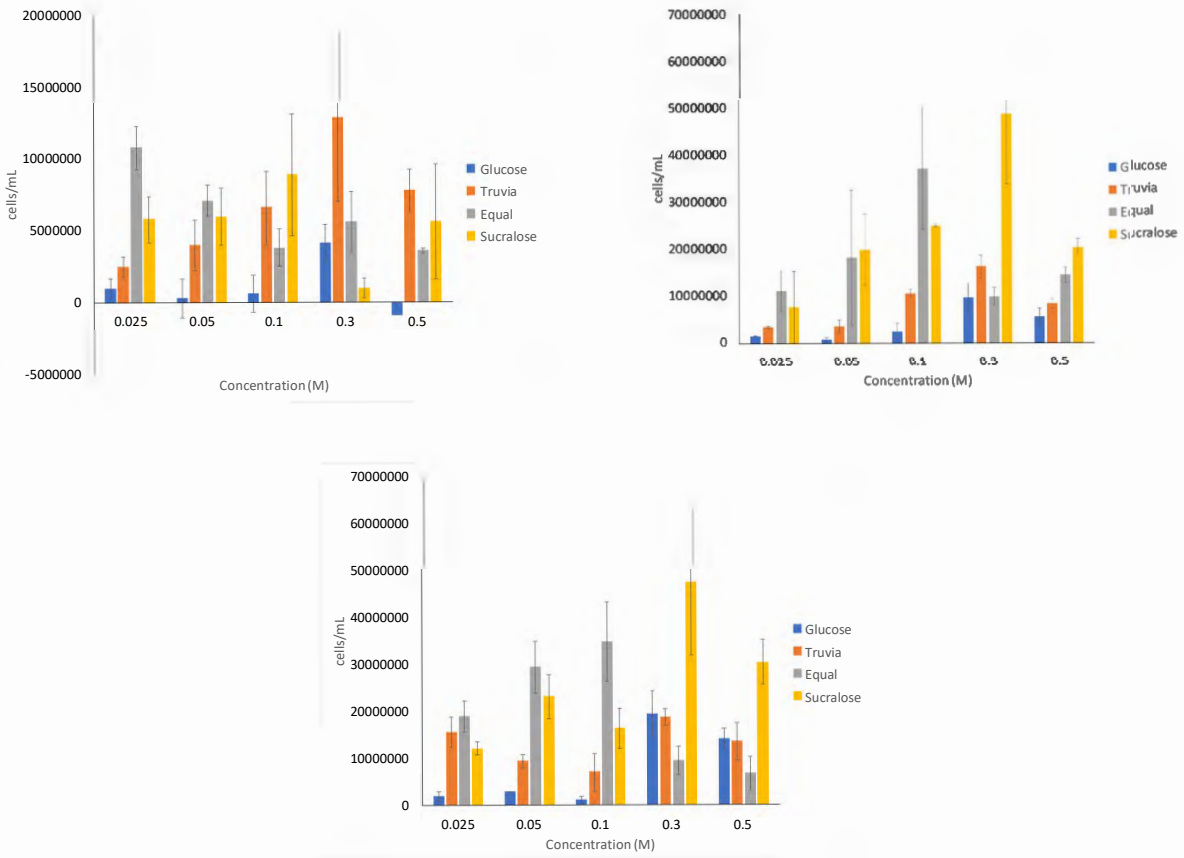

Figure 2, 3, and 4 show the number of yeast cells per mL at various concentrations of each sugar at 24 (top left), 48 (top right), and 72 hours (bottom)

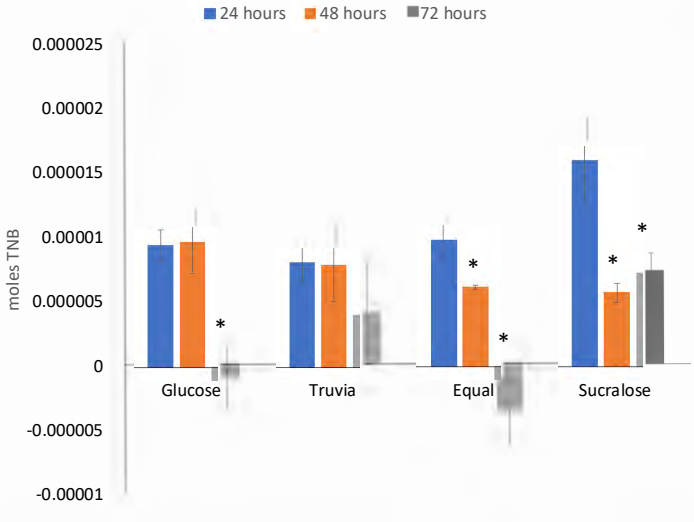

Figure 5 shows moles of TNB for each sugar at 0.3 M concentration.

** Indicates a statistical difference of less than .05 in comparison to the 24 hour mark for each respective sugar*

Discussion

Yeast is typically grown in 0.1 M glucose, and from our results on figures 2,3, and 4 there is not a large difference between the number of cells at each concentration of glucose at 24, 48, or 72 hours. Growth of yeast cells does not seem to be inhibited by Truvia at 24, 48, or 72 hours, but there is a decline of growth at the highest concentration of 0.5 M. At early times, Truvia shows increased growth at higher concentrations, but at longer periods there is less of an effect. Overall, the three alternative sweeteners showed more cell growth than glucose at early times.

Glucose, Equal, and Sucralose had statistically significant changes from 24 hour when compared with the extinction coefficient of TNB indicating the presence of an oxidizing environment and possibly an increase in the presence of ROS (Figure 5). The most pronounced change was in Equal and was expected due to the products of metabolism increasing ROS as discussed in the background section. We know that ROS are naturally occurring in the cell. However, if ROS are moderately increased for a period of time, the cell can stimulate increased uptake of glucose and consequently produce even more ROS as depicted by the results with glucose in figure 5.[5] Sucralose passes through the body undigested.[10] Therefore, the significant indication of ROS is unexplained by literature of metabolism. In order to test this further, sucralose concentration may be examined at 24, 48, and 72 hours. If sucralose is not found, observing what products are available after uptake by the cells may be beneficial.

Steviol glycosides, such as those in Truvia, pass through the upper GI tract intact. In the large intestines, bacteria remove glucose and leave the steviol backbone. This backbone is

absorbed in humans, modified in the liver, and excreted as urine. If the same is true in yeast cells, this may explain the lack of statistically significant indication of ROS. [7]

References

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