

TRICK OR TREAT? MULTIPARAMETRIC INVESTIGATION OF THE  
BIOLOGICAL EFFECTS OF SUCRALOSE SIGNALING IN THE GUT

BY

SUN MIN PARK

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## Abstract

Obesity is one of the greatest healthcare challenges facing today's society that constitutes a significant portion of the burden related to various chronic diseases. Consequently, there has been a concerted effort to create new food additives, such as non-nutritive sweeteners, that could successfully mimic the sweet taste of sugars, without actually contributing to caloric intake. Since its creation, sucralose among other non-nutritive sweeteners has become a staple in the global market of sugar-free food and beverages as a safe and effective alternative for sugar. However, despite their widespread use, growing evidence raises concerns about the potentially toxic effects of sucralose that may disrupt metabolic processes, thereby negatively impacting the gut microbiome and physiology. Although the safety of sucralose has been under extensive and rigorous investigation over the years, the conflicting research findings in the current literature are contributing to the never-ending debate of whether sucralose is a "trick" or "treat". To help address this big knowledge gap, we seek to qualitatively and quantitatively evaluate the various parameters of cell health (cell morphology, metabolic activity, viability, clonogenic growth, and oxidative stress) in human gut cell line (HCT116) upon sucralose exposure in the present study, using a panel of biochemical assays and cell physiology measurements. The findings illustrated that NNS sucralose is, in fact, more cytotoxic in human gut cells in a concentration- and time-dependent manner compared to the natural sugar sucrose. While sucralose was shown to negatively impact overall HCT116 cell health and survival, it did not seem to induce oxidative stress, suggesting that the mechanism underlying cell death seen in the present study is not likely through the production and/or accumulation of reactive oxygen species. Given that the cytotoxic effects seen in the present study are heavily dependent on the context of concentration and time, further research examining across a wide range of concentrations, timepoints, and duration, as well as various other cell health parameters will allow for a more thorough and comprehensive phenotyping of sucralose cytotoxicity and help ensure the safety of food additives currently on the market. Until then, the controversy still remains: is sucralose a *trick* or *treat*?

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Holm-Šídák multiple comparisons using one-way ANOVA are highlighted with \*,  $p < 0.05$  vs. untreated control within treatment.

## List of Abbreviations

AB	Apoptotic bodies
ANOVA	Analysis of variance
DCF	Dichlorofluorescein
DCF-DA	Dichlorofluorescein diacetate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
CB	Cell bursting
CC	Chromatin condensation
CR	Cell rounding
CS	Cell shrinkage
CV	Crystal violet
FBS	Fetal bovine serum
FDA	United States Food & Drug Administration
GI	Gastrointestinal
GPCR	G protein-coupled receptors
MB	Membrane blebbing
MTT	Thiazolyl blue tetrazolium bromide
NB	Nuclear blebbing
NNS	Non-nutritive sweeteners
NS	Nuclear swelling
PBS	Phosphate buffered saline
PR	Phenol red
ROS	Reactive oxygen species
SEM	Standard error of mean

# **1. Introduction**

## **1.1. Emergence of Non-nutritive Sweeteners**

As one of the greatest healthcare challenges, obesity constitutes a significant portion of the burden related to various chronic diseases, including type 2 diabetes, cardiovascular disease, and certain types of cancer (O'Neill et al., 2019). This makes obesity prevention and management an important public health priority in Canada and worldwide. While growing evidence suggests that a series of complex physiological and lifestyle risk factors contribute to the multifactorial etiology of different metabolic diseases, high sugar consumption in diets has always been widely regarded as a prominent contributing risk factor. With taste being one of the essential senses driving our perception of food quality and our innate desire for sweet taste (Grotz and Munro, 2009; Weihrauch and Diehl, 2004), the increase in obesity-related morbidity and mortality, therefore, has shifted the attention towards creating new food additives that could successfully imitate the sweet taste of sugars without actually contributing to caloric intake (Whitehouse et al., 2008). This brought about the surge of low-calorie diets and food additives such as non-nutritive sweeteners (NNSs), better known as artificial sweeteners, that are favoured by many consumers searching for food products that offer the sweet taste of sugar without calories. Since its creation, NNSs have become increasingly more common in foods and beverages among people of all ages (Garriguet, 2009; Hamel et al., 2022; Warren et al., 2022).

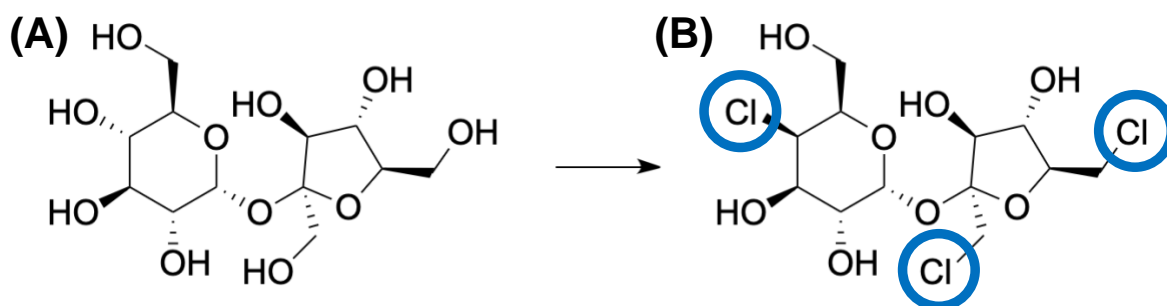
## **1.2. Non-nutritive Sweeteners**

The NNSs include chemical substances categorized into several different classes according to their source (natural or artificial) and other intrinsic functional properties such as nutritional value (caloric or non-caloric) and sweetness potency, which typically exceed the sweetness of the natural sugar, sucrose, by a factor of 30 to 13,000 times (Risdon et al., 2021; Whitehouse et al., 2008). The most common NNSs currently approved and introduced in Canada's food and beverage market are acesulfame potassium, aspartame, and sucralose, to name a few (Ahmad et al., 2020c). Of these, sucralose, more commonly referred to by its brand name Splenda®, has been a staple of the global NNS market and,

therefore, is the focus of the present study. It was first approved by Health Canada and the United States Food & Drug Administration (FDA) for commercial uses in 1991 and 1998, respectively, followed by approvals of different public health authorities worldwide (Knight, 1994; Whitehouse et al., 2008). In 2016 alone, it accounted for 30% of the \$2.3 billion worth of total NNS sales worldwide (Risdon et al., 2021). Global sucralose sales are only projected to increase by tens of billions over the coming years (Alsunni, 2020; Risdon et al., 2021; Sylvetsky and Rother, 2016; Wang et al., 2018).

### 1.2.1. Non-nutritive Sweeteners: Sucralose

Sucralose (1,6-dichloro-1,6-dideoxy-beta-D-fructofuranosyl-4-chloro-4-deoxy-alpha-D-galactopyranoside) stands out among the other NNSs currently on the market as it is the only sweetener structurally derived and synthesized from the natural sugar, sucrose; the three primary hydroxyl groups at the 1', 4', and 6' positions on the sucrose molecule are substituted for chlorine (Fig. 1.1).



**Figure 1.1: Comparison of molecular structures.** (A) The natural disaccharide sucrose and (B) chlorinated disaccharide sucralose.

This chemical modification of sucrose through chlorination not only makes the sweetness potency of sucralose 600 times greater than that of sucrose (Magnuson et al., 2016), but the substitution is also known to help sucralose maintain its molecular structure throughout the metabolic process (Power, 2020, unpublished). The chlorine substitutions essentially lead to a conformational change, such that glycosidic enzymes responsible for hydrolyzing sucrose and other carbohydrates are rendered unable to cleave sucralose, resulting in a minimal breakdown of sucralose into conjugates (Magnuson et al., 2016). With little to no digestion and metabolism for energy, sucralose, therefore, also adds no

calories (Magnuson et al., 2016). In other words, sucralose can produce a comparable amount of sweet taste even at an extremely low concentration and intake, without contributing to caloric intake.

### **1.2.2. What makes sucralose an ideal sweetener?**

A battery of extensive pharmacological and toxicological studies have been conducted in several species, including mice, rats, dogs, rabbits and humans, to evaluate further the absorption, distribution, metabolism, and excretion of sucralose (John et al., 2000a; John et al., 2000b; Roberts et al., 2000; Sims et al., 2000; Sylvetsky et al., 2017; Wood et al., 2000). Specifically in humans, peak plasma concentrations following sucralose consumption were low at around 262.3 ng/mL (0.0006  $\mu$ M) and 365.6 ng/mL (0.0009  $\mu$ M) in children and adults, respectively (Sylvetsky et al., 2017). Further evidence of low sucralose absorption with little to no metabolism in humans, among other species, are also available in a study which investigated the metabolic fate of radio-labelled sucralose given orally (Roberts et al., 2000). More than 90% of the radioactivity was present as unchanged sucralose excreted in urine and feces. In contrast, the small proportion that had been absorbed and excreted were later identified as glucuronide conjugates of sucralose which got metabolized within the body tissues as opposed to the gut lumen (Roberts et al., 2000). This is also supported by the hydrophilic properties of sucralose. Similar to sucrose, the multiple hydroxyl groups present in sucralose, despite the chlorine substitutions, tend to form hydrogen bonds with water (Magnuson et al., 2017). This ultimately results in high hydrophilicity and comparatively low fat solubility of the sucralose molecule, which further contributes to the lack of retention and build-up of sucralose in the body (Magnuson et al., 2017).

Furthermore, rigorous testing using radio-labelled sucralose once again illustrated that sucralose is extremely stable across a wide range of temperatures and pH (Barndt and Jackson, 1990; Grotz and Munro, 2009; Magnuson et al., 2016). Its excellent stability at elevated temperatures and in low pH products helps to maintain product sweetness following food processing (e.g., cooking, baking, pasteurization) and contribute to a longer shelf-life, respectively (Grotz and Munro, 2009; Magnuson et al., 2016).

A multitude of findings that were used to garner approval from health authorities, including the FDA, highlight the biological inertness of sucralose which, along with its other qualities (i.e., high thermostability and solubility, low production cost), unsurprisingly make sucralose a “treat” for the consumers and food manufacturing industry alike (Thomson et al., 2019). Consequently, sucralose has been widely marketed and used as a safe and effective alternative to sugar not only for those who have and are at risk of obesity and diabetes, but also for the general population. This dramatically changed the paradigm of how we eat and how we tackle against the increasing threat of metabolic diseases and their adverse health outcomes.

### **1.3. How does sweet taste from sucralose signal?**

#### **1.3.1. Signal Transduction**

Cellular communication is integral to the function and survival of any organisms (Hancock, 2017). The chemical communication that takes place within and between cells at all stages from signal reception, transduction to response is what results in an integrated and efficient exchange of information that is essential to generate coordinated physiological responses in organisms (Perbal, 2003; Valls and Esposito, 2022). In other words, how individual cells and organisms adjust to and survive in ever-changing environments or how their dysfunction causes cancer cells to develop, proliferate, and invade other tissues or how we taste food, for example, are mechanisms that are ultimately underpinned by signal transduction events through a complex network of cell signaling pathways (Hancock, 2017). Specifically, cell-surface receptors, located at the interface of the extracellular environment, are the key components of these networks (Cattaneo et al., 2014). They play crucial roles in converting extracellular signals into intracellular signals via intracellular signaling pathways in order to relay cellular communication from external to internal environment (Hilger et al., 2018). There are three different classes of cell-surface receptors: ion-channel receptors, enzyme-coupled receptors, and G protein-coupled receptors (GPCRs) (Hilger et al., 2018). The GPCR family are second to none with respect to the breadth of receptor distribution and thus have a paramount role in mediating a wide array of physiological processes, including in taste physiology (Ahmad and Dalziel, 2020).

### **1.3.2. G protein-Coupled Receptors in Sweet-Taste Signaling**

Growing evidence from studies conducted on the biological fate of sweet molecules points to GPCRs as the receptors responsible for initiating the molecular pathways that sense and respond to sweet taste and subsequently mediating physiological responses that result in our perception of sweet taste (DuBois, 2016). Specifically, in rats and humans, the single heterodimeric complex of two taste-sensing class C GPCRs, T1R2/T1R3 mediate the sweet stimuli (Li et al., 2002; Nelson et al., 2001). When T1R2/T1R3 combine, they form a sweet taste heterodimer receptor that responds to all different classes of sweet tastes, including those produced by natural caloric sugars, NNS sucralose, and various other sweet organic compounds (Shil et al., 2020). These receptors then activate the heterotrimeric G proteins, such as G $\alpha$ -gustducin, to initiate a series of downstream signal transduction cascades, resulting in an increased intracellular Ca<sup>2+</sup> concentration. Consequently, this depolarization induces the release of adenosine triphosphate, which subsequently triggers an action potential to activate the gustatory cortex in the brain and relay the sweet taste stimuli (Ahmad and Dalziel, 2020).

Despite our understanding of signal transduction involved in taste physiology, the ways in which the gut senses nutrients have, until recently, been mysterious. While these sweet taste receptors were once thought to be mainly expressed within the taste buds of the tongue (Kojima and Nakagawa, 2011), growing evidence illustrates the presence of the T1R2/T1R3 receptors beyond the oral cavity. As a matter of fact, they are found across the body and predominantly within the gastrointestinal (GI) epithelium, exerting their effects throughout the whole digestive system (Bezencon et al., 2007; Kojima and Nakagawa, 2011). Recent studies also illustrate the presence of the receptors specifically localized within the enteroendocrine cells along the murine and human GI tract (Dyer et al., 2005; Sutherland et al., 2007). Stimulation of these sweet taste receptors in the GI tract activates an intracellular gustatory signaling pathway, similar to that of the mouth (Sclafani, 2007). This consequently plays an important role in detecting and metabolizing different sweet molecules in the intestinal lumen, triggering physiological responses that regulate sugar absorption, gut motility, nutrient absorption, and metabolism in the body (Ahmad et al., 2020; Margolskee et al., 2007).

#### **1.4. Physiological Response to Sucralose**

Despite their widespread consumption, there is, however, a big controversy and disagreement questioning the safety of sucralose, suggesting that sucralose is not an inert compound as once thought. As such, the claims that replacing sugars with sucralose and other NNSs in foods and beverages can help improve metabolic function have been constantly challenged over time, as their negative effects on the gut microbiota, glucose tolerance, and increased cancer risk has gained a lot of attention and raised a lot of concerns (Abou-Donia et al., 2008; Ahmad et al., 2020a; Ahmad et al., 2020c; Bornemann et al., 2018; Grice and Goldsmith, 2000; Li et al., 2020; Mezitis et al., 1996; Pepino, 2015; Qin, 2002; Qin, 2012; Romo-Romo et al., 2018; Wang et al., 2018). Given the growing consumption of sucralose and incidences of chronic metabolic diseases and cancer, it is imperative to assess our physiological and metabolic responses to sucralose. Therefore, what follows is a brief review that maps the currently available evidence about health outcomes possibly associated with sucralose consumption.

##### **1.4.1. Effect of Sucralose Exposure in the Gut**

First and foremost, according to a recent study using murine models, sucralose was shown to get metabolized in the gut, absorbed in the plasma, producing at least two fat-soluble compounds in urine and feces (Bornemann et al., 2018). These metabolites are also acetylated, meaning they dissolve easily in fat and thus, remain in the body for a longer period of time (Bornemann et al., 2018). In addition, sucralose itself was further detected in adipose tissues of rats even weeks after the rats had stopped consuming sucralose (Bornemann et al., 2018). In humans, while the majority of sucralose consumed is excreted in feces, a significant portion is also reported to get absorbed by the small intestine and get circulated in plasma (Harrington et al., 2018). These contradict the many previous studies looking at the biological fate of sucralose. Despite being known as a molecule of hydrophilic nature, sucralose extraction resulted in polar protic and aprotic solvents as well as a non-polar solvent, providing further evidence of the amphiphilicity of sucralose with both lipid and water solubility (Bornemann et al., 2018). The absence of the acetylated sucralose metabolites in earlier toxicity studies could be attributed to low solubility of the metabolites in methanol, which was used as a solvent in the urine and fecal extractions

(Bornemann et al., 2018). When extracted in the presence of methanol, the sucralose metabolites may have undergone transesterification resulting in the failed detection of sucralose metabolites (Bornemann et al., 2018). Moreover, the use of linear analyzer methods, such as radio thin layer chromatography, comes with sensitivity and resolution limitations (Bornemann et al., 2018). This could have, therefore, further contributed to the difference in findings observed in the present study conducted with ultra-high performance liquid chromatography tandem mass spectrophotometry. These findings raise a serious cause for concern given that they differ considerably from that of the studies initially used to garner regulatory approval for the commercial use of sucralose, which reported that sucralose was not metabolized by the body (Bornemann et al., 2018).

Growing body of knowledge also specifically raises concerns about the potentially toxic effects of sucralose that may negatively affect the gut microbiome and physiology. The gut microbiota has many different physiological roles, including the proper maintenance of the immune system, regulation of GI tract motility, drug metabolism, defense against foreign microbes and pathogens, to name a few (Ahmad et al., 2020b; Wu and Wu, 2012). Hence, any changes in the composition of the gut microbiota can bring about gut dysbiosis and other disruption to gut homeostasis associated with many metabolic diseases such as diabetes, obesity, inflammatory bowel diseases, and colorectal cancer (Chassaing et al., 2015; Lee et al., 2022; Suez et al., 2014).

While there are many potential genetic and lifestyle risk factors that are proposed to negatively influence the gut microbiota, the impact of changes in one's diet through the introduction of new nutrients and dietary chemicals has recently gained a lot of attention (Mendonça et al., 2018; Rapozo et al., 2017). This is because the functions and the broader composition of the gut microbiota are primarily governed by diet (Lee et al., 2022). Low-calorie food additives like NNS sucralose have long been proposed to be active in the GI tract where they influence host control mechanisms that can inhibit gut microbes, resulting in a serious gut dysbiosis (Qin, 2002; Qin, 2012). In fact, sucralose inhibited growth and caused cytotoxicity in model *E. coli* (Harpaz et al., 2018; Wang et al., 2018), whereas chronic consumption of sucralose significantly decreased populations of beneficial anaerobic bacteria within the gut microbiome, including proteobacteria in rats (Li et al., 2020). It is thought that some microbes may have the ability to metabolize NNSs; thus,

sweetener intake may exert a strong bacteriostatic effect on certain gut microbes, causing a harmful shift in gut microbiome composition (Ahmad et al., 2020c; Wang et al., 2018).

Although many studies showed a positive association of sucralose with adverse health risks particularly in the GI tract, resulting in a serious gut dysbiosis; many other studies conducted have also shown that sucralose bears no such risk (Jeffrey and Williams, 2000; Weihrauch and Diehl, 2004). Following sucralose consumption at concentrations mirroring the high average diet soda intake, the fecal microbiota screened from healthy participants before and after consuming sucralose-contained drinks demonstrated no change in gut microbiota richness and evenness (Ahmad et al., 2020c). The relative ratios of the most abundance bacterial phyla and genus-level taxa were also parallel before and after treatments (Ahmad et al., 2020c), supporting the safety of sucralose.

In addition to causing gut dysbiosis, the presence of sucralose within the GI tract exacerbated inflammation and damage in the gut. More specifically, increased sucralose consumption resulted in histopathological changes in rat colons, which in turn induced infiltration of inflammatory lymphocytes (Abou-Donia et al., 2008). Murine models of ulcerative colitis and Crohn's disease, which are already susceptible to gut dysbiosis and inflammation, also further revealed that rats fed with increased concentration of sucralose in their drinking water experienced a more severe dysbiosis and inflammation as well as an aggravated damage to their gut barrier and tissues (Li et al., 2020).

#### **1.4.2. Effect of Sucralose Exposure on Cancer Risk**

The relationship between NNSs and cancer remains quite controversial. The carcinogenic potential of these sweeteners has been continuously questioned, probed, challenged, and investigated over time through a range of *in vivo* studies. However, these studies, once again, revealed contradicting results on the basis of lack of carcinogenicity and genotoxicity (Berry et al., 2016; Chappell et al., 2020; Mann et al., 2000a; Mann et al., 2000b).

Although a few previous studies have found no conclusive evidence that sucralose causes cancer in humans even at ranges far exceeding beyond the anticipated and/or recommended daily intake (Berry et al., 2016), findings from a recent study in murine models raise questions regarding the potentially carcinogenic effects of sucralose,

particularly within the GI tract (Li et al., 2020). The same murine study which demonstrated that increased sucralose consumption resulted in exacerbated gut dysbiosis and inflammation also highlighted that sucralose promoted and intensified colorectal cancer tumourigenesis (Li et al., 2020). Along with pathological and compositional changes in the gut microbiota, murine models of azoxymethane/dextran-sulfate sodium-induced colorectal cancer treated with sucralose exhibited significant increases in the number and size of tumours (Li et al., 2020). Sucralose caused pronounced epithelial destruction, infiltration of inflammatory cells, crypt deformation, marked increase in abundance of mitotic cells, and a high degree of dysplasia, indicating the presence of inflammation and highly differentiated adenocarcinoma of the colon (Li et al., 2020). Furthermore, the expression levels of pro-inflammatory cytokines and key tumour-associated genes within the TLR4/MyD88/TRAF6/NF- $\kappa$ B signaling network in the colon were significantly upregulated in these murine models as well (Li et al., 2020).

While the effect of other NNSs on tumourigenesis have been investigated often, *in vivo* studies specifically investing the effect of sucralose exposure on tumourigenesis within the GI tract are very limited. With incidences of cancer-related morbidity and mortality on the rise worldwide and cancer diagnosis and treatment imposing a huge burden, there is a perennial desire to ensure the safety of chemicals added to foods and beverages. Therefore, it is crucial to spur more research in the field to further evaluate the potential association of sucralose and carcinogenicity.

#### **1.4.3. Effect of Sucralose Exposure on Glucose Homeostasis**

With NNSs established to be active in the GI tract, the alterations in gut microbiome have shown to bring about concerning metabolic impairments. Specifically, increased NNS intake increases insulin secretion, posing a negative impact on obesity and glucose tolerance (Brown et al., 2010; van Eyk, 2015). More recent findings further suggest a positive correlation between sucralose consumption and significant decrease in insulin sensitivity and insulin clearance, despite the higher glucose and insulin concentrations (Romo-Romo et al., 2018). This finding gives cause for serious concerns considering that sucralose and other NNSs are recommended as a healthier and safer alternative to help

diabetic and/or obese individuals, as well as those who are at high risk of developing metabolic disease improve their health.

In comparison to findings highlighting adverse effects of sucralose on glucose homeostasis, other studies have found no concrete evidence to establish a link between sucralose consumption and glucose homeostasis. Findings from a murine study showed no adverse effects of sucralose on fasting glucose levels, even at supraphysiologic concentrations for up to two years (Grice and Goldsmith, 2000). Furthermore, sucralose consumption at high doses for two weeks also did not affect plasma glucose, insulin, glucagon-like peptide 1 or leptin concentrations in healthy participants (Ahmad et al., 2020a; Grotz et al., 2003; Ma et al., 2009). Additional findings from studies conducted in humans with and without diabetes found that high doses of sucralose above the maximum recommended intake did not affect blood glucose, C-peptide, or HbA1c concentrations (Mezitis et al., 1996; Pepino, 2015). Again, given the opposing research findings, more research is needed to better elucidate the effect of sucralose in glucose homeostasis. Having a better understanding of the mechanisms by which sucralose and other NNSs may contribute to metabolic regulation or dysregulation is crucial to combat the increasing threat of metabolic diseases.

### **1.5. Experimental Objectives: Is sucralose a “trick” or “treat”?**

While several previous assessments demonstrated the safety of sucralose, growing evidence from more recent epidemiological and experimental studies in human, animal, and cell culture studies raise questions regarding the potentially pathogenic role of sucralose. Despite the safety of sucralose being the subject of rigorous and extensive investigation, the conflicting findings brought about the current state of controversy regarding the safety of sucralose consumption, contributing to this ongoing debate of whether sucralose is a “trick” or “treat”. This represents a big knowledge gap that needs to be addressed as it is unclear whether sucralose is a part of the solution or is what perpetuates the problem in a catch-22 scenario, highlighting the need to further examine the effect of sucralose to ensure the safety of food additives currently in use and on the market.

Although the consumption of foods containing NNSs is increasing, mostly due to the growing economic drive to satisfy increasing consumer demand for products with

reduced calories but high palatability, large gaps still remain in our knowledge regarding the effects of NNSs on human physiology, particularly within the GI tract. Therefore, the aim of the present study is to further investigate the biological effects of sucralose signaling in the gut. To address a part of this broad research question, we treated human gut cells with physiologically relevant concentrations of sucralose prior to examining various parameters of cell health in order to determine whether sucralose consumption negatively affects human gut cells and if so, to what extent.

The qualitative and quantitative investigation into the biological effects of sucralose signaling in the gut followed a multiparametric approach using a panel of biochemical assays and cell physiology measurements. Specifically, sucralose effects on human epithelial gut cell morphology, viability, metabolic activity, clonogenic growth, and oxidative stress were thoroughly examined to complete the cytotoxicity profiling of sucralose with the goal to have it serve as a foundation to potentially help elucidate the mechanisms underlying its signaling in the future.

## **2. Materials and Methods**

### **2.1. Cell Culture and Maintenance**

Human colon cancer cell line (HCT116), gifted by Hannah Cahill (Dalhousie University), was maintained in high glucose (4.5 g L<sup>-1</sup>) complete Dulbecco's Modified Eagle Medium cell culture medium with (DMEM; Corning, 10-013-CV) supplemented with 10% fetal bovine serum (FBS; VWR, 89510-186) in a humidified atmosphere of 95% air, 5% CO<sub>2</sub> at 37°C. All experiments were carried out while the cells were in exponential growth phase with cells cultured in plates for two to three days prior to experiments. The cells were harvested from the culture plates at 80 to 90% confluency by rinsing with 1X phosphate buffered saline (PBS) and trypsinizing with 0.25% trypsin/2.21 mM ethylenediaminetetraacetic acid (Corning, 25-053-CI) prior to incubating for 4 minutes at 37°C and 5% CO<sub>2</sub>. Detached cells were mixed with complete DMEM and centrifuged for five minutes at 500 x g. Cell pellets were resuspended in complete DMEM, diluted, and reseeded in appropriate ratio prior to incubation at 37°C and 5% CO<sub>2</sub>. Cells were maintained in culture for up to 50 passages. After 50 passages, new cells were expanded from frozen stock vials.

### **2.2. Sucralose and Sucrose Treatments**

Sucralose (TCI Chemicals, 56038-13-2) and sucrose (TCI Chemicals, 57-50-1) chemicals were dissolved in complete DMEM at a stock concentration of 24 mM prior to being filter-sterilized. Treatment stocks were diluted serially on the day of treatment in appropriate medium at concentrations ranging from 0 to 18 mM.

### **2.3. Biochemical Assays and Cell Physiology Measurements**

#### **2.3.1. Microscopy: Cell Morphology, Health, and Survival**

HCT116 cells were seeded at 5,000 cells well<sup>-1</sup> in complete DMEM in a 96-well plate and incubated overnight at 37°C and 5% CO<sub>2</sub>. DMEM was removed and replaced with 100 µL of sucralose and sucrose treatments in complete DMEM for 24 hours (acute exposure) and 72 hours (chronic exposure) at 37°C and 5% CO<sub>2</sub>. Treatments were replaced with 50 µL of Hoechst 33342 nuclear staining dye (Thermo Fisher, H3570) diluted 1:2000 in complete DMEM (5 µg mL<sup>-1</sup>) and incubated for 15 minutes at room temperature. The

Hoechst-stained cells were visualized at 10X magnification using both the brightfield microscopy under transmitted light and the DAPI (370/450 nm) filter with fluorescent imaging on the ImageXpress Pico Automated Microscope (Pico; Molecular Devices) to examine changes in cell and nuclei morphological features, respectively. Following visualization, cells were counted using the automated fluorescence “cell count” protocol on CellReporterXpress (Molecular Devices) software to determine number of cells, nuclear size, total and average cell area, and intensity. Five independent biological assays, each with three technical replicates, were performed.

### **2.3.2. MTT Cytotoxicity Assay: Cellular Metabolism (Viability)**

Treatment cytotoxicity was determined by the thiazolyl blue tetrazolium bromide (MTT) assay. HCT116 cells were seeded at 5,000 cells well<sup>-1</sup> in complete DMEM in a 96-well plate and incubated for 24 hours at 37°C and 5% CO<sub>2</sub>. DMEM was replaced with 100 µL of sucralose and sucrose treatments in complete DMEM for 20 hours (acute exposure) and 68 hours (chronic exposure) at 37°C and 5% CO<sub>2</sub>. 20 µL of MTT (VWR, 298-93-1) reagent (5 mg mL<sup>-1</sup>) diluted 1:2 in complete DMEM was added to each well and incubated for an additional four hours at 37°C and 5% CO<sub>2</sub>. The supernatant was removed gently, and the remaining formazan crystals were solubilized in 100 µL of dimethyl sulfoxide (DMSO; VWR, 67-68-5) with shaking for three to five minutes at room temperature before measuring the absorbance at 570 nm using a Synergy HT microplate reader (BioTek). Raw absorbance values were adjusted to the blank prior to calculation of HCT116 metabolic activity (viability) in percentage relative to the untreated control. Five independent biological assays, each with five technical replicates, were performed.

### **2.3.3. Crystal Violet Colony Forming Assay: Clonogenic Growth**

HCT116 cells were seeded at 100 cells well<sup>-1</sup> in complete DMEM in a 96-well plate and incubated for four hours at 37°C and 5% CO<sub>2</sub> to allow for the cells to adhere to the plate. Following cell adherence, DMEM was removed and replaced with 100 µL of sucralose and sucrose treatments in complete DMEM for five days at 37°C and 5% CO<sub>2</sub>. Following the five-day incubation, treatments were replaced with 50 µL of crystal violet (CV; BioShop, CRY422) dye (0.2% CV in 2% ethanol) prior to a 10-minute incubation in

the dark at room temperature. Excess CV was removed by washing the plate three times *via* water submergence and further incubated for one hour at 37°C and 5% CO<sub>2</sub>. Following the incubation, the CV-stained cells were visualized on the Pico to examine HCT116 colony formation prior to obtaining the number and approximate area of colonies formed using ImageJ (National Institutes of Health) software. Five independent biological assays, each with three technical replicates, were performed.

#### **2.3.4. DCF-DA Reactive Oxygen Species Assay: Oxidative Stress**

HCT116 cells were seeded at 5,000 cells well<sup>-1</sup> in complete phenol-red free DMEM (PR-free DMEM; Corning, 17-205-CV) in a black 96-well plate and incubated overnight at 37°C and 5% CO<sub>2</sub>. PR-free DMEM was removed and replaced with 100 µL of dichlorofluorescein diacetate (DCF-DA; Sigma, D6665-5G) reagent diluted to 100 µM in complete PR-free DMEM immediately before use, prior to a 30-minute incubation at 37°C and 5% CO<sub>2</sub>. Following the incubation, DCF-DA was removed and replaced with 100 µL of sucralose and sucrose treatments in complete PR-free DMEM for 24 hours (acute exposure) and 72 hours (chronic exposure) at 37°C and 5% CO<sub>2</sub>. Treatments were removed gently, and cells were washed twice with 1X PBS prior to adding 100 µL of complete PR-free DMEM and measuring fluorescence with excitation and emission filters set at 485 nm and 530 nm, respectively. Raw fluorescence values were adjusted to the blank prior to calculation of fold change in dichlorofluorescein (DCF) fluorescence in sucralose- and sucrose-treated cells relative to the untreated control. Cisplatin treatments at concentrations ranging from 0 to 0.1 µM were used as a positive control for the detection of reactive oxygen species (ROS). Five independent assays, each with three replicates, were performed.

#### **2.4. Data Visualization and Statistical Analysis**

GraphPad Prism 9 software (GraphPad) was used to log transform the data prior to fitting a log(inhibitor) concentration response curve on XY plots with the mean of each treatment group and error bars indicating the standard error of the mean (SEM). For experiments with MTT and DCF-DA assays specifically, raw absorbance values were adjusted to the blank prior to calculation of % metabolic activity (viability) and ROS

production in sucralose- and sucrose-treated cells relative to the untreated vehicle control and the subsequent data visualization.

The GraphPad Prism 9 software was used to evaluate lognormal distribution of data using a Shapiro-Wilk test. If assumptions were not met, outliers were identified using the software. If the normality assumptions were met, an ordinary one-way analysis of variance (ANOVA) was conducted with using the Brown-Forsythe test to check for equal population variances. If the assumptions were not met, Brown-Forsythe version of one-way ANOVA assuming unequal variances was conducted. If the assumptions were all met, data was analyzed using one-way ANOVA followed by multiple comparisons corrected with the recommended Holm-Šídák or Dunnett tests, as indicated, were interpreted to compare within and between sucralose and sucrose treatment groups. Finally, if the assumptions still did not pass, data was analyzed using one-way ANOVA followed by multiple comparisons corrected with the non-parametric Kruskal Wallis test. Comparisons were deemed statistically significant if  $p < 0.05$ .

### **3. Results**

A panel of biochemical assays and cell physiology measurements were conducted to examine the cellular mechanisms of cytotoxicity induced by sucralose. The range of assays measured various parameters of HCT116 cell health, including morphology, metabolic activity (viability), clonogenic growth, and oxidative stress.

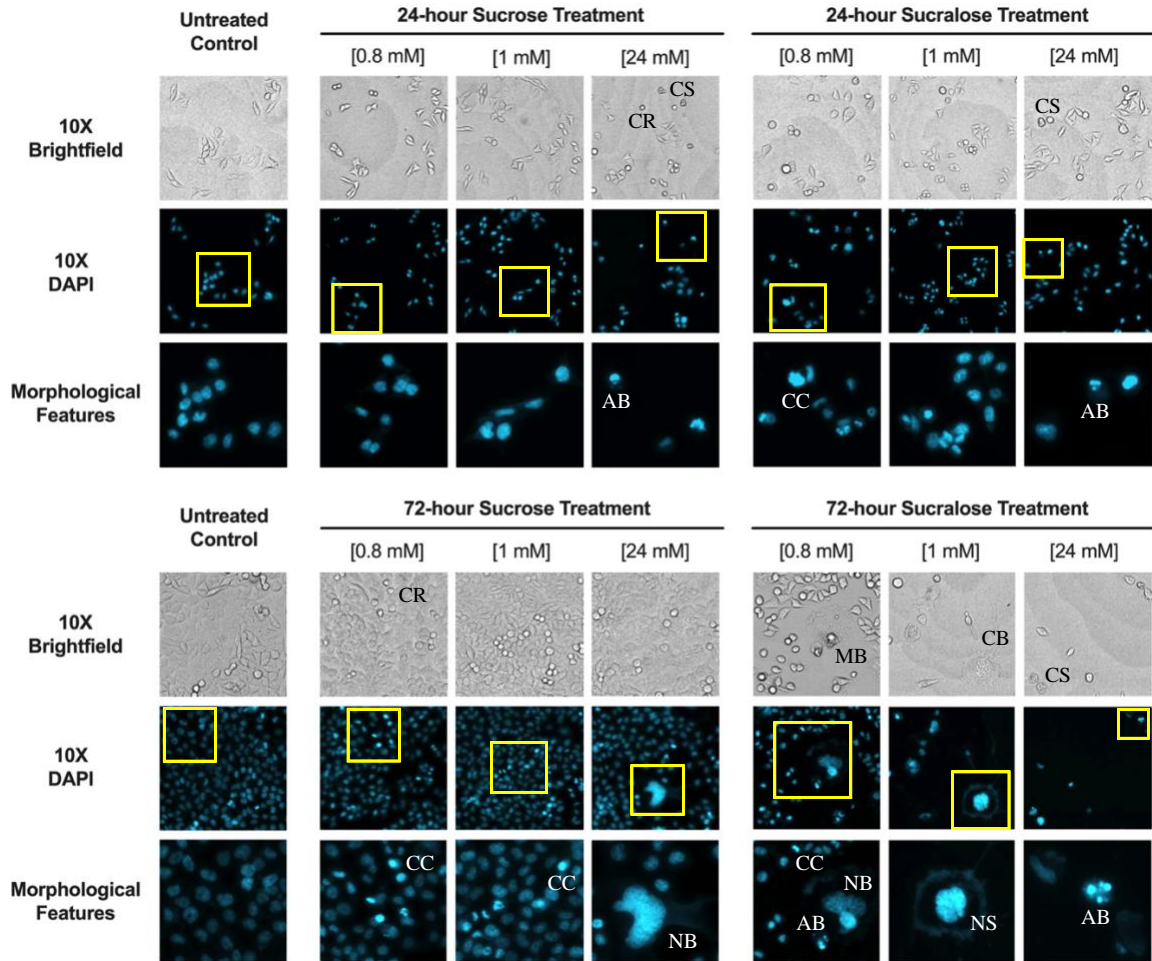
#### **3.1. Sucralose induces morphological changes in HCT116 cells and nuclei**

HCT116 cells stained with Hoechst nuclear staining were visualized under brightfield and fluorescent microscopy to observe the changes in the morphological features of the cells and their nuclei, following exposures to varying sucralose and sucrose concentrations (Fig. 3.1).

Following both 24- and 72-hour incubations with no sucralose treatment, most of the untreated control HCT116 cells are polygonal in shape with a healthy adherent appearance and normal epithelial cell morphology. Their nuclei are also consistent and regular in shape and dimensions. This consistency in cell and nuclei morphology are also seen in cells treated with sucrose for 24 and 72 hours; although, these cells did have minimal occurrences of cell rounding and pyknosis (chromatin condensation) as seen by the bright, round spots with stronger fluorescence intensity as well as nuclear blebbing (protrusions of the nucleus), indicating early- to mid-stage apoptosis and necrosis.

On the other hand, morphological features consistent with cell death become visible with exposures to low sucralose concentrations at 0.8 and 1 mM. After the 24-hour sucralose treatment, signs of cell death become evident with increased cell detachment and rounding as seen by the retraction of pseudopod projections as well as chromatin condensation. At the highest concentration of 24 mM, cells treated with sucralose began to show signs of late-stage apoptosis that are characterized by cellular fragmentation and shrinkage into membrane-bound apoptotic bodies. These changes in morphological features of HCT116 cells and nuclei were even more pronounced following the 72-hour sucralose treatment. These characteristic features of cell death became more apparent and abundant with increased presence of morphological features consistent with cell death. In particular, there is also further evidence of mid- to late-stage necrosis in cells treated with sucralose for 72 hours at concentration as low as 0.8 mM. As opposed to cells undergoing

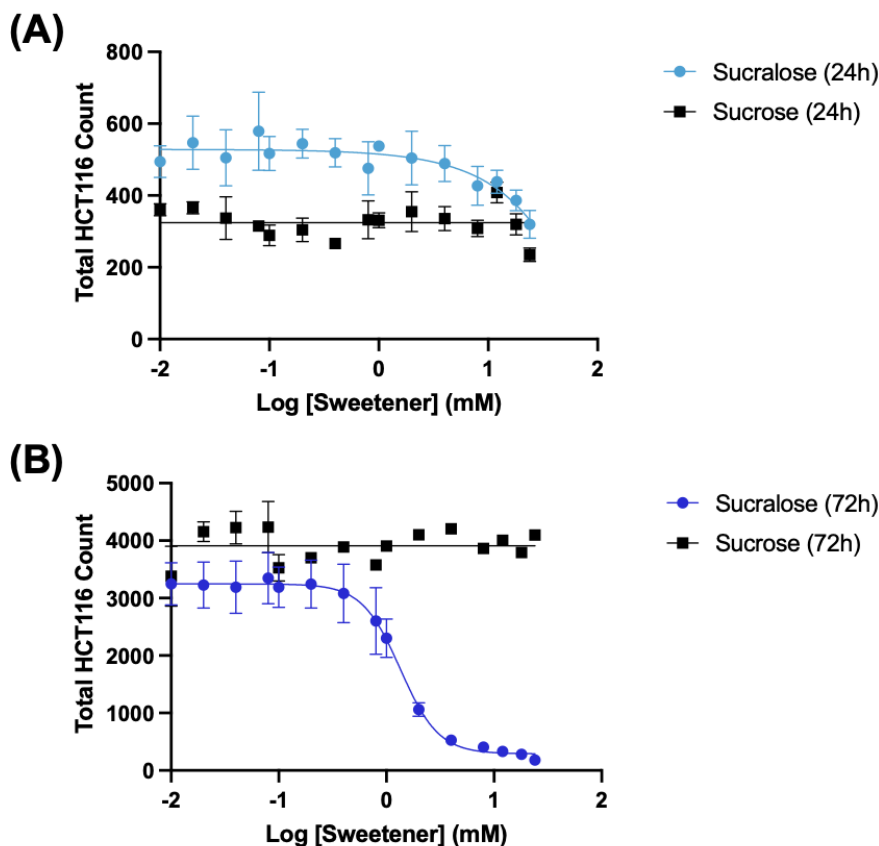
apoptosis, a hallmark of necrotic cell death seen here can be characterized by the nuclear and cytoplasmic swelling, eventually resulting in the rupture of the plasma membrane, loss of membrane integrity, and uncontrolled release of inflammatory cellular contents. These qualitative observations suggest that sucralose triggers concentration- and time-dependent cell death in the forms of apoptosis and necrosis.



**Figure 3.1: Sucralose induces morphological features of apoptosis and necrosis in HCT116 cells.** Images obtained using ImageXpress Pico Automated Microscope show nuclear-stained cells using Hoechst staining dye visualized at 10X magnification under transmitted light in brightfield and the DAPI (370/450 nm) filter with fluorescent imaging following 24- and 72-hour sucralose and sucrose treatments. Individual cells representative of morphological changes seen in respective treatment are magnified under morphological features. (CR = cell rounding; CS = cell shrinkage; MB = membrane blebbing; CB = cell bursting; CC = chromatin condensation; NB = nuclear blebbing; NS = nuclear swelling; AB = apoptotic bodies).

### 3.2. Sucralose decreases cellular metabolism and viability of HCT116 cells

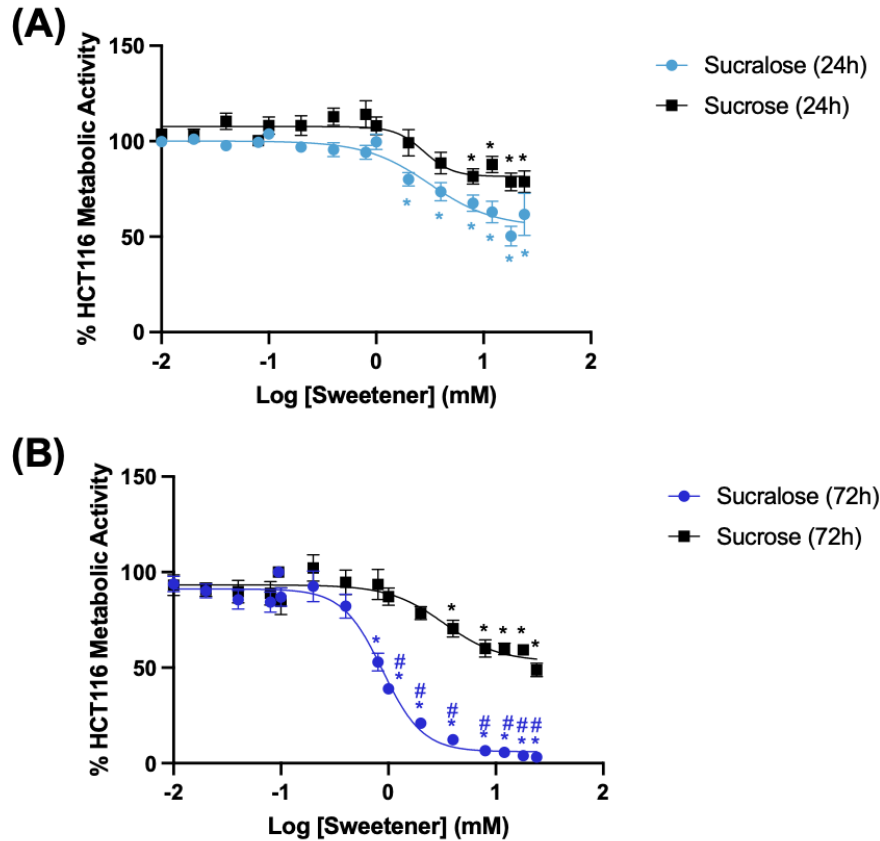
Hoechst-stained HCT116 cells were also counted using an automated fluorescence cell counting protocol following morphology observation to examine cell viability after exposures to varying sucralose and sucrose concentrations (Fig. 3.2.1).



**Figure 3.2.1: Sucralose decreases total HCT116 cell count.** The overall count of Hoechst-stained HCT116 cells following (A) 24 and (B) 72 hours post sucralose and sucrose exposure obtained using the automated fluorescence cell counting protocol on CellReporterXpress software on the ImageXpress Pico Automated Microscope.

HCT116 cells treated with sucralose resulted in decreasing overall cell counts with increasing concentrations. The decreases seen following the 72-hour treatment demonstrated a distinct dose-dependent response ( $IC_{50} = 1.35$  mM) as opposed to that following the 24-hour treatment ( $IC_{50} = N/A$ ), whereas the HCT116 cells treated with sucrose for 24 and 72 hours illustrated no specific trends in cell count changes. However, given the limitations with fluorescence cell counting, MTT assays were conducted simultaneously to evaluate cellular metabolism by spectrophotometrically assessing the

metabolic conversion of MTT to the purple formazan crystals, as a proxy for HCT116 cell viability (Fig. 3.2.2).



**Figure 3.2.2: Sucralose concentration- and time-dependently decreases HCT116 cell viability following MTT assay.** The % metabolic activity of HCT116 cells, as an indirect measure of cell viability, (A) 24 and (B) 72 hours post sucralose and sucrose exposure. Cellular metabolic activity (viability) is presented as a percentage relative to that of the untreated control and data are shown as mean  $\pm$  SEM. Significant decreases obtained from Dunnett's multiple comparisons using one-way ANOVA are highlighted with \*,  $p < 0.05$  vs. untreated control within treatment; and #,  $p < 0.05$  vs. sucrose.

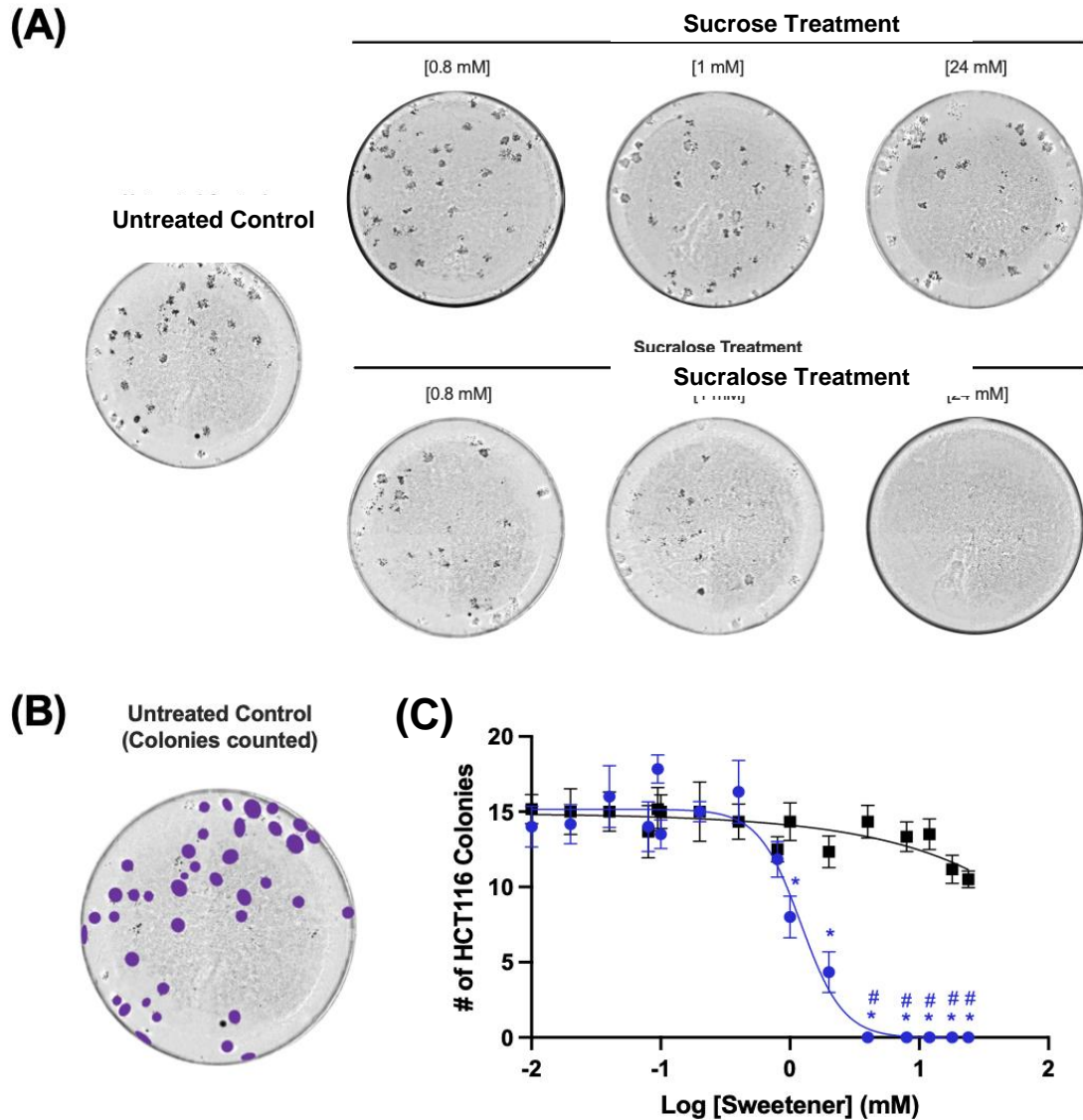
The MTT assay revealed that there was a significant, dose-dependent reduction in HCT116 cell viability following both 24 and 72 hours of sucralose exposure. After 24 hours of treatment, sucralose was toxic to HCT116 cells; viability was significantly reduced at concentrations exceeding 2 mM ( $IC_{50} = 3.14$  mM,  $p = 0.043$ ) with a maximal cell viability loss of 40 to 45%. The 72-hour sucralose treatment resulted in more potent viability reductions at doses exceeding 0.8 mM with a maximal cell viability loss of 90 to 95% ( $IC_{50} = 0.883$  mM,  $p = 0.041$ ). Meanwhile, both the 24- and 72-hour sucrose treatments

also led to reduced HCT116 cell viability ( $p = 0.004$  and  $p = 0.0021$ , respectively). However, these reductions were less potent in comparison to the respective sucralose treatments. Altogether, the findings here suggest that 72-hour sucralose treatment evoked the most drastic cytotoxic effects on cell viability as lower doses caused much larger reductions in cell viability than any other treatment conditions.

### **3.3. Sucralose impairs HCT116 cell survival into colonies**

Previous assays demonstrated sucralose exhibits severe cytotoxic effects in HCT116 cell health if the exposure is long enough. As the observed reduction in overall cell health can be due to a number of causes, we used a multiparametric approach to further assess sucralose cytotoxicity and identify the mechanisms causing cell death. Consequently, colony forming assays were conducted to qualitatively and quantitatively evaluate the cells' ability to survive and reproduce into colonies after sucralose exposure ([Fig. 3.3](#)).

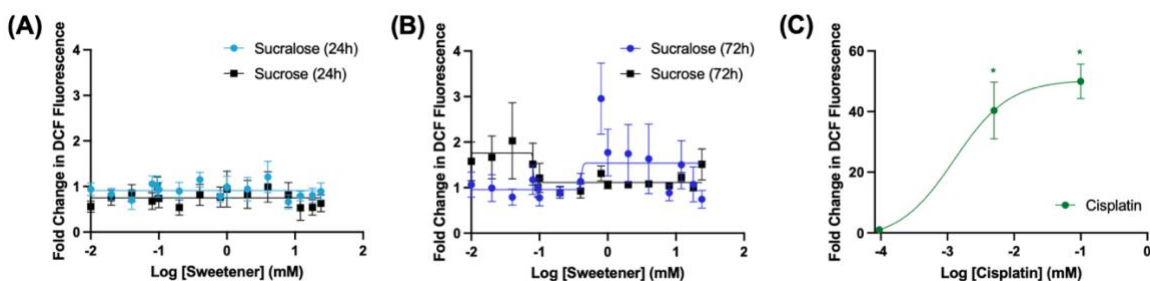
Without any treatments, the untreated control plate showed formation of individual colonies evenly spread throughout the plate. This was also seen in cells treated with sucrose, where the cells did not undergo any significant reduction in the number of colonies even with increasing concentrations. In contrast, HCT116 cell abundance gradually decreased with increasing sucralose treatment concentrations, resulting in a significant reduction in the number of colony formation at concentrations exceeding 0.8 mM ( $p = 0.041$ ), suggesting that sucralose impaired HCT116 colony growth in a concentration-dependent manner.



**Figure 3.3: Sucralose concentration-dependently impairs HCT116 clonogenic growth following crystal violet colony forming assay.** (A) Images obtained using ImageXpress Pico Automated Microscope show crystal violet-stained plates visualized at 4X magnification under transmitted light in brightfield following sucralose and sucrose treatments. (B) A schematic example with purple circles representing the number of HCT116 colonies (> 50 cells/colony) counted in the untreated control plate on Image J. (C) The number of HCT116 colonies counted post sucralose and sucrose exposure is presented and data are shown as mean  $\pm$  SEM. Significant decreases obtained from Kruskal-Wallis multiple comparisons using one-way ANOVA are highlighted with \*,  $p < 0.05$  vs. untreated control within treatment; and #,  $p < 0.05$  vs. sucrose.

### 3.4. Sucralose does not induce oxidative stress in HCT116 cells

Finally, DCF-DA assays were conducted to determine whether sucralose exposure induces any oxidative stress. This was completed by spectrophotometrically assessing the fold change of DCF-DA oxidation into DCF through fluorescence measurements, which are proportional to the formation of reactive oxygen species production and/or accumulation (Fig. 3.4).



**Figure 3.4: Sucralose does not induce oxidative stress in HCT116 cells following DCF-DA assay.** The DCF fluorescence of HCT116 cells, as an indirect measure of reactive oxygen species production, (A) 24 and (B) 72 hours post sucralose and sucrose exposure in comparison to (C) cells treated with cisplatin as a positive control for cell death and reactive oxygen species production, is presented as a fold change relative to that of the untreated control and data are shown as mean  $\pm$  SEM. Significant decreases obtained from Holm-Šídák multiple comparisons using one-way ANOVA are highlighted with \*,  $p < 0.05$  vs. untreated control within treatment.

HCT116 cells exposed to sucralose and sucrose for 24 and 72 hours both exhibited no significant differences in the relative DCF fluorescence between all treatment conditions and the untreated control. Cells treated with cisplatin as a positive control for cell death, on the other hand, resulted in a significant increase in DCF-DA oxidation at treatment concentrations ranging from 0.005 and 0.1 mM, with the highest treatment group corresponding to approximately a 50-fold increase in relative reactive oxygen species production levels compared to the untreated controls. Based on comparisons of reactive oxygen species production between sucralose- and cisplatin-treated HCT116 cells, the findings suggest that the cells had no response to sucralose in terms of reactive oxygen species production and/or accumulation.

## 4. Discussion

Although previously considered to be biologically inert, there is a growing body of evidence highlighting that sucralose may disrupt metabolic processes thereby negatively impacting the gut microbiome and physiology. Given the controversial research findings surrounding the safety of sucralose obtained over the years, it warrants a need to revisit and reassess the biological effects elicited by sucralose in the GI tract. The present study, therefore, aimed to conduct an *in vitro* investigation of the biological effects of sucralose signaling in the gut at the cellular level by characterizing a diverse array of parameters of HCT116 cell health including morphology, metabolic activity (viability), clonogenic growth, to oxidative stress in response to sucralose exposure. Such multiparametric analysis not only provides valuable insight into different parameters of overall cell health, but it helps to develop a more comprehensive understanding of sucralose cytotoxicity. Through a panel of biochemical assays and cell physiology measurements, sucralose was shown to exhibit concentration- and time-dependent toxicity in HCT116 cells, negatively impacting overall cell health and survival. However, sucralose did not seem to induce oxidative stress in the gut cells, and the natural sugar sucrose was minimally toxic in the gut cells.

### 4.1. Cell Morphology

The more qualitative characterization of the sucralose phenotype in gut cells highlighted that the cells underwent extensive morphological changes upon sucralose exposure (Fig. 3.1). The control cells and the cells treated with sucrose mostly illustrated healthy epithelial cell morphology with very minimal occurrences of early-stage apoptotic cell death. However, to a much greater extent than other treatment conditions, the cells treated with sucralose showed typical morphological features consistent with apoptosis and necrosis, the two most common forms of cell death (Fink and Cookson, 2005). These morphological changes became even more severe, pronounced, and abundant with increasing concentrations and increased exposure at 72 hours. These results indicate that the sucralose treatment has little to moderate impact at lower dosages and shorter exposure times but are extremely cytotoxic to the HCT116 cells at higher concentration and longer exposure time. To further these findings with a more comprehensive, stage-

specific quantification of cell death, conducting apoptosis and necrosis assays to specifically detect and quantify the cellular events associated with various stages of cell death is recommended.

#### **4.2. Cell Metabolic Activity (Viability)**

A crucial parameter of cell health is cell viability, which is the cells' ability to continue performing metabolic functions after exposure to chemicals of interest. As such, the cell death observed in morphology was first quantified using the automated fluorescence cell count protocol on the Pico microscope (Fig. 3.2.1). The cells treated with sucralose for 72 hours demonstrated a distinct concentration-dependent response in comparison to the 24-hour treatment. On the other hand, cells treated with sucrose showed no specific trends in cell count with increasing concentrations.

While the automated fluorescent cell counting technique provides a valuable instrument for assessing cell numbers, it is prone to several practical limitations. For instance, with fluorophores slowly losing their fluorescence as it starts to fade over time, it becomes difficult to get an accurate cell count by simply measuring fluorescence intensity. Furthermore, the technical complexity associated with the analysis software which involves manual operations of parameters such as area selection, white balance, calibration, and colour correction can also lead to systematic “operator effect” (Morelli et al., 2021). Without optimized settings that are consistent throughout replicates, fluorescence readings can result in non-negligible variations between the samples. This imposes further challenges and limitations to the fluorescent quantification, resulting in less accurate cell counting.

For these reasons, the MTT assay was simultaneously conducted to evaluate whether sucralose induced any changes in HCT116 cell viability (Fig. 3.2.2). Given that only the living cells are able to facilitate the metabolic conversion of the yellow MTT dye into purple formazan crystals, which can then be measured as an increase in absorbance when quantified on a spectrophotometer, this assay has been the gold standard technique in a diverse array of cytotoxicity studies to indirectly measure cell viability (Ghasemi et al., 2021). The results indicate that HCT116 cell viability significantly decreased in a time- and dose-dependent manner relative to the untreated control and cells treated with sucrose,

where the mitochondrial ability to metabolize the MTT dye through oxidoreductase enzymes was significantly affected for HCT116 cells with increasing concentrations and increased exposure at 72 hours. On the other hand, cells treated with sucrose resulted in a much smaller and slower magnitude of reduction in cell viability in all treatment conditions.

While there are no known previous *in vitro* studies for the HCT116 cell line, similar studies examining the effect of sucralose (1 to 50 mM) on cell viability have been conducted on different human gut cell lines (Caco-2 and HT-29) which are known to be great research models of human enterocytes (van Eyk, 2015). Their findings, in a similar fashion, also revealed statistically significant losses in cell viability in concentration- and time-dependent manners. However, there were marked differences in the magnitude of cell viability reductions in these cell lines particularly after 72-hour sucralose treatment. For instance, only 39% and 12% of HCT116 cells treated with 1 mM and 4 mM sucralose relative to the untreated control, respectively, were viable. Yet, HT-29 cells treated with 1 mM and 4 mM sucralose for 72 hours resulted in 90% and 98% of cell viability, respectively, relative to the untreated control (van Eyk, 2015). In contrast, Caco-2 cells treated for 72 hours with 1 mM sucralose initially revealed a slight increase in cell viability at 101% prior to undergoing a gradual decrease in cell viability, starting with 66% at 4 mM sucralose (van Eyk, 2015). Comparing results across different gut cell lines suggests a higher sensitivity of HCT116 cells to sucralose exposure compared to Caco-2 and HT-29 cell lines.

So far, the findings from previous assays suggest that sucralose is exhibiting a dose-dependent toxicity in the cells if the exposure is long enough. However, these reductions in overall cell health and survival can have a variety of causes and furthermore, while measuring cell survival with fluorometric and colourimetric dyes can provide an insight into the cell survival in a population at a specific time, it does not differentiate cells that are actively dying or marked for future death. Consequently, we continued with our multiparametric investigation to further assess sucralose cytotoxicity and identify the possible mechanisms causing this death.

### 4.3. Clonogenic Growth

In many cases, to get an idea of potential mechanisms that could have contributed to the reduction in overall cell health and survival, it is insightful to examine the proliferative capacity of a single cell to grow into a large colony through clonal expansion. The findings from the colony forming assay showed that cells treated with sucralose had low colony-forming efficiency and thus, exhibited the weakest replicative potential. This was indicated by significant reduction in HCT116 colony formation by approximately 50% at 0.8 mM, and the lowest overall response to sucralose resulted in a 100% reduction in colony formation (Fig. 3.3).

The colony forming assay employed in the present study is used routinely for measuring the cytotoxic effects of chemicals of interest *in vitro* in the pharmacology and toxicology fields. However, the 96-well format used in the current technique, as opposed to the conventional 6-well format, to allow for more biological replicates and avoid plate effect made the quantification of colonies much more challenging. For instance, the 4X magnification chosen due to its time-efficient visualization resulted in an extremely low resolution where there were no marked differences in contrast between the cells and the plate. This consequently made it difficult for the automated brightfield cell counting analysis software on the Pico microscope to distinguish and detect the cells from the background noise. Furthermore, the low resolution also made the subsequent manual measurement of the size of single cells to roughly approximate the number of cells per colony on ImageJ demanding. Not to mention, this method also does not account for cells growing on top of each other.

As a result, to overcome the variations that are prone to this quantification technique as well as in the interest of increased efficiency, absorption-based spectrophotometry quantification can be used alternatively. This method is based on fast and complete solubilization of the crystal violet dye absorbed by cells during staining through the use of 10% acetic acid as a solvent, prior to reading the absorbance of the dissolved solution at a wavelength of 590 nm (Guzmán et al., 2014; Kueng et al., 1989). This is consistent with the growing body of literature favouring the use of 10% acetic acid as a solvent over the more traditional solvent, DMSO, as the dye was solubilized in matter of

minutes as opposed to several hours (Guzmán et al., 2014; Kueng et al., 1989; Mabate et al., 2023; Okada et al., 2016).

With mounting incidences of cancer within the GI tract emerging as a cause for serious concern, being able to carry out the colony forming assay appropriately is critical to accurately investigate the cells' ability to survive and proliferate into colonies following sucralose exposure. Since tumour proliferation and metastasis have become a focus in scientific and clinical cancer research, understanding whether sucralose can impact the cells' ability to move, migrate, and clonally expand can further provide valuable insights into whether sucralose harbours any potentially carcinogenic effects or not.

#### **4.4. Oxidative Stress**

In the MTT assay, a significant reduction in cell viability was seen with increasing sucralose concentrations and increased exposure at 72 hours, as indicated by the reduced conversion of MTT dye into purple formazan crystals. Given that the mitochondria facilitate this metabolic conversion and also serve as important regulators of energy homeostasis by producing ROS through mitochondrial oxidative metabolism when necessary (Bórquez et al., 2021; Nogueira et al., 2008), we next conducted the DCF-DA assay to examine the oxidation levels of DCF-DA into the fluorescent DCF to indirectly assess whether sucralose induces any oxidative stress in HCT116 cells ([Fig. 3.4](#)).

Apoptosis is often correlated with severe damages to DNA, lipid, and proteins among other things (Circu and Aw, 2010; De Zio et al., 2013), which happen as a result of the accumulation of ROS that were not detoxified by the cells. A wide variety of ROS such as peroxy, alkoxy, and carbonate groups, to name a few, are initially produced when there are mismatches in the electron flow, leading to electrons leaking out of the electron transport system (ETS) before subsequently coming in contact with molecular oxygen, instead of being passed onto the next electron carrier in the ETS (Onukwufor et al., 2019). Therefore, I predicted that there would be an increase in ROS production in the cells upon sucralose exposure and thus, an increase in the relative DCF fluorescence, reflecting the cell death seen previously. However, the present study did not support this assumption as the cells exhibited no significant differences in the relative DCF fluorescence between all treatment conditions and the untreated control. Cells treated with cisplatin as a positive

control for cell death and ROS production, on the other hand, showed a markedly different concentration-dependent increase in relative DCF fluorescence. This suggests that (1) the DCF-DA assay used to examine ROS production in the present study actually worked, (2) ROS can accumulate in HCT116 cells (as seen in cells treated with cisplatin) without perhaps being cleared by the cells' machinery, and (3) the mechanism of cell death seen previously in HCT116 cells in response to sucralose is likely not mediated through ROS. This is in line with a recent *in vitro* study where the authors conducted DCF-DA assays on Caco-2, a different human gut cell line known to be a great research model of human enterocytes, which has also shown that sucralose did not increase ROS production (Shil et al., 2020), but it differs from previous *in vitro* and *in vivo* studies which presented that sucralose promoted accumulation of ROS in human adipose tissue-derived mesenchymal stromal cells and murine liver, respectively (Bian et al., 2017; Kundu et al., 2020). The conflicting findings, yet again, contribute to the ongoing “trick or treat” debate surrounding sucralose and highlight the need to conduct more thorough research to fully understand the effects of sucralose in oxidative stress.

#### **4.5. Other potential mechanism underlying HCT116 cell death**

In the meantime, it is also possible that sucralose altered mitochondrial membrane potential in HCT116 cells, as it has been shown to in Caco-2 cells (Bórquez et al., 2021), which could have potentially triggered endoplasmic reticulum (ER) stress. When cells experience ER stress, this can result in an accumulation of misfolded and unfolded proteins within the lumen of the ER (Osowski and Urano, 2011). This leads to the activation of the unfolded protein response (UPR) signaling to help alleviate this stress and restore homeostasis within the ER to stimulate cell survival (Osowski and Urano, 2011). But with prolonged stress from long exposures to chemicals, the UPR can also activate signaling pathways that can ultimately induce ER stress-mediated cell death (Osowski and Urano, 2011). Although it has been recently established that sucralose can induce ER stress in the human liver cell line (HepG2) and hypothalamic cell line (mHypoE-N43/5) (Park et al., 2019; Wu et al., 2022), it is not yet known whether sucralose can do so in gut cells. This opens door for exciting future studies that could investigate ER stress and the activation of

the UPR signaling network in gut cells to examine whether these are the mechanisms underlying cell death observed in response to sucralose exposure.

Additionally, despite the findings which do not illustrate ROS as a possible mechanism underlying the cell death seen previously and the fact that mechanisms whereby sucralose induces apoptosis and necrosis still remain elusive, the cells' response to sucralose at least in terms of ROS production is interesting. With sugars and other carbohydrates emerging as non-enzymatic antioxidants (Matros et al., 2015; Peshev et al., 2013), it raises curiosity about whether sugar-derived sweetener that is sucralose could potentially be involved in the detoxification of ROS as a “scavenger”. However, in general, more research is needed to fully comprehend the effect of sucralose in oxidative stress and ROS production to better understand its potential implications in metabolic diseases.

#### **4.6. Future Directions: Sucralose Signaling in the Gut**

Now, we can see that sucralose, in fact, does elicit biological effects despite being known that it does not get metabolized in the body. However, how sucralose elicits biological effects in the gut microbiome is poorly understood, given that there is currently no established mechanism as to how and why this might happen. However, based on previous research by Rourke Lab, the underlying molecular signaling, while outside of the scope of the present study, could potentially be attributed to an orphan GPCR called GPR52.

While mechanisms that sense and respond to “sweet” stimuli by T1R2/T1R3 receptors are well-conserved across different species, recent *in vitro* studies suggest the presence of additional GPCRs responsible for sensing natural and artificial sweeteners (Fernandez, 2020). Given that class A GPCRs are traditionally involved in food metabolite sensing and regulation of energy homeostasis (Husted et al., 2017), other unrevealed sweet taste receptors within the metabolically active class A family that are capable of initiating signaling cascades in response to sweet stimuli, independent of T1R2/T1R3, could potentially exist (Fernandez, 2020; Power, 2020). Through previous quantification studies using the high throughput  $\beta$ -arrestin recruitment2 (PRESTO-Tango) assay, we identified a novel receptor-mediated mechanism that can distinguish between natural sweet molecules (*e.g.*, sucrose) and selectively detect the NNS sucralose. HTLA (HEK293 cell line stable expressing a tTA-dependent luciferase reporter and a  $\beta$ -arrestin2-TEV fusion

gene) cells transiently transfected with GPR52 and treated with sucralose resulted in a concentration-dependent activation of the receptor at doses exceeding 0.02 mM relative to the untreated control and 0.08 mM relative to sucrose treatment.

Furthermore, though GPR52 is an orphan receptor, its expression in the central nervous system and its promising role in regulating various brain functions through activation of cAMP-dependent pathways are well-established in the current literature (Komatsu et al., 2014; Wang et al., 2020). However, not much was known about GPR52 expression patterns beyond the brain in other organs and tissues until we discovered that GPR52 was most highly expressed in the whole intestinal tissues of the stomach and lower GI tract, while it was moderately expressed in the cortex and cerebellum.

Altogether, these evidences suggest that the biological effects of sucralose could potentially be mediated through GPR52. Therefore, it would be informative and insightful to further assess whether and to what extent sucralose signaling through GPR52 can impact cell health and function in the human gut cell line, HCT116. The characterization of sucralose/GPR52 signaling can be achieved by repeating the cellular phenotyping of sucralose following the inhibition of the *GPR52* gene through lentiviral shRNA-mediated knockdown and pharmacological inhibition of the receptor action. This will not only help tap the “untapped” potential of the orphan GRP52 receptor, but also enhance our understanding of the biological relevance of sucralose/GPR52 signaling in the gut and most importantly, help inform pharmacological approaches to design versatile NNSs with reduced metabolic side effects.

## 5. Conclusions

Sucralose, among many other NNSs, quickly made its mark in the market of sugar-free food and beverages as a safe and effective alternative for sugar, without contributing to any caloric intake. However, to date, approval of such food additives has never adequately taken into consideration their impact on gut health. Moreover, studies conducted post-approval have shown inconsistent results time and time again, leading to growing controversy and disagreement surrounding the safety of sucralose. The findings of this *in vitro* study illustrate that NNS sucralose is, in fact, cytotoxic in the human gut cell line, HCT116, and most importantly, in a concentration- and time-dependent manner. Although sucralose negatively impacts overall HCT116 cell health and survival, it does not seem to induce oxidative stress, suggesting that the mechanism underlying cell death seen here is not likely through the production and/or accumulation of ROS. On the other hand, the natural sugar sucrose appears to be minimally toxic in the gut cells in comparison to sucralose.

Nonetheless, it is important to acknowledge that cytotoxic effects seen in the present study are heavily dependent on the context of concentration and time. This, along with the presence of other conflicting research findings, speak volume about the need for continued research into the effects of sucralose on a wide range of concentrations, timepoints, and duration as well as various other cell/tissue health parameters to ensure the safety of food additives currently on the market. Given the widespread and increasing consumption of sucralose and other NNSs, as well as the continued emergence of chronic metabolic diseases with alarming prevalence and consequences, it is crucial to revisit the safety and regulations surrounding the consumption of sucralose and other NNSs in order to tackle the complex paradoxes of human health that are facing today's society. In the meantime, this is a complex issue, and the controversy still remains: is sucralose a *trick* or *treat*?

## 6. References

- Abou-Donia, M. B., El-Masry, E. M., Abdel-Rahman, A. A., McLendon, R. E. and Schiffman, S. S.** (2008). Splenda alters gut microflora and increases intestinal p-glycoprotein and cytochrome p-450 in male rats. *J Toxicol Environ Health A* **71**, 1415–1429.
- Ahmad, R. and Dalziel, J. E.** (2020). G Protein-Coupled Receptors in Taste Physiology and Pharmacology. *Front Pharmacol* **11**, 587664.
- Ahmad, S. Y., Friel, J. K. and MacKay, D. S.** (2020a). The effect of the artificial sweeteners on glucose metabolism in healthy adults: a randomized, double-blinded, crossover clinical trial. *Appl Physiol Nutr Metab* **45**, 606–612.
- Ahmad, S. Y., Friel, J. K. and Mackay, D. S.** (2020b). Effect of sucralose and aspartame on glucose metabolism and gut hormones. *Nutr Rev* **78**, 725–746.
- Ahmad, S. Y., Friel, J. and Mackay, D.** (2020c). The Effects of Non-Nutritive Artificial Sweeteners, Aspartame and Sucralose, on the Gut Microbiome in Healthy Adults: Secondary Outcomes of a Randomized Double-Blinded Crossover Clinical Trial. *Nutrients* **12**, 3408.
- Alsunni, A. A.** (2020). Effects of Artificial Sweetener Consumption on Glucose Homeostasis and Its Association with Type 2 Diabetes and Obesity. *Int J Gen Med* **13**, 775–785.
- Barndt, R. L. and Jackson, G.** (1990). Stability of sucralose in baked goods. *Food Technology (Chicago)* **44**, 62–66.
- Berry, C., Brusick, D., Cohen, S. M., Hardisty, J. F., Grotz, V. L. and Williams, G. M.** (2016). Sucralose Non-Carcinogenicity: A Review of the Scientific and Regulatory Rationale. *Nutrition and Cancer* **68**, 1247–1261.
- Bezencon, C., le Coutre, J. and Damak, S.** (2007). Taste-Signaling Proteins Are Coexpressed in Solitary Intestinal Epithelial Cells. *Chemical Senses* **32**, 41–49.
- Bian, X., Chi, L., Gao, B., Tu, P., Ru, H. and Lu, K.** (2017). Gut Microbiome Response to Sucralose and Its Potential Role in Inducing Liver Inflammation in Mice. *Front Physiol* **8**, 487.
- Bornemann, V., Werness, S. C., Buslinger, L. and Schiffman, S. S.** (2018). Intestinal Metabolism and Bioaccumulation of Sucralose In Adipose Tissue In The Rat. *Journal of Toxicology and Environmental Health, Part A* **81**, 913–923.
- Bórquez, J. C., Hidalgo, M., Rodríguez, J. M., Montaña, A., Porrás, O., Troncoso, R. and Bravo-Sagua, R.** (2021). Sucralose Stimulates Mitochondrial Bioenergetics in Caco-2 Cells. *Frontiers in Nutrition* **7**,.

- Brown, R. J., De Banate, M. A. and Rother, K. I.** (2010). Artificial sweeteners: a systematic review of metabolic effects in youth. *International Journal of Pediatric Obesity* **5**, 305–312.
- Cattaneo, F., Guerra, G., Parisi, M., De Marinis, M., Tafuri, D., Cinelli, M. and Ammendola, R.** (2014). Cell-Surface Receptors Transactivation Mediated by G Protein-Coupled Receptors. *International Journal of Molecular Sciences* **15**, 19700–19728.
- Chappell, G. A., Borghoff, S. J., Pham, L. L., Doepker, C. L. and Wikoff, D. S.** (2020). Lack of potential carcinogenicity for sucralose – Systematic evaluation and integration of mechanistic data into the totality of the evidence. *Food and Chemical Toxicology* **135**, 110898.
- Chassaing, B., Koren, O., Goodrich, J. K., Poole, A. C., Srinivasan, S., Ley, R. E. and Gewirtz, A. T.** (2015). Dietary emulsifiers impact the mouse gut microbiota promoting colitis and metabolic syndrome. *Nature* **519**, 92–96.
- Circu, M. L. and Aw, T. Y.** (2010). REACTIVE OXYGEN SPECIES, CELLULAR REDOX SYSTEMS AND APOPTOSIS. *Free Radic Biol Med* **48**, 749–762.
- De Zio, D., Cianfanelli, V. and Cecconi, F.** (2013). New Insights into the Link Between DNA Damage and Apoptosis. *Antioxid Redox Signal* **19**, 559–571.
- Dyer, J., Salmon, K. S. H., Zibrik, L. and Shirazi-Beechey, S. P.** (2005). Expression of sweet taste receptors of the T1R family in the intestinal tract and enteroendocrine cells. *Biochem. Soc. Trans.* **33**, 302–305.
- Fink, S. L. and Cookson, B. T.** (2005). Apoptosis, Pyroptosis, and Necrosis: Mechanistic Description of Dead and Dying Eukaryotic Cells. *Infect Immun* **73**, 1907–1916.
- Garriguet, D.** (2009). Impact of identifying plausible respondents on the under-reporting of energy intake in the Canadian Community Health Survey. *Health reports / Statistics Canada, Canadian Centre for Health Information = Rapports sur la santé / Statistique Canada, Centre canadien d'information sur la santé* **19**, 47–55.
- Ghasemi, M., Turnbull, T., Sebastian, S. and Kempson, I.** (2021). The MTT Assay: Utility, Limitations, Pitfalls, and Interpretation in Bulk and Single-Cell Analysis. *International Journal of Molecular Sciences* **22**, 12827.
- Grice, H. C. and Goldsmith, L. A.** (2000). Sucralose--an overview of the toxicity data. *Food Chem Toxicol* **38 Suppl 2**, S1-6.
- Grotz, V. L. and Munro, I. C.** (2009). An overview of the safety of sucralose. *Regulatory toxicology and pharmacology* **55**, 1–5.

- Grotz, V. L., Henry, R. R., McGill, J. B., Prince, M. J., Shamoan, H., Trout, J. R. and Pi-Sunyer, F. X.** (2003). Lack of effect of sucralose on glucose homeostasis in subjects with type 2 diabetes. *J Am Diet Assoc* **103**, 1607–1612.
- Guzmán, C., Bagga, M., Kaur, A., Westermarck, J. and Abankwa, D.** (2014). ColonyArea: An ImageJ Plugin to Automatically Quantify Colony Formation in Clonogenic Assays. *PLOS ONE* **9**, e92444.
- Hamel, V., Nardocci, M., Flexner, N., Bernstein, J., L'Abbé, M. R. and Moubarac, J.-C.** (2022). Consumption of Ultra-Processed Foods Is Associated with Free Sugars Intake in the Canadian Population. *Nutrients* **14**, 708.
- Hancock, J. T.** (2017). *Cell Signalling*. Oxford University Press.
- Harpaz, D., Yeo, L. P., Cecchini, F., Koon, T. H. P., Kushmaro, A., Tok, A. I. Y., Marks, R. S. and Eltzov, E.** (2018). Measuring Artificial Sweeteners Toxicity Using a Bioluminescent Bacterial Panel. *Molecules* **23**, 2454.
- Harrington, E. O., Vang, A., Braza, J., Shil, A. and Chichger, H.** (2018). Activation of the sweet taste receptor, T1R3, by the artificial sweetener sucralose regulates the pulmonary endothelium. *Am J Physiol Lung Cell Mol Physiol* **314**, L165–L176.
- Hilger, D., Masureel, M. and Kobilka, B. K.** (2018). Structure and dynamics of GPCR signaling complexes. *Nat Struct Mol Biol* **25**, 4–12.
- Husted, A. S., Trauelsen, M., Rudenko, O., Hjorth, S. A. and Schwartz, T. W.** (2017). GPCR-Mediated Signaling of Metabolites. *Cell Metabolism* **25**, 777–796.
- Jeffrey, A. M. and Williams, G. M.** (2000). Lack of DNA-damaging activity of five non-nutritive sweeteners in the rat hepatocyte/DNA repair assay. *Food and Chemical Toxicology* **38**, 335–338.
- John, B. A., Wood, S. G. and Hawkins, D. R.** (2000a). The pharmacokinetics and metabolism of sucralose in the mouse. *Food Chem Toxicol* **38 Suppl 2**, S107-110.
- John, B. A., Wood, S. G. and Hawkins, D. R.** (2000b). The pharmacokinetics and metabolism of sucralose in the rabbit. *Food Chem Toxicol* **38 Suppl 2**, S111-113.
- Knight, I.** (1994). The development and applications of sucralose, a new high-intensity sweetener. *Can. J. Physiol. Pharmacol.* **72**, 435–439.
- Kojima, I. and Nakagawa, Y.** (2011). The Role of the Sweet Taste Receptor in Enteroendocrine Cells and Pancreatic  $\beta$ -Cells. *Diabetes Metab J* **35**, 451–457.
- Komatsu, H., Maruyama, M., Yao, S., Shinohara, T., Sakuma, K., Imaichi, S., Chikatsu, T., Kuniyeda, K., Siu, F. K., Peng, L. S., et al.** (2014). Anatomical Transcriptome of G Protein-Coupled Receptors Leads to the Identification of a

Novel Therapeutic Candidate GPR52 for Psychiatric Disorders. *PLoS ONE* **9**, e90134.

- Kueng, W., Silber, E. and Eppenberger, U.** (1989). Quantification of cells cultured on 96-well plates. *Analytical Biochemistry* **182**, 16–19.
- Kundu, N., Domingues, C. C., Patel, J., Aljishi, M., Ahmadi, N., Fakhri, M., Sylvetsky, A. C. and Sen, S.** (2020). Sucralose promotes accumulation of reactive oxygen species (ROS) and adipogenesis in mesenchymal stromal cells. *Stem Cell Res Ther* **11**, 250.
- Lee, J.-Y., Tsolis, R. M. and Bäuml, A. J.** (2022). The microbiome and gut homeostasis. *Science* **377**, eabp9960.
- Li, X., Staszewski, L., Xu, H., Durick, K., Zoller, M. and Adler, E.** (2002). Human receptors for sweet and umami taste. *Proceedings of the National Academy of Sciences* **99**, 4692–4696.
- Li, X., Liu, Y., Wang, Y., Li, X., Liu, X., Guo, M., Tan, Y., Qin, X., Wang, X. and Jiang, M.** (2020). Sucralose Promotes Colitis-Associated Colorectal Cancer Risk in a Murine Model Along With Changes in Microbiota. *Front. Oncol.* **10**, 710.
- Ma, J., Bellon, M., Wishart, J. M., Young, R., Blackshaw, L. A., Jones, K. L., Horowitz, M. and Rayner, C. K.** (2009). Effect of the artificial sweetener, sucralose, on gastric emptying and incretin hormone release in healthy subjects. *American Journal of Physiology-Gastrointestinal and Liver Physiology* **296**, G735–G739.
- Mabate, B., Daub, C. D., Pletschke, B. I. and Edkins, A. L.** (2023). Comparative Analyses of Fucoidans from South African Brown Seaweeds That Inhibit Adhesion, Migration, and Long-Term Survival of Colorectal Cancer Cells. *Marine Drugs* **21**, 203.
- Magnuson, B. A., Carakostas, M. C., Moore, N. H., Poulos, S. P. and Renwick, A. G.** (2016). Biological fate of low-calorie sweeteners. *Nutrition Reviews* **74**, 670–689.
- Magnuson, B. A., Roberts, A. and Nestmann, E. R.** (2017). Critical review of the current literature on the safety of sucralose. *Food and Chemical Toxicology* **106**, 324–355.
- Mann, S. W., Yuschak, M. M., Amyes, S. J. G., Aughton, P. and Finn, J. P.** (2000a). A combined chronic toxicity/carcinogenicity study of sucralose in Sprague–Dawley rats - ScienceDirect. *Food Chem Toxicol* **38**, 71–89.
- Mann, S. W., Yuschak, M. M., Amyes, S. J. G., Aughton, P. and Finn, J. P.** (2000b). A carcinogenicity study of sucralose in the CD-1 mouse. *Food and Chemical Toxicology* **38**, 91–97.

- Matros, A., Peshev, D., Peukert, M., Mock, H.-P. and Van den Ende, W.** (2015). Sugars as hydroxyl radical scavengers: proof-of-concept by studying the fate of sucralose in Arabidopsis. *The Plant Journal* **82**, 822–839.
- Mendonça, L. A. B. M., dos Santos Ferreira, R., de Cássia Avellaneda Guimarães, R., de Castro, A. P., Franco, O. L., Matias, R. and Carvalho, C. M. E.** (2018). The Complex Puzzle of Interactions Among Functional Food, Gut Microbiota, and Colorectal Cancer. *Frontiers in Oncology* **8**.
- Mezitis, N., Maggio, C., Koch, P., Quddoos, A., Allsion, D. and Pi-Sunyer, F. X.** (1996). Glycemic Effect of a Single High Oral Dose of the Novel Sweetener Sucralose in Patients With Diabetes | Diabetes Care | American Diabetes Association. *Diabetes Care* **19**, 1004–1005.
- Morelli, R., Clissa, L., Amici, R., Cerri, M., Hitrec, T., Luppi, M., Rinaldi, L., Squarcio, F. and Zoccoli, A.** (2021). Automating cell counting in fluorescent microscopy through deep learning with c-ResUnet. *Sci Rep* **11**, 22920.
- Nelson, G., Hoon, M. A., Chandrashekar, J., Zhang, Y., Ryba, N. J. P. and Zuker, C. S.** (2001). Mammalian Sweet Taste Receptors. *Cell* **106**, 381–390.
- Nogueira, V., Park, Y., Chen, C.-C., Xu, P.-Z., Chen, M.-L., Tonic, I., Unterman, T. and Hay, N.** (2008). Akt determines replicative senescence and oxidative or oncogenic premature senescence and sensitizes cells to oxidative apoptosis. *Cancer Cell* **14**, 458–470.
- Okada, K., Hakata, S., Terashima, J., Gamou, T., Habano, W. and Ozawa, S.** (2016). Combination of the histone deacetylase inhibitor depsipeptide and 5-fluorouracil upregulates major histocompatibility complex class II and p21 genes and activates caspase-3/7 in human colon cancer HCT-116 cells. *Oncology Reports* **36**, 1875–1885.
- O’Neill, M., Kornas, K. and Rosella, L.** (2019). The future burden of obesity in Canada: a modelling study. *Can J Public Health* **110**, 768–778.
- Onukwufor, J. O., Berry, B. J. and Wojtovich, A. P.** (2019). Physiologic Implications of Reactive Oxygen Species Production by Mitochondrial Complex I Reverse Electron Transport. *Antioxidants (Basel)* **8**, 285.
- Osowski, C. M. and Urano, F.** (2011). Measuring ER stress and the unfolded protein response using mammalian tissue culture system. *Methods Enzymol* **490**, 71–92.
- Park, S., Sethi, S. and Bouret, S. G.** (2019). Non-nutritive Sweeteners Induce Hypothalamic ER Stress Causing Abnormal Axon Outgrowth. *Front Endocrinol (Lausanne)* **10**, 876.
- Pepino, M. Y.** (2015). METABOLIC EFFECTS OF NON-NUTRITIVE SWEETENERS. *Physiol Behav* **152**, 450–455.

- Perbal, B.** (2003). Communication is the key. *Cell Commun Signal* **1**, 3.
- Peshev, D., Vergauwen, R., Moglia, A., Hideg, É. and Van den Ende, W.** (2013). Towards understanding vacuolar antioxidant mechanisms: a role for fructans? *J Exp Bot* **64**, 1025–1038.
- Qin, X. F.** (2002). Impaired inactivation of digestive proteases by deconjugated bilirubin: the possible mechanism for inflammatory bowel disease. *Med Hypotheses* **59**, 159–163.
- Qin, X.** (2012). Etiology of inflammatory bowel disease: A unified hypothesis. *World J Gastroenterol* **18**, 1708–1722.
- Rapozo, D. C. M., Bernardazzi, C. and de Souza, H. S. P.** (2017). Diet and microbiota in inflammatory bowel disease: The gut in disharmony. *World J Gastroenterol* **23**, 2124–2140.
- Risdon, S., Battault, S., Romo-Romo, A., Roustit, M., Briand, L., Meyer, G., Almeda-Valdes, P. and Walther, G.** (2021). Sucralose and Cardiometabolic Health: Current Understanding from Receptors to Clinical Investigations. *Adv Nutr* **12**, 1500–1513.
- Roberts, A., Renwick, A. G., Sims, J. and Snodin, D. J.** (2000). Sucralose metabolism and pharmacokinetics in man. *Food Chem Toxicol* **38 Suppl 2**, S31-41.
- Romo-Romo, A., Aguilar-Salinas, C. A., Brito-Córdova, G. X., Gómez-Díaz, R. A. and Almeda-Valdes, P.** (2018). Sucralose decreases insulin sensitivity in healthy subjects: a randomized controlled trial. *The American Journal of Clinical Nutrition* **108**, 485–491.
- Sclafani, A.** (2007). Sweet taste signaling in the gut. *Proceedings of the National Academy of Sciences* **104**, 14887–14888.
- Shil, A., Olusanya, O., Ghufoor, Z., Forson, B., Marks, J. and Chichger, H.** (2020). Artificial Sweeteners Disrupt Tight Junctions and Barrier Function in the Intestinal Epithelium through Activation of the Sweet Taste Receptor, T1R3. *Nutrients* **12**, 1862.
- Sims, J., Roberts, A., Daniel, J. W. and Renwick, A. G.** (2000). The metabolic fate of sucralose in rats. *Food Chem Toxicol* **38 Suppl 2**, S115-121.
- Suez, J., Korem, T., Zeevi, D., Zilberman-Schapira, G., Thaiss, C. A., Maza, O., Israeli, D., Zmora, N., Gilad, S., Weinberger, A., et al.** (2014). Artificial sweeteners induce glucose intolerance by altering the gut microbiota. *Nature* **514**, 181–186.

- Sutherland, K., Young, R. L., Cooper, N. J., Horowitz, M. and Blackshaw, L. A.** (2007). Phenotypic characterization of taste cells of the mouse small intestine. *Am J Physiol Gastrointest Liver Physiol* **292**, G1420-1428.
- Sylvetsky, A. C. and Rother, K. I.** (2016). Trends in the consumption of low-calorie sweeteners. *Physiology & Behavior* **164**, 446–450.
- Sylvetsky, A. C., Bauman, V., Blau, J. E., Garraffo, H. M., Walter, P. J. and Rother, K. I.** (2017). Plasma concentrations of sucralose in children and adults. *Toxicological and environmental chemistry* **99**, 535.
- Thomson, P., Santibañez, R., Aguirre, C., Galgani, J. E. and Garrido, D.** (2019). Short-term impact of sucralose consumption on the metabolic response and gut microbiome of healthy adults. *British Journal of Nutrition* **122**, 856–862.
- Valls, P. O. and Esposito, A.** (2022). Signalling dynamics, cell decisions, and homeostatic control in health and disease. *Current Opinion in Cell Biology* **75**, 102066.
- van Eyk, A. D.** (2015). The effect of five artificial sweeteners on Caco-2, HT-29 and HEK-293 cells. *Drug Chem Toxicol* **38**, 318–327.
- Wang, Q.-P., Browman, D., Herzog, H. and Neely, G. G.** (2018). Non-nutritive sweeteners possess a bacteriostatic effect and alter gut microbiota in mice. *PLOS ONE* **13**, e0199080.
- Wang, P., Felsing, D. E., Chen, H., Stutz, S. J., Murphy, R. E., Cunningham, K. A., Allen, J. A. and Zhou, J.** (2020). Discovery of Potent and Brain-Penetrant GPR52 Agonist that Suppresses Psychostimulant Behavior. *Journal of Medicinal Chemistry*.
- Warren, C., Hobin, E., Manuel, D. G., Anderson, L. N., Hammond, D., Jessri, M., Arcand, J., L'Abbé, M., Li, Y., Rosella, L. C., et al.** (2022). Socioeconomic position and consumption of sugary drinks, sugar-sweetened beverages and 100% juice among Canadians: a cross-sectional analysis of the 2015 Canadian Community Health Survey–Nutrition. *Can J Public Health* **113**, 341–362.
- Weihrauch, M. and Diehl, V.** (2004). Artificial sweeteners—do they bear a carcinogenic risk? *Annals of Oncology* **15**, 1460–1465.
- Whitehouse, C. R., Boullata, J. and McCauley, L. A.** (2008). The potential toxicity of artificial sweeteners. *AAOHN J* **56**, 251–259; quiz 260–261.
- Wood, S. G., John, B. A. and Hawkins, D. R.** (2000). The pharmacokinetics and metabolism of sucralose in the dog. *Food Chem Toxicol* **38 Suppl 2**, S99-106.
- Wu, H.-J. and Wu, E.** (2012). The role of gut microbiota in immune homeostasis and autoimmunity. *Gut Microbes* **3**, 4–14.

**Wu, H.-T., Lin, C.-H., Pai, H.-L., Chen, Y.-C., Cheng, K.-P., Kuo, H.-Y., Li, C.-H. and Ou, H.-Y.** (2022). Sucralose, a Non-nutritive Artificial Sweetener Exacerbates High Fat Diet-Induced Hepatic Steatosis Through Taste Receptor Type 1 Member 3. *Front Nutr* **9**, 823723.