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Impact of Non-Nutritive Sweeteners on Placental Function: An *In Vitro* Study Using HTR-8/SVneo Cells

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Impact of Non-Nutritive Sweeteners on Placental Function: An *In Vitro* Study Using HTR-8/SVneo Cells

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Summary

The consumption of non-nutritive sweeteners (NNSs) has been increasing over the years, with the promise of sweetening foods while keeping them sugar-free. They have become an alternative to traditional sugar, and pregnant women consume as much or even more NNSs than the general population. Although these compounds are generally considered safe within the established acceptable daily intake limits, concerns remain regarding their potential effects on fetal development, particularly because some NNSs have been detected in maternal and fetal compartments.

During the first weeks of pregnancy, the placenta is formed through a tightly regulated process known as placentation, in which extravillous trophoblasts (EVTs) migrate and invade the maternal decidua, contributing to spiral artery remodelling and an adequate supply of oxygen and nutrients to the fetus. Alterations in the function of these cells may compromise placental development and potentially affect pregnancy outcome.

Accordingly, this research aimed to determine whether three NNSs (acesulfame-K, saccharin and sucralose) could compromise the behaviour of HTR-8/SVneo cells, an *in vitro* cell model of first-trimester extravillous trophoblasts. For this, cells were exposed for 24 h to different concentrations of each NNSs, selected to approximate values reported in the literature for attainable plasma levels in humans. Mitochondrial metabolic activity, cell membrane integrity, proliferation, migration, oxidative stress, nutrient (glucose and methionine) and MPP^+ (an organic cation) cellular uptake, and cell cycle distribution were evaluated using MTT, LDH release, ^{3}H-thymidine incorporation, wound healing, TBARS, ^{3}H-deoxi-glucose, L-^{14}C-methionine and ^{3}H-MPP^+ uptake, and flow cytometry assays, respectively.

Results indicated that while metabolic activity was generally unafected (MTT assay), some concentrations of NNSs induced membrane damage, particularly sucralose, as shown by increased LDH release. TBARS analysis demonstrated that sucralose increased and acesulfame-K and saccharin reduced MDA levels at selected concentrations. Cell migration was significantly reduced by saccharin and sucralose at the highest concentrations, whereas acesulfame-K had no significant effect. DNA synthesis was differentially affected, with sucralose increasing and saccharin reducing ^{3}H-thymidine incorporation at higher concentrations. These NNSs also slightly altered ^{3}H-deoxi-glucose and L-^{14}C-methionine uptake, showing weaker effects than classical inhibitors of transporters of these compounds

(BAY-876 and phloridzin for ^{3}H-deoxi-glucose, L-arginine and phenylalanine for L-^{14}C-methionine). Similarly, ^{3}H-MPP^{+} uptake was not significantly affected by NNS exposure, while cell cycle analysis revealed only small changes in specific phases, without a consistent redistribution indicative of a clear change in cell proliferation. To conclude, these findings suggest that NNSs modulate multiple aspects of HTR-8/SVneo cell function and homeostasis, with the most consistent effects observed on membrane integrity and migratory capacity, while effects on proliferation, oxidative status and substrate uptake were more variable and compound-dependent.

Keywords: Edulcorants; Extravillous trophoblasts; Placentation; Maternal-fetal interface

Resumo

O consumo de adoçantes não nutritivos (NNSs) tem vindo a aumentar ao longo dos anos, com a promessa de adoçar os alimentos mantendo-os isentos de açúcares. Estes compostos tornaram-se uma alternativa ao açúcar tradicional, e as mulheres grávidas consomem quantidades semelhantes ou até superiores de NNSs relativamente à população em geral. Embora estes compostos sejam geralmente considerados seguros dentro dos limites estabelecidos de ingestão diária admissível, subsistem preocupações quanto aos seus potenciais efeitos durante o desenvolvimento fetal, particularmente porque alguns NNSs foram detetados nos compartimentos maternos e fetais.

Durante as primeiras semanas de gravidez, a placenta forma-se através de um processo rigorosamente regulado conhecido como placentação, no qual os trofoblastos extravilositários (EVTs) migram e invadem a decídua materna, contribuindo para a remodelação das artérias espiraladas e para um aporte adequado de oxigénio e nutrientes ao feto. Por conseguinte, alterações no comportamento destas células podem comprometer o desenvolvimento placentário e afetar potencialmente o desfecho da gravidez.

Assim, esta investigação teve como objetivo determinar se três NNSs (o acessulfame-K, a sacarina e a sucralose) poderiam comprometer o comportamento das células HTR-8/SVneo, um modelo *in vitro* de trofoblastos extravilositários do primeiro trimestre. Para tal, as células foram expostas durante 24 h a diferentes concentrações de cada NNS, selecionadas de modo a aproximarem-se das concentrações plasmáticas alcançáveis em humanos. A atividade metabólica mitocondrial, a integridade da membrana plasmática, a proliferação, a migração, o stress oxidativo, a captação celular de nutrientes (glicose e metionina) e de MPP^+ (um catião orgânico), e a distribuição do ciclo celular foram avaliadas através de ensaios de MTT, libertação de LDH, incorporação de ^{3}H-timidina, *wound healing*, TBARS, captação de ^{3}H-desoxi-glucose, L-^{14}C- metionina e ^{3}H-MPP^+, e citometria de fluxo, respetivamente.

Os resultados indicaram que, embora a atividade metabólica se mantivesse globalmente estável (ensaio MTT), algumas concentrações dos NNSs, particularmente de sucralose, induziram danos na membrana celular, conforme demonstrado pelo aumento da libertação de LDH. A análise de TBARS revelou que a sucralose aumentou e o

acessulfame-K e a sacarina reduziram os níveis de MDA em concentrações específicas. A migração celular foi significativamente reduzida pela sacarina e pela sucralose nas concentrações mais elevadas, enquanto o acessulfame-K não teve qualquer efeito. A incorporação de ^{3}H-timidina foi afetada de forma distinta pelos diferentes NNSs, aumentando com a sucralose e diminuindo com a sacarina nas concentrações mais elevadas. Além disso, foram observadas apenas alterações pontuais na captação de ^{3}H-desoxi-glucose e L-^{14}C-metionina após a exposição aos NNSs, sem um padrão consistente e com efeitos menos pronunciados do que os induzidos pelos inibidores clássicos dos transportadores desses compostos (BAY-876 e floridzina para a ^{3}H-desoxi-glucose e a L-arginina e L-fenilalanina para a L-^{14}C-metionina). De forma semelhante, a captação de ^{3}H-MPP^{+} não foi significativamente afetada pela exposição aos NNSs, enquanto a análise do ciclo celular revelou apenas pequenas alterações em fases específicas, sem uma redistribuição consistente indicativa de uma alteração clara na proliferação celular. Em conclusão, estes resultados sugerem que os NNSs estudados modulam múltiplos aspetos da função das células HTR-8/SVneo, tendo os efeitos mais consistentes sido observados ao nível da integridade da membrana e da capacidade migratória, enquanto os efeitos na proliferação, no estado oxidativo e na captação de substratos foram mais variáveis e dependentes do composto.

Palavras-chave: Edulcorantes; Trofoblastos extravilositários; Placentação; Interface materno-fetal

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Abbreviations

ADI - Acceptable Daily Intake

ADME – Absorption, distribution, metabolism and excretion

ANOVA - Analysis of Variance

AS – Artificial Sweetener

BMI – Body Mass Index

BSA – Bovine Serum Albumin

CAT – Catalase

DMSO – Dimethyl sulfoxide

DNA – Deoxyribonucleic Acid

DOHaD – Developmental Origins of Health and Disease

eEVTs – Endovascular extravillous trophoblasts

EFSA – European Food Safety Authority

EVTs – Extravillous Trophoblasts

FBS – Fetal Bovine Serum

FDA – Food and Drug Administration

GLUT – Glucose Transporter

GLUT1 – Glucose Transporter 1

GPx – Glutathione Peroxidase

GSH – Glutathione

^{3}H-DG – 2-deoxy-D-[^{3}H]glucose

HTR-8/SVneo – Human first-trimester extravillous trophoblast cell line

HPLC – High-performance liquid chromatography

iEVTs – Interstitial extravillous trophoblasts

IL-1β – Interleukin-1 beta

IL-6 – Interleukin-6

LAT – L-type Amino Acid Transporter

LAT1 – L-type Amino Acid Transporter 1

LAT2 – L-type Amino Acid Transporter 2

L-[^{14}C]Met – L-[^{14}C]methionine

LDH – Lactate dehydrogenase

MDA – Malondialdehyde

miRNA – MicroRNA

MPP^+ – 1-methyl-4-phenylpyridinium

mTOR – Mechanistic Target Of Rapamycin

MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NAD^+ – Oxidized Nicotinamide Adenine Dinucleotide

NADH – Reduced Nicotinamide Adenine Dinucleotide

NF-κB – Nuclear Factor Kappa B

NLRP3 – NLR Family Pyrin Domain Containing 3

NNS – Non-Nutritive Sweeteners

OCTs – Organic Cation Transporters

8-OHdG – 8-Hydroxy-2'-deoxyguanosine

PBS – Phosphate-Buffered Saline

PI – Propidium Iodide

PMAT – Plasma Membrane Monoamine Transporter

PPARGC1A – Peroxisome Proliferator-activated Receptor Gamma Coactivator 1-Alpha

ROS – Reactive Oxygen Species

RPMI – Roswell Park Memorial Institute medium

SERT – Serotonin Transporter

SEM – Standard Error of the Mean

SGLT – Sodium-Glucose Cotransporter

SGLT1 – Sodium-Glucose Cotransporter 1

SGLT2 – Sodium-Glucose Cotransporter 2

SOD – Superoxide Dismutase

SV40 – Simian virus 40

TBARS – Thiobarbituric Acid Reactive Substances

TCA – Trichloroacetic Acid

TGs – Triglycerides

TNF-α – Tumour Necrosis Factor Alpha

WHO – World Health Organisation

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Table 1 - Acceptable daily intake (ADI) values established by the European Food Safety Authority (EFSA) and the U.S. Food and Drug Administration (FDA), for selected non-nutritive sweeteners (NNSs). Adapted from EFSA and FDA [6,8] 8

Introduction

1. Non-nutritive Sweeteners in Modern Diets

The emergence of healthier lifestyle trends, including structured training plans, diverse dietary patterns, and an increased focus on mental health have become progressively more common in modern society [1]. This shift is driven not only by a greater awareness of health benefits, but also by the increasing influence of social media and the widespread dissemination of information through digital platforms [1]. Considering how quickly individuals rely on these platforms to choose the information they apply to their daily lives, it becomes essential to understand whether these so-called trends represent opportunities for positive change or, conversely, may mislead more vulnerable populations [2].

The increasing worldwide prevalence of obesity and type 2 diabetes has contributed to a growing public interest regarding excessive sugar consumption and its associated metabolic consequences. As a result, dietary trends have promoted the replacement of sugar with alternative sweeteners [3]. These sweeteners are currently widely present in our daily diet, due to their appeal as low-calorie alternatives to sugar and their widespread inclusion in products marketed as healthier or low-calorie options [4].

1.1 Overview and classification of sweeteners

Sweeteners may be classified as nutritive or non-nutritive, according to their caloric contribution [5]. The nutritive sweetener group, that provides energy, includes sugars and sugar-alcohols, whereas non-nutritive sweeteners (NNSs) provide intense sweetness with little or no caloric contribution, making them attractive alternatives for reducing caloric intake [5]. Within non-nutritive sweeteners, these compounds may be classified according to their origin as either artificial sweeteners (AS), when chemically synthesized in the laboratory, or natural sweeteners (NS), when derived directly from plant sources [5]. Artificial sweeteners include compounds such as aspartame, saccharin, sucralose, neotame, acesulfame-K, and advantame. In contrast, natural sweeteners are mainly represented by stevia, obtained from extracts of the plant *Stevia rebaudiana*, and monk fruit (Luo Han Guo) [5].

The sweetening intensity of these compounds, when compared to table sugar (sucrose), varies considerably, with advantame and neotame being thousands of times sweeter than sucrose, whereas saccharin, sucralose, acesulfame-K and aspartame exhibit lower sweetening intensities [6].

1.2 Regulatory Perspectives

NNSs are commonly found in a variety of foods available on the market, such as non-alcoholic beverages (including diet sodas and flavoured waters), yogurts, jams, and nutritional supplements [7]. The U.S. Food and Drug Administration (FDA) and the European Food Safety Authority (EFSA) have already set acceptable daily intake (ADI) levels based on animal and human studies, as described in Table 1 [6,8]. Of note, these limits vary slightly between regulatory agencies due to differences in evaluation approaches [6,8].

Table 1 - Acceptable daily intake (ADI) values established by the European Food Safety Authority (EFSA) and the U.S. Food and Drug Administration (FDA), for selected non-nutritive sweeteners (NNSs). Adapted from EFSA and FDA [6,8].

Non-Nutritive Sweetener (NNS)	ADI (mg/kg bw/day) EFSA	ADI (mg/kg bw/day) FDA
Aspartame	40	50
Acesulfame-K	15	15
Sucralose	15	5
Saccharin	9	15
Neotame	10	0.3
Advantame	5	32.8

However, in response to the increasing concerns regarding the long-term metabolic effects of NNSs, the World Health Organization (WHO) published in 2023 guidelines advising against the use of these compounds for weight control purposes in the general population [9]. These recommendations reflect the growing scientific debate regarding the safety of these compounds and emphasize the importance of further mechanistic and long-term studies [9].

2. The Placenta and Fetal Programming

The human life starts with fertilization and involves a series of coordinated events that ultimately lead to the placenta formation and fetal growth. The placenta is a transient, but vital organ for fetal development, allowing the transport of nutrients and other molecules to the fetus, through its chorionic villi [10]. This organ has several other important functions during pregnancy, including hormone production, immune protection, and waste elimination. Therefore, an adequate placental development is essential for a healthy pregnancy [10].

2.1 Placental development and trophoblast differentiation

During early placental development, the blastocyst implants into the endometrium. Its outermost layer, called the trophectoderm, differentiates into trophoblast cells, which subsequently form distinct trophoblast populations [11]. During this period, the placenta develops in a low-oxygen environment, and the placental villi are lined by an inner layer of cytotrophoblasts and an outer layer of syncytiotrophoblasts, which constitute the primary interface for exchange between the mother and the fetus [12]. Cytotrophoblasts are highly proliferative cells that serve as a source for further differentiation, including the formation of syncytiotrophoblasts through cell fusion [13]. In contrast, syncytiotrophoblasts exhibit limited proliferative capacity and protect the fetus from the maternal immune response and act as a nutrient and metabolite exchange barrier [14]. Syncytiotrophoblasts are also responsible for the synthesis of several proteins, including human chorionic gonadotropin, somatomammotropin and placental growth hormone, as well as steroid hormones such as progesterone and estrogens [15].

Additionally, cytotrophoblasts can differentiate into extravillous trophoblasts (EVTs), which acquire an invasive phenotype and migrate into the maternal decidua, contributing to the remodelling of uterine spiral arteries [16]. This process converts the spiral arteries into low-resistance, high-capacitance vessels, capable of ensuring adequate maternal blood flow and oxygen delivery to the developing fetus [16]. Figure 1 presents a schematic representation of the early stages of placentation and EVT invasion. Furthermore, EVTs invade the uterine glands, establishing connections with the intervillous space and supporting fetal nutritional demands during first trimester

[17]. EVTs can be divided into two main phenotypes: interstitial EVTs (iEVTs), which invade the decidua after detaching from the placental villi, and endovascular EVTs (eEVTs), that directly participate in spiral artery remodelling [18,19].

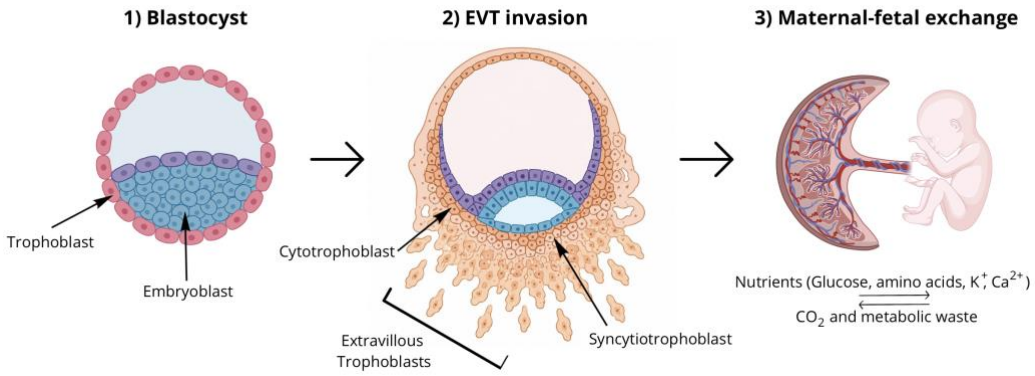


Figure 1 - Simplified schematic representation of early placentation and maternal–fetal exchange during pregnancy. Created using BioRender.com based on concepts described in [19].

The first trimester of fetal development is critical for both the mother and the fetus, as fertilization, implantation and organogenesis occur during this period [20]. All major organs begin to form at this stage, making it a highly sensitive phase to disruptions caused by nutritional deficiencies, toxins or hormonal imbalances [21]. This stage is also associated with an increased risk of pregnancy complications, as impaired trophoblast invasion and abnormal placentation have been linked to disorders such as preeclampsia, fetal growth restriction, and preterm birth [22].

2.2 Placentał functions and maternal-fetal nutrient exchange

Besides its endocrine and immunological functions, one of the most important roles of the placenta is to act as the main interface between the maternal and fetal circulations, being therefore involved in gas, nutrients and metabolic waste exchange between the mother and the fetus. Placental nutrient transport depends on several factors, including placental size, morphology, nutrient transporter availability and capacity, as well as uteroplacental and fetoplacental blood flow [23].

To sustain appropriate fetal development, the maternal diet must provide adequate nutrients such as glucose, amino acids and fatty acids, that are transported across the placenta to the fetus [23]. Glucose is the main placental-fetal unit energy substrate, being required for fetal and placental growth and is transported through facilitated diffusion [24]. Although the GLUT family comprises 14 members, only GLUT-1 is

expressed abundantly from early pregnancy to term, representing the main placental glucose transporter in humans [25,26]. Amino acids are also very important for fetal tissue development, participating in protein synthesis, nutrient sensing, oxidative stress regulation, and cell signalling pathways [23]. They are transported across the placenta against a fetal-maternal concentration gradient, requiring the use of active transport mechanisms mediated by multiple amino acid transporters [23]. Among them, several amino acids, including glutamine, glutamate, arginine, leucine, tryptophan, and methionine, are involved in various metabolic and regulatory pathways during pregnancy [27]. For instance, arginine serves as a substrate for nitric oxide and polyamine synthesis, contributing to placental angiogenesis and fetal development, while leucine activates mTOR signalling to promote protein synthesis and cell growth [28,29]. Tryptophan is involved in immunomodulatory pathways through metabolites such as kynurenine and serotonin, and methionine has an important role in protein synthesis and methylation-related processes [28,29]. Additionally, fatty acids are also fundamental for fetal growth, particularly for brain development and adipose tissue accumulation [30]. In the maternal circulation, these fatty acids are mainly found in triglycerides (TGs), phospholipids and cholesterol esters [23]. In addition to nutrient transport, this organ also expresses transport systems involved in the transfer of xenobiotics, including organic cations transporters (OCTs), that may affect fetal exposure to therapeutic drugs and other bioactive compounds [31].

2.3 Fetal programming and developmental origins of disease

Emerging evidence highlights a strong relationship between prenatal maternal nutrition and the developmental programming of chronic diseases in offspring [32]. This concept, known as fetal programming or the Developmental Origins of Health and Disease (DOHaD), was first proposed by Barker and colleagues, and suggests that the intrauterine environment may influence the long-term health of the offspring, through metabolic and physiological adaptations [33]. As one of the most important regulators of the intrauterine environment, the placenta is fundamental in this process. Therefore, this organ is no longer seen solely as a passive barrier to molecular exchange between the mother and the fetus, but is now recognised as an active participant in fetal programming [34]. Acting as the primary fetal-maternal interface, the placenta can respond to variations in nutrient supply, blood flow, oxygen tension, and endocrine

signals [34]. In this way, this organ can translate the maternal environment into developmental responses that may have lasting consequences for the child health [35].

A number of factors have been reported to influence fetal programming, and maternal nutritional status is recognized as one of its main determinants [36]. Both undernutrition and overnutrition during pregnancy may disrupt maternal metabolic homeostasis and alter placental nutrient supply to the fetus, thus affecting fetal nutrient availability [36]. These changes can contribute to excessive fetal growth, hypoglycaemia, fetal fat accretion, and fetal macrosomia [36]. Moreover, these changes may increase susceptibility to obesity, insulin resistance, type 2 diabetes, and cardiovascular disease later in life [37]. For instance, studies in genetically obesity-prone rats demonstrated that maternal overnutrition was associated with increased postnatal adiposity in the offspring compared with offspring from normally nourished dams [38]. Gestational diabetes mellitus is another important example of an altered intrauterine metabolic environment [39]. In this condition, the fetus may be exposed to abnormal concentrations of glucose, lipids, and hormones, that can influence developmental adaptations during fetal life [40]. Particularly, maternal diabetes may affect fetal pancreatic development and insulin production, leading to alterations in β-cell mass and glucose homeostasis that may persist beyond birth [40]. Simultaneously, psychological and environmental factors have also been implicated in fetal programming [41]. Among these factors, stress, infections, exposure to environmental contaminants and endocrine-disrupting chemicals, may contribute to adverse metabolic phenotypes in the offspring [41]. For example, animal studies have shown that exposure to chronic stress during gestation may increase the offspring susceptibility to adiposity, insulin resistance, and other metabolic disturbances [41]. A variety of mechanisms are known to underlie fetal programming, including alterations in placental nutrient transport, endocrine signalling, oxidative stress, inflammation, and epigenetic regulation [42]. Among these mechanisms, epigenetic regulation has received particular attention due to its ability to induce long-term changes in gene expression without altering the DNA sequence [43]. Epigenetic mechanisms include DNA methylation, histone modifications, chromatin remodelling, and microRNA expression [44]. For example, maternal pre-pregnancy BMI has been associated with altered methylation of the *PPARGC1A* promoter in umbilical cord tissue [44]. Given the role of this gene in

mitochondrial metabolism, these epigenetic modifications may influence the metabolic health of the mother and baby [44].

3. Biological Effects of Non-Nutritive Sweeteners

During pregnancy, exposure to dietary compounds, including NNSs, may have significant implications for both maternal and fetal health. Therefore, understanding patterns of NNS consumption and their potential effects throughout this period is fundamental [45]. Among NNSs, artificial compounds such as acesulfame-K, saccharin, and sucralose, have gained increasing attention due to their widespread use in our diet (Figure 2) [46].

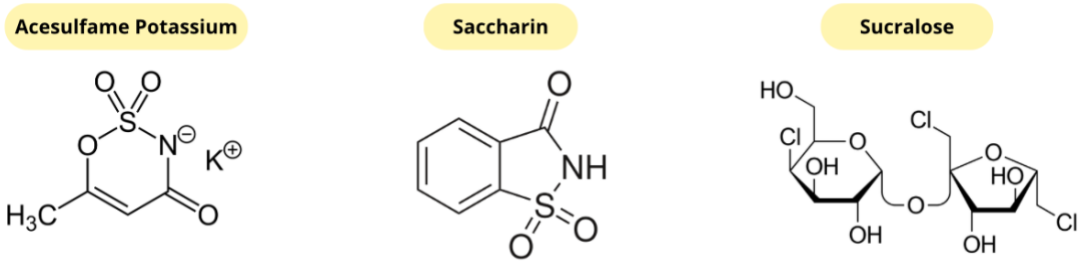


Figure 2 - Chemical structures of the non-nutritive sweeteners (NNSs) investigated in this study: acesulfame-K, saccharin, and sucralose, respectively. Source: Sigma-Aldrich (Merck).

These three artificial NNSs are characterized by a high sweetening power and stability over a wide range of pH and temperature conditions, making them suitable for industrial food production. In addition, these compounds exhibit favourable physico-chemical properties, being generally colourless, odourless, and highly soluble in water [6].

Acesulfame-K is a high-intensity sweetener, approximately 200 times sweeter than sucrose [6]. This compound is frequently utilized, due to its high thermal stability, often in combination with other sweeteners to improve flavour profiles and sweetness perception [47]. Saccharin, discovered in 1879, is one of the oldest and most widely used NNSs [48]. It is approximately 200–700 times sweeter than sucrose and is commercially available mainly as sodium, calcium, and ammonium salts [6,49]. Despite their high sweetening capacity, saccharin and acesulfame-K are recognized for having a bitter or metallic aftertaste at elevated concentrations [50]. Sucralose is produced by the selective chlorination of sucrose, replacing three hydroxyl groups by

chlorine atoms [51]. It exhibits a sweetening power approximately 600 times greater than sucrose and is characterized by high thermal and chemical stability [6,52].

3.1 NNS Consumption by pregnant women

Recent studies have shown that pregnant women consume as much or even more NNSs than the general population, with sucralose, acesulfame-K, and saccharin being among the most ingested ones [53]. The majority of pregnant women do not exceed the ADI of NNSs, but unintentional consumption is widespread, especially in countries with high NNS prevalence in foods [54]. Different cohort studies and meta-analyses have been performed, indicating that consumption of beverages, dairy and candy products are the main sources of NNSs during pregnancy (Figure 3) [55].



Figure 3 - Common dietary sources of NNSs and maternal exposure during pregnancy. Created with Biorender.com.

The metabolic fate and ADME profile of NNSs vary according to their physico-chemical properties, and these compounds indeed differ considerably in their absorption, metabolism, and excretion profiles [56,57]. As summarized in Figure 4, the pharmacokinetic profile of acesulfame-K, saccharin and sucralose following maternal ingestion differs among them. Namely, acesulfame-K is rapidly absorbed (99%) and almost completely excreted unchanged in the urine, with less than 1% eliminated in the feces and no evidence of accumulation in the body [56]. Similarly, approximately 85–95% of ingested saccharin enters the systemic circulation before being primarily eliminated through urine, while the remaining fraction (5-15%) is excreted in feces [56]. In contrast, sucralose is poorly absorbed, with only 10% of the ingested dose reaching the bloodstream [56]. The majority of this compound is subsequently excreted unchanged in feces [56]. Thus, after absorption, acesulfame-K and saccharin remain essentially unchanged, whereas sucralose undergoes minimal degradation, as evidenced by the detection of two small unidentified metabolites via high-performance liquid chromatography (HPLC) [56,58].

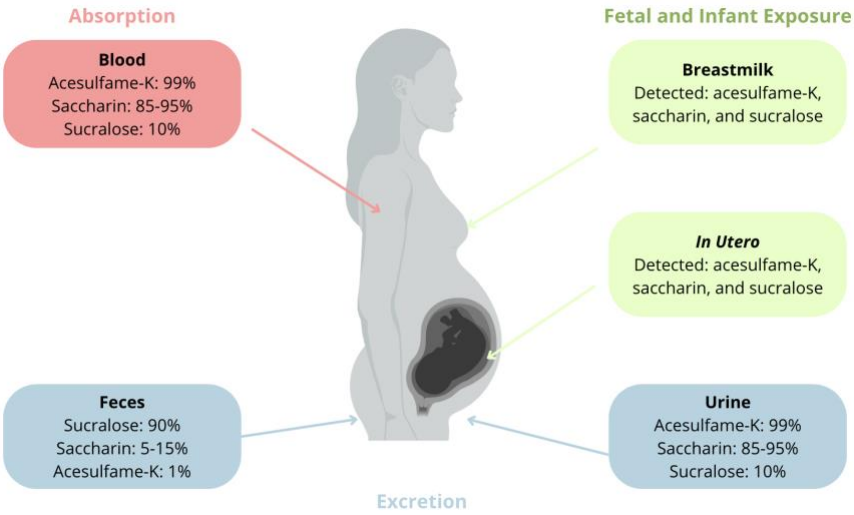


Figure 4 - Overview of the absorption, excretion, and distribution of NNSs during pregnancy. The percentages shown for blood, urine, and feces refer to the rates of absorption and excretion of each NNSs, while for breast milk and in utero, the results are presented qualitatively (as detected). Adapted from [56].

Once present in the maternal circulation, these compounds may reach the placenta and subsequently be transferred to the fetus. As also illustrated in Figure 4, previous experimental and clinical studies have reported the detection of sucralose, acesulfame-K, and saccharin in breast milk and *in utero* [56,59]. Moreover, these NNSs have been detected in various fetal/newborn-related biological samples, including blood cord, amniotic fluid, newborn urine, and fetal bladder [60,61].

Although these findings demonstrate fetal exposure to these NNSs, they do not quantify the extent of placental transfer. More recently, Leth-Møller et al. directly quantified maternal–fetal transfer of these NNSs by measuring paired maternal and fetal plasma concentrations [60]. A positive correlation between these concentrations was observed, confirming that placental transfer occurs and suggesting that passive diffusion may contribute to this process [60]. However, the data regarding the exact extent of this transfer appears to differ among NNSs. Specifically, fetal plasma concentrations reached approximately 80% of maternal levels for acesulfame-K, 65% for saccharin, and 57% for sucralose [60].Together, these findings suggest that exposure to maternally consumed NNSs may begin *in utero* and persist into early postnatal life, supporting the hypothesis that these compounds may influence developmental processes during critical windows of metabolic programming [62].

3.2 Metabolic and cellular effects of NNSs

NNSs were introduced into the human diet as low-calorie alternatives to sugar and were initially perceived as metabolically inert compounds [63]. However, it has been increasingly reported that these compounds may interfere with various metabolic pathways [63]. In the context of pregnancy, NNSs are often consumed as sugar substitutes to help control caloric intake and reduce the risk of excessive gestational weight gain and gestational diabetes mellitus, particularly due to the awareness regarding the adverse effects associated with sugar intake [64]. Nevertheless, in a prospective cohort study, daily intake of NNSs during pregnancy was associated with increased risk of large-for-gestational-age infants and higher BMI z-scores during childhood, pointing to a role of NNS exposure in fetal metabolic programming [65].

Recently, the effects of NNSs on the gut microbiota have attracted attention due to their possible impact on insulin resistance, obesity, and inflammation [66]. Several studies have suggested that the gut microbiota may mediate some of the physiological effects of NNSs, emphasizing the need to clarify their interactions with the host and the microbiota [67]. Moreover, these compounds are also known to interact with sweet-taste receptors located not only in the oral cavity, but also throughout the gastrointestinal tract, and the activation of these receptors may influence hormone secretion, glucose absorption, and signalling pathways involved in energy homeostasis [68]. Even so, it remains unclear whether these NNSs could in any way interfere with other targets (e.g. receptors) in the placenta [69].

Oxidative stress is also an important theme to consider when investigating the metabolic effects associated with NNSs. Recent literature has demonstrated that these compounds may interfere with cellular redox homeostasis [70]. For instance, studies performed in animal models, as well as in human cell lines such as microglial and adipose-derived stromal cells, have shown that the presence of NNSs may increase oxidative stress markers, including reactive oxygen species (ROS), malondialdehyde (MDA), and 8-hydroxydeoxyguanosine (8-OHdG) [70–73]. Furthermore, the antioxidant defence mechanisms, involving molecules such as glutathione (GSH), glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase (CAT) have also been reported to be altered under these conditions [70].

In addition, prolonged exposure to NNSs may also be associated with an increased expression of inflammatory mediators, including TNF-α, IL-1β and IL-6, as well as with the activation of NF-κB and NLRP3-related pathways [72,74]. Since oxidative stress and inflammatory pathways are intimately interconnected processes, these alterations may contribute to impaired cellular function and viability [75]. Nevertheless, it remains unclear whether there may be any effects of NNSs on placental and trophoblast biology, as most of the available evidence derives from animal and non-placental *in vitro* cell models.

3.3 Effects of NNSs on placental and trophoblast biology

As previously discussed, NNSs have been associated with changes in glucose homeostasis, gut microbiota composition, sweet taste receptor activation and the potential induction of oxidative stress and inflammatory pathways, presumably contributing to metabolic disorders [68,70,74,76,77]. When consumed during pregnancy, they have also been linked with structural and functional changes in the placenta [46,78]. Specifically, experimental studies in animal models have shown that maternal exposure to aspartame can induce structural alterations in placental tissue, including changes in the basal and labyrinth zones, increase oxidative stress markers, and impair mitochondrial function and biogenesis within the organ [46,78]. These observations show that NNSs may interfere with placental homeostasis, likely affecting nutrient exchange and fetal growth [46,78].

Despite the accumulating evidence, there's still little information about the present and long-term effects of their consumption and their direct impact on human EVTs, which are fundamental cells during the first trimester of pregnancy. Nevertheless, a previous study, using the HTR-8/SVneo first-trimester EVT cell line, investigated the effects of aspartame and its metabolites (L-aspartic acid, L-phenylalanine, and methanol) on placentation-related characteristics of EVTs, and demonstrated that aspartame exposure could interfere with cell proliferation, glucose uptake, metabolic activity, and cell cycle progression, while also increasing oxidative stress [79]. These findings suggested that this NNSs may interfere with EVTs function and consequently affect pregnancy outcomes [79]. Since these processes are essential for normal placentation, it is important to further explore other widely consumed NNSs, such as saccharin, sucralose, and acesulfame-K, to understand if they could also affect EVTs [80,81].

To summarize, there are still several knowledge gaps regarding the consequences of NNSs consumption during pregnancy, and specifically on their effects on the placenta, that need to be addressed. Importantly, most available evidence is based on observational and animal studies, and information regarding long-term effects and dose-response relationships is still limited.

Aims

Considering the growing interest in the consumption of NNSs by pregnant women and the increasing evidence regarding their effects on several physiological and cellular processes, it is important to investigate their direct impact on early placental cells. Since EVTs actively contribute to the placentation process, changes in their function will influence fetal development, in line with the concept of fetal programming [11]. Therefore, understanding the effects of NNSs on these cells is important to clarify the safety of their consumption during pregnancy and provide further scientific evidence on this topic [60].

Thus, this project aimed to study the effects of three distinct NNSs (acesulfame-K, saccharin and sucralose) in an EVT cell line, by evaluating their impact on characteristic features such as proliferation, viability, migration, cell cycle distribution, oxidative stress levels and nutrient uptake capacity. The obtained results provide insights on their potential ability to compromise the placentation process and, consequently, fetal development.

Materials and Methods

1. Materials

BAY-876; Bradford reagent; Desipramine hydrochloride; Dimethyl sulfoxide (DMSO); Dipotassium phosphate (K_2HPO_4); Fluoxetine hydrochloride; L-arginine; L-phenylalanine; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT); N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES); Phloridzin; Phosphate-buffered saline (PBS); Reduced nicotinamide adenine dinucleotide (NADH); RPMI 1640 medium; Sodium hydroxide (NaOH); Sodium pyruvate (Sigma-Aldrich, St. Louis, MO, USA).

Penicillin/Streptomycin/Amphotericin B Mix; Fetal bovine serum (FBS), heat-inactivated; Trypsin–ethylenediaminetetraacetic acid (EDTA) solution (PAN-Biotech, Aidenbach, Germany).

Propidium iodide (PI)/RNase (Immunostep, Salamanca, Spain).

Acesulfame-K; Saccharin; Sucralose (TCI Europe, Zwijndrecht, Belgium).

Calcium chloride ($CaCl_2$); D-glucose; Disodium hydrogen phosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$); Hydrochloric acid (HCl); Magnesium chloride ($MgCl_2$); Magnesium sulfate ($MgSO_4$); 2-(N-morpholino)ethanesulfonic acid (MES); Potassium chloride (KCl); Potassium dihydrogen phosphate (KH_2PO_4); Sodium bicarbonate ($NaHCO_3$); Sodium chloride (NaCl); Thiobarbituric acid (TBA); Trichloroacetic acid (TCA); Triton X-100 (Merck, Darmstadt, Germany).

Bovine serum albumin (BSA) (NZYTech, Lisbon, Portugal).

^{3}H-thymidine ([methyl-^{3}H]-thymidine; specific activity 79 Ci/mmol) (GE Healthcare GmbH, Freiburg, Germany).

^{3}H-MPP$^+$ ([methyl-^{3}H]-1-methyl-4-phenylpyridinium; specific activity 80 Ci/mmol); D-[^{3}H]-deoxy-D-glucose ([1,2-^{3}H]-deoxy-D-glucose; specific activity 60 Ci/mmol); L-[^{14}C]-methionine (L-[^{14}C]-methionine; specific activity 55 mCi/mmol) (American Radiolabeled Chemicals Inc., St Louis, MO, USA). Scintillation liquid (PerkinElmer, Waltham, MA, USA).

2. Methods

2.1 Cell Culture

The human trophoblast cell line HTR-8/SVneo (American Type Culture Collection, ATCC CRL-3271) was used as an *in vitro* model of first-trimester extravillous trophoblasts. These cells were isolated from the placenta of a 6-to-12-week-old embryo and were immortalized by transfection with a plasmid encoding the simian virus 40 (SV40) large T antigen placenta. Cells were grown in RPMI 1640 medium supplemented with 5% fetal bovine serum (FBS) and 1 % (v/v) of antibiotic/antimycotic solution and were used between passage number 14 to 34. This culture medium was prepared from RPMI 1640 powder (Sigma R6504, containing 2 mM L-Glutamine, without $NaHCO_3$ and HEPES), supplemented with D-Glucose (25 mM), $NaHCO_3$ (17 mM), HEPES (10 mM), and sodium pyruvate (1 mM). The medium was prepared with Mili-Q water, adjusted to pH 7.2, and sterilized by filtration. The cells were cultured in 21 cm^2 plates (21 cm^2; diameter 60 mm; TPP®, Trasadingen, Switzerland) and incubated at 37°C. To maintain the appropriate pH, cells were kept in a humidified atmosphere containing 5% CO_2. The culture medium was changed every 3–4 days, and the culture was split every 7 days. For subculturing, cells were washed with phosphate-buffered saline (PBS) to wash away any residual medium and then were enzymatically removed (0.25% trypsin–EDTA, 4 min, 37°C). Afterwards, the detached cells were collected, re-suspended into fresh RPMI medium, and sub-cultured in plastic culture dishes (21 cm^2, TPP®).

For MTT and LDH experimental procedures, HTR-8/SVneo cells were cultured in 96-well plates (~0.34 cm^2, diameter 6.6 mm; TPP®) with a 1:6 split ratio and were used after 3 days. For the ^{3}H-thymidine incorporation, wound-healing and nutrient uptake assays, cells were seeded in 24-well plates (2 cm^2; diameter 16 mm; TPP®) with a 1:5 split ratio (for ^{3}H-thymidine incorporation and cellular uptake assays) or 1:6 split ratio (for wound-healing assay), and were used after 4 days (for ^{3}H-thymidine incorporation assay) or after 3 days (for the remaining assays). Finally, to evaluate lipid peroxidation, cells were seeded on 12-well plates (3.6 cm^2; diameter 22 mm; TPP®) with a 1:6 split ratio and were used after 3 days. For cell cycle analysis, cells were seeded in 21 cm^2 culture dishes with a 1:8 split ratio and were allowed to grow for 3 days prior to treatment.

2.2 Treatment of the cells

The NNSs used in the experiments were purchased from Tokyo Chemical Industry (TCI Europe, Zwijndrecht, Belgium) and were readily soluble in water. Stock solutions were prepared in sterile water prior to dilution in culture medium.

A range of five concentrations (1 µM, 10 µM, 100 µM, 1000 µM, and 5000 µM) was selected based on reported human plasma levels of these compounds, as well as previous *in vitro* studies investigating exposure to these NNSs. Plasma concentrations were measured following oral consumption of these NNSs (primarily through beverages) and ranged from 848.5–1548 ng/mL (~4.2–7.7 µM) for acesulfame-K, 45.6–134 ng/mL (~0.1–0.3 µM) for sucralose, and 101.8–1118 ng/mL (~0.4–1.5 µM) for saccharin [59,60,82,83]. On the other hand, a wide range of concentrations of these NNSs has been used in *in vitro* studies, from micromolar up to 10-50 mM [72,84,85]. The selected concentrations therefore include both physiologically relevant levels (low micromolar range) and higher concentrations, allowing the assessment of potential concentration-dependent effects. Acceptable Daily Intake (ADI) values established by the EFSA (9 mg/kg/day for saccharin, 15 mg/kg/day for sucralose and acesulfame-K) were also considered as general safety reference points, although they do not directly reflect circulating concentrations. During the experiments, the range of concentrations was progressively refined, with greater focus on physiologically relevant levels.

In the experimental design, cells were assigned to a control group (water, used as the solvent) or to treatment groups, being exposed to one of the NNSs at concentrations of 1, 10, 100, 1000, or 5000 µM. HTR-8/SVneo cells were seeded and allowed to adhere and reach the desired confluence for 3 or 4 days prior to treatment. In all the experiments, cells were exposed to each treatment (or solvent) for 24 h (chronic exposure), in serum-free culture medium. Each condition was tested in 2-3 technical replicates per experiment, and all experiments were independently repeated 3-4 times (biological replicates). In some of the assays, data were normalized to total protein content (Bradford method-see below).

2.3 MTT assay – Assessment of cell viability (metabolic activity)

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay is based on the ability of a yellow tetrazolium salt (MTT) to be reduced by metabolically active cells, forming purple formazan crystals. Therefore, this procedure evaluates cell viability by quantifying metabolically active cells. Following the 24 h of treatment, cells were incubated for 3 h at 37ºC with MTT solution (5 mg/mL) added to each well in 200 µL of FBS-free culture medium. After incubation, the resulting formazan crystals were dissolved in 200 µL DMSO. The absorbance was measured at 550 nm, and the background absorbance at 650 nm was subtracted. This allowed the determination of the absorbance of formazan crystals formed by MTT reduction via mitochondrial dehydrogenases, which is proportional to the number of viable cells.

2.4 LDH assay – Assessment of cell viability (membrane integrity)

When the plasma membrane is damaged, lactate dehydrogenase (LDH), an oxidoreductase enzyme, is released into the extracellular medium. This enzyme has the capacity to catalyse the conversion of pyruvate to lactate, coupled to the oxidation of NADH to NAD^+. The equation of this reaction is:

$$\text{Pyruvate} + \text{NADH} + H^+ \rightleftharpoons \text{Lactate} + NAD^+$$

Given that extracellular release of LDH is associated with cell death, cell membrane integrity was measured by quantifying LDH activity. The quantification was determined spectrophotometrically by measuring the decrease in absorbance of NADH during the reduction of pyruvate to lactate. At the end of the 24 h treatment period, 50 µL of extracellular medium were mixed with 1.5 mL of phosphate/pyruvate buffer (50 mM phosphate buffer, 0.63 mM sodium pyruvate, pH 7.5) and 25 µL of NADH solution (11.3 mM), and absorbance values were monitored at 340 nm for 2 min. Total LDH activity was measured after cell lysis with 500 µL of 0.1% (v/v) Triton X-100 for 60 min at 37ºC. Finally, LDH levels released into the extracellular medium were expressed as a percentage of total LDH activity.

2.5 Incorporation of ^{3}H-thymidine assay - Assessment of cell proliferation

The ^{3}H-thymidine incorporation assay, which quantifies DNA synthesis, was used to determine the effect of the NNSs on cellular proliferation. For the assay, incubation with radioactive thymidine (^{3}H-thymidine, 0.417 µCi/mL) was carried out in the last 4 h of the 24 h treatment period. After incubation, the excess of ^{3}H-thymidine was rinsed off, twice, with 300 µL of 10% TCA, and the remaining material was then solubilized with 1 M NaOH for 15 min. The wells were then scraped, and 250 µL of the solution were transferred into scintillation vials. As NaOH is a strong base, neutralization was required prior to addition of scintillation fluid, where 50 µL of HCl 5 mM were added first to each vial. Finally, the radioactivity in the samples was quantified by liquid scintillation counting (LKB Wallac Liquid Scintillation Counter 1209, Turku, Finland). The rate of cellular DNA synthesis was expressed relative to protein content (mCi/mg protein).

2.6 Wound injury assay – Assessment of cell migration

The wound injury assay was used to evaluate the effect of the selected NNSs on cell migration ability, by assessing the rate of wound closure over time. On the day of treatment, the culture medium was first removed, and a linear scratch was created in the cell monolayer using a sterile pipette tip. Fresh medium containing the treatments was added, and cells were exposed for 24 h to a reduced range of concentrations, selected based on previous findings from viability and proliferation assays: acesulfame-K (1, 100, and 1000 µM), sucralose (1, 10, and 100 µM), and saccharin (1, 100, and 1000 µM), or to the corresponding control (water). Afterwards, using the LEICA Microscope, images of the scratch area were immediately acquired (0 h) and after 24 h. The wound area was quantified using the ImageJ software (version 1.54g, National Institutes of Health, USA).

2.7 TBARS assay – Assessment of lipid peroxidation

The TBARS assay quantifies oxidative stress levels through the detection of lipid peroxidation products. In detail, thiobarbituric acid (TBA) reacts with these products, particularly MDA, forming a coloured complex that can be measured spectrophotometrically. After treatment of HTR-8/SVneo cells for 24 h with NNSs (in the same concentrations used in the wound healing assay), the culture medium was removed from the wells, and cells were washed with 500 µL of PBS and detached with

300 µL of trypsin-EDTA. Afterwards, 150 µL of homogenization solution (KH_2PO_4 62.5 mM, $Na_2HPO_4.2H_2O$ 50 mM, Triton X-100 0.1%, pH=7) was added and the cells were resuspended. From this resuspension, 200 µL were transferred into Eppendorf tubes and 130 µL of TCA 50% was added to each tube, followed by vortexing. The samples were left at room temperature for 5 min and then centrifuged at 10000 rpm for 2 min at 4°C. Afterwards, 300 µL of the supernatant was carefully transferred to a new tube and mixed with 300 µL of TBA 1% solution. The samples were then incubated at 97°C for 40 min. Afterwards, 150–200 µL from each sample were transferred in triplicate to a 96-well plate and read spectrophotometrically at 535 nm.

2.8 Determination of cellular uptake

The cellular uptake of several compounds (glucose, methionine and organic cations) was evaluated, using ^{3}H-deoxy-D-glucose (^{3}H-DG), L-^{14}C-methionine, and ^{3}H-1-methyl-4-phenylpyridinium (^{3}H-MPP^{+}). For this, HTR-8/SVneo cells were treated for 24 h with a more focused range of concentrations (1 and 100 µM for acesulfame-K and saccharin, and 1 and 10 µM for sucralose). The uptake experiments were performed in GF-HBS buffer (composition, in mM: 140 NaCl, 5 KCl, 20 HEPES, 2.5 $MgSO_4$, 1 $CaCl_2$; pH 7.4) for ^{3}H-DG uptake experiments and in HMBS buffer (composition, in mM: 140 NaCl, 5 KCl, 12.5 HEPES, 12.5 MES, 5 glucose, 2.5 $MgSO_4$, 1 $CaCl_2$), for L-^{14}C-methionine and ^{3}H-MPP^{+} uptake experiments. Firstly, the culture medium was removed, and 300 µL of buffer at 37°C (GF-HBS or HMBS) were added to each well. Cells were pre-incubated for 20 min in this buffer to allow cell adaptation. Uptake was then initiated by incubating the cells for 6 min in 200 µL of buffer containing either 20 nM ^{3}H-DG, 250 nM L-^{14}C-methionine or 50 nM ^{3}H-MPP^{+}. After 6 min, the radioactive solution was removed, and 500 µL of ice-cold buffer was added to stop the reaction. Next, 300 µL of 0.1 % (v/v) Triton X-100 (in 5 mM Tris-HCl, pH 7.4) were added to each well to solubilize the cells, and the plate was stored at 4 °C overnight. Radioactivity was then measured by liquid scintillation counting (LKB Wallac Liquid Scintillation Counter 1209, Turku, Finland).

NNS treatments were present during both the pre-incubation and uptake periods. In addition, specific transport inhibitors were present also in both periods: BAY-876 and phloridzin for ^{3}H-DG uptake, L-phenylalanine and L-arginine for L-^{14}C-methionine uptake, and fluoxetine and desipramine for ^{3}H-MPP^{+} uptake. These inhibitors were selected based on previous literature and studies performed by the group [86–89].

2.9 Cell cycle analysis

Cell cycle analysis provides information about cell proliferation and possible cell cycle arrest by evaluating cell distribution across various phases (G1, S, and G2/M). For this experiment, cells were exposed to NNSs for 24 h (100 µM acesulfame-K and saccharin and 10 µM sucralose). Following treatment, cells were collected by centrifugation at 300 g for 5 min. The resulting pellet was then fixed in 200 µL of 70% ethanol and kept at 4ºC. To prepare cells for staining, the cells were washed with 1 mL of PBS supplemented with 2% BSA, followed by another 5-min centrifugation cycle. After discarding the supernatant, the cells were resuspended in 75 µL of a propidium iodide/RNase solution. This mixture was incubated for 15 min at room temperature before being analysed on a BD LSRFortessa™ flow cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Data processing was handled through FlowJo software (v. 10.7.1), with results expressed as % of total cells arrested in each phase in the cell cycle.

2.10 Protein determination

The protein content was determined using the Bradford protein assay [90]. In this protocol, 2.45 mL of Bradford reagent were mixed with 50 µL of sample, and the absorbance was measured at 595 nm after 2 min.

2.11 Statistical analysis

All data are presented as mean ± standard error of the mean (S.E.M.). The value of n represents the number of individual experiments (biological replicates), each performed with 2-3 technical replicates. Statistical analysis was performed using Student's t-test for comparison between 2 groups, and the ANOVA test, followed by the *Tukey's* test when needed to evaluate statistical significance between three or more groups. The results were considered statistically significant when $p < 0.05$. All analyses were performed using the GraphPad Prism version 10.3.1 software (San Diego, CA, USA).

Results

1. Effect of NNSs on cell viability (MTT and LDH assay)

Firstly, it was important to determine if the selected NNSs could impair the viability of first-trimester EVTs. The MTT assay evaluated cellular metabolic function by measuring mitochondrial dehydrogenase activity. As illustrated in Figure 5, acesulfame-K and saccharin did not change viability at any concentration, with results close to the control. Likewise, sucralose maintained a similar pattern at lower concentrations, except at 5000 µM, where it significantly increased MTT reduction. Overall, no cytotoxic effects were observed under the tested conditions.

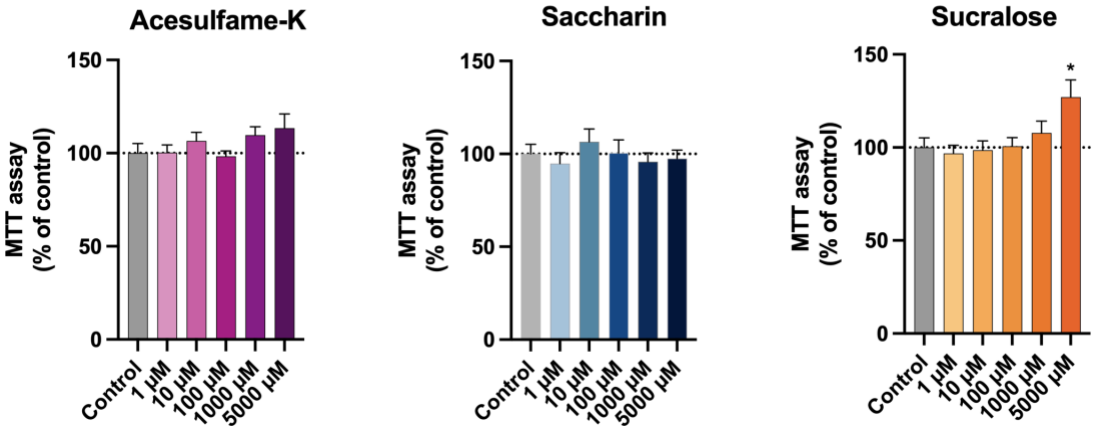


*Figure 5 - Effect of NNSs on cellular metabolic activity (MTT assay). Sucralose at 5000 µM increased MTT reduction compared with control cells (*p < 0.05 vs. control). Data are shown as arithmetic means ± S.E.M. (n = 12, 3 independent experiments).*

Subsequently, an LDH assay was conducted in order to assess cell membrane integrity. As shown in Figure 6, all tested NNSs induced a concentration-dependent increase in LDH release. Acesulfame-K showed a clear cytotoxic effect at 100 µM and higher. Instead, saccharin significantly increased LDH release from 10 µM onwards, whereas sucralose induced a higher release even at 1 µM. Among these three NNSs, sucralose demonstrated the highest cytotoxicity, reaching nearly 200% of control values at 5000 µM.

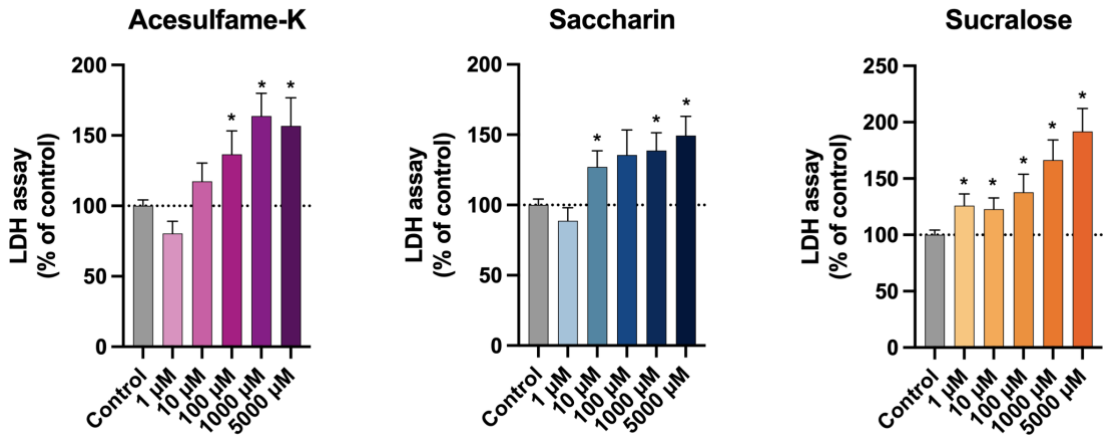


*Figure 6 - Effect of NNSs on cell membrane integrity (LDH assay). All NNSs tested increased LDH release in a concentration-dependent manner compared with control cells (*p < 0.05 vs. control). Data are shown as arithmetic means ± S.E.M. (n = 12; 3 independent experiments).*

2. Effect of NNSs on DNA synthesis (^{3}H-thymidine incorporation)

To measure the proliferation rate of HTR-8/SVneo cells when exposed to the NNSs, we performed the ^{3}H-thymidine incorporation assay. As seen in Figure 7, the effects of NNSs exposure on cell proliferation were not consistent across all concentrations. Acesulfame-K induced a slight increase in ^{3}H-thymidine incorporation at 100 µM, although no significant differences were observed at the remaining concentrations. In a similar way, saccharin did not significantly affect cell proliferation at most concentrations, despite a slight reduction in incorporation at 5000 µM. In contrast, sucralose caused a significant increase in ^{3}H-thymidine incorporation at higher concentrations (1000 and 5000 µM).

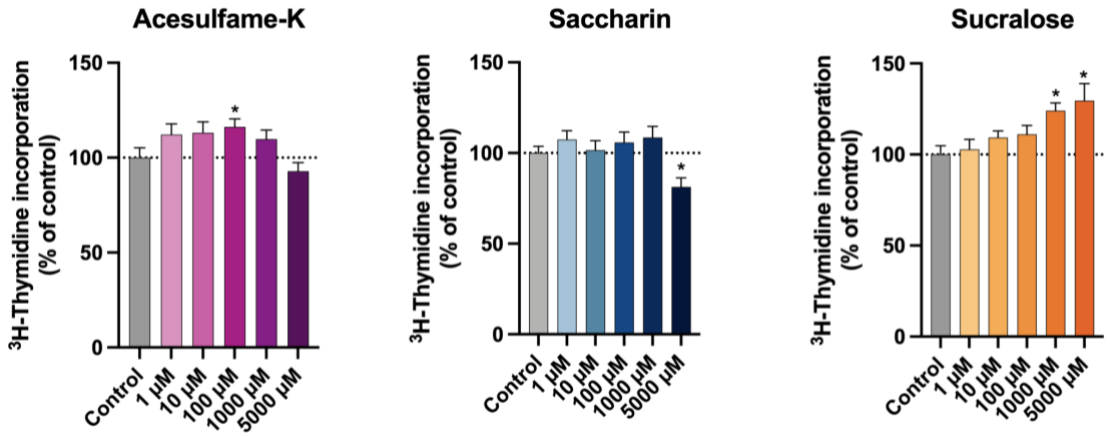


*Figure 7 - Effect of NNSs on cell proliferation (^{3}H-thymidine incorporation assay). Proliferation was differentially affected, with sucralose enhancing and saccharin reducing DNA synthesis at higher concentrations (*p < 0.05 vs. control). Data are shown as arithmetic means ± S.E.M. (n = 9; 3 independent experiments).*

3. Effect of NNSs on cell migration (wound healing assay)

The wound healing assay was performed to evaluate the migration rates of EVT cells in the presence of NNSs. As evidenced in Figure 8, acesulfame-K had no significant effect on the cell migratory capacity at any concentration tested, although a tendency for a slight decrease in cell motility was noted. In contrast, saccharin at 1000 µM and sucralose at 100 µM significantly decreased cell migration.

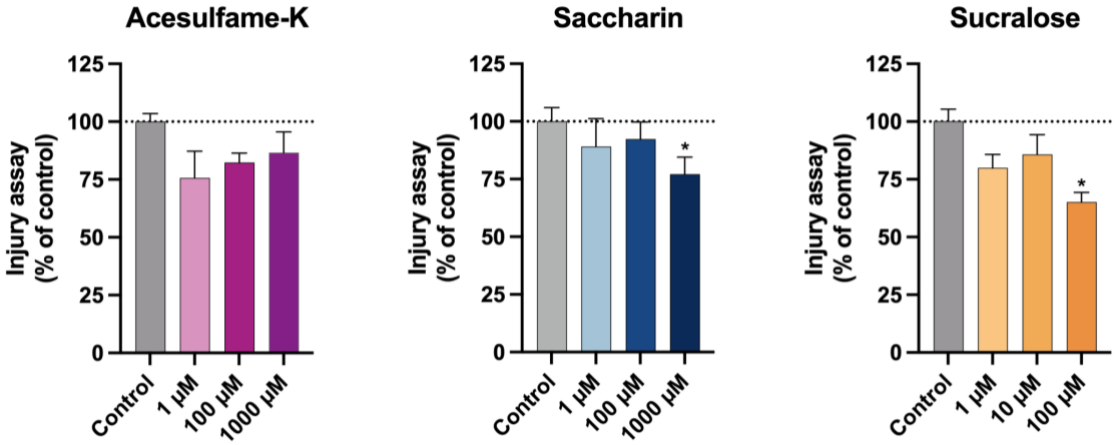


*Figure 8 - Effect of NNSs on cell migration (wound healing assay). Cell migration was significantly reduced by saccharin and sucralose at the highest concentrations, whereas acesulfame-K had no significant effect (*p < 0.05 vs control). Data are shown as arithmetic means ± S.E.M. (n = 9; 3 independent experiments).*

4. Effect of NNSs on oxidative stress levels (TBARS assay)

The TBARS assay was selected to investigate the effect of the NNSs on lipid peroxidation. As shown in Figure 9, distinct effects were observed among the different NNSs. Acesulfame-K and saccharin significantly decreased MDA levels at 1000 µM. In contrast, sucralose significantly increased lipid peroxidation, as reflected by higher MDA levels at the lower concentration tested (1 µM).

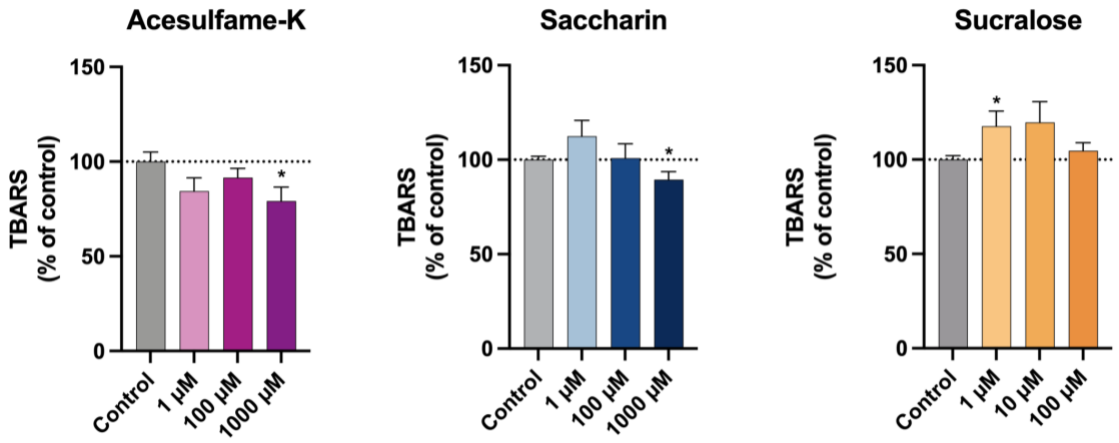


*Figure 9 - Effect of NNSs on lipid peroxidation (TBARS assay). TBARS analysis demonstrated compound-specific effects on lipid peroxidation, with sucralose increasing and acesulfame-K and saccharin reducing MDA levels at selected concentrations. (*p < 0.05 vs control). Data are shown as arithmetic means ± S.E.M. (n = 8–10; 4–5 independent experiments).*

5. Effect of NNSs on cellular uptake mechanisms

To evaluate the effect of NNSs on glucose, methionine, and organic cation uptake, we quantified the cellular uptake of ^{3}H-deoxy-D-glucose (^{3}H-DG), L-^{14}C-Methionine (L-^{14}C-Met), and ^{3}H-1-methyl-4-phenylpyridinium (^{3}H-MPP^{+}), respectively.

5.1 ^{3}H-Glucose uptake

^{3}H-deoxy-D-glucose is a non-metabolizable glucose analogue that is efficiently transported across trophoblast cell membranes by glucose transporters, especially GLUT-1 [25,91]. As illustrated in Figure 10, under basal conditions (absence of inhibitors), exposure to acesulfame-K (1 and 100 µM), saccharin (100 µM), and sucralose (10 µM) did not significantly alter ^{3}H-DG uptake compared to control cells. In contrast, treatment with 1 µM saccharin and 1 µM sucralose induced small but significant decreases in ^{3}H-DG uptake. The effect of NNSs was also evaluated in the presence of two glucose transport inhibitors, BAY-876 and phloridzin. BAY-876 strongly reduced ^{3}H-DG uptake (by 50-60%). Similarly, phloridzin also lowered ^{3}H-DG uptake by 50-60%. Moreover, we could also observe that there were no significant differences between inhibitor-treated control cells and the corresponding inhibitor-treated NNS groups (Figure 10). So, none of the NNSs was able to interfere with ^{3}H-DG uptake, both in the absence and in the presence of specific transport inhibitors.

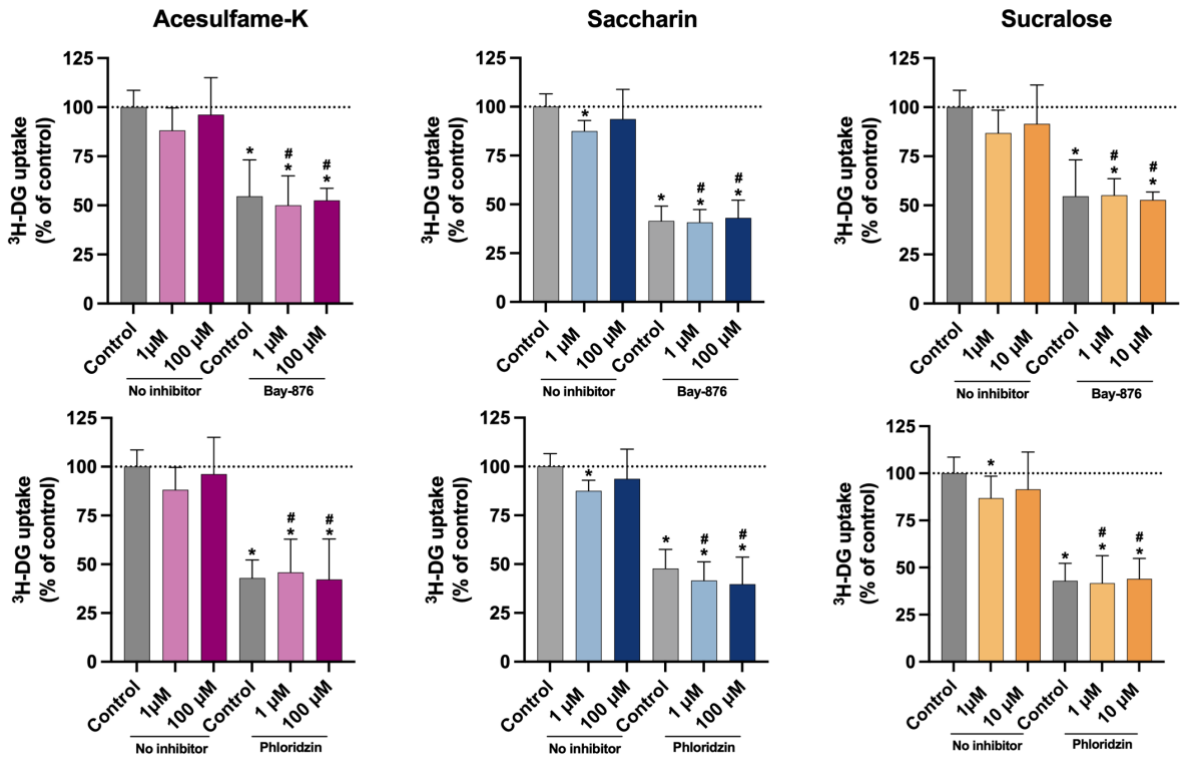


*Figure 10 - Effect of NNSs on glucose uptake (^{3}H-DG uptake assay). The NNSs produced modest and inconsistent effects on ^{3}H-DG uptake, both in the absence and presence of BAY-876 or phloridzin. *p < 0.05 vs. respective control (no inhibitor); $^{\#}$p < 0.05 vs. corresponding concentration without inhibitor. Data are shown as arithmetic means ± S.E.M. (n = 8, 4 independent experiments).*

5.2 L-^{14}C-Methionine uptake

Methionine is an essential amino acid involved in placental metabolism and cellular methylation through its conversion to S-adenosylmethionine [80,92]. To study if NNSs affect methionine uptake, L-^{14}C-Met incorporation was measured in the presence or absence of the competitive inhibitors L-arginine and L- phenylalanine (Figure 11).

When no transport inhibitors were present (basal condition), acesulfame-K (1 and 100 µM) did not significantly affect L-^{14}C-Met uptake compared with control cells. In contrast, saccharin (1 and 100 µM) and sucralose (1 and 10 µM) led to a significant increase in L-^{14}C-Met uptake. In comparison, both L-arginine and L-phenylalanine significantly reduced L-^{14}C-Met uptake in all experimental groups (by 50-60 and 70-80%, respectively), but there were no significant differences observed between NNS-treated and non-treated cells under these conditions (Figure 11).

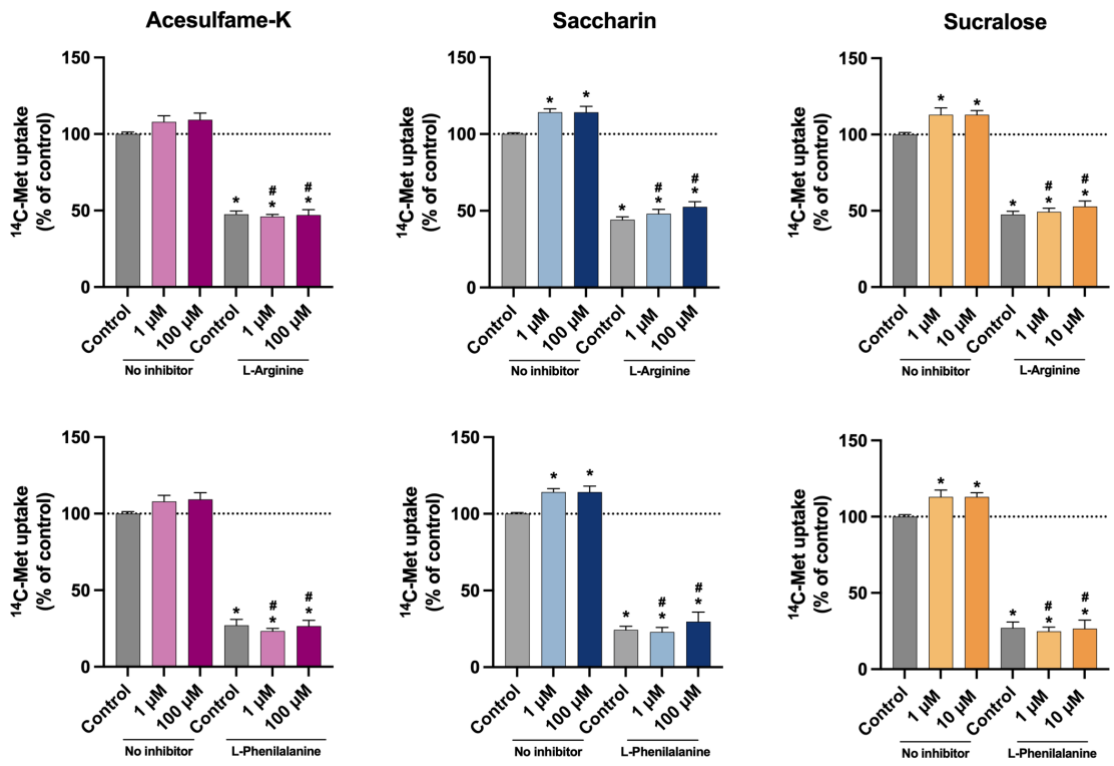


*Figure 11 - Effect of NNSs on methionine uptake (L-^{14}C-methionine uptake assay). NNSs modestly altered L-^{14}C-methionine (L-^{14}C-Met) uptake, both in the absence and presence of L-arginine or L-phenylalanine. *p < 0.05 vs. respective control (no inhibitor); $^{\#}$p < 0.05 vs. corresponding concentration without inhibitor. Data are shown as arithmetic means ± S.E.M. (n = 6–8, 3–4 independent experiments).*

5.3 ^{3}H-MPP^{+} uptake

^{3}H-MPP^{+} (1-methyl-4-phenylpyridinium) is a radiolabelled cationic compound often used as a model substrate to quantify monoamine and organic cation transport [93].

To explore the transport systems involved in ^{3}H-MPP^{+} uptake and if the NNSs interfere with them, HTR-8/SVneo cells were exposed to acesulfame-K, saccharin, or sucralose in the presence or absence of desipramine and fluoxetine (Figure 12). Under basal conditions, exposure to acesulfame-K, saccharin, or sucralose did not significantly affect ^{3}H-MPP^{+} uptake compared with control cells. As to desipramine, it did not significantly alter ^{3}H-MPP^{+} uptake in either control or NNS-treated cells. Finally, fluoxetine alone caused only a small increase in ^{3}H-MPP^{+} uptake, and the same lack of effect was observed when cells were simultaneously exposed to acesulfame-K or saccharin (1 and 100 µM). In contrast, combination of fluoxetine with sucralose (10 µM) led to a small but significant increase in ^{3}H-MPP^{+} uptake (Figure 12).

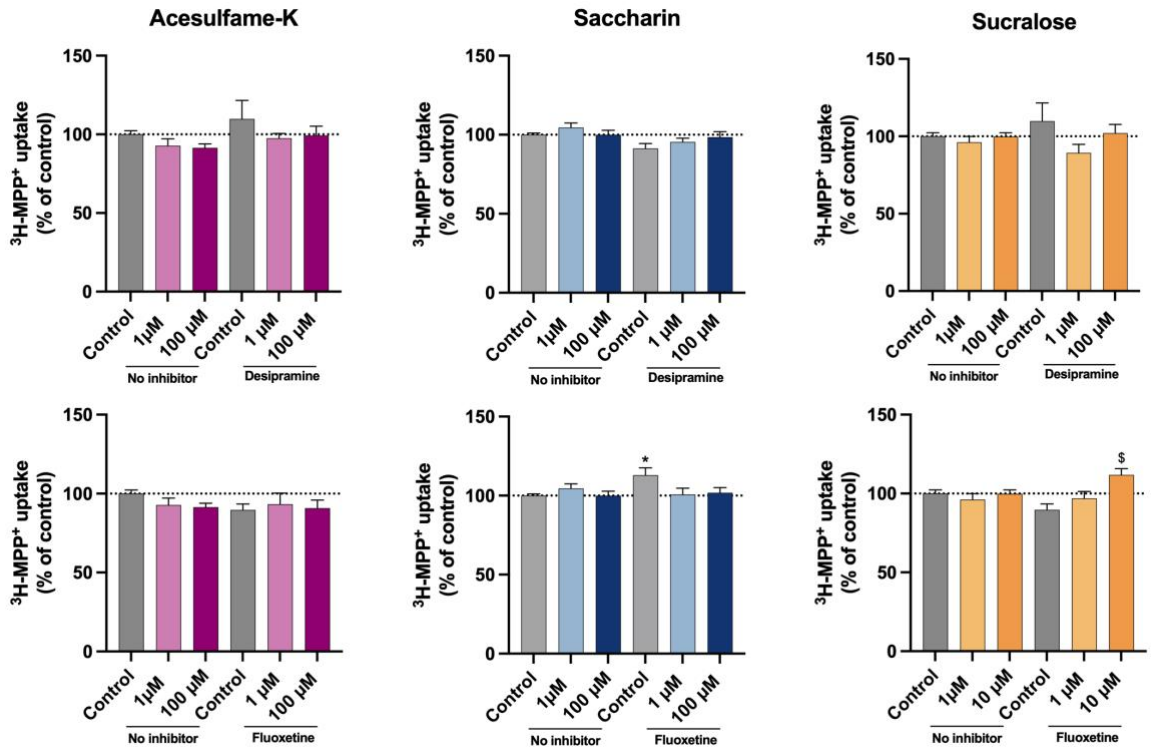


*Figure 12 - Effect of NNSs on MPP$^+$ uptake (^{3}H-MPP$^+$ uptake assay). NNSs did not alter ^{3}H-MPP$^+$ uptake, either in the absence or presence of desipramine or fluoxetine, except for an isolated change observed with sucralose under fluoxetine-treated conditions. *$p < 0.05$ vs. respective control (no inhibitor); $^{\$}p < 0.05$ vs. corresponding fluoxetine-treated control. Data are shown as mean ± S.E.M. ($n = 8$, 4 independent experiments).*

6. Effect of NNSs on cell cycle

Cell cycle analysis through flow cytometry allowed the distinction between G1, S, and G2/M phases, based on cellular DNA content, as determined by fluorescence intensity. As evidenced in Figure 13, when cells were treated with acesulfame-K (100 µM), there was a significant decrease of the percentage of cells in G1 phase and a significant increase in the G2/M phase, even though no differences were detected in the S phase. Moreover, saccharin (100 µM) did not cause significant differences in the percentage of cells in any cell cycle phase and sucralose (10 µM) significantly decreased the percentage of cells in the G2/M phase, while no significant changes were observed in the G1 or S phases. Figure 14 shows the representative histograms of each experimental group.

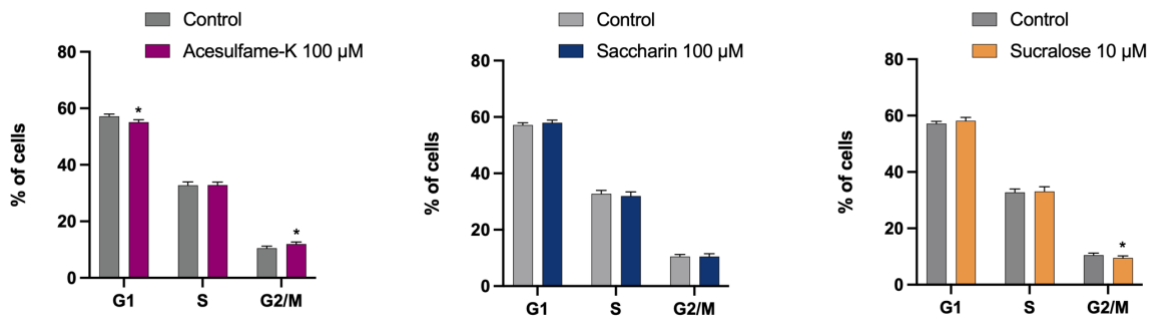


*Figure 13 - Effect of NNSs on cell cycle distribution. Acesulfame-K and sucralose induced significant changes in the percentage of cells in specific cell cycle phases (*p < 0.05 vs. control). Data are shown as arithmetic means ± S.E.M. (n = 8; 4 independent experiments).*

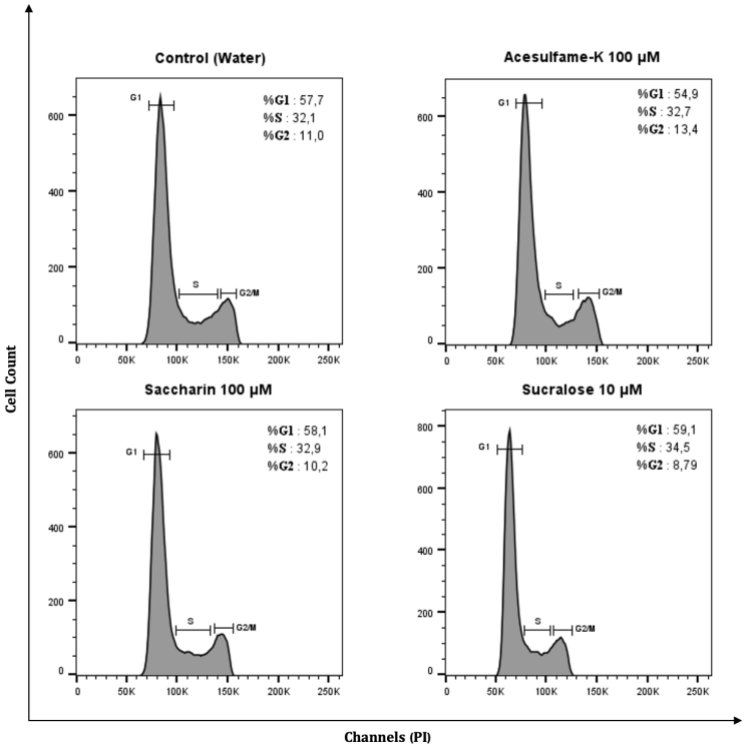


Figure 14 - Representative DNA content histograms obtained by flow cytometry following treatment with acesulfame-K, saccharin and sucralose. HTR-8/SVneo cells were stained with propidium iodide (PI), and representative histograms show the distribution of cells in the G1, S, and G2/M phases of the cell cycle.

Discussion

The influence of maternal diet on fetal growth and development throughout pregnancy has been a topic of great interest. This concern has led to increasing research into specific dietary compounds that may, to some extent, interfere with the placental function and, consequently, fetal development. Due to the rising prevalence of obesity and metabolic disorders, and considering the different efforts to reduce sugar intake, the consumption of NNSs has become increasingly common, even during pregnancy. However, although these compounds are considered safe within the acceptable intake limits, it is now known that some NNSs can cross the placenta, possibly affecting the fetus and the function of the organ itself. Consequently, there are still some concerns regarding their impact not only on fetal development, but also on fetal programming.

Therefore, a better understanding of the mechanisms underlying the effects of NNSs on placental cells, particularly extravillous trophoblasts, is important to clarify whether these compounds may influence placental function. Accordingly, the present study evaluated the effects of acesulfame-K, saccharin and sucralose on HTR-8/SVneo cells, focusing on metabolic activity, cytotoxicity, proliferation, migration, cell cycle analysis, oxidative stress, and nutrient and organic cation transport.

In order to determine whether these compounds influenced distinct aspects of cellular function, mitochondrial metabolic activity and plasma membrane integrity were evaluated using the MTT and LDH assays, respectively. Using the MTT assay, we verified no consistent cytotoxic profile, since only sucralose, at the highest concentration tested (5000 μM), increased MTT reduction, suggesting higher mitochondrial reductive capacity or an alteration in metabolic function, rather than a cytotoxic response. These results contrast with previous studies performed in other cellular models, where exposure to NNSs, particularly at high concentrations or after longer exposure periods, was associated with reduced cell viability, morphological changes and genotoxic effects. For instance, exposure of Caco-2 and HT-29 cells, used as intestinal barrier models, as well as human embryonic kidney-derived HEK-293 cells, to several NNSs (including saccharin, sucralose and acesulfame-K) at concentrations ranging from 1 to 50 mM for 24–72 h was associated with decreased MTT-based cell viability, particularly at concentrations above 10 mM [94]. Similarly,

acesulfame-K, tested at concentrations ranging from approximately 0.04 to 1.19 mM, decreased MTT-based viability in HepG2 cells [95]. Also, our group verified that exposure of HTR-8/SVneo cell line to aspartame was associated with decreased metabolic activity [77]. In contrast, sucralose exposure at concentrations ranging from 0.5 to 50 mM for 3–24 h increased MTT reduction in Caco-2 cells. Notably, the increase observed at 5 mM is consistent with the effect detected at the same concentration in the present study [96]. These contrasting findings suggest that the effects of NNSs on MTT reduction vary depending on compound, concentration, exposure duration and cellular model.

In contrast with the MTT results, when these cells were exposed to NNSs, there was a concentration-dependent increase in LDH release. Considering the three NNSs tested, it is worth noting that acesulfame-K showed a significant effect from 100 µM onwards, whereas saccharin induced this effect from 10 µM onwards. Sucralose appeared to induce the most pronounced cytotoxic response in this assay, increasing LDH release even at the lowest concentration tested and showing a marked effect at the highest concentrations. Since LDH release into the extracellular medium is commonly used as a marker of plasma membrane damage, the observed increase is consistent with compromised membrane integrity, despite the absence of a marked reduction in mitochondrial metabolic activity [97]. Previous studies also verified an effect of NNSs on membrane integrity. For instance, exposure to sucralose was shown to increase LDH release in human endothelial progenitor cells, indicating loss of plasma membrane integrity [98]. Although this study was not performed in trophoblast cells, it supports the idea that sucralose seems to compromise the cell membrane. However, a different pattern was reported in the 2022 study performed in the same HTR-8/SVneo cell line with aspartame and its metabolites, where none of the tested compounds induced a significant increase in LDH [79]. Together, these findings suggest that the effects on membrane integrity may depend on the specific NNSs and cellular model. This pattern further suggests that NNSs may primarily affect membrane integrity without detectably impairing mitochondrial function in extravillous trophoblasts, at least during the first 24 h.

To further understand if the cellular effects suggested by the MTT and LDH assays caused any alterations in proliferative behaviour, we evaluated ^{3}H-thymidine

incorporation and analysed the cell cycle distribution. This is particularly relevant since EVTs proliferate from the cytotrophoblast layer of the anchoring villi and contribute to placental development, with impaired trophoblast proliferation being associated with obstetric complications such as preeclampsia and fetal growth restriction [99,100]. The ^{3}H-thymidine incorporation provided information about DNA synthesis and, consequently, of proliferative activity. In this study, the tested NNSs did not induce a consistent effect on ^{3}H-thymidine incorporation across all concentrations, although sucralose significantly enhanced and saccharin reduced DNA synthesis at higher concentrations. Further, cell cycle analysis enabled the assessment of cell distribution across the G1, S, and G2/M phases. While acesulfame-K 100 μM decreased the percentage of cells in G1 phase and increased the percentage of cells in G2/M phase, and sucralose 10 μM decreased the percentage of cells in G2/M phase, these alterations were not accompanied by a strong or consistent redistribution of cells towards S phase. Saccharin, in turn, did not markedly alter cell cycle distribution under the tested conditions. Previous work using the same HTR-8/SVneo cell model reported that exposure to aspartame and its metabolites decreased trophoblast proliferation, supporting the idea that some NNSs interfere with proliferative behaviour [79]. In other cellular models, sucralose has also been associated with altered proliferative responses, including reduced proliferation in human microglial cells and impaired T-cell proliferation and differentiation after high sucralose exposure [72,101]. Although the changes observed in G1 and G2/M may indicate subtle effects on cell cycle progression, the absence of a consistent increase in S phase limits the interpretation of these findings as a clear proliferative response. Therefore, the changes in ^{3}H-thymidine incorporation induced by sucralose and saccharin should be interpreted with caution, as they may reflect subtle modulation of DNA synthesis or cell cycle progression rather than a robust alteration in the proliferative behaviour of HTR-8/SVneo cells.

Having investigated the proliferative behaviour of HTR-8/SVneo cells, it was also important to assess the effect of the three NNSs on cell migration. Overall, these compounds showed a tendency to reduce wound closure, with statistically significant effects observed for saccharin and sucralose at the highest tested concentrations (1000 and 100 μM, respectively). Acesulfame-K also induced a slight reduction in wound closure, although this effect did not reach statistical significance. These observations are pertinent, since EVT migration and invasion are essential for decidual invasion and

spiral artery remodelling during early placentation, and defects in these processes have been associated with pregnancy complications [102,103]. In this context, these results suggest that, under the experimental conditions tested, saccharin and sucralose appear to affect the migratory behaviour of HTR-8/SVneo cells. Of note, sucralose has also been reported to impair migration and neovasculogenic activity in human endothelial progenitor cells, suggesting that this compound can affect cell motility-related processes in other cellular models [98]. Interestingly, in the 2022 study performed in the same HTR-8/SVneo cell line with aspartame and its metabolites, aspartame itself did not affect migratory ability, whereas its metabolite methanol markedly reduced cell migration [79]. Wound closure is not determined exclusively by migration, as it can also be influenced by cell proliferation, adhesion, and overall cell viability [104]. Therefore, these results should be interpreted together with the ^{3}H-thymidine incorporation, cell cycle and LDH data. Since no consistent decrease in proliferative behaviour was observed, the reduction in wound closure is more likely to reflect an effect on cell motility rather than being solely explained by reduced proliferation. Nevertheless, given the increase in LDH release, particularly after sucralose exposure, some contribution of membrane disturbance or cytotoxic stress cannot be excluded.

The involvement of oxidative stress in the regulation of trophoblast function has been increasingly recognized, since changes in the redox balance can affect trophoblast metabolism [105,106]. During pregnancy, increased ROS generation is generally counterbalanced by antioxidant defences, allowing the maintenance of redox homeostasis [107]. However, when ROS formation exceeds the cellular antioxidant capacity, oxidative stress may contribute to lipid peroxidation and cellular damage [107]. In this context, MDA, a stable end-product of lipid peroxidation, is commonly used as an indirect marker of lipid oxidative damage, and increased MDA levels have been reported in pregnancy complications such as preeclampsia and pregnancy-induced hypertension [108,109]. Therefore, in the present study, the TBARS assay was used to evaluate whether exposure to NNSs affects lipid peroxidation in HTR-8/SVneo cells. Nevertheless, since other reactive aldehydes and sample components may also react with thiobarbituric acid, the results obtained with this assay should be interpreted with caution [108]. In this study, exposure to the three NNSs did not induce a uniform increase in MDA levels. Instead, the effects appeared to be compound- and concentration-dependent, with acesulfame-K and saccharin decreasing MDA levels at

higher concentrations, and sucralose increasing them at lower concentrations. Therefore, no clear linear or consistent pattern of lipid peroxidation was observed among the three NNSs. Previous work from our group in the same HTR-8/SVneo cell model provides an additional example of the compound-specific nature of these responses. In that study, aspartame and two of its metabolites (L-phenylalanine and L-aspartate) did not markedly affect lipid peroxidation, whereas a third metabolite (methanol) significantly increased MDA levels [79]. Together, these observations suggest that the effects of NNSs on lipid peroxidation are compound-specific and do not follow a uniform pattern in this trophoblast cell model. The decrease in MDA levels observed with acesulfame-K and saccharin may indicate reduced lipid peroxidation. However, because the TBARS assay does not directly assess antioxidant capacity or antioxidant defence mechanisms, these findings alone cannot be interpreted as evidence of an antioxidant effect. In contrast, the increase in MDA levels induced by sucralose at lower concentrations may indicate a slight increase in lipid peroxidation, although the absence of this effect at higher concentrations does not support a clear dose-dependent oxidative response. When interpreted together with the LDH and migration results, these findings suggest that lipid peroxidation is unlikely to be the main mechanism underlying the alterations in membrane integrity or wound closure observed in this study. Likewise, although changes in redox balance can influence trophoblast migration, the present TBARS results do not support a clear association between the impaired migratory response observed under some conditions and increased lipid peroxidation [106].

After evaluating the effects of NNSs on HTR-8/SVneo cellular function and oxidative status, uptake assays were performed to understand whether these compounds could also interfere with the transport of selected substrates, namely glucose, methionine and MPP^+. One of the most energy-rich and important substrates for fetal development is glucose. Since gluconeogenesis is limited in the uteroplacental-fetal unit, fetal glucose availability depends mainly on maternal supply and placental glucose transport [110]. In the human placenta, this process is largely mediated by facilitative glucose transporters, particularly GLUT1 [25,111]. In HTR-8/SVneo cells, glucose uptake has previously been characterized as time-dependent, saturable, sodium-independent, and sensitive to the glucose transport inhibitors cytochalasin B, BAY-876, phloretin and phloridzin [112,113]. In the present study, acesulfame-K did not significantly alter ^{3}H-

DG uptake, under basal conditions, while saccharin and sucralose induced only a small decrease at 1 μM, without a consistent effect at higher concentrations. In a previous study from our group, HTR-8/SVneo cells showed that aspartame, L-aspartic acid and methanol reduced ^{3}H-DG uptake [79]. Considered together, these findings support compound-specific effects of NNSs and their metabolites on glucose cellular uptake, The literature on the effects of NNSs on glucose uptake in trophoblast cells remains very limited. However, evidence from other cell models suggests that these compounds can modulate glucose transport depending on the cellular context and experimental conditions. For instance, acesulfame-K increased glucose uptake in Caco-2 and RIE-1 cells, but only under high-glucose conditions and at a specific uptake interval [85], whereas a later study in Caco-2/TC7 cells found that acesulfame-K and sucralose did not alter glucose transport under the tested acute conditions [114]. In adipocyte models, sucralose and saccharin reduced insulin-stimulated glucose uptake by impairing GLUT4 translocation [115]. In our study, the modest and non-consistent changes observed under basal conditions suggest that these NNSs have a limited overall impact on glucose uptake in HTR-8/SVneo cells. BAY-876 and phloridzin further helped to clarify the contribution of glucose transport systems in this model. BAY-876 is a strong inhibitor of GLUT1, and, as expected, markedly reduced ^{3}H-DG uptake, supporting a contribution of GLUT1 to glucose uptake in HTR-8/SVneo cells [116]. Phloridzin, a competitive inhibitor of sodium-glucose cotransporters 1 and 2 (SGLT1 and SGLT2), also reduced ^{3}H-DG uptake, indicating the presence of a phloridzin-sensitive glucose transport component [117]. Importantly, saccharin and sucralose did not produce an additional reduction in ^{3}H-DG uptake when combined with either BAY-876 or phloridzin, as uptake remained similar to that observed in cells treated with the respective inhibitor alone. This lack of an additive effect suggests that the modest basal effects of saccharin and sucralose on ^{3}H-DG uptake involve their interference with transport processes sensitive to these inhibitors. In the BAY-876 experiments, this is compatible with a possible involvement of the GLUT1-mediated component. Nevertheless, because a similar pattern was observed with phloridzin and neither inhibitor completely abolished ^{3}H-DG uptake, the effects cannot be conclusively attributed to a specific transporter. Overall, these findings suggest that saccharin and sucralose exert only modest effects on glucose uptake in HTR-8/SVneo cells, rather than causing a strong or generalized impairment of glucose transport.

As amino acid transport is essential for placentation and fetal growth, it was also important to assess their uptake. Methionine is particularly relevant, given that it is required for protein synthesis and is also involved in one-carbon metabolism and methylation reactions [118,119]. In human placental trophoblasts, L-methionine uptake has been shown to be mediated predominantly by the System L transporter LAT1, with a smaller contribution from the System y^+L transporter y^+LAT2 [86,118,120]. In contrast to glucose uptake, saccharin and sucralose increased L-^{14}C-methionine uptake in the absence of inhibitors, while acesulfame-K produced only a slight, non-significant increase. This suggests that some NNSs, particularly saccharin and sucralose, may stimulate methionine uptake under basal conditions. This study provides, to our knowledge, the first evidence of NNS effects on methionine transport in a placental trophoblast cell model. To further investigate the transport systems involved, L-phenylalanine and L-arginine were used as methionine transport inhibitors, based on previous studies examining L-^{14}C-methionine transport systems in placental trophoblast cells [86]. L-phenylalanine is a substrate of System L amino acid transporters, including LAT1 and LAT2, whereas L-arginine interacts with cationic amino acid transport systems, including y^+L and $b^{0,+}$ [86,121]. When either L-arginine or L-phenylalanine was added, L-^{14}C-methionine uptake was markedly reduced, supporting the important contribution of amino acid transport pathways sensitive to these substrates in L-^{14}C-methionine uptake. Importantly, the increases in L-^{14}C-methionine uptake observed with saccharin and sucralose under basal conditions were no longer evident in the presence of either competing substrate, as uptake values were similar to those observed in the respective L-arginine- or L-phenylalanine-treated controls. These findings suggest that the increases induced by saccharin and sucralose may involve methionine transport components sensitive to L-arginine and L-phenylalanine. Thus, in contrast to the limited changes observed in glucose uptake, saccharin and sucralose modestly increased methionine uptake under basal conditions. Besides, despite the increase in LDH release observed under some conditions, glucose and methionine uptake remained broadly preserved, suggesting that nutrient transport was not markedly impaired under the experimental conditions tested.

In addition to nutrient uptake, we also considered relevant to assess ^{3}H-MPP^+ uptake, as this compound is commonly used as a model substrate for organic cation and monoamine-related transport systems [89]. In the human placenta, OCT3 is localized

to the basal membrane of the syncytiotrophoblast and has been shown to contribute substantially to MPP^+ uptake. In the trophoblast-derived JAR cells, MPP^+ uptake involves SERT and an additional unidentified transporter [31,89]. In this assay, NNS exposure did not induce a consistent alteration in basal ^{3}H-MPP^+ uptake in HTR-8/SVneo cells. To our knowledge, these findings provide the first assessment of the effects of NNS exposure on organic cation uptake in an extravillous trophoblast cell model. To further explore the involvement of monoamine-related transport mechanisms, desipramine and fluoxetine were used as MPP^+ transport inhibitors based on previous findings in JAR cells [89]. Unexpectedly, these inhibitors did not interfere with ^{3}H-MPP^+ uptake. However, HTR-8/SVneo and JAR cells represent different trophoblast-related models, and the expression or functional relevance of MPP^+ transporters may differ between cell lines. Therefore, the limited effect of these inhibitors observed in the present study may indicate that the transport pathways targeted by desipramine and fluoxetine are not highly active in HTR-8/SVneo cells, or that ^{3}H-MPP^+ uptake in this model is mainly mediated by other mechanisms that are not efficiently inhibited by these compounds. Nevertheless, some compound-specific changes were observed in the presence of fluoxetine. In the saccharin series, fluoxetine increased ^{3}H-MPP^+ uptake in control cells, but this effect was not observed in cells exposed to saccharin. In addition, sucralose at 10 µM increased ^{3}H-MPP^+ uptake in the presence of fluoxetine. Therefore, these isolated alterations are insufficient to establish a clear effect of any of the tested NNS on ^{3}H-MPP^+ uptake in HTR-8/SVneo cells.

Although evidence regarding the effects of acesulfame-K, sucralose and saccharin in extravillous trophoblast models remains limited, animal and human studies provide a broader context for the potential consequences of NNS exposure during pregnancy. Previous studies have raised concerns regarding NNS consumption during pregnancy, although the mechanisms involved remain unclear. For example, in mice, the consumption of an acesulfame-K-sweetened solution during pregnancy was associated with maternal glucose intolerance, fetal hypoglycemia and reduced fetal weight, suggesting metabolic alterations in both the mother and the fetus [122]. In another mouse study, exposure to sucralose and acesulfame-K during pregnancy and lactation altered metabolism and gut microbiota-related profiles in the offspring, demonstrating the possibility of metabolic programming effect [123]. In line with these findings, human cohort studies have reported associations between maternal consumption of

NNSs during pregnancy and a higher infant BMI, alterations in the infant gut microbiota, and an increased risk of overweight [124–126]. However, it remains unclear whether these outcomes result from direct placental effects, changes in maternal metabolism and microbiome, or a combination of these factors.

One important aspect to consider is the physiological relevance of the concentrations tested. Although the lower concentrations may be representative of potential maternal systemic levels after NNS consumption, the higher concentrations used in this study most likely exceed the levels expected in maternal and fetal plasma following dietary intake. Therefore, the present findings should not be directly extrapolated to *in vivo* pregnancy exposure. Nevertheless, they remain relevant as an experimental approach to assess the sensitivity of HTR-8/SVneo cells to NNS exposure and to identify cellular processes that may deserve further investigation under more physiologically relevant conditions.

Despite the relevance of these findings, it is important to recognise the limitations of this study. One of the main constraints is the use of the HTR-8/SVneo cell line, which, although widely used as a trophoblast model, does not fully replicate the phenotype and complexity of primary extravillous trophoblasts or the *in vivo* placental environment. As a 2D *in vitro* model, this approach does not account for the structural organization of the placenta, mother–fetus exchanges, cell–cell interactions, or the influence of other cell types present in the placenta and maternal organism. The exposure conditions used in this study should also be considered, as HTR-8/SVneo cells were exposed to the different NNSs for only 24 hours, representing an acute exposure that does not fully mimic the repeated or chronic exposure that may occur during pregnancy. In addition to exposure time, the range of concentrations tested should also be considered as a limitation, particularly the higher concentrations, which may exceed the levels expected in maternal plasma following dietary intake. The methodological limitations should also be acknowledged. The MTT assay reflects cellular metabolic activity and does not necessarily indicate changes in cell viability or cell number. Similarly, TBARS provides only an indirect measure of lipid peroxidation and does not fully quantify oxidative stress levels. Regarding migration measurements, the wound healing should also be interpreted with caution, since wound closure may be influenced not only by cell migration, but also by changes in proliferation or cell viability. Furthermore, this

study did not assess transporter expression, intracellular signalling pathways, inflammatory responses, or other molecular mechanisms that could help explain the observed effects. Finally, this study did not assess combined exposure to mixtures of NNSs, despite the possibility that these compounds may coexist in the diet. Therefore, the findings may not fully reflect real-world dietary exposure and should be interpreted considering the experimental model and exposure conditions used.

Taken together, the results obtained with this study suggest that NNSs do not trigger a uniform cellular response in HTR-8/SVneo, but rather caused specific effects depending on the assay evaluated. Some of the results indicate cellular alterations, in particular the increase in LDH release, that suggests some membrane disturbance, as well as the changes observed in the migratory capacity. Through these findings, we have an indication that NNSs may affect EVTs homeostasis, metabolic activity and behavior. At the same time, these changes were not accompanied by a consistent increase in lipid peroxidation, a disruptive proliferative response or even alterations in cellular uptake of diverse compounds. Therefore, this study does not support a marked disruption of cellular responses, but it does suggest that NNS exposure may induce subtle alterations in specific cellular processes, rather than a generalized impairment of cellular function. In this way, considering the main role of EVTs in placental development, even modest changes in these cellular processes may be relevant and should be further explored in more complex placental models.

Conclusion

In conclusion, this work characterized the effects of the NNSs acesulfame-K, saccharin and sucralose on multiple aspects of HTR-8/SVneo cell function, using this cell line as an *in vitro* model of EVTs. Overall, these compounds did not markedly reduce cellular metabolic activity, but increased LDH release, suggesting possible effects on membrane integrity. In addition, saccharin and sucralose reduced wound closure at the highest tested concentrations, while the effects on proliferation, lipid peroxidation and substrate uptake were less consistent and depended on the compound, concentration and assay evaluated.

Although no uniform or strongly disruptive cellular response was observed, this study demonstrated that NNS exposure may induce subtle alterations in specific cellular processes relevant to trophoblast function. Considering the importance of EVT behaviour for placental development, particularly in processes such as migration and interaction with the maternal environment, these findings highlight the need for further investigation, particularly in the context of maternal dietary exposure during pregnancy, and reinforce the importance of a cautious approach to excessive NNS consumption during this period.

Future Perspectives

Further studies should explore the effects of NNSs on placental cells using more physiologically relevant experimental models and a broader range of exposure conditions. In particular, it would be important to validate these findings in primary extravillous trophoblasts, placental explants, 3D trophoblast spheroids or other models that better reproduce the interface between the mother and the fetus. In addition, future studies should test these compounds under prolonged or repeated exposure conditions, using periods longer than 24 h, such as 48 h or 72 h.

The mechanisms responsible for the effects observed in this study should also be investigated. This could include further assessment of glucose and amino acid transporters, including GLUT, SGLT and LAT transporters, as well as transport systems potentially involved in MPP^+ uptake. Their expression could be evaluated by RT-qPCR, Western blot or immunofluorescence, allowing the uptake results obtained in the present study to be related to changes in transporter expression or localisation. Oxidative stress could also be further characterised using additional markers, such as intracellular ROS production, antioxidant enzyme activity, glutathione balance or mitochondrial oxidative stress. Moreover, assays evaluating mitochondrial function, apoptosis and inflammatory signalling, such as mitochondrial membrane potential, caspase activation, Annexin V/PI staining, or the expression of inflammatory mediators, could help clarify whether NNSs affect trophoblast homeostasis through these pathways. Finally, considering that dietary exposure may involve the simultaneous intake of different sweeteners, future research should also assess the effects of combined exposure to mixtures of NNSs, rather than evaluating each compound individually.

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