https://doi.org/10.31925/farmacia.2023.3.17 **ORIGINAL ARTICLE**

AN *IN VITRO* MODEL OF ASPARTAME CYTOTOXICITY *VIA* HETEROLOGOUS EXPRESSION OF NMDA RECEPTORS

SHUROOQ ASAAD ABDULAMEER SHAHER [1,2]*, BIANCA GALATEANU [1], ARIANA HUDITA [1], ALBAJY MAITHAM [1,3], DAN FLORIN MIHAILESCU [1], BOGDAN AMUZESCU [1]

[1]*Department Biophysics and Physiology/Biochemistry, Faculty of Biology, University of Bucharest, Romania*
[2]*Department Medical laboratories, Babylon Technical Institute, Alfurat Al Awsat Technical University, Iraq*
[3]*National Centre for Occupational Health and Safety / Dhi Qar Governorate, University of Thi-Qar College of Science, Nasiriyah, Iraq*

**corresponding author: drshuruq73@gmail.com*

Manuscript received: February 2023

Abstract

Within the last 40 years, aspartame (L-aspartyl-L-phenylalanyl-methylester) became a very popular artificial sweetener, being introduced in over 6000 food products worldwide, including solid foods, beverages, and drugs for oral administration. However, its safety and toxicity have been subject of concern since its discovery, due to potentially harmful effects of its intestinal decomposition products: aspartate, phenylalanine, and methanol. Neuropsychological effects have been reported occasionally, such as headache, blurred vision, insomnia, torpor, memory loss, nausea, speech impairment, personality changes, loss of energy, hyperactivity, hearing problems. Our aim in the present study was to determine the effects of aspartame at various concentrations on the viability of cells transiently transfected with the combination of N-methyl-D-aspartate (NMDAR) ionotropic glutamate receptor subunits NR1+NR2b, as well as to prove direct activation of NMDAR currents by aspartame *via* whole-cell patch-clamp experiments. CHO-K1 cells transfected with NR1+NR2b *via* plasmid constructs containing the two genes fused with enhanced green fluorescent protein (eGFP) gene were exposed for 2 - 4 h to aspartame in progressive decimal dilutions (0.1 μM to 10 mM) and assessed by flow cytometry after propidium iodide (PI) staining, showing increased percentages of dead cells (PI+) at aspartame concentrations of 1 μM or higher, while non-transfected cells featured increased PI+ percentages at aspartame concentrations above 1 mM. We also showed in HEK293T cells transfected by electroporation with the same plasmid combination (NR1+NR2b) transient outward NMDAR currents at +40 mV elicited by brief pulses of glutamate 100 μM or aspartame 100 μM.

Rezumat

În ultimii 40 ani, aspartamul a devenit un îndulcitor artificial popular, fiind introdus la nivel mondial în peste 6000 alimente, băuturi și medicamente pentru administrare orală. Totuși, siguranța și toxicitatea sa au fost subiecte de interes încă de la descoperire, datorită potențialelor efecte nocive ale produșilor săi de descompunere intestinală: aspartat, fenilalanină, metanol. Efecte neuropsihice au fost raportate uneori, precum cefalee, vedere neclară, insomnie, torpoare, pierderi de memorie, greață, tulburări de vorbire, schimbarea personalității, pierderea energiei, hiperactivitate, tulburări auditive. Scopul nostru a fost de a determina efectele aspartamului la diverse concentrații asupra viabilității celulelor transfectate tranzient cu combinația de subunități de receptor de N-metil-D-aspartat (NMDAR) NR1+NR2b, și de a demonstra direct activarea de către aspartam a NMDAR în experimente de *whole-cell patch-clamp*. Celule CHO-K1 transfectate cu NR1+NR2b prin plasmide conținând cele 2 gene cuplate cu eGFP au fost expuse 2 - 4 h la diluții decimale progresive de aspartam (0,1 μM-10 mM) și măsurate prin citometrie în flux după marcare cu iodură de propidiu (PI), arătând procentaje crescute de celule moarte (PI+) la concentrații de aspartam peste 1 μM, în timp ce celulele netransfectate au avut procentaje crescute PI+ la concentrații de aspartam peste 1 mM. De asemenea, am evidențiat în celule HEK293T transfectate prin electroporare cu aceleași plasmide (NR1+NR2b), curenți *outward* prin receptori NMDA la +40 mV declanșați de pulsuri scurte de glutamat 100 μM sau aspartam 100 μM.

Keywords: aspartame, NMDAR, cell viability, flow cytometry, patch-clamp, electroporation

Introduction

One of the most popular non-nutritive sweeteners, aspartame, is currently used in more than 6000 food and drug products worldwide [30]. Aspartame's sweet taste was accidentally discovered by biochemist James Schlatter in 1965 [32]. The compound is a methyl ester of a dipeptide made up of L-aspartic acid and L-phenylalanine, two naturally occurring amino acids. Despite being approximately 200 times sweeter than sucrose, it generates the same amount of energy when metabolized – 4 kcal (17 kJ) *per* gram – and thus the energy intake brought by the amount needed for sweetening is significantly lower. Several intestinal enzymes (peptidases and esterases) entirely break down ingested aspartame into three major components: phenylalanine (50%), aspartic acid (40%) and methanol

(10%) [22]. Although at the beginning of industrial production, commercialization and widespread use aspartame was perceived as an excellent solution to limit calorie and sugar consumption, particularly in obese subjects or those at risk of developing diabetes mellitus, subsequent in-depth studies revealed several side effects that may jeopardize its effectiveness and safety.

Phenylalanine is an amino acid that may cross the blood-brain barrier, interfering with absorption of other large neutral amino acids like tryptophan, valine and tyrosine and subsequently reducing brain levels of essential neurotransmitters such as dopamine and serotonin [9]. Although methanol *per se* could be perceived as a toxic product due to conversion into formaldehyde and formic acid and raised concerns in earlier studies [23], subsequent studies found that the amounts generated by aspartame intake below the ADI (admitted daily intake) are in fact smaller than those resulted by ingestion of many other common foods, for example fruits containing pectins [42]. Stegink *et al*. obtained by acute administration of aspartame 100 mg/kg in infants peak methanol levels of 1.02 ± 0.28 mg/dL at 90 min post-ingestion, and in adults for the same amount 1.27 ± 0.20 mg/dL at 60 min [49], while Davoli *et al.* obtained by administering in human male subjects 500 mg aspartame (equivalent to 6 - 8.7 mg/kg) plasma methanol peak levels of 1.06 mg/L at 45 min, and in rats given aspartame 34 mg/kg peak plasma levels of 3.1 mg/L 1 h after ingestion [14]. For reference, the lethal methanol dose in adult human subjects is 6 g or about 100-fold the methanol dose provided by consuming the maximal allowed daily intake of aspartame (40 - 50 mg/kg/ day) [33].

Numerous studies have demonstrated that aspartame consumption can result in migraines, seizures, immune system difficulties, allergies, behavioural and cognitive issues [6]. Aspartate may lead to excitotoxicity, which can harm nerve cells in the brain. In the central nervous system, it binds to and activates N-methyl D-aspartate (NMDA) ionotropic glutamate receptors, allowing Ca^{2+} to enter and then activate neuronal nitric oxide synthase (nNOS), resulting in the production of nitric oxide (NO) and free oxygen/nitrogen radicals peroxynitrite ($ONOO^-$) and $NO^\bullet$ [1].

In vitro investigations of aspartame or aspartate binding to NMDA receptors have been carried out to determine whether pharmacological modulation of ligand-gated ion channels can improve nervous system function or reduce aberrant activity that may be the cause of nervous system illnesses [24, 28, 35, 37, 38]. Aspartate, resulted from aspartame intestinal breakdown, can efficiently activate NMDARs [9], in addition to other neurotransmitter imbalances caused by aspartate and phenylalanine [10, 22]. Aspartame and L-aspartate have been shown to directly interact with the NMDAR glutamate recognition sites in brain synaptic membranes in early *in vitro* tests [35]. Rats lacking folate and given aspartame had altered amounts of phosphorylated NMDAR1 subunits, increased iNOS and nNOS expression and enhanced NO generation [23]. Through the intricate process of long-term potentiation (LTP), which includes receptor phosphorylation caused by calcium influx in response to repeated stimulation, the N-methyl-D-aspartate receptors (NMDAR) are essential molecular components of learning and memory [40].

Numerous pathological circumstances, including ischemic stroke and neurodegenerative illnesses, can cause excitotoxicity, consisting in excessive synaptic glutamate release, excessive NMDAR activation and enormous neuronal Ca^{2+} inflow [34]. Olney also postulated that the developing human brain during embryogenesis is more exposed to excitotoxic compounds such as those present in foods (*e.g*. monosodium glutamate) due to an immature blood-brain barrier [34]. It could be possible under certain circumstances to raise similar concerns regarding the usage of aspartame, due to the substance itself and its break-down products [9, 31].

The objective of the present experimental *in vitro* study is to evaluate the effects of aspartame at various concentrations on the cell viability of an excitotoxicity model consisting in cultured CHO-K1 (Chinese hamster ovary) cells transfected with rat NMDAR subunits NR1 and NR2b, and to assess NMDAR current activation by aspartame *via* patch–clamp experiments on HEK293T (Human embryonic kidney) cells co-transfected with NR1+NR2b.

Materials and Methods

Cell culture

CHO-K1 and HEK293T cells were cultured in DMEM:F12 (1:1) medium buffered with 15 mM HEPES, containing glucose 3.15 g/L and Na pyruvate 0.055 g/L (Sigma-Aldrich D8900) supplemented with $NaHCO_3$ 1.2 g/L, 10% Foetal Bovine Serum (FBS), 2 mM glutamine and 1% penicillin-streptomycin (Sigma-Aldrich P4333), and maintained at 37°C in a humidified atmosphere with 5% CO_2. The cells were grown in 25 cm^2 sterile flasks and for experiments in 24-well plates, with medium changes every 2 - 3 days and weekly passages after detachment with trypsin-EDTA solution.

Plasmid multiplication and isolation

We used for experiments two plasmid constructs containing rat NMDAR subunits 1 and 2b having inserted in the N-terminal region the fluorescent marker gene eGFP: Addgene plasmids #45446 (pCI-eGFP-NR1) and #45447 (pCI-eGFP-NR2b) were a gift from Andres Barria and Robert Malinow, being developed and used for transfection in rat hippocampal slices neurons and HEK293 cells [3]. We used a standard protocol for multiplication consisting in seeding

plasmid-containing *E. coli* colonies on semisolid LB-agar medium containing ampicillin 100 μg/mL, picking individual colonies with a 200 μL sterile pipette tip and reseeding them in sterile liquid LB broth supplemented with ampicillin at the same concentration in Erlenmeyer flasks kept at 37°C under continuous shaking. Plasmids were isolated from the bacterial suspension with a DNA plasmid purification kit (PureLink™ HiPure Plasmid Midiprep Kit, Invitrogen-Thermo-Fisher) following manufacturer's instructions.

Transfection

CHO-K1 cells plated in 24-well plates at convenient density such as to reach 50 - 70% confluence at 24 h were transfected with NR1 4 μg + NR2b 2 μg *per* well using Polyplus Jet OPTIMUS® following the standard procedure, in culture medium supplemented with competitive NMDAR inhibitor 5-APV 200 μM (Sigma-Aldrich A5282). For electrophysiology experiments cultured HEK293T cells detached with trypsin-EDTA were resuspended in a hypoconductive electroporation buffer [7]. 50 μL of cell suspension supplemented with plasmids NR1 8 μg + NR2b 4 μg were placed in an electroporation cell with interelectrode distance 1 mm and transfected by electroporation with a βtech Electro Cell B10 instrument using two series of 4 rectangular positive pulses of 120 V 100 μs 1 ms start-to-start interval, followed by 4 x 10 V 50 ms 200 ms start-to-start interval. After electroporation the cells were transferred in 24-well plates in the same supplemented medium as CHO-K1 cells and cultured for 48 h.

Aspartame exposure and cell viability assessment by flow cytometry

After 48 h CHO-K1 eGFP expression was checked by fluorescence microscopy, and then medium in wells was replaced with a HEPES-buffered Ringer solution (similar to that used for patch-clamp experiments) without Mg^{2+}, but with 1 mM Ca^{2+} and 10 μM glycine, supplemented with aspartame (Supelco® 47135) at different concentrations (0.1 μM, 1 μM, 10 μM, 100 μM, 1 mM, 10 mM) prepared from a 50 mM aqueous stock solution. After 2 - 4 h of exposure to aspartame, the Ringer solution in each well was refreshed and cells were incubated for 10 minutes with propidium iodide (PI – Sigma-Aldrich P4170) 10 μg/ml in relative darkness, then detached with trypsin-EDTA, centrifuged (1000g 3 min), re-suspended in 100 - 200 μL Ringer solution and assessed by flow cytometry using a Beckman-Coulter CytoFLEX instrument driven by the CytExpert software. Gating intervals for the green fluorescence (FITC-A) and red fluorescence (PE-A) channels were set using as controls transfected CHO-K1 cells non-exposed to PI and non-transfected cells exposed to PI, respectively (Figure 1), with lower limits of 2.5 x 10^3 relative units for the FITC-A channel and 10^4 relative units for the PE-A channel. The same settings were used for analysis of all experiments, which included a selected polygonal area of non-fragmented cells defined on the forward scattering/side scattering plot.

Patch-clamp experiments

We performed whole-cell patch–clamp experiments on HEK293T cells co-transfected with NR1+NR2b by electroporation, after detachment by trypsin-EDTA, centrifugation and replating in Petri dishes 35 mm in diameter, in external solution. Individual transfected cells were selected *via* epifluorescence using blue light excitation provided by the inverted microscope of the patch-clamp setup (Olympus IMT-2). Patch-clamp pipettes with 2 - 3 MΩ resistance in solution were prepared from GC150F-10 borosilicate glass capillaries (Harvard Apparatus, USA) by 4-step pulling and fire-polishing, followed by filling with pipette solution. The currents were recorded in voltage-clamp mode with a resistive feedback patch-clamp amplifier (WPC-100, ESF electronic, Göttingen, DE) connected to a computer interface (Digidata 1322A) controlled by the Clampex module of the pClamp 8.2 software (Axon Instruments, Molecular Devices, Sunnyvalle, CA, USA). The external solution was composed of (concentrations in mM): NaCl 140 mM, KCl 2.5 mM, $CaCl_2$ 1 mM, Glycine 10 μM, EDTA 1 μM, HEPES 10, glucose 10 mM, sucrose 15 mM, pH 7.40 with NaOH, and the pipette solution contained CsCl 130 mM, Na_4BAPTA 5 mM, HEPES 10, pH 7.20 with CsOH [19]. Brief pulses (200 ms) of L-glutamate 100 μM or aspartame 100 μM in external solution were applied by a home-made application system with gravitational flow (100 - 200 μL/min) with electro-magnetic valves controlled by the Clampex software *via* a narrow common outlet placed near the approached cell, and the whole-cell currents elicited by these pulses were recorded at +40 mV and coherently mediated for several repeated sweeps to minimize experimental noise.

Statistical analysis

Values are expressed as mean ± SD or mean ± SEM, as appropriate, with *n* indicating the number of individual experiments. The difference between control and aspartame treatment was evaluated using one-way ANOVA for independent samples with Tukey post-hoc tests. A *p* value less than 0.05 was considered statistically significant.

Results and Discussion

Effects of aspartame on cell viability

We tested the effects of aspartame exposure in the concentration range of 0.1 μM to 10 mM on cell viability (assessed with PI) of CHO-K1 cells transfected with NMDAR subunits NR1+NR2b (as described in Methods), along with transfected cells non-exposed to aspartame and PI and non-transfected cells exposed to PI as negative controls. Fluorescence histograms on the red channel (PE-A) and green channel (FITC-A)

and forward-side scattering plots for the two negative controls and the samples exposed to aspartame 1 μM and 100 μM in one individual experiment are shown in Figure 1, while the average (± SEM) of relative percentages of PI-positive cells from the total of transfected or non-transfected cells of each sample in $n = 5$ individual experiments are shown in Figure 2. For the transfected cells, a repeated-measures one-way ANOVA test resulted in a F value of 0.9182 ($p = 0.4774$), but the pairing was statistically significant ($F = 23.99$, $p < 0.0001$). Likewise, for the non-transfected cells (lacking eGFP fluorescence), the one-way ANOVA resulted in $F = 0.9052$, $p = 0.4842$, with statistically significant pairing ($F = 15.05$, $p < 0.0001$). We assume the lack of statistical significance in differences between groups (as shown in Figure 2) is the result of high variability in percentage values of PI-positive cells for both transfected and non-transfected CHO-K1 cells in individual experiments. However, the trend towards increased percentages of apoptotic (PI-positive) cells for the transfected groups appears at low micromolar concentrations of aspartame and peaks at 1 mM, while for the non-transfected cells groups the increase appears above 1 mM.

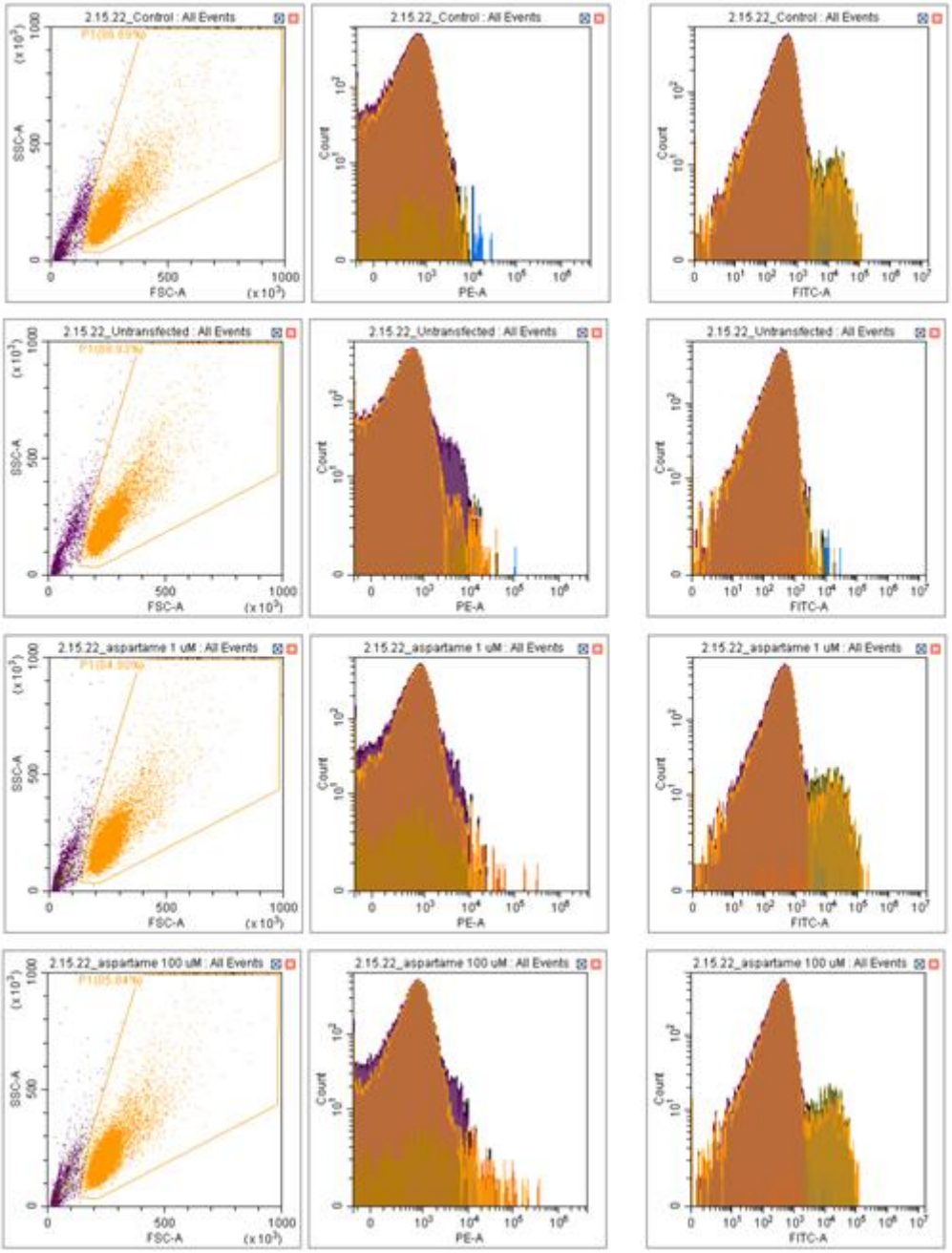

Figure 1.
Effect of aspartame on cell viability (by PI staining) of CHO-K1 cells co-transfected with NMDAR subunits NR1+NR2b assessed *via* flow cytometry

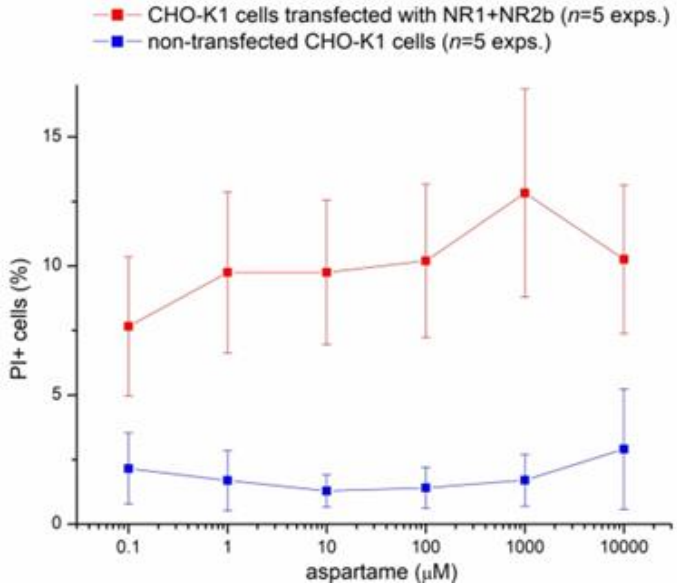

Figure 2.
Results of PI viability assays on CHO-K1 cells co-transfected with NMDAR subunits NR1+NR2b by JetOptimus® (average ± SEM of relative percentages of PI+ cells in *n* = 5 experiments)

Patch-clamp experiments on HEK293T cells co-transfected with NR1+NR2b

We performed whole-cell patch-clamp experiments on HEK293T cells transfected with NR1+NR2b *via* electroporation using a special combination of external and pipette solution (Cs-based and buffered with BAPTA), and we succeeded to record NMDAR transient outward currents at +40 mV elicited by brief (200 ms) pulses of glutamate 100 μM and aspartame 100 μM (in the presence of glycine 10 μM), proving direct NMDAR activation by aspartame.

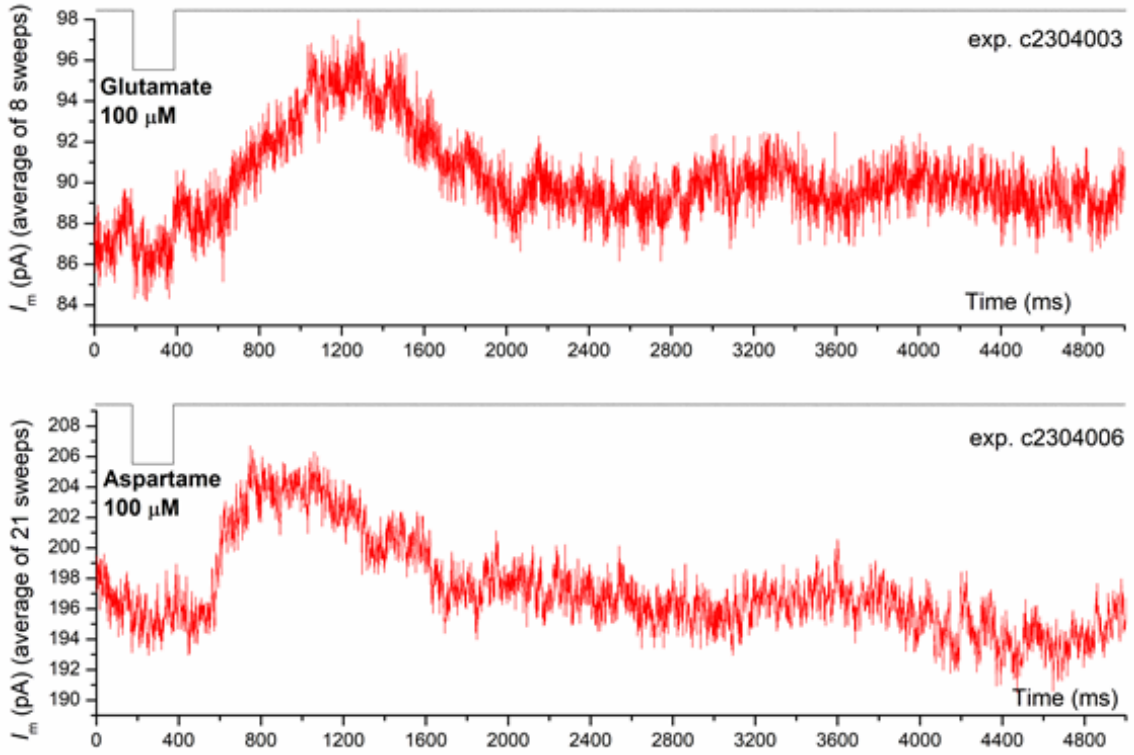

Figure 3.
NMDAR currents elicited by brief pulses of glutamate 100 μM and aspartame 100 μM in the presence of Gly 10 μM in a HEK293T cell transfected with NR1+NR2b (voltage-clamp recordings at +40 mV, coherent averages of multiple sweeps)

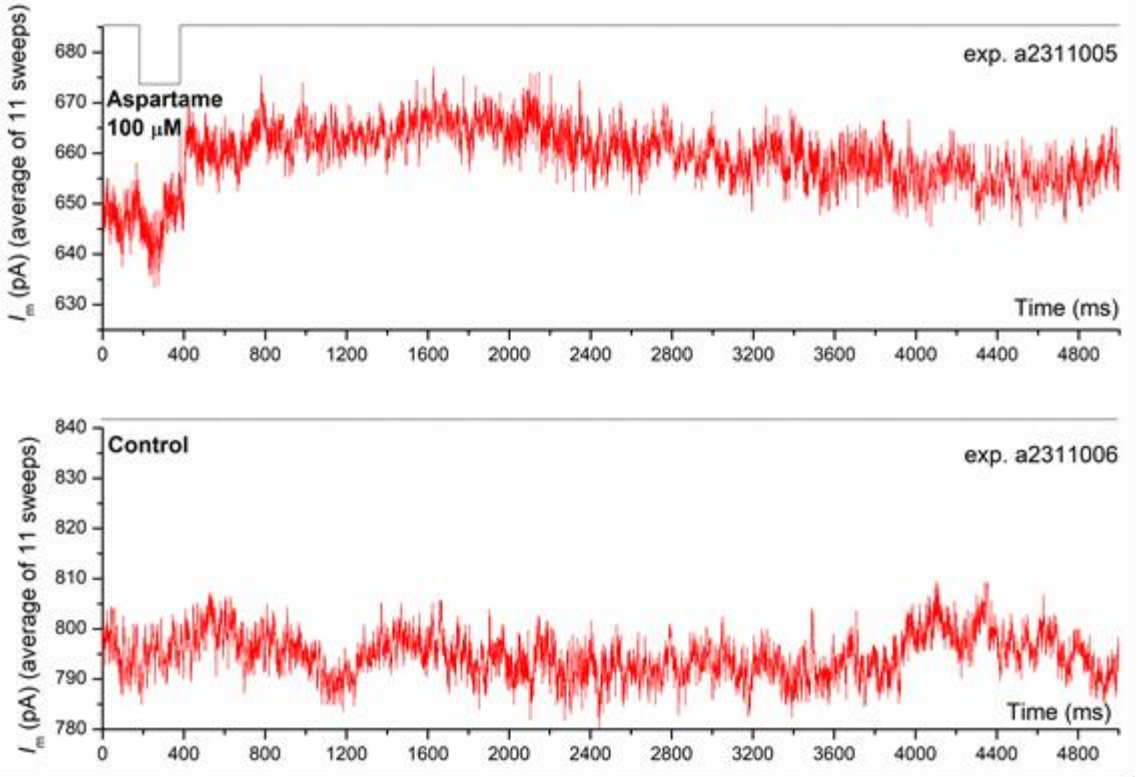

Figure 4.
NMDAR currents elicited by brief pulses of aspartame 100 µM in the presence of Gly 10 µM plus a control recording without aspartame application in a HEK293T cell transfected with NR1+NR2b (voltage-clamp recordings at +40 mV, coherent averages of multiple sweeps)

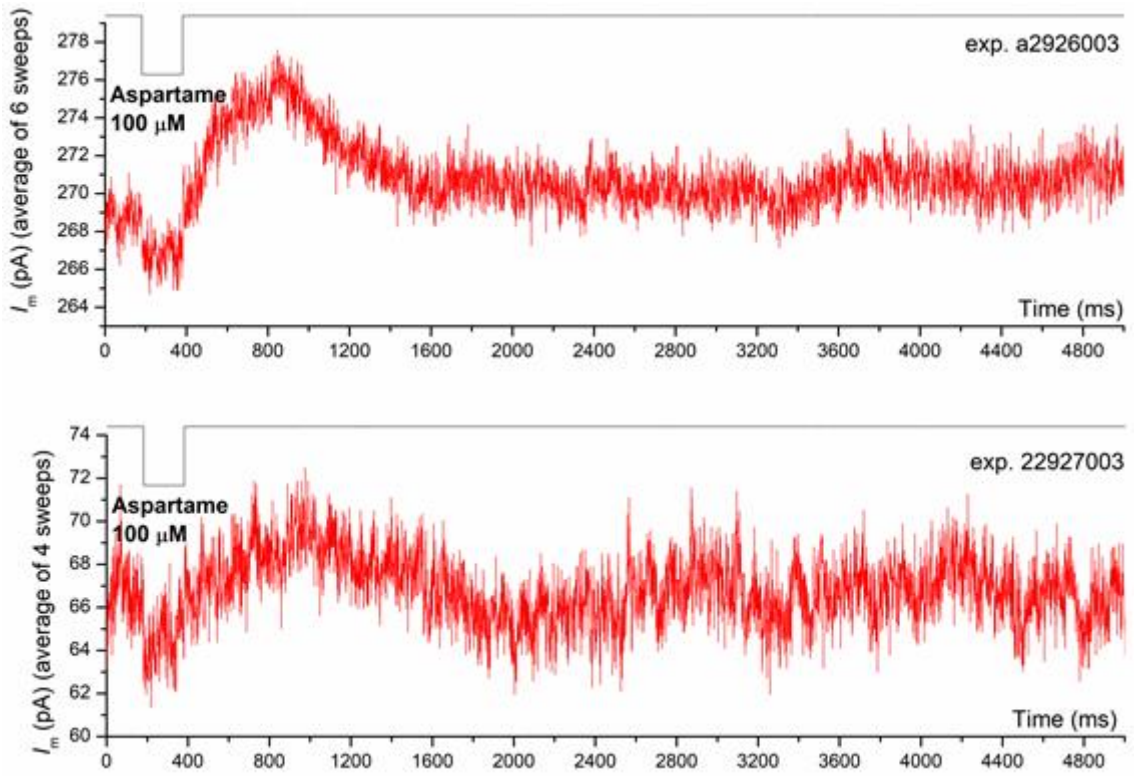

Figure 5.
NMDAR currents elicited by brief pulses of aspartame 100 µM in the presence of Gly 10 µM in HEK293T cells transfected with NR1+NR2b (voltage-clamp recordings at +40 mV, coherent averages of multiple sweeps)

Figures 3, 4 and 5 illustrate whole-cell currents recorded in voltage-clamp mode at +40 mV with synchronized application of NMDAR agonists. Multiple individual traces were coherently averaged to improve the signal-to-noise ratio. Although the amplitudes of transient outward currents elicited by either glutamate or aspartame at 100 μM are small, their time course and kinetics is specific for activation of NMDARs in these conditions, and in negative control experiments no significant transient was activated upon solution pathway switch, as shown in Figure 4 lower panel. The amplitudes and time constants of activation and decay of NMDAR transient currents elicited by aspartame or glutamate 100 μM are summarized in Table I.

Table I

Amplitudes and kinetics of NMDAR transient currents elicited by 200-ms pulses of glutamate or aspartame in HEK293T cells transfected with NR1 + NR2b

Experiment	NMDAR peak amplitude (pA)	τ rise (ms)	τ decay (ms)	Agonist	Number of averaged sweeps
c2304003	9.0	277.2	428.4	glutamate 100 μM	8 sweeps
c2304006	9.0	75.9	626.5	aspartame 100 μM	21 sweeps
a2311005	24.0	86.9	1864.9	aspartame 100 μM	11 sweeps
a2926003	10.0	152.5	319.2	aspartame 100 μM	6 sweeps
22927003	5.0	349.5	705.4	aspartame 100 μM	4 sweeps
Average	**12.0**	**166.2**	**879.0**		
SD	**8.3**	**126.8**	**678.0**		

Using an *in vitro* model of heterologous NMDAR expression, we succeeded to evidence increased cytotoxicity triggered by exposure to micromolar concentrations of aspartame, as well as direct activation by aspartame of NMDAR transient currents in cells transfected with a combination of subunits NR1 and NR2b. Flow cytometry results showed an aspartame-induced dose-dependent increase in PI staining (indicative of cell apoptosis) in transfected cells, but high experimental variability in percentages of transfection and cell condition precluded reaching a statistical significance. An interesting finding related to these cell viability experiments is the fact that transfection *per se* tends to increase the percentage of apoptotic cells three-fold on average (Figure 2), but for transfected cells aspartame exposure exerts a supplementary proapoptotic effect at doses above 1 μM, while in non-transfected cells cytotoxic effects are noticed at much higher concentrations (above 1 mM), confirming earlier results on viability of HeLa cells exposed to aspartame *in vitro* [36]. This pattern of cytotoxicity in NMDAR-expressing cells is consistent with experimental data on aspartame and aspartate binding affinity of native NMDARs obtained in earlier studies [35].

The viability study was supplemented with patch-clamp recordings of NMDAR transient outward currents at +40 mV elicited by brief pulses of glutamate 100 μM and aspartame 100 μM (in the presence of glycine 10 μM), proving direct NMDAR activation by aspartame. The time constant of NMDAR current activation obtained in our experiments (166.2 ± 126.8 ms, Table I) is larger compared to other studies using heterologously expressed NMDAR subunits, *e.g.* < 10 ms at -65 mV [19]. This relatively slow activation could be due to the peculiar type of application system and solution switching used in our experiments; with a multi-barrel perfusion system driven by a stepping motor, time constants of solution exchange of 27.4 ± 6.6 ms could be measured [20]. Another factor influencing activation kinetics in our experiments may have been the glutamate or aspartame concentration used (100 μM), smaller than 1 mM glutamate and 100 μM glycine as used in the above-mentioned studies to assure that all measurements are performed at saturating agonist concentrations [50, 52]. However, the average decay time constant found by us (879.0 ± 678.0 ms, Table I) is consistent with previous results obtained in cells expressing the combination NR1 + NR2b, *e.g.* 421 ms at -65 mV [19]. It has been also shown that the decay time constant of NMDAR transient currents is influenced by the phosphorylation status of the receptors and cell cycle phase [45].

The *in vivo* effects of NMDAR activation by aspartame, or, more likely, by its intestinal hydrolysis product L-aspartate, are more difficult to assess. Daily aspartame ingestion at high doses (but lower than the maximal admitted daily intake) resulted in neuro-physiological symptoms including cognitive impairments, headache, migraines, vision problems, tinnitus, irritable mood, anxiety, depression and insomnia [27]. Aspartame is decomposed by intestinal enzymes into a number of chemical components, including aspartic acid, phenylalanine and methanol, the latter being further metabolized into formaldehyde and formic acid [34]. Aspartate, as an analogue and precursor of glutamate, may act as a neurotoxin. Glutamate acts on neuronal presynaptic metabotropic glutamate receptors (mGluR receptors) and postsynaptic N-methyl D-aspartate (NMDA) receptors, leading to hyperexcitability of cells and free radicals release [46]. Aspartic acid is a precursor of glutamate and a direct agonist at NMDAR [18]. Aspartame is associated with the activation of microglia, which causes the production of nitric oxide and other inflammatory mediators such as peroxynitrite leading to oxidative stress [1]. Oxidative stress in different regions of rat brain has been linked to aspartame consumption [25]. Another effect of NMDAR

and other glutamate receptors activation in brain and other tissues is related to carcinogenesis. Some *in vitro* studies showed cytotoxic effects of exposure to high concentrations of aspartame in human colorectal carcinoma cell line HT-29 [29] using vital staining techniques [43]. The association of aspartame usage and increased risk of developing cancer is still highly controversial [26, 51], although several epidemiology follow-up studies on large cohorts have demonstrated increased frequency of different types of cancers in high-dose chronic aspartame consumers [4, 16, 39, 47]. Recent cohort studies also evidenced increased risk for cardiovascular diseases associated to high aspartame intake [15].

Several *in vivo* and *in vitro* studies have shown increased production of reactive oxygen/nitrogen species, lipid peroxydation and changes in scavenging systems in erythrocytes or serum of aspartame-treated animals [2, 11, 41] or in human neuroblastoma cells [21]. *Via* similar effects, aspartame can also influence inflammatory and immune cells such as neutrophils and lymphocytes, decreasing neutrophil adhesion and phagocytic index, and increasing antibody titres and soluble immune complexes [11]. Effects on some key enzymes, such as the inhibition of lipoxygenase-1 isoform by aspartame with half-inhibitory concentrations of 50 μM [8], may contribute to these complex immunomodulatory effects.

Other neurobiochemical and behavioural changes induced by aspartame consumption, particularly at high doses and over extended periods of time, have been reported [12]. At high concentrations, phenyl-alanine may compete with cerebral tyrosine to be hydroxylated by cerebral tyrosine hydroxylase, blocking the conversion of tyrosine to L-DOPA [48]. Dopamine deficiency may result in prefrontal cortex deficiency which is linked to brain dysfunction and impaired cognitive function [5]. Also NMDA glutamate receptors activation has been associated with multiple neuronal functions ranging from synapse formation to synaptic plasticity, learning and memory [17]. Like glutamate, aspartic acid may act as excitatory neurotransmitter at glutamate receptor sites in NMDAR [44]. The excessive activation of glutamate receptors and oxidative stress are sequential and interact with the processes that cause cell vulnerability in the brain, resulting in neurophysiological symptoms [13].

Conclusions

We performed cell viability studies on CHO-K1 cells transfected with NMDAR subunits NR1+NR2b (with or without co-transfection with a GFP plasmid); the transfected cells were exposed for 2 - 4 h at different concentrations of aspartame (0.1 μM to 10 mM), then stained with PI (10 μg/mL for 10 min), detached and assessed with a CytoFlex flow cytometer. Longer exposures to aspartame led to advanced cell degradation, making experiments too difficult. A similar degradation occurred in the absence of an effective specific inhibitor (such as 5-APV 200 μM) added to the cell culture medium. Flow cytometry results showed an aspartame-induced dose-dependent increase in PI staining (indicative of cell apoptosis) in transfected cells, but high experimental variability in percentages of transfection and cell condition precluded reaching a statistical significance.

We also performed whole-cell patch-clamp experiments on HEK293T cells transfected with NR1+NR2b *via* electroporation using a special combination of external and pipette solution (Cs-based and buffered with BAPTA), and we succeeded to record NMDAR transient outward currents at +40 mV elicited by brief pulses of glutamate 100 μM and aspartame 100 μM (in the presence of glycine 10 μM), proving direct NMDAR activation by aspartame, with amplitudes, rise and decay kinetics similar to those of currents activated by glutamate at the same concentration.

Conflict of interest

The authors declare no conflict of interest.

References

1. Abdel-Salam OM, Salem NA, Hussein JS, Effect of aspartame on oxidative stress and monoamine neurotransmitter levels in lipopolysaccharide-treated mice. *Neurotox Res.*, 2012; 21(3): 245-255.
2. Anbara H, Kian M, Darya GH, Sheibani MT, Long-term intake of aspartame-induced cardiovascular toxicity is reflected in altered histochemical parameters, evokes oxidative stress, and trigger P53-dependent apoptosis in a mouse model. *Int J Exp Pathol.*, 2022; 103(6): 252-262.
3. Barria A, Malinow R, Subunit-specific NMDA receptor trafficking to synapses. Neuron 2002; 35(2): 345-353.
4. Bassett JK, Milne RL, English DR, Giles GG, Hodge AM, Consumption of sugar-sweetened and artificially sweetened soft drinks and risk of cancers not related to obesity. *Int J Cancer*, 2020; 146(12): 3329-3334.
5. Brozoski TJ, Brown RM, Rosvold HE, Goldman PS, Cognitive deficit caused by regional depletion of dopamine in prefrontal cortex of rhesus monkey. *Science (New York, NY.)*, 1979; 205(4409): 929-932.
6. Butchko HH, Stargel WW, Aspartame: scientific evaluation in the postmarketing period. *Regul Toxicol Pharmacol.*, 2001; 34(3): 221-233.
7. Čepurnienė K, Ruzgys P, Treinys R, Šatkauskienė I, Šatkauskas S, Influence of plasmid concentration on DNA electrotransfer *in vitro* using high-voltage and low-voltage pulses. *J Membr Biol.*, 2010; 236(1): 81-85.
8. Chontzopoulou E, Papaemmanouil CD, Kolokouris D, Kiriakidi S, Konstantinidi A, Gerogianni I, Tselios T, Kostakis IK, Chrysina ED, Hadjipavlou-Litina D, Tzeli D, Tzakos AG, Mavromoustakos T, Molecular investigation of artificial and natural sweeteners as potential anti-inflammatory agents. *J Biomol Struct Dyn.*, 2021; 1-13.

9. Choudhary AK, Lee YY, Neurophysiological symptoms and aspartame: What is the connection?. *Nutr Neurosci.*, 2017; 21(5): 306-316.
10. Choudhary AK, Lee YY, The debate over neurotransmitter interaction in aspartame usage. *J Clin Neurosci.*, 2018; 567-615.
11. Choudhary AK, Rathinasamy S, Sundareswaran L, Role of antioxidant enzymes in oxidative stress and immune response evaluation of aspartame in blood cells of Wistar albino rats. *Int Food Res J (Malaysia)*, 2014; 21(6): 2263-2272.
12. Coulombe RA Jr, Sharma RP, Neurobiochemical alterations induced by the artificial sweetener aspartame (NutraSweet). *Toxicol Appl Pharmacol.*, 1986; 83(1): 79-85.
13. Coyle JT, Puttfarcken P, Oxidative stress, glutamate, and neurodegenerative disorders. *Science (New York, NY.)*, 1993; 262(5134): 689-695.
14. Davoli E, Cappellini L, Airoldi L, Fanelli R, Serum methanol concentrations in rats and in men after a single dose of aspartame. *Food Chem Toxicol.*, 1986; 24(3): 187-189.
15. Debras C, Chazelas E, Sellem L, Porcher R, Druesne-Pecollo N, Esseddik Y, de Edelenyi FS, Agaësse C, De Sa A, Lutchia R, Fezeu LK, Julia C, Kesse-Guyot E, Allès B, Galan P, Hercberg S, Deschasaux-Tanguy M, Huybrechts I, Srour B, Touvier M, Artificial sweeteners and risk of cardiovascular diseases: results from the prospective NutriNet-Santé cohort. BMJ. 2022; 378e071204.
16. Debras C, Chazelas E, Srour B, Druesne-Pecollo N, Esseddik Y, Szabo de Edelenyi F, Agaësse C, De Sa A, Lutchia R, Gigandet S, Huybrechts I, Julia C, Kesse-Guyot E, Allès B, Andreeva VA, Galan P, Hercberg S, Deschasaux-Tanguy M, Touvier M, Artificial sweeteners and cancer risk: Results from the NutriNet-Santé population-based cohort study. *PLoS Med.*, 2022; 19(3): e1003950.
17. Dingledine R, Borges K, Bowie D, Traynelis SF, The glutamate receptor ion channels. *Pharmacol Rev.*,1999; 51(1): 7-61.
18. Garthwaite J, Glutamate, nitric oxide and cell-cell signalling in the nervous system. *Trends Neurosci.*, 1991; 14(2): 60-67.
19. Glasgow NG, Johnson JW, Whole-cell patch-clamp analysis of recombinant NMDA receptor pharmacology using brief glutamate applications. *Methods Mol Biol.*, 2014; 2014: 118323-118341.
20. Glasgow NG, Wilcox MR, Johnson JW, Effects of $Mg^{(2+)}$ on recovery of NMDA receptors from inhibition by memantine and ketamine reveal properties of a second site. *Neuropharmacol.*, 2018; 2018: 137344-137358.
21. Griebsch LV, Theiss EL, Janitschke D, Erhardt VKJ, Erhardt T, Haas EC, Kuppler KN, Radermacher J, Walzer O, Lauer AA, Matschke V, Hartmann T, Grimm MOW, Grimm HS, Aspartame and its metabolites cause oxidative stress and mitochondrial and lipid alterations in SH-SY5Y cells. *Nutrients*, 2023; 15(6): 1467.
22. Humphries P, Pretorius E, Naudé H, Direct and indirect cellular effects of aspartame on the brain. *Eur J Clin Nutr.*, 2008; 62(4): 451-462.
23. Iyaswamy A, Kammella AK, Thavasimuthu C, Wankupar W, Dapkupar W, Shanmugam S, Rajan R, Rathinasamy S, Oxidative stress evoked damages leading to attenuated memory and inhibition of NMDAR-CaMKII-ERK/CREB signalling on consumption of aspartame in rat model. *J Food Drug Anal.*, 2018; 26(2): 903-916.
24. Iyaswamy A, Rathinasamy S, Effect of chronic exposure to aspartame on oxidative stress in the brain of albino rats. *J Biosci.*, 2012; 37(4): 679-688.
25. Iyaswamy A, Rathinasamy S, Neurobehavioral changes and activation of neurodegenerative apoptosis on long-term consumption of aspartame in the rat brain. *J Nutr Intermed Metabol.*, 2015; 2(3-4): 76-85.
26. Landrigan PJ, Straif K, Aspartame and cancer - new evidence for causation. *Environ Health*, 2021; 20(1): 42.
27. Lindseth GN, Coolahan SE, Petros TV, Lindseth PD, Neurobehavioral effects of aspartame consumption. *Res Nurs Health*, 2014; 37(3): 185-193.
28. Magalhães PCG, Abadie-Guedes R, da Costa Mendonça MAB, de Souza AD, Guedes RCA, Behavioral and electrophysiological brain effects of aspartame on well-nourished and malnourished rats. *Metab Brain Dis.*, 2019; 34(2): 651-658.
29. Maghiari AL, Coricovac D, Pinzaru IA, Macașoi IG, Marcovici I, Simu S, Navolan D, Dehelean C, High Concentrations of Aspartame Induce Pro-Angiogenic Effects *in Ovo* and Cytotoxic Effects in HT-29 Human Colorectal Carcinoma Cells. *Nutrients*, 2020; 12(12): 3600.
30. Magnuson BA, Burdock GA, Doull J, Kroes RM, Marsh GM, Pariza MW, Spencer PS, Waddell WJ, Walker R, Williams GM, Aspartame: a safety evaluation based on current use levels, regulations, and toxicological and epidemiological studies. *Crit Rev Toxicol.*, 2007; 37(8): 629-727.
31. Maher TJ, Wurtman RJ, Possible neurologic effects of aspartame, a widely used food additive. *Environ Health Perspect.*, 1987; 7553-7557.
32. Mazur RH, Discovery of aspartame. In Aspartame: Physiology and Biochemistry, Stegink, L.D., Filer, L.J.J., Eds.; Marcel Dekker: New York, 1984; 3-9.
33. Monte WC, Aspartame: methanol and the public health. *J Appl Nutr.*, 1984; 36(1): 1-9.
34. Olney JW, Farber NB, Spitznagel E, Robins LN, Increasing brain tumor rates: is there a link to aspartame?. *J Neuropathol Exp Neurol.*, 1996; 55(11): 1115-1123.
35. Pan-Hou H, Suda Y, Ohe Y, Sumi M, Yoshioka M, Effect of aspartame on N-methyl-D-aspartate-sensitive L-[3H] glutamate binding sites in rat brain synaptic membranes. *Brain Res.* 1990; 520(1-2): 351-353.
36. Pandurangan M, Enkhtaivan G, Kim DH, Cytotoxic effects of aspartame on human cervical carcinoma cells. *Toxicol Res (Camb.)*, 2016; 5(1): 45-52.
37. Park CH, Choi SH, Piao Y, Kim S, Lee YJ, Kim HS, Jeong SJ, Rah JC, Seo JH, Lee JH, Chang K, Jung YJ, Suh YH, Glutamate and aspartate impair memory retention and damage hypothalamic neurons in adult mice. *Toxicol Lett.*, 2000; 115(2): 117-125.
38. Park S, Sethi S, Bouret SG, Non-nutritive Sweeteners Induce Hypothalamic ER Stress Causing Abnormal

Axon Outgrowth. *Front Endocrinol (Lausanne)*, 2019; 2019: 10876.
39. Pavanello S, Moretto A, La Vecchia C, Alicandro G, Non-sugar sweeteners and cancer: Toxicological and epidemiological evidence. *Regul Toxicol Pharmacol.*, 2023; 2023: 139105369.
40. Polizzi S, Pira E, Ferrara M, Bugiani M, Papaleo A, Albera R, Palmi S, Neurotoxic effects of aluminium among foundry workers and Alzheimer's disease. *Neurotoxicol.*, 2002; 23(6): 761-774.
41. Prokić MD, Paunović MG, Matić MM, Djordjević NZ, Ognjanović BI, Štajn AS, Saičić ZS, Prooxidative effects of aspartame on antioxidant defense status in erythrocytes of rats. *J Biosci.*, 2014; 39(5): 859-866.
42. Pyrogianni V, Comments from the International Sweeteners Association (ISA) in response to "Revisiting the safety of aspartame" by Arbind Kumar Choudhary and Etheresia Pretorius Nutrition Reviews 2017; 75: 718-730. *Nutr Rev.*, 2018; 76(11): 857-858.
43. Radu IC, Hudiţă A, Zaharia C, Negrei C, Burcea-Dragomiroiu GTA, Popa DE, Costache M, Iovu H, Georgescu M, Ginghină O, Gălăţeanu B, Silk fibroin nanoparticles reveal efficient delivery of 5-FU in a HT-29 colorectal adenocarcinoma model *in vitro*. *Farmacia*, 2021; 96(1): 113-122.
44. Robinson MB, Coyle JT, Glutamate and related acidic excitatory neurotransmitters: from basic science to clinical application. *FASEB J.*, 1987; 1(6): 446-455.
45. Rosendo-Pineda MJ, Vicente JJ, Vivas O, Pacheco J, Loza-Huerta A, Sampieri A, Wordeman L, Moreno C, Vaca L, Phosphorylation of NMDA receptors by cyclin B/CDK1 modulates calcium dynamics and mitosis. *Commun Biol.*, 2020; 3(1): 020-01393.
46. Rycerz K, Jaworska-Adamu JE, Effects of aspartame metabolites on astrocytes and neurons. *Folia Neuropathol.*, 2013; 51(1): 10-17.
47. Schernhammer ES, Bertrand KA, Birmann BM, Sampson L, Willett WC, Feskanich D, Consumption of artificial sweetener- and sugar-containing soda and risk of lymphoma and leukemia in men and women. *Am J Clin Nutr.*, 2012; 96(6): 1419-1428.
48. Shiman R, Akino M, Kaufman S, Solubilization and partial purification of tyrosine hydroxylase from bovine adrenal medulla. *J Biol Chem.*, 1971; 246(5): 1330-1340.
49. Stegink LD, Brummel MC, Filer LJJ., Baker GL, Blood methanol concentrations in one-year-old infants administered graded doses of aspartame. *J Nutr.*, 1983; 113(8): 1600-1606.
50. Traynelis SF, Wollmuth LP, McBain CJ, Menniti FS, Vance KM, Ogden KK, Hansen KB, Yuan H, Myers SJ, Dingledine R, Glutamate receptor ion channels: structure, regulation, and function. *Pharmacol Rev.*, 2010; 62(3): 405-496.
51. Voiculescu DI, Ostafe V, Isvoran A, Computational assessment of the pharmacokinetics and toxicity of the intensive sweeteners. *Farmacia*, 2021; 69(6): 1032-1041.
52. Wild AR, Dell'Acqua ML, Potential for therapeutic targeting of AKAP signaling complexes in nervous system disorders. *Pharmacol Ther.*, 2018; 185: 99–121.