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Investigation of cyto-genotoxic effects of a food sweetener Acesulfame potassium

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ABSTRACT

Acesulfame potassium (ACE-K) is an artificial sweetener widely used in many foods. This investigation assessed the cytotoxic effect of ACE-K using MTT assay in human hepatocellular carcinoma (HepG2) cell line and the genotoxic effect using chromosomal aberrations (CAs), micronucleus (MN), and comet assays in human lymphocytes. 7.5-240 µg/mL concentrations of ACE-K were applied to cells. ACE-K notably decreased the cell viability on HepG2 cells, especially at 120 and 240 µg/mL at 24 and 48 h. It also significantly reduced the mitotic index (MI) at 60, 120, and 240 µg/mL at both treatments (24 and 48 h) in human lymphocytes. The frequency of the CAs significantly increased at 60, 120, and 240 µg/mL for 48 h treatment compared to control. However, no difference was observed in the frequency of MN and nuclear division index (NDI) at all the treatments. ACE-K also induced comet tail length, tail intensity, and moment at 15 µg/mL in isolated human lymphocytes. Therefore, ACE-K showed a cytotoxic effect in HepG2 cells as well as human lymphocytes at higher concentrations. It also exhibits a mild genotoxic effect by increasing the frequency of CAs at long-term treatment and DNA damaging effect only at 15 µg/mL.

Keywords: Acesulfame potassium, Food sweetener, MTT assay, Chromosomal aberrations, Micronucleus, Comet assay

Introduction

Sweeteners are used as food additives in all kinds of foods and beverages to make foods tastier (Cruz-Rojas et al., 2019; Schiano et al., 2021). They entered the food industry around the 1800s and are the basis of foodstuffs today (Carocho et al., 2017). Due to their low calorie, cost, and having a higher sweetness than natural table sugar, they are increasingly being included in foods and beverages. Sweeteners are added to foods and beverages as sugar substitutes such as artificial sweeteners, sugar-free sweets, and sugar-free sodas (Cao et al., 2020). They can be grouped into artificial sweeteners, modified sugars, sugars, and sugar alcohols, natural caloric sweeteners, and zero-calorie sweeteners (Hernández-Pérez et al., 2020). Artificial sweeteners are obtained by chemical synthesis and are primarily used in food, beverages, pharmaceuticals, and animal feeds with high sweetening power without extra energy (Li et al., 2020; Schiano et al., 2021). There are more than 3000 additives approved for use around the world (Whitehouse et al., 2008; Cao et al., 2020). These sweeteners are aspartame, acesulfame potassium, neotame, saccharin, cyclamate, sucralose, and neohespiridin dihydrochalcone (Kokotou et al., 2012; Heredia-García et al., 2019; Li et al., 2020).

Acesulfame potassium (ACE-K) (also known as Sweet One, and Sunett) is a widely-used artificial sweetener worldwide (Cruz-Rojas, 2019; Belton et al., 2020). It is one of several low- and no-calorie sweeteners used as an alternative to sugar. In 1967, ACE-K was discovered by chemist Karl Clauss. In 1988, the Food and Drug Administration (FDA) was approved as a food/drink sweetener (Whitehouse et al., 2008; Belton et al., 2020). Additionally, the acceptable daily intake (ADI) value for ACE-K has been recommended by FDA is 15 mg/kg body-weight in the same year. The ADI value has been determined by the Joint Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO) Expert Committee on Food Additives (JECFA) to be 0–15 mg/kg body-weight in 1991, and by the European Scientific Committee on Food to be 0-9 mg/kg body-weight in 2000 (WHO 1980; JECFA 1991; Belton et al., 2020; Chappell et al., 2020). ACE-K, whose sweetness is nearly 200 times higher than that of sucrose, is known as a potential sweetener due to its superior sweet taste and high water solubility (Magnuson et al., 2016; Ibi et al., 2018). Additionally, ACE-K is often mixed with other sweeteners (aspartame or sucralose) and exhibits a synergistic effect that makes the mixture sweeter than its components (Chattopadhyay et al., 2014). It can be included in some foods like baked goods, frozen desserts, candies, chewing gum, desserts, non-alcoholic beverages, and breath mints (Whitehouse et al., 2008; Fındıklı and Türkoglu, 2014). It is not metabolized in the body and is excreted unaltered in the urinary (Whitehouse et al., 2008; van Eyk, 2015; Najam et al., 2017). ACE-K contains the chemical methylene chloride, which has a known carcinogenic effect (Fındıklı and Türkoglu 2014).

Some *in vitro* and *in vivo* studies revealed that ACE-K exhibited cytotoxic and genotoxic effects (Mukherjee and Chakrabarti 1997; Bandyopadhyay et al., 2008; van Eyk, 2015). Cytotoxicity studies on cancer cell lines related to ACE-K have been observed to be very limited. van Eyk (2015) determined that ACE-K had a cytotoxic effect at concentrations >10 mM in colon HT-29 and Caco-2 cells and kidney HEK-293 cells using MTT assay. However, it had no significant effect on DNA damage using comet assay at all treatments. Mukherjee and Chakrabarti (1997) demonstrated that ACE-K (60, 450, 1500, and 2250 mg/kg) caused an increase in the frequency of chromosomal aberrations in *vivo* Swiss albino male mice. However, Mukhopadhyay et al. (2000) determined that the combination of ACE-K (1.5, 15, and 150 mg/kg) and Aspartame (3.5, 35, and 350 mg/kg) did not show any genotoxic effect using chromosomal aberration assay in Swiss albino male mice. Bandyopadhyay et al., (2008) demonstrated that ACE-K induced DNA damage in bone marrow cells of mice at 150, 300, and 600 mg/kg concentrations with the comet assay. This result is consistent with other results obtained in isolated human lymphocytes at 2.5 and 5 ppm using comet assay (Fındıklı and Türkoglu, 2014). Additionally, Silva et al. (2008) reported that ACE-K and Aspartame (ACE-K+AS) mixture increased the DNA tail length in human lymphocytes at 0.5% and 5% concentrations using the comet assay.

In literature, limited studies have been conducted on the genotoxic effects of ACE-K despite its frequently used in foods. Moreover, there are not enough studies about the cytotoxic/antiproliferative effects of ACE-K on human hepatocellular carcinoma (HepG2) cells. Therefore, this study purposed to investigate the cytotoxic effect of ACE-K in HepG2 cells using MTT assay as well as genotoxic effects in human lymphocytes using chromosomal aberrations (CAs), micronucleus (MN), and comet (SCGE) assays in human lymphocytes.

Materials and Methods

Chemicals

The test substance Acesulfame Potassium (ACE-K) (Cas. No: 55589-62-3) was obtained from Merck. The molecular formula of ACE-K is $C_4H_4KNO_4S$ and the molecular weight is

201.24. It was dissolved in distilled water. LymphoPlus Medium was obtained from Cegrogen Biotech. Dulbecco's Modified Eagle Medium with phenol red and without phenol red (DMEM, Cas. No: F0445, Cas. No: F0475, respectively), fetal bovine serum (FBS, Cas. No: S0613), PBS (Cas. No: L1825), trypsin (Cas. No: L2163), L-glutamine (Cas. No: K0283) and penicillin/streptomycin (Cas. No: A2213) were obtained from Biochrome. *In vitro* toxicology assay kit (MTT based, Tox-1), mitomycin-C (MMC, Cas. No: 50-07-7), cytochalasin-B (Cyt-B, Cas. No: 14930-96-2), NaCl (Cas. No: 7647-14-5), colchicine (Cas. No: 64-86-8) were obtained from Sigma. Low Melting Agarose (Cas. No: 9012-36-6), Normal Melting Agarose (Cas. No: 9012-36-6), NaOH (Cas. No: 1310-73-2), Tris (Cas. No: 77-86-1), Triton X-100 (Cas. No: 9002-93-1), EtBr (Cas. No: 1239-45-8), DMSO (Cas. No: 67-68-5), EDTA (Cas. No: 6381-92-6) and H_2O_2 (Cas.No: 7722-84-1) were obtained from Applichem.

MTT Assay

Human hepatocellular carcinoma (HepG2) cells were cultured according to ATCC protocols (https://www.atcc.org/products/hb-8065). The cytotoxicity of ACE-K was examined using an MTT assay with *In Vitro* Toxicology Assay kit. The experiment was carried out according to the method of Mossman (1983) with some modifications (Mamur et al. 2018). The grown cells were seeded in 96-multi-well plates, including $5x10^3$ cells per well, and cultured in a CO_2 incubator for 24 h. The cells were treated with 7.5, 15, 30, 60, 120, and 240 µg/mL concentrations of ACE-K for 24 and 48 h. A negative control was also maintained.

The absorbance (ABS) values of the wells were read at 570 nm wavelength in the ELISA microplate reading device (Molecular Devices Spectramax, M5). Then, the cell viability (%) values and also IC_{50} values that killed half of the cell population were determined. All experiments were repeated three times independently.

Lymphocyte Cultures

In this study, human peripheral lymphocyte cells from three healthy volunteers (a male and two female, non-alcoholic, nonsmokers, aged 24–27 years) were used. This study was confirmed by the Ethical Committee of the Faculty of Medicine, Gazi University (11.11.2019/144). Cells were treated with between 7.5 and 240 µg/mL concentrations of ACE-K. A positive control (Mitomycin-C; MMC, 0.20 µg/mL for CAs and MN; Hydrogen peroxide; H_2O_2, 100 µM for comet assay) and negative control (distilled water) were also maintained.

Chromosomal Aberrations (CAs) Assay

For the CAs assay, Evans's (1984) method was applied with some modifications according to Yuzbasıoglu et al. (2022). A blood sample (with heparin added to prevent clotting) was added to 2.5 mL of LymphoPlus medium. The cells were treated with 7.5, 15, 30, 60, 120 and 240 µg/mL concentrations of ACE-K for 24 and 48 h.

In CA analysis, a total of 300 (100 metaphases for each donor) metaphases per concentration were examined. In addition, the mitotic index (MI) was determined by scoring a total of 3000 cells per concentration (1000 cells from each donor).

Micronucleus (MN) Assay

The methods of Fenech (2000) and Palus et al. (2003) were followed for the preparation of MN with some modifications (Yuzbasioglu et al. 2022). Whole blood (0.2 mL) was added to 2.5 mL the LymphoPlus medium and incubated for 72 h at 37°C. Human lymphocytes were treated with 7.5, 15, 30, 60, 120 and 240 µg/mL of ACE-K at 48 h treatment period. Both of a negative control (distilled water) and positive control (Mitomycin C) were also maintained.

MN was scored from 1000 binucleated cells (BN) from each donor (a total of 3000 BN per concentration). The nuclear division index (NDI) was determined via analyzing 500 cells from each donor (a total of 1500 cells per concentration) according to the methods of Surrales et al (1995).

Comet (Single cell gel electrophoresis, SCGE) Assay

The alkaline comet assay was applied according to the methods of Singh et al. (1988) with some modifications (Erikel et. al. 2020). Lymphocytes were treated with six different concentrations of ACE-K (7.5, 15, 30, 60, 120, and 240 µg/mL) for 1 h at 37°C. In addition, negative and positive (100 µM H_2O_2) controls were also maintained.

Finally, the slides were evaluated using a fluorescence microscope (Olympus) equipped with an excitation filter (546 nm) and a barrier filter (590 nm), at 400× magnification. For each ACE-K concentration, the tail length (µm), intensity (%) and moment of the randomly selected 300 (100 cells from each donor) comets were determined using a specialized image analysis system (Comet Assay IV, Perceptive Instruments Ltd., Haverhill, UK).

Statistical Analysis

MTT assay was analysed by using One Way ANOVA followed by Dunnet's multiple comparison test and a p value less than 0.05 was considered as statistically significant. z-test was performed for statistical analysis of the percentage of abnormal cells, CAs/cell number, MN frequency, MI and

NDI results. For the analysis of Comet assay results, t-test was used. Besides, correlation and regression analysis were performed in order to designate the concentration-response relationship for the experiment groups.

Results and Discussion

Recently, artificial sweeteners are widely used by millions of people worldwide in various substances including pharmaceuticals, diet drinks and foods, and in consumer products such as toothpaste (Oldfield et al., 2020). Food consumers often select those foods with sweeteners because they want the taste of sweetness without added calories (Chattopadhyay et al., 2014). Artificial sweeteners supply the sweetness of sugar without the calories (Whitehouse et al. 2008). However, the safety of these sweeteners has been contradictory issue and there is some concern about their health effects (Cao et al., 2020). Several previous studies reported that artificial sweetener consumption can be related to psychotic conditions (Lindseth et al., 2014), oxidative stress (Ashok et al., 2017), type 2 diabetes mellitus (De Koning et al., 2011), weight gain (Fowler et al., 2008), obesity (Fowler, 2016), metabolic syndrome (Lutsey et al., 2008), coronary heart disease (Fung et al., 2009) and even cancer (Soffritti et al., 2006). It has been determined that the frequent use of these additives is also a risk factor for higher-grade tumors (Sturgeon et al., 1994). Another study reported that there is a positive association between the consumption of artificial sweeteners and well-differentiated thyroid carcinoma (Singh et al., 2020). In addition, artificial sweeteners are also known as environmental pollutants that occur permanently in aquatic environments (Dong et al., 2020; Yang et al., 2021). ACE-K has been identified as a new type of environmental pollutant because it is ubiquitous in the ecosystem and is extremely persistent (Cruz-Rojas et al., 2019; Dong et al., 2020). Considering the human health and food safety point, determining the potential cytotoxicity and genotoxicity of ACE-K have the utmost necessary. However, no other cytotoxicity study of ACE-K with HepG2 cells was available. Moreover, limited studies conducted on the genotoxic potential of ACE-K in human lymphocytes. Therefore, the main goal of the present study is to investigate the potential cytotoxic and genotoxic effects of ACE-K *in vitro*.

The potential cytotoxic effect of ACE-K was analyzed in HepG2 cell line using MTT assay. The cell viability was significantly and concentration-dependently decreased at 15, 60, 120, and 240 µg/mL for 24 h and at 30, 120 and 240 µg/mL for 48 h compared to negative control (24 h r= -0.68; 48 h r=-0.67, respectively) (Table 1). IC_{50} value was detected as 120 µg/mL for 24 h and >240 µg/mL for 48 h (Figure 1). The MTT assay is noted as the "gold standard" for cytotoxicity testing (van Tonder et al., 2015; Pintor et al., 2020). The results of the MTT assay demonstrated that ACE-K exhibited the cytotoxic effect in HepG2 cells, especially at 120 and 240 µg/mL at both treatments. HepG2 cells are frequently used in *in vitro* models (Choi et al. 2015). There is only one study evaluated the cytotoxic effect of ACE-K on the different cell lines. Van Eyk (2015) has investigated the cytotoxic effect of ACE-K on Caco-2, HT-29 (colon), and HEK-293 (kidney) cells at 1, 4, 10, 20, and 50 mM concentrations for 24, 48, and 72 h by using the MTT assay. The cell viability decreased at concentrations >10 mM for both concentrations- and treatment-dependently in all cell lines. The results obtained from this study are consistent with previous results. This study also evaluated the potential cytotoxic effect of ACE-K in human peripheral lymphocytes using MI and NDI which are other techniques for evaluating cellular proliferation and its kinetics (Eroglu et al. 2007). Our study revealed that ACE-K significantly decreased the MI at 60, 120, and 240 µg/mL for 24 h and at 30, 60, 120, and 240 µg/mL for 48 h versus control (Table 2). It has been determined that these decreases were in a concentration-dependent manner in both treatments (24 h r = -0.90; 48 h r= -0.88). The decrease in the rate of MI may be due to a blockage in G2, preventing the cell from entering mitosis, or a decrease in ATP level and pressure from the energy production center (Jain and Sorbhoy, 1988). Contrary to the results regarding MI in this study, NDI values did not verify other cytotoxicity results.

Genotoxicity is related to serious health effects and contains different types of DNA lesions, gene mutations, structural, and numerical chromosomal abnormalities comprising breakage and/or rearrangement of chromosomes (Dusinska et al., 2019). Considering the close relationship between genotoxicity and carcinogenesis, various assays have been developed to detect genetic damage (Souza et al., 2019). The CAs, MN, and Comet assays are commonly used measurement techniques for detecting the genotoxic, mutagenic, and carcinogenic effects (Stice et al., 2019). Clastogenicity and aneugenicity can be assessed by a CA test that identifies agents that cause chromosomal or chromatid breaks, dicentrics, and other chromosomal abnormalities (Dusinska et al., 2019).

In the present study, the effects of ACE-K on the frequency of CAs and CAs/cells were shown in Table 2. ACE-K significantly and concentration-dependently increased the CAs and CAs/cell frequency for 48 h treatment at 60, 120 and 240 µg/mL (r=0.78; r=0.79, respectively) (Table 2). However, ACE-K did not affect the frequency of CAs in 24 h treatment compared to the negative control (Table 2). Six types of structural abnormalities were observed such as chromatid breaks (31.95%), chromosome break (7.73%),

sister chromatid union (35.05%), dicentric chromosomes (5.15%), fragment (2.06%), chromatid exchange (9.27%) and one type of numerical abnormality polyploidy (8.76%) in cultured human lymphocytes. The commonly observed structural aberrations were sister chromatid unions (35.05%) and chromatid breaks (31.95%). The formation of structural chromosomal aberrations (CAs) can be caused by unrepaired or insufficiently repaired DNA double-strand breaks (Vodicka et al., 2018). Chromosomal aberrations and chromosomal instability are often associated with human cancers (Bach et al., 2019). In *in vivo* study, Mukherjee and Chakrabarti (1997) investigated the potential genotoxic and clastogenic effects of ACE-K. Swiss albino male mice were exposed to ACE-K by gavage using concentrations of 15, 30, 60, 450, 1500, and 2250 mg/kg (bw). ACE-K caused a significant raise in the frequency of chromosomal aberrations at the concentrations of 60, 450, 1500, and 2250 mg/kg (bw). Authors reported that it exhibited clastogenic and genotoxic effects. However, Mukhopadhyay et al. (2000) reported that the combination of ACE-K (1.5, 15, and 150 mg/kg) and Aspartame (3.5, 35, and 350 mg/kg) was not genotoxic in Swiss albino male mice using CAs assay.

Table 1. Cytotoxic effect of ACE-K on HepG2 cells

Test Substance			24 hour	48 hour
	Concentration (µg/mL)	N	Mean±SD	Mean±SD
Control	0.00	3	2.833±0.054	3.423±0.187
ACE-K	7.5	3	2.657±0.715	3.212± 0.091
	15	3	1.693±0.616 *	2.674± 0.234
	30	3	2.093±0.138	2.068± 0.560 *
	60	3	1.794±0.407 *	3.029± 0.000
	120	3	1.408±0.184 *	2.536± 0.454 *
	240	3	1.514±0.101 *	1.925±0.286 *

ACE-K: Acesulfame potassium, HepG2: Human hepatocellular carcinoma cell line, SD: Standard deviation
N: The number of repetitions
* Significantly different from the control P < 0.05 (One-way ANOVA-Dunnet Test).

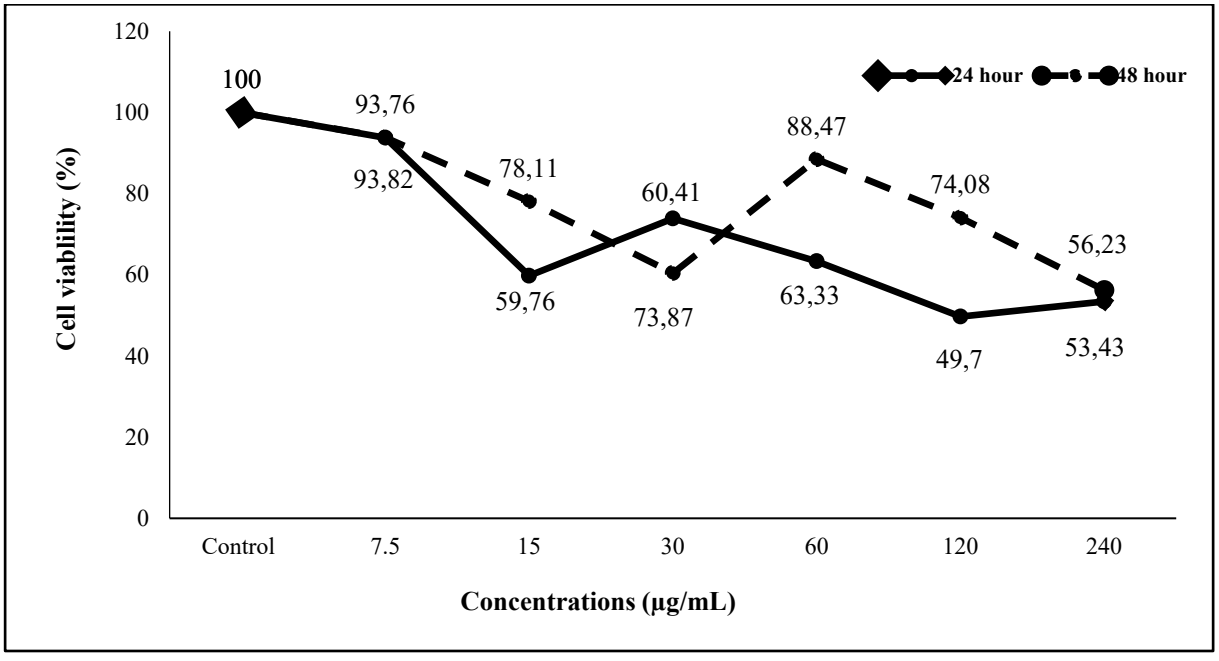

Figure 1. Cell viability of ACE-K on HepG2 cells

Table 2. The effect of ACE-K on the frequencies of chromosomal abnormalities and mitotic index in cultured human lymphocytes

Test substance	Treatment		Abnormalities							Abnormal cell ± SE (%)	CA/Cell ± SE	MI ±SE
	Time (hour)	Concent. (µg/mL)	ctb	csb	scu	cte	dic	f	p			
Control	24	0	-	1	2	2	-	-	-	1.66±0.73	0.016±0.007	7.23±0.47
PC (MMC)	24	0.20	15	2	7	2	2	-	2	9.66±1.70	0.100±0.017	4.00±0.35
ACE-K	24	7.5	2	-	3	-	-	-	2	2.33±0.87	0.023±0.008	6.80±0.45
		15	1	-	6	1	-	-	-	2.66±0.92	0.026±0.009	6.23±0.44
		30	-	-	6	1	-	-	1	2.66±0.92	0.026±0.009	6.10±0.43
		60		1	2	1	3	-	2	3.00±0.98	0.030±0.010	5.73±0.42 *
		120	1	-	5	1	1	1	2	3.66±1.08	0.036±0.010	5.73±0.42 *
		240	2	-	4	2	-	1	2	3.33±1.08	0.036±0.010	4.66±0.38 ***
Control	48	0	1	-	-	-	1	1	-	1.00±0.57	0.010±0.005	7.26±0.46
PC (MMC)	48	0.20	18	2	4	6	-	-	-	10.0±1.73	0.100±0.017	4.00±0.35
ACE-K	48	7.5	4	1	2	-	-	-	1	2.66±0.92	0.026±0.009	6.70±0.45
		15	4	-	6	-	-	-	-	3.33±1.03	0.033±0.010	6.30±0.44
		30	2	-	3	1	-	1	1	2.66±0.92	0.026±0.009	5.96±0.43 *
		60	6	2	4	-	1	-	1	4.66±1.21*	0.046±0.011*	5.73±0.42 *
		120	3	4	6	-	2	-	1	5.33±1.29**	0.053±0.012**	5.83±0.42 *
		240	3	2	8	1	-	-	2	5.33±1.29**	0.053±0.012**	4.70±0.38 ***
The percentage of chromosomal abnormalities (%)			31.95	7.73	35.05	9.27	5.15	2.06	8.76			

PC (MMC): Positive control- Mytomycin-C, ACE-K: Acesulfame potassium , SE: Standard error, MI: Mitotic index,
ctb: chromatid break, csb: chromosome break, scu: sister chromatid union, cte: chromatid exchange, dic: dicentric chromosome, f: fragment, p: polyploidy
*Significantly different from the control $p < 0.05$ (z test)
**Significantly different from the control $p < 0.01$ (z test)
***Significantly different from the control $p < 0.001$ (z test)

Micronuclei are one of the most studied biomarkers of DNA damage and chromosomal instability in humans. The most commonly used cells are lymphocytes due to a better understanding and easy attainment of MN formation (Fenech et al. 2020). It has been documented that MN formed in these cells is a reliable biomarker for the prediction of cancer risk in human (Setayesh et al., 2020). In this study, ACE-K did not caused significant difference on the frequency of MN at all concentrations. ACE-K slightly increased the MN frequency in all treatments versus negative control. However, it was not significant. Therefore, it did not exhibit clastogenic and aneugenic effects in human lymphocytes *in vitro*. The nuclear division index (NDI) was not induced at all concentrations of ACE-K (Table 3).

The Comet assay is a simple method commonly used to determine DNA breaks *in vitro* as well as *in vivo* (Dusinska et al., 2019). In comet assay, ACE-K significantly and concentration-dependently increased the comet tail intensity at 7.5 µg/mL and 15 µg/mL versus control ($r = 0.43$) in isolated human lymphocytes. Besides, a statistically significant increase in comet tail length and tail moment were observed only at 15 µg/mL concentration. However, this induction was not concentration-dependent manner (Table 4).

These current results were consistent with previous reports. Fındıklı and Türkoglu (2014) showed that ACE-K caused DNA damage by increasing tail DNA and tail moment parameters at 2.5 and 5 ppm concentrations in human lymphocytes treated for three hours by the comet assay. In another study, van Eyk (2015) investigated the genotoxic effect of ACE-K on human colon carcinoma (Caco-2 and HT-29) cells and human embryonic kidney HEK-293 cells by the treatment with 1, 4, 10, 20, and 50 mM concentrations for 24, 48 and 72 h using the comet assay. The author indicated that the cells treated with ACE-K had no or little DNA fragmentation at all treatment times and all the cells tested. In *in vivo* study, Bandyopadhyay et al. (2008) assessed the genotoxic effect of ACE-K (150, 300, and 600 mg/kg body weight) in mouse bone marrow cells using comet assay and the mutagenic effect using Ames assay. They found that ACE-K increased the tail DNA and tail extent values in mice bone marrow cells at all treatments. However, it did not cause any mutagenic effect using the Ames assay. Additionally, Jeffrey and William (2000) determined that ACE-K caused DNA damage in hepatocytes of F-344 and Sprague-Dawley rats using a hepatocyte/DNA repair assay. However, ACE-K did not affect DNA damage in rat hepatocytes. Silva et al.

(2008) found that a significant increase was observed in DNA damage of the alone ACE-K (5% concentration) and the combination of ACE-K+Aspartame at 0.5% and 5% concentrations in human peripheral lymphocytes using the comet assay. It was concluded that alone ACE-K did not exert genotoxic activity at low concentrations. Najam et al. (2017) have evaluated the *in vitro* genotoxic effect of alone ACE-K (100, 200, 400, 800, and 1600 µg/mL in concentrations) and combination with Sitagliptin (100+190, 200+380, 400+760, 800+1520, and 1600+3040 µg/mL in concentrations) on lymphocytes by Comet assay and mutagenic effect by Ames assay. ACE-K induced a significant and concentration-dependent DNA damage in lymphocytes compared to the negative control. It also showed the mutagenic effect at the concentrations of 800 µg/plate and 1600 µg /plate. The combination of ACE-K + Sitagliptin showed a mutagenic potential at the combined concentrations of 760+400 µg/plate and 1520+800 µg/plate.

Table 3. Frequencies of the micronucleus and nuclear division index in cultured human lymphocytes treated with ACE-K

Test sub-stance	Treatment		The number of counted BN cell	Distribution of BN cells according to the No. of MN			MN ± SE (%)	NDI±SE
	Time (hour)	Concentration (µg/mL)		(1)	(2)	(3)		
Control	48	0	3000	8	1	-	0.33±0.10	1.54±0.31
PC (MMC)	48	0.20	3000	40	2	-	1.40±0.21	1.40±0.30
ACE-K	48	7.5	3000	8	-	1	0.36±0.11	1.70±0.33
		15	3000	9	1	-	0.36±0.11	1.55±0.31
		30	3000	12	1	-	0.46±0.12	1.57±0.32
		60	3000	17	-	-	0.56±0.13	1.51±0.31
		120	3000	14	1	-	0.53±0.13	1.53±0.31
		240	3000	15	1	-	0.56±0.13	1.40±0.30

PC (MMC): Positive Control-Mytomycin C, ACE-K: Acesulfame Potassium, SE: Standard error, BN: Binucleat, MN: Micronucleus, NDI: Nuclear division index.

Table 4. DNA damage caused by ACE-K in isolated human lymphocytes

Test sub-stance	Treatment		Tail intensity (%)	Tail Length (µm)	Tail Moment
	Time (hour)	Concent. (µg/mL)			
Control	1	0.00	3.92±0.29	37.80±0.52	0.77±0.06
PC (H_2O_2)	1	100 (µM)	17.16±1.16	64.82±1.10	4.55±0.37
ACE-K	1	7.5	4.88±0.34 *	38.40±0.46	0.92±0.07
		15	5.17±0.46 *	39.44±0.47 *	1.01±0.09 *
		30	3.93±0.31	37.67±0.40	0.76±0.06
		60	4.52±0.36	38.38±0.42	0.88±0.07
		120	4.23±0.32	37.11±0.45	0.80±0.06
		240	3.90±0.31	38.19±0.58	0.78±0.08

PC (H_2O_2): Positive Control (Hydrogen peroxide)

* Significantly different from the control $p < 0.05$ (t- test)

Conclusion

Considering all the results, ACE-K showed a cytotoxic effect in HepG2 cells as well as human lymphocytes, especially at higher concentrations. This sweetener exhibited a mild genotoxic effect by increasing the frequency of CAs at 60-240 µg/mL in long-term treatment and DNA damaging effect especially at 15 µg/mL using the comet assay. Since the health effects of artificial sweeteners are not yet fully known, foods containing these substances should not be consumed excessively. In this regard, more *in vivo* studies would be needed.

Compliance with Ethical Standard

Conflict of interests: The author declares that for this article they have no actual, potential, or perceived conflict of interests.

Ethics committee approval: This study was confirmed by the Ethical Committee of the Faculty of Medicine, Gazi University (11.11.2019/144).

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