Full Length Research Paper

Determination of the effects of some artificial sweeteners on human peripheral lymphocytes using the comet assay

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In this study, the genotoxicity of the artificial sweeteners acesulfame potassium, aspartame, saccharin, and sorbitol, which are used in food industry and by patients with diabetes, was investigated in human peripheral lymphocyte cells using the single-cell gel electrophoresis (comet) technique. Human lymphocyte cells were treated with the substances for three hour at each of the three dosages (1.25, 2.5, and 5 ppm). The chemical additives were studied, and the related DNA damages in the study group were compared to the control group for each of the treatment dosages. The DNA breakages observed in the comet assay were assessed in terms of tail moment and tail DNA percent using the comet parameters. The statistical and photographic analyses were performed using SPSS 15 and BAB BS 200 Pro software, respectively. Based on the results for the short-term in vitro treatments, the 4 different food flavorings were found to have genotoxic effects.

Key words: Comet assay, DNA damages, artificial sweeteners, human peripheral lymphocytes.

INTRODUCTION

Sugar-free food products are sweetened by sugar substitutes that are commonly referred to as non-nutritive sweeteners, low calorie sweeteners, artificial sweeteners, or alternative sweeteners. Irrespective of their name, all sugar substitutes taste similar to sugar, but contain few to no calories and produce a low glycemic response. These sweeteners are widely used in processed foods, including baked goods, carbonated beverages, powdered drink mixes, candy, puddings, canned foods, jams, jellies, and dairy products.

Artificial sweeteners have been the subject of intense scrutiny for decades. Critics of artificial sweeteners maintain that sweeteners cause a variety of health problems, including cancer. Their arguments are based on studies dating to the 1970s, which linked saccharin to bladder cancer in laboratory rats. Because of these studies, saccharin once had a warning label stating that the product might be hazardous to human health.

Rapid increases in the consumption of sugar and sugar-containing foods have led to the emergence of certain health problems. High sugar consumption is associated with dental caries, obesity, and cardiovascular

disease, which occur because of a rapid increase in blood sugar levels and cause deleterious effects, especially in patients with diabetes (Howard and Wylie-Rosett, 2002). Considering the effect of sugar on diabetes patients, the most effective management, aside from medical treatment, involves a diet with limited quantities of sugar and sugary foods. Therefore, it is undesirable to use natural sweeteners to replace sugar.

The most popular artificial sweeteners are acesulfame potassium, aspartame, sorbitol, cyclamate, saccharin, sucralose, alitame, neotame, and neohesperidin dihydrochalcone. The latter is a semi-synthetic sweetener. In this study, the genotoxic effects of acesulfame potassium, aspartame, saccharin, and sorbitol were investigated.

Acesulfame potassium is a high-intensity, non-nutritive sweetener that is 200 times sweeter than sucrose. It is non-carcinogenic and stable under high temperatures and has an excellent shelf life. Acesulfame potassium is used as a sweetener in many foods, including chewing gums, baked goods, dessert and dairy products, alcoholic beverages, canned foods, candies, and over 4000 other products sold in approximately 90 countries, including Turkey, Australia, Canada, and Germany (Calorie Control Council, 2006). Acesulfame potassium is not metabolized or stored in the body. It is quickly absorbed and then excreted without undergoing modification. The results of several long-term animal studies that tested significantly higher amounts of acesulfame potassium than normally consumed by humans found no evidence of the development of cancers or tumors. Acesulfame potassium contains the chemical methylene chloride, a known carcinogen. Long-term exposure to methylene chloride can cause headaches, depression, nausea, mental confusion, liver and kidney effects, and cancer in humans (ATSDR, 1998; HSDB, 1993, Graves et al., 1994). Another one of the byproducts of acesulfame potassium's breakdown in the body is acetoacetamide, which is toxic at high doses. Center for Science in the Public Interest (CSPI) notes that acetoacetamide has been shown to cause tumor growth in the thyroid gland in rats, rabbits, and dogs after administration of only 1% acetoacetamide in the diet for three months (http://www.cspinet.org/reports/asekquot.html).

Aspartame has a sugar-like taste that is used to enhance fruit and citrus flavors. It can be safely heated to high temperatures with some loss of sweetness, and it is non-carcinogenic. In addition, aspartame is approximately 200 times sweeter than sucrose. Since its approval, aspartame has been used in over 6000 different types of products, including soft drinks, dessert mixes, frozen desserts and yogurt, chewable multi-vitamins, breakfast cereals, table top sweeteners, and pharmaceuticals (Rencüzoğulları et al., 2004). It is consumed by millions of people around the world (Butchko et al., 2002; Fry, 1999). Upon digestion, aspartame breaks down into small amounts of methanol

and the amino acids aspartic acid and phenylalanine. Chronic exposure to aspartame has been reported to cause the following symptoms: headaches, blurred vision, epileptic fits, brain tumors, eye problems, numbness, insomnia, memory loss, nausea, slurred speech, loss of energy, hyperactivity, hearing problems, neurological problems, and behavioral disturbances (Humphries et al., 2008).

Saccharin is an artificial sweetener that has been used for over a century to sweeten foods and beverages without adding calories or carbohydrates. It is found in food such as soft drinks, baked goods, chewing gum, canned fruit, salad dressings, cosmetic products, and pharmaceuticals. Saccharin has been approved for use in more than 100 countries. After ingestion, saccharin is neither absorbed nor metabolized; instead, it is excreted unmodified via the kidneys. Because saccharin is not metabolized, the Food and Drug Administration (FDA) of the USA considers it safe.

Sorbitol, also known as glucitol, is a sugar alcohol that is slowly metabolized by the human body. It is often used in diet foods, mints, cough syrups, and sugar-free chewing gum. Sorbitol contains fewer calories than sugar and has minimal effects on blood sugar levels. However, sorbitol consumption is associated with side effects, especially when ingested in large quantities. One common side effect of sorbitol is diarrhea. Johannes et al. (1992) reported that sorbitol induced DNA fragmentation in Chinese hamster ovary cells.

Consistent ingestion of food additives has been reported to induce toxic, genotoxic, and carcinogenic effects (Demir et al., 2010; Hobbs et al., 2012; Güngörmüş and Kılıç, 2012; Jeffrey and Williams, 2000; Kumar and Srivastava, 2011; Saad et al., 2014 Zengin et al., 2011). The DNA damage induced by food additives depends on their transport across cellular/nuclear membranes, the activation and deactivation of intracellular enzymatic processes, the levels of radical scavengers, and the repair mechanisms in the target cell population.

The comet assay has been used to determine the effects of these cellular processes on the amount of DNA damage induced (Kasamatsu et al., 1996; Szeto et al., 2002; Tice et al., 2000).

This assay is a powerful tool for determining genotoxicity, because it is simple and highly sensitive, has a short response time, and requires a relatively small number of cells and test substances (Adegoke et al., 2012; Benedetti et al., 2013; Čabarkapa et al., 2014; Fabiani et al., 2012; Liman et al., 2011; Severin et al., 2010).

Food sweeteners are widely used in food, but little is known about their genotoxic effects. Thus, the purpose of this study was to evaluate the potential genotoxic effects of food sweeteners, such as acesulfame potassium, aspartame, saccharin, and sorbitol, on isolated human lymphocytes using the comet assay.

MATERIALS AND METHODS

Acesulfame potassium (CAS No: 55589-62-3), aspartame (CAS No: 22839-47-0), saccharin (CAS No: 81-07-2), and sorbitol (CAS No: 50-70-4) were purchased from Sigma Chemical Co.

On each day of the analysis, fresh human peripheral blood was obtained by venipuncture from three healthy male human volunteers and placed into BD Vacutainer collection tubes containing heparin as an anticoagulant. The volunteers were non-smokers, unmedicated, and had an average age of 23 ± 1 years. Fresh blood (2 ml) was diluted with an equal volume of PBS, and 2 ml of the diluted blood was layered gently over 2 ml of Histopaque and incubated for 25 min. The tube was centrifuged at 1000 rpm for 40 min, and the buffy coat was aspirated into 4 ml of PBS. The resulting mixture was centrifuged at 1000 rpm for 10 min. The supernatant was then discarded, and the lymphocyte pellet was resuspended at a concentration of 10^6 cells/ml in RPMI-1640 medium. The isolated lymphocytes were incubated with various concentrations of the different food sweeteners at 37°C for 3 h.

The concentrations of the four sweeteners were selected on the basis of permissible sweetener levels (Turkish Food Codex, 2011). The following food sweetener concentrations were used: 1.25, 2.5, and 5 ppm. The samples prepared were incubated at 37°C for 3 h. After incubation, the lymphocytes were harvested by centrifugation at 2000 rpm for 10 min, and the cell pellet was resuspended in PBS.

The comet assay was performed according to the methods of Singh et al. (1989), Tice et al. (1991), and Ghosh et al. (2010) with slight modifications. To visualize DNA damage, slides were examined at 400× magnification using a fluorescence microscope, and 100 cells were randomly selected for analysis in each sample (BAB Bs200ProP/BsComet DNA Comet Assay). The tail DNA (%) and tail moment were used to measure DNA damage because they give the most meaningful results in genotoxicity studies (Kumaravel and Jha, 2006). This study was performed in accordance with the Declaration of Helsinki and with the approval of the local ethics committee (No. 2010-04/04).

Statistical analysis was performed using SPSS (version 15.0). The percentage of DNA in the tail (% DNA tail) and tail moment (μm) were measured as comet parameters. All data were presented as arithmetic mean ± standard error. The statistical approach was analysis of variance (ANOVA), which was used to evaluate the significance of the difference in DNA damage between the control and treated cells. Results were considered statistically significant at $p < 0.05$.

RESULTS

The results of the comet assay are as shown in Tables 1 to 4. The percent tail DNA and tail moment for human lymphocytes exposed to different doses of acesulfame potassium or to distilled water (control group) are compared as shown in Table 1. Acesulfame potassium at 2.5 and 5 ppm increased the tail moment to 7.87 ± 1.30 and 17.60 ± 1.58, respectively. The same concentrations of acesulfame potassium increased the tail DNA to 9.21 ± 1.02% and 19.01 ± 0.63%, respectively. Thus, these concentrations of acesulfame potassium increased DNA damage.

Exposure to 1.25, 2.5, and 5 ppm of aspartame increased the tail moment and tail DNA (Table 2). Among these doses, the highest DNA damage was observed after treatment with 2.5 ppm of aspartame (tail moment: 17.62 ± 1.00, tail DNA: 18.92 ± 1.87%).

All of the saccharin concentrations tested increased DNA damage relative to that observed in the control group (Table 3). The group treated with 2.5 ppm of saccharin exhibited the greatest DNA damage.

Table 4 summarizes the comet assay data, which are expressed as the tail moment and tail DNA (%), for human lymphocytes exposed for 3 h to different concentrations of sorbitol. Cells treated with 2.5 ppm of sorbitol exhibited a significant increase in single-strand breaks when measured by the tail moment (25.85 ± 1.64) and tail DNA (27.44% ± 2.17%).

DISCUSSION

Acesulfame potassium, aspartame, saccharin, and sorbitol are commonly used as sweeteners in the food and pharmaceutical industries. These sweeteners are low in calories and provide sweetness with little to no intake of food energy. According to a 2007 survey published by the Calorie Control Council, 86% of Americans consume low-calorie, reduced sugar, or sugar-free foods and beverages (Calorie Control Council, 2007).

Food additives are widely used in factory-made foods. Therefore, they must be completely safe for human consumption. Nevertheless, scientific studies on these additives have yielded unfavorable results, especially in gene toxicity and carcinogenicity tests. Genotoxicity pertains to all types of DNA damage. Agents that interact with DNA and/or associated cellular components (e.g., the spindle apparatus) or enzymes (e.g., topoisomerases) are considered genotoxins (Dearfield et al., 2002; Jouyban and Parsa, 2012; Robinson, 2010).

Acesulfame potassium, aspartame, saccharin, and sorbitol are used as sweeteners in food products and pharmaceuticals. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the European Union's Scientific Committee have found these sweeteners to be safe for use in food. However, using the comet assay, we determined that these sweeteners were genotoxic.

Reports on the genotoxicity of acesulfame potassium, aspartame, saccharin, and sorbitol are inconsistent. Very little research has been done on acesulfame potassium, which was considered once under scrutiny by the FDA for being a potentially dangerous compound with adverse side effects. The few studies on acesulfame potassium were incomplete and inconclusive, but found the compound to be genotoxic and clastogenic in mice (Mukherjee and Chakrabarti, 1997), non-mutagenic in mammalian cells (Marquardt, 1978), and non-cytotoxic and non-genotoxic in *in vivo* and *in vitro* experiments (Baeder et al., 1977). Bandyopadhyay et al. (2008) used the comet assay to determine the mutagenic activity of acesulfame potassium. Additional research on the effects

Table 1. Effect of acesulfame potassium on DNA damage measured as comet percent tail DNA and tail moment (mean± SE).

Dose (ppm)	Tail DNA* (%)	Tail moment*
Control	3.05±0.14[a]	2.19±1.16[a]
1.25	2.96±1.55[a]	2.08±0.92[a]
2.50	9.21±1.02[b]	7.87±1.36[b]
5.00	19.01±0.63[c]	17.60±1.58[c]

*Means with the same letters do not significantly differ at 0.05 level.

Table 2. Effect of aspartame on DNA damage measured as comet percent tail DNA and tail moment (mean±SE).

Dose (ppm)	Tail DNA* (%)	Tail moment*
Control	3.05±0.14[a]	2.19±1.16[a]
1.25	13.88±1.58[b]	12.55±1.76[b]
2.50	18.92±1.87[c]	17.62±1.00[c]
5.00	10.94±1.23[d]	9.99±1.15[d]

*Means with the same letters do not significantly differ at 0.05 level.

Table 3. Effect of saccharin on DNA damage measured as comet percent tail DNA and tail moment (mean±SE).

Dose	Tail DNA* (%)	Tail moment*
Control	3.05±0.14[a]	2.19±1.16[a]
1.25 ppm	16.63±1.52[b]	15.49±1.85[b]
2.50 ppm	21.96±3.03[c]	20.48±1.74[c]
5.00 ppm	13.00±2.27[d]	11.61±2.46[d]

*Means with the same letters do not significantly differ at 0.05 level.

Table 4. Effect of sorbitol on DNA damage measured as comet % tail DNA and tail moment (mean±SE).

Dose	Tail DNA* (%)	Tail moment*
Control	3.05±0.14[a]	2.19±1.16[a]
1.25	4.63±1.54[b]	3.39±1.11[a]
2.50	27.44±2.17[c]	25.85±1.64[b]
5.00	14.22±1.06[d]	12.35±1.35[c]

*Means with the same letters do not significantly differ at 0.05 level.

of acesulfame potassium on mice revealed chronic use over a period of 40 weeks resulted in a moderate but limited effect on neurometabolic function. These results suggest chronic usage of acesulfame potassium may alter neurological function (Cong et al., 2013).

Creppy et al. (1998) suggested that aspartame had antigenotoxic activity. According to Trocho et al. (1998), aspartame consumption might be hazardous because of its contribution to the formation of formaldehyde adducts. Furthermore, Soffritti et al. (2006, 2007, 2010, 2014) have demonstrated the carcinogenic potential of this compound. The safety of aspartame and its metabolic breakdown products (phenylalanine, aspartic acid and methanol) was investigated *in vivo* using chromosomal aberration (CA) test and sister chromatid exchange (SCE) test in the bone marrow cells of mice. Treatment

with aspartame induced dose dependently chromosome aberrations at all concentrations while it did not induce sister chromatid exchanges. On the other hand, aspartame did not decrease the mitotic index (MI). However, statistical analysis of the results show that aspartame is not significantly genotoxic at low concentration (AlSuhaibani, 2010). Mukhopadhyay et al. (2000) reported that blends of aspartame and acesulfame potassium did not increase chromosomal aberrations in the bone marrow of Swiss albino mice. Sasaki et al. (2002) examined the *in vivo* genotoxicity of aspartame in eight mouse organs after 3 and 24 h using the comet assay. Chromosome aberrations and the results of a micronucleus test performed on human lymphocytes indicated that aspartame had genotoxic effects. However, the Ames/*Salmonella*/microsome test detected no mutagenic effects (Rencüzoğulları et al., 2004). Aspartame has also been administered orally to pregnant rats, and cytogenetic effects were observed in the mother rats and their offspring (Abd El Fatah et al., 2012). In addition, mutagenicity studies on acesulfame potassium (Mukherjee and Chakrabarti, 1997) indicated that when mice were administered doses within the acceptable daily intake of 15 mg/kg body weight, the number of chromosomal aberrations was not statistically different from the number in control mice. However, at high doses, acesulfame potassium was clastogenic and genotoxic. Taken together, these studies show that, depending on the dose, acesulfame potassium interacts with DNA and causes genetic damage.

Saccharin has also been the subject of extensive scientific research and debate. It is one of the most studied food ingredients. Although studies indicate that saccharin is safe for human consumption; there has been controversy over its safety. Bladder tumors have been reported in some male rats fed high doses of saccharin (Arnold et al., 1980; Schoenig et al., 1985). In an *in vitro* clastogenicity assay, high doses of saccharin induced chromosomal aberrations and sister chromatid exchange in Chinese hamster ovary and lung cells (IARC, 1999). Furthermore, Sasaki et al. (2002) used the comet assay to assess the ability of 39 currently used food additives, including saccharin and sodium saccharin, to induce DNA damage in mice. They observed that DNA damage in the colon increased a statistically significant amount 3 h after saccharin exposure. Sorbitol is a sugar alcohol that is slowly metabolized by the human body. After it is absorbed in the body, sorbitol oxidizes to fructose, which is subsequently metabolized to fructose-1-phosphate. Sorbitol has been shown to induce DNA damage in Chinese hamster ovary (CHO) cells (Johannes et al., 1992). However, to our knowledge, the genotoxicity of sorbitol in human lymphocytes has not been reported.

In this study, comet assay was used to determine whether four sweeteners induced DNA damage in human peripheral lymphocytes. This assay has been used as a rapid and sensitive tool to assess chemically induced DNA damage. The results showed that all selected concentrations of the four sweeteners significantly increased the level of DNA damage ($p < 0.05$ vs. control). Especially, cells treated with the highest concentration of acesulfame potassium showed a significant increase of the percent tail DNA values compared to the control and other food sweeteners. Acesulfame potassium is stable in foods, beverages and cosmetic preparations under normal storage conditions. Under extreme conditions of pH and temperature, detectable decomposition may occur leading to the formation of acetone, CO_2, and ammonium hydrogen sulfate, or amido-sulfate, as final decomposition products; under acid (pH 2.5) conditions, minute quantities of acetoacetamide and acetoacetamide N-sulfonic acid are formed as unstable intermediate decomposition products, while under alkaline (pH 3-10.5) conditions, acetoacetic acid and acetoacetamide N-sulfonic acid can be detected (http://www.inchem.org/documents/jecfa/jecmono/v16je0 2.htm). These degradation products may cause DNA strand breaks. Another reason of observed high damage rate on 5 ppm dosage of acesulfame potassium may be smaller size of fractures and consequently faster movement in electrophoresis.

The food sweeteners induced a concentration-dependent increase in DNA single-strand breaks, as evident by comet formation. The DNA damage detected in this study may have originated from DNA single-strand breaks, DNA double-strands breaks, DNA adducts formations, and DNA-DNA and DNA-protein cross-links (Mitchelmore and Chipman, 1998) that result from the interactions of sweeteners or their metabolites with DNA. The mechanism by which acesulfame potassium, aspartame, saccharin, and sorbitol exposure induces DNA strand breaks which is poorly understood, and little is known about these sweeteners or the metabolites that are responsible for DNA strand breaks.

Studies on the interactions between small molecules and DNA will be valuable for the development of disease treatments and the prevention of disease, because DNA is a major drug target and can be damaged by harmful chemicals. The DNA damage caused by sweeteners may be associated with the generation of free radicals (reactive oxygen species), which cause DNA strand breaks and irreversible damage to proteins involved in DNA replication, repair, recombination, and transcription (Lin et al., 2007).

Conclusion

This study demonstrated that acesulfame potassium, aspartame, saccharin, and sorbitol cause DNA damage. Commonly used food sweeteners may be toxic at high concentrations in the long term. Therefore, the effects of sweeteners on human health should be extensively investigated, especially when used at high concentrations

in foods and beverages.

Conflicts of İnterest

The authors declare that there are no conflicts of interest.

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Abbreviations: ADI, Acceptable Daily Intake; **FDA,** Food and Drug Administration; **JECFA,** Joint FAO/WHO Expert Committee on Food Additives (JECFA).

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