MAKÜ FEBED
ISSN Online: 1309-2243
http://dergipark.gov.tr/makufebed
DOI: 10.29048/makufebed.377982

Mehmet Akif Ersoy Üniversitesi Fen Bilimleri Enstitüsü Dergisi 9(2): 129-135 (2018)
The Journal of Graduate School of Natural and Applied Sciences of Mehmet Akif Ersoy University 9(2): 129-135 (2018)

Araştırma Makalesi / Research Paper

Aspartame-Induced Mitochondrial DNA Damage in Drosophila

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Geliş Tarihi (Received): 12.01.2018, Kabul Tarihi (Accepted): 11.04.2018
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ABSTRACT

Artificial sweeteners are added to many products sold in the market today, such as food, beverages, medicines, hygiene products. The possible toxic effects of any of these products, because they are used by many people in the community, can pose a risk for public health. DNA mutations generated by some artificial sweeteners have been investigated by some researchers but there is no information in the literature about the effects of these substances on mtDNA. mtDNA damage potentially is more important than deletions in nDNA because the whole mitochondrial genome is expressed without intron regions. Also, somatic mtDNA mutations have been increasingly observed in primary human cancers. The aim of the current study, detection of the effects of some artificial sweeteners on mtDNA damage and copy number in *Drosophila melanogaster*. The Quantitative PCR method was used for this purpose. According to our results, Aspartame created statistically significant mtDNA damage. There was no mtDNA damage in Saccharine+Cyclamate, Saccharine, Aceculfam K and Sucralose application groups. These results indicate that the effects of aspartame in human should carefully detect.

Keywords: mtDNA damage, Aspartame, Saccharine, Acesulfam K, Sucralose, Cyclamate

Drosophila'da Aspartam Tarafından Oluşturulan Mitokondrial DNA Hasarı

ÖZ

Yapay tatlandırıcılar çok çeşitli yiyecek, içecek, ilaç ve hijyen ürünlerine eklenir. Bu ürünlerin herhangi birinin toksik etkilerinin olması, toplumdaki birçok insan tarafından kullanıldığı için, halk sağlığı açısından risk oluşturabilir. Bazı yapay tatlandırıcıların nüklear DNA üzerindeki etkileri araştırılmış, ancak bu maddelerin mtDNA üzerindeki etkileri hakkında herhangi bir çalışmaya literatürde rastlanmamıştır. Mitokondrial genomdaki tüm bölgeler ifade edildiğinden, mtDNA'daki mutasyonlar, nDNA'daki delesyonlardan potansiyel olarak daha önemli olabilir. Ayrıca, primer insan kanserlerinde somatik mtDNA mutasyonları gittikçe artan bir şekilde gözlenmektedir. Bu çalışmanın amacı, bazı yapay tatlandırıcıların mtDNA hasarı ve kopya sayısına etkilerinin *Drosophila melanogaster*'de saptanmasıdır. Bu amaçla kantitatif PCR yöntemi kullanılmıştır. Sonuçlarımıza göre, Aspartam istatistiksel olarak anlamlı derecede mtDNA hasarı oluşturmuştur. Sakkarin + Siklamat, Sakkarin, Asesulfam K ve Sukraloz uygulama gruplarında ise mtDNA hasarı saptanmamıştır. Bu sonuçlar, Aspartamın insanlar üzerinde yapabileceği olası etkilerinin dikkatle saptanması gerektiğini göstermektedir.

Anahtar Kelimeler: mtDNA hasarı, Aspartam, Sakkarin, Asesulfam K, Sukraloz, Siklamat

INTRODUCTION

Artificial sweeteners are added to many products sold in the market today, such as food, beverages, medicines, hygiene products (Weihrauch and Diehl, 2004). In recent years, increasing obesity rates in the world, particularly in developed countries, has also increased the popularity of diets and products for weight loss. Non-nutritive sweeteners have been used for many years in weight loss and diabetic diets (Shankar et al., 2013). Although many people use these products for weight loss, there have been some studies about the weight gain of these products (Yang, 2010).

Saccharin, cyclamate and aspartame are defined as first-generation sweeteners, while others such as acesulfame K, sucralose, alitame, and neotame are described as second-generation sweeteners. The toxic effects of any of these products, because they are used by many people in the community, can pose a risk for public health (Weihrauch and Diehl, 2004).

The aim of the current study, detection of the effects of saccharin, cyclamate, aspartame, acesulfam K and sucralose on mtDNA damage and copy number in Drosophila melanogaster. The chemical formulas of these artificial sweeteners are given in Figure 1.

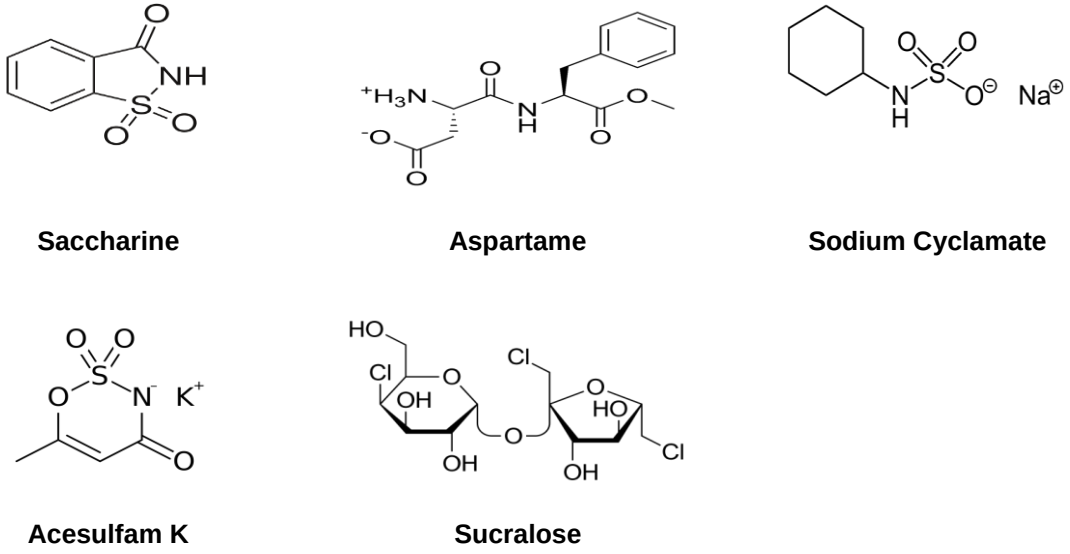

Figure 1. The Molecular formulas of artificial sweeteners used in this investigation.

DNA mutations generated by some artificial sweeteners have been investigated by some researchers (AlSuhaibani 2010; Bandyopadhyay et al., 2008; Brusic et al., 2010; Demir et al., 2014; Jeffrey and Williams, 2000; Kashanian et al., 2013; Rencüzoğulları et al., 2004; Sasaki et al. 2002) but there is no information in the literature about the effects of these substances on mtDNA. mtDNA damage could potentially be more important than deletions in nDNA because the entire mitochondrial genome codes for genes are expressed (Liang and Godley, 2003). Also, somatic mtDNA mutations have been increasingly observed in primary human cancers (Chatterjee et al. 2006). In this research, the QPCR method was used to measure mtDNA damage.

MATERIAL AND METHODS

Wild type Drosophila melanogaster larvae were used in studying mtDNA damage. Flies were fed on corn meal (water, corn flour, sugar, yeast and agar) in glass bottles at 24 ±1 °C.

Artificial sweeteners instead of sugar were added to the corn meal of the experimental groups. In the experimental groups treatments applied were: “Aspartame 72 mg/100 ml”; “Na saccharine + Na cyclamate 12,5 mg+125 mg/100 ml”; “Na saccharine 50 mg/100 ml”; “Sucralose 16 mg/100 ml”; “Acesulfam K 80 mg/100 ml” corn meal. The amounts of artificial sweeteners applied are selected based on the amount of sugar put into the control group and the amounts of artificial sweeteners which will give the same degree of sugar tastes are preferred.

Adult female and male flies were placed in experimental medium and breeding was provided. Approximately 3-4 days after the fertilized egg is released, the third instar larvaes to appear in the bottle. DNA isolation of the third instar larvae was conducted. Twelve larvae were analyzed from each group. SIGMA G1N350 Genomic DNA kits were used for total DNA isolation using

the methods indicated in the technical bulletin. Invitrogen (Molecular Probes) Pico Green dsDNA quantitation dye and QUBIT 2.0 fluorometer were used for template DNA quantitation and for the fluorometric analysis of PCR products. A crucial step of the QPCR method is the concentration of the DNA sample. The accuracy of the assay relies on initial template quantity because all of the samples must have exactly the same amount of DNA. The Pico Green dye has not only proven to be an efficient method for template quantitation but also for PCR product analysis (Santos et al., 2002). DMSO (4% of total volume) was added to 5 ng of template total DNA in each PCR tube. Thermostabil polymerase used was Thermo Phire hot start II DNA polymerase.

Primers for *Drosophila* mtDNA small fragment (100 bp) were (Mutlu 2012A, Mutlu 2012B, Mutlu 2013, Mutlu 2017);

11426 5'- TAAGAAAATTCCGAGGGATTCA - 3'
11525 5'- GGTCGAGCTCCAATTCAAGTTA - 3'

Primers for large fragment (10629 bp) were;

1880 5'- ATGGTGGAGCTTCAGTTGATTT - 3'
12508 5'- CAACCTTTTTGTGATGCGATTA - 3'

For long fragment PCR amplification, DNA was denatured initially at 98°C for 1 minute; the material then underwent 21 PCR cycles of 98°C for 10 seconds, 52°C for 45 seconds, and 68°C for 5 minutes. Final extension was allowed to proceed at 68°C for 5 minutes.

For small fragment PCR amplification, DNA was denatured initially at 98°C for 1 minute; the material then underwent 21 PCR cycles of 98°C for 10 seconds, 55°C for 45 seconds, and 72°C for 10 seconds. Final extension was allowed to proceed at 72°C for 2 minutes.

The QPCR method was used to measure mtDNA damage. The lesion presents in the DNA blocked the progression of any thermostable polymerase on the template, so a decrease in DNA amplification was observed in damaged templates. The QPCR method is highly sensitive to measurements of DNA damage and repair. mtDNA damage was quantified by comparing the relative efficiency of amplification of long fragments of DNA and normalizing this to gene copy numbers by the amplification of smaller fragments, which have a statistically negligible likelihood of containing damaged bases (Yakes and Van Houten, 1997; Santos et al., 2002; Venkatraman et al., 2004). To calculate normalized amplification, the long QPCR values were divided by the corresponding short QPCR results to account for potential copy number differences between samples (the mtDNA/total DNA value may be different in the 5-ng template of total DNA in each PCR tube). Decreased abundance of amplification is an indicator of the damaged DNA. The copy number results do not indicate damage.

Minitab Release 13.0 software was used for statistical analysis. The results were analyzed using the Mann–Whitney Test.

RESULTS AND DISCUSSION

According to the results of our work, Aspartame caused statistically significant mtDNA damage. There was no significant mtDNA damage in groups treated with Saccharine+Cyclamate, Saccharine, Aceculfam K and Sucralose. There was also a significant increase in the number of mtDNA copies in the aspartame treated group (Table 1).

Table 1. Relative amplification and mtDNA copy number results.

Groups	Relative Amplification ± SE	mtDNA copy number ± SE
Control	3,49 ± 0,23	131,2 ± 5,42
Aspartame	2,86 ± 0,15*	158,1 ± 3,88*
Sodium Saccharine	3,04 ± 0,15	144,78 ± 7,05
Sucralose	3,33 ± 0,18	147,22 ± 5,63
Sodium Saccharine + Sodium Cyclamate	3,25 ± 0,10	136,5 ± 3,78
Acesulfam K	3,22 ± 0,20	144,2 ± 4,97

*Statistically significant ($p<0,05$).

To date, the effects of artificial sweeteners on nuclear DNA (nDNA) have been investigated (AlSuhaibani 2010; Bandyopadhyay et al., 2008; Brusic et al., 2010; Demir et al., 2014; Jeffrey and Williams, 2000; Kashanian et al., 2013; Rencüzoğulları et al., 2004; Sasaki et al. 2002). There was no information on the effects of artificial sweeteners on mtDNA in the literature.

In a study by Jeffrey and Williams, nDNA damage activities was investigated in rats for aspartame, cyclamate, saccharin, acesulfam K and sucralose. Data from this study documented that resulting in no DNA damage (Jeffrey and Williams, 2000).

Others showed that aspartame, acesulfame K and saccharin increased DNA damage in bone marrow cells and this damage caused acesulfame K and saccharine was bigger than aspartame did. However, in the Ames / Salmonella / Microsome test conducted in the same study, it was suggested that none of them were potential mutagens (Bandyopadhyay et al., 2008). In a study using COMED in mice, cyclamate, saccharin and sucralose were found to induce DNA damage in gastrointestinal organs, whereas acesulfame K and aspartame did not increase DNA damage (Sasaki et al., 2002). Previous study have documented that acesulfam K, saccharin and sucralose were not genotoxic by COMED test in Drosophila while genotoxic effect of aspartame was detected (Demir et al., 2014).

Cytogenetic studies have been carried out in mice by treating acesulfam K. It has been observed that acesulfam K does not cause chromosomal disturbances at low doses (15 mg / kg body weight) in one of these studies, but it is clastogenic and genotoxic in high doses (60, 450, 1100, 2250 mg / kg) (Whitehouse et al., 2008). It has been shown in some studies that it can interact with DNA at high doses but not at normal doses. These doses are much more than the recommended doses for daily intake (Whitehouse et al., 2008).

The formation of DNA damage by a compound often creates strong suspicions that the compound may be carcinogenic. In this context, studies on the genotoxicity and carcinogenicity of artificial sweeteners have been intensively conducted. In 1977, one of the earliest studies of the relationship between artificial sweeteners and cancer has been shown to increase the risk of bladder cancer in men using saccharine (Howe et al., 1977). The study by Morrison and Buring, published in 1980, found opposite results and showed that the use of artificial sweeteners did not increase the risk of lower urinary tract cancers (Morrison and Buring, 1980). Other study published two months after this study suggested that artificial sweeteners are not a risk in bladder cancer (Hoover and Strasser, 1980). Other earlier study revaled that there was no relationship between artificial sweetener use and bladder cancer (Wynder and Stellman, 1980).

Studies with saccharin have produced both positive and negative results. Studies of two generations showed frequent increases in the risk of bladder cancer in males, especially in groups exposed to 5% or 7.5% diets containing saccharin. Saccharin is not metabolized, it is nucleophilic, but it does not bind to DNA. Therefore it does not behave like a typical carcinogen. However, its suppression impact on production of antibodies has been shown. Two-fold studies are useful to investigate the potential toxic effects of compounds. But most of the studies about effect of saccharin is for examining a single exposed group (Whitehouse et al., 2008).

Weihrauch reviewed more than 50 studies on effects of saccharine in rats, and found that no more neoplasms were observed in any group of first-generation rats except one study. Since the rats used in this positive study may contain Trichosomoides crassiconda, which is common bladder parasites, the results of the study are questionable. Carcinogenic effects of saccharin have not been observed in monkey studies (Wiehrauch and Diehl, 2004).

Cyclamate is metabolized to cyclohexylamine. This metabolite has been shown to cause testicular atrophy and spermatogenesis problems in rats and dogs. In a monkey study, reserchers detected malignancies and benign tumors in some animals in the cyclamate treated group. The authors commented that this was not evidence of carcinogenesis of sodium cyclamate. Because the tumors seen in the cyclamate group have different histologies in different organs and also the incidence of tumor in monkeys is very high (Weihrauch and Diehl, 2004).

At normal doses, there is a broad consensus regarding the safety of acesulfam K (Shankar et al., 2013). Toxicological studies of sucralose have shown that this molecule is very reliable (Whitehouse et al., 2008). There are many studies that report that sucralose is not carcinogenic or genotoxic (Brusick et al., 2010, Shankar et al., 2013). Because it is not absorbed from the intestines, it lacks bioaccumulation and bioreactivity and is considered safe for long-term use. However, it has been shown in some studies that the sucralose causes a decrease of the beneficial bacteria in the intestines, resulting in weight gain (Shankar et al., 2013). Despite the fact that artificial sweeteners are the cause of weight gain, the opposite is also true. In a study comparing sucrose and artificial sweeteners, it was found that the sucrose group increased much mo-

re in terms of body weight and fat mass (Raben et al., 2002).

Aspartame is one of the most controversial artificial sweetener. There are some studies that have carcinogenic potential. In this first study of Soffritti and colleagues 2006, the multipotential carcinogenic effects of aspartame in rats have been experimentally demonstrated for the first time. In this study aspartame exhibited these effects even at a daily doses of less than the acceptable daily intake (20 mg / kg body weight) (Soffritti et al., 2006). Soffritti and colleagues' work in 2007 showed that this molecule has carcinogenicity at doses close to the daily acceptable doses of intake. The study was conducted in rats and in aspartame-treated groups, the incidence of malignant tumors, lymphomas and leukemia increased significantly in males and females, and in females, the incidence of breast cancer increased (Soffritti et al., 2007).

Belpoggi et al. (2006) showed that aspartame caused an increase in the incidence of malignant tumor-bearing animals as well as an increase in lymphoma and leukemia (Belpoggi et al., 2006). It is also believed that aspartame induces angiogenesis and therefore, use in cancers and cancer suspected cases may produce undesirable consequences (Yeşildal et al., 2004). In a study published in 2010, it was found that aspartame induces liver and lung cancers in male mice, but not in female (Soffritti et al., 2010).

Results of in vitro binding studies of Aspartame with calf DNA showed that this molecule has high binding affinity to DNA. This binding is not of intercalative type. This interaction is thought to be related to the binding to the grooves of DNA (Kashanian et al., 2013). AlSuhaibani observed increased chromosomal abnormalities in the bone marrow of the aspartame treated rats, which was found to be highly significant at high concentrations while not being statistically significant at low concentrations (AlSuhaibani, 2010). Rencüzoğulları and colleagues found that aspartame only induces chromosomal aberrations and micronucleus formation at high concentrations and shows cytotoxic effects by mitotic index reduction (Rencuzoğulları et al., 2004).

Although the genotoxic, cytotoxic and carcinogenic effects of aspartame has been shown in many studies, it has been reported that aspartame is harmless in some reviews written before 2006. Weihrauch and Diehl argued in their papers written in 2004 that animal studies show that aspartame is not a cancer-inducing effect even at high doses.

A study on the effect of aspartame on gene expression was published in 2007. According to this study, aspartame increases expression of both oncogen and tumor suppressor genes in bone marrow and kidney in mice (Gombas et al., 2007). It has been observed that aspartame causes oxidative stress in rats, especially in the liver, which increases lipid peroxidation. It has also been observed that superoxide dismutase (SOD) and catalase enzyme activity are reduced in liver and kidney tissues. Reduced glutathione (GSH) levels were reduced in these mice while glutathione-S-transferase (GST) activities were found to increase (Mourad, 2011).

There are conflicting and mixed results about the reliability of aspartame. All industry-funded studies show that aspartame is safe, while 92% of independently funded trials reported that aspartame had adverse health effects (Shankar et al., 2013).

According to the results of our study, Aspartame caused mtDNA damage in larvae of Drosophila ($p < 0,05$) at a statistically significant level. The level of mtDNA damage in the other groups treated with artificial sweeteners was not statistically significant compared to the control group. The effects of aspartame on mtDNA can be mediated through methanol. Because one of the products of metabolism of aspartame is methanol. In her study published in 2012, Mutlu found that methanol induced mtDNA damage in Drosophila (Mutlu, 2012).

CONCLUSIONS

Many of the cellular energies are produced by the mitochondrial electron transport system and oxidative phosphorylation events. Moreover, mitochondria are the central regulator of apoptosis. Each mammalian cell can contain hundreds of mitochondria and thousands of mtDNA. When a new mtDNA mutation occurs in the cell, a mixed intracellular population of mtDNA forms and is called heteroplasmia. When the percentage of mutant mtDNA increases, the mitochondrial energetic capacity decreases, the production of ROS and the probability of the apoptosis increases. Brain, heart, skeletal muscle, endocrine system and kidney are most sensitive to loss of mitochondrial function (Wallace, 2002).

The lack of mitochondrial nucleotide excision repair makes mtDNA more susceptible to mutagenesis (Pettepher et al., 1991). Damage to mitochondrial DNA can potentially be more important than deletions in nDNA. Because there are no introns in the mitochondrial genome. Furthermore, unlike nDNA, mtDNA replicates continuously and continues to multiply even in fully differentiated cells such as neurons and cardiomyocytes. Thus, somatic mutations in mtDNA may have more deleterious effects on cellular functions than nDNA mutations (Liang and Godley, 2003). Also, in recent

years, many mtDNA mutations have been identified in various types of human cancer.

Aspartame created mtDNA damage in Drosophila according to this study. These results indicate that the effects of aspartame in human should carefully detect.

ACKNOWLEDGEMENTS

This study was supported by the Mehmet Akif Ersoy University Scientific Research Projects Unit (0305-NAP-16).

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