Komal Ahire
Dinesh Kumar
Ahmad Ali

Authors' addresses:
Department of Life Sciences, University of Mumbai, Vidyanagari, Santacruz (East) Mumbai, Maharashtra, India.

Correspondence:
Ahmad Ali
Department of Life Sciences, University of Mumbai, Vidyanagari, Santacruz (East) Mumbai, Maharashtra, India.
Tel.: +919870941656
e-mail: ahmadali@mu.ac.in

Differential glycation of arginine and lysine by glucose and inhibition by acesulfame potassium

ABSTRACT

Glycation is a non-enzymatic process between the reactive carbonyl group of sugars and free amino groups of proteins especially arginine and lysine residues. This process leads to the formation of a group of compounds called as Amadori products and advanced glycation products. These products have been implicated in many secondary complications of diabetes. In the last few years, the intake of sweetener has increased for various health reasons like control of hyperglycaemia and obesity. The present study was designed to evaluate the effect of Acesulfame-K, a well-known and widely used sweetener, on glycation system of arginine-glucose and lysine-glucose. The number of glycation products generated in the presence and absence of acesulfame potassium was measured by established methods such as browning, Fructosamine assay, and determination of carbonyl content. The effect of acesulfame potassium was also checked on the glycation of DNA by agarose gel electrophoresis method. The results indicate that lysine is more potent in causing glycation as compared to arginine. Acesulfame potassium could significantly decrease the number of glycation products in the glycation systems, arginine-glucose and lysine-glucose. It can be concluded that Acesulfame-K has anti-glycation potential as it decreased the formation of Amadori products and AGEs. This study is significant in understanding the role of artificial sweetener in the process of glycation.

Key words: Amadori products, Advanced glycation end products (AGEs), artificial sweetener, Acesulfame-K, DNA damage.

Introduction

Glycation is a multistep process which begins with a covalent interaction between the reactive carbonyl group of sugars and amino groups of proteins and other biomolecules. Initially, an unstable Schiff base is formed which undergoes modification to generate early glycation products known as Amadori products (Sharma et al., 2002). These products rearrange themselves in a series of reaction to generate various harmful products, advanced glycation end products (AGEs) (Monnier & Cerami, 1981). There have been continuous efforts by the group of researchers to understand the mechanism of formation of AGEs as they have been implicated in various secondary complications of diabetes and neurodegenerative disorders (Cerami et al., 1985; Singh et al., 2014). Glycation has been also a field of great concern for the food industry because of the browning of the food items during processing and resulting loss of food quality.

The process of glycation is dependent on several factors: concentration and reactivity of sugars and compounds contributing amino groups (basic amino acids like arginine and lysine), duration of interaction between the sugars and proteins, pH etc. During the hyperglycaemic conditions, the number of glycation products and their deleterious effects increase several fold. AGEs have been also shown to be involved in the generation of reactive oxygen/carbonyl species (ROS/RCS) (Son, 2012). These free radicals have been shown to be involved in damaging biomolecules especially nucleic acids and proteins (Ali et al., 2014).

The use of sweeteners has increased several folds in last few decades because of the industrial and pharmacological reasons. There are five artificial sweeteners (saccharin, aspartame, sucralose, neotame, and acesulfame-K) which are considered as safe for human use by the Food and Drug Administration (FDA) (Lorenzo et al., 2015). However, there are still reports in the literature which suggest their potential effects on human health. However, there are very few reports suggesting the role of artificial sweeteners in the process of glycation (Ali & Devrukhkar, 2016; Ali et al., 2017).

The main focus of the present study was to study the effect of acesulfame potassium on different glycation

systems. In a previous study, Ali et al. (2017) had reported the antiglycating property of this sweetener on the lysine-glucose system. However, arginine is another basic amino acid which is found in many proteins and has been shown to interact with sugars during the glycation process. Therefore a comparative study of the differential glycation of arginine and lysine was designed. The results indicate that lysine is more reactive towards glucose as a higher amount of early and advanced glycation end products were generated as compared to arginine. It was also observed that acesulfame potassium prevents the glycation-mediated DNA damage in the presence of both the glycation systems.

This study is significant in understanding the probable role of artificial sweeteners in the process of glycation and the subsequent effect on macromolecular alteration.

Materials and Methods

Materials

The following chemicals were purchased as follows: Glucose from MERCK chemicals; Arginine and lysine from Sigma Aldrich; Acesulfame-K purchased from NeelChem Mumbai; pBR322 from Thermo Fisher. All other chemicals used were of a high analytical grade.

In vitro glycation of lysine/arginine with glucose and artificial sweetener

An aqueous solution of lysine/arginine (100 mg/mL each) was incubated with glucose (100 mg/mL) and the artificial sweetener Acesulfame-K (100 mg/mL) at 37°C for five days. All the incubations were carried out in 0.1 mol/L phosphate buffer, pH 7.4 and contained 3.0 mmol/L sodium azide to prevent bacterial contamination.

Measurement of browning

Browning was measured at 420 nm by using Shimadzu UV-Vis1800 Spectrophotometer (Rondeau et al., 2007).

Measurement of Fructosamine

The concentration of Fructosamine, an Amadori product, was measured by Nitro blue tetrazolium (NBT) assay (Meeprom et al., 2013) with slight modifications. The glycated sample (10.0 μL) was incubated with 100 μL of 0.5 mmol/L NBT in 0.1 mol/L carbonate buffer (pH 10.4) at 37°C for 15 min. The volume of the reaction mixture was made up to 1.0 mL with distilled water (D/W). The absorbance was measured at 530 nm using a Shimadzu UV 1800 spectrophotometer.

Determination of carbonyl content

By the conventional 2, 4-dinitrophenylhydrazine (DNPH) method the carbonyl group in the glycated sample was determined (Meeprom et al., 2013). In this method, 400 μL of 10 mmol/L DNPH in 2.5 mol/L HCl was added to 100 μL of glycated samples and incubated in dark for an hour. Then, 500 μL of 20.00% (w/v) Trichloroacetic acid was added to the tubes and kept on ice for 5 min for proteins to precipitate. Centrifugation at 10,000 rpm (14800g) for 10 min at 4°C was carried out, following which the protein pellet was washed with 500 μL of ethanol/ethyl acetate (1:1) mixture three times. Lastly, the pellet was resuspended in 100 μL of 6.0 mol/L guanidine hydrochloride and 1.0 mL D/W was added. Spectroscopic measurement at 370 nm was taken by using a Shimadzu UV 1800 spectrophotometer.

In vitro glycation of plasmid DNA in the presence of acesulfame-K

The role of sweetener (acesulfame-K) in the glycation-mediated DNA strand breakage was performed according to a previous publication with minor modifications (Ali et al., 2014). pBR322 plasmid DNA (0.5 μg) in 0.1 mol/L phosphate buffer (pH 7.4) was incubated with lysine/arginine (100 mg/mL), glucose (100 mg/mL), ferric chloride (100 μmol/L) in the presence and absence of acesulfame-K (100 mg/mL), in a 10 μL reaction system, at 37°C for 3 h.

Agarose gel electrophoresis of glycated plasmid DNA sample

Ten microliters of samples were mixed with 2.0 μL 6 X gel loading dye and loaded on to 1.0% agarose gel. Electrophoresis carried out initially at 100 V. Once the dye band reached two-thirds of gel length, electrophoresis was terminated and the gel was stained using ethidium bromide solution (final concentration 5.0 μg/mL) for 20 min in dark (Ali et al., 2017). Subsequently, the gel was visualized under gel documentation system and analyzation of bands did with the help of control.

Statistical analysis

Statistical analysis was performed using Microsoft Excel 2010. Results of each experiment were presented as means + SE. Student's *t*-test (paired) was used to analyze the data for significance, where results with p value $\leq$ 0.05 were accounted as statistically significant.

Results

Browning of lysine/arginine incubated with glucose and artificial sweetener

The intensity of browning of glycation system (lysine/arginine + glucose) and/or artificial sweetener is presented in Figure 1. Lysine/Arginine was incubated with Acesulfame-K at 37°C for five days and the intensity of the brown colour was measured at 420 nm. It can be seen from the graph that the extent of browning was almost the same in both the glycation systems. However, the intensity of browning of arginine mixture decreased more in the presence of Acesulfame-K as compared to lysine system. The extent of

Table 1: *Measurement of Glycation Parameters in the presence of individual components*

Samples	Browning	Fructosamine	Carbonyl
Arg	0.025 ± 0.000	0.123 ± 0.008	1.028 ± 0.213
Lys	6.672 ± 0.164	0.414 ± 0.198	42.040 ± 0.213
GLU	0.250 ± 0.000	7.207 ± 0.192	7.319 ± 0.312
ACE-K	0.291 ± 0.041	0.421 ± 0.129	4.656 ± 0.595

browning was negligible when sugar and amino acids were incubated alone (Table 1).
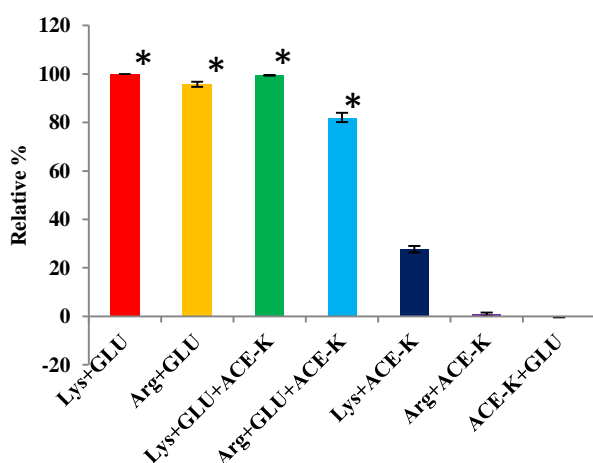

Figure 1. *Measurement of browning. Arginine/Lysine (100 mg/mL) was incubated with glucose (100 mg/mL); Acesulfame-K (100 mg/mL) was incubated with sodium azide (3.0 mmol/L) at 37°C for five days. Absorbance was measured at 420 nm post-incubation. Lys + GLU tube was used as control (100.0%) for calculating the relative percentage of other tubes. Results are expressed as means $\pm$ SEM (n =3) *, Statistical significance, p < 0.05. [ACE-K, acesulfame potassium; Arg, arginine; GLU, glucose; Lys, lysine].*

Measurement of Fructosamine

In the early stages of glycation, unstable Schiff's bases are formed and turned into Amadori products such as Fructosamine. Fructosamine measurement was performed to access the level of Amadori product formed during the glycation process (Figure 2). It was found that there was less formation of early glycation products in the arginine + glucose system (40.0%) as compared to lysine + glucose. Acesulfame-potassium caused inhibition of the formation of early glycation products in both the systems. However, the inhibition was more significant in the lysine glycation system (by 40.0%) as compared to arginine system (15.0%). The number of fructosamines was very low in the samples where sugar and amino acids were incubated alone (Table 1).

Determination of carbonyl content

Oxidation of the glycated samples was measured by the determination of carbonyl content (Figure 3). It can be observed from the graph that the carbonyl content of the arginine system was almost negligible as compared to lysine system. Acesulfame-K caused a decrease in the carbonyl content significantly (by almost 50.0%). When the sugar and amino acids were incubated alone the amount of carbonyl content was comparatively much lower than the glycated samples (Table 1).
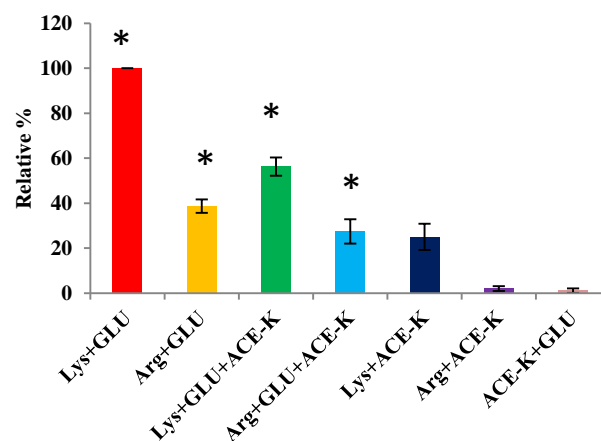

Figure 2. *Measurement of Fructosamines. Arginine/Lysine (100 mg/mL) was incubated with glucose (100 mg/mL); Acesulfame-K (100 mg/mL) was incubated with sodium azide (3.0 mmol/L) at 37°C for five days. Amount of early Amadori product determined using Nitro blue tetrazolium assay, with the resultant absorbance measured at 530 nm. Each value expressed as relative percentage considering Lys + GLU as 100.0%. Results are expressed as means $\pm$ SEM (n = 3) *, Statistical significance, p < 0.05. [ACE-K, acesulfame potassium; Arg, arginine; GLU, glucose; Lys, lysine].*

Effect of Acesulfame-K on Glycated DNA

The effect of glycation was checked on the structure of DNA. It can be seen from the figure 4 that DNA was damaged more significantly in the presence of metal (Fe) in both the glycation systems (Figure 4; Lane 4 & 7). Acesulfame-K prevented the extent of DNA damage induced by glycation systems (Figure 4; Lane 5 & 8).

Disscusion

The process of glycation is an unavoidable non-enzymatic reaction in the body. It leads to the generation of harmful products, AGEs. Many of these AGEs have been identified and implicated in pathophysiological conditions. The deleterious effects of these products are more significantly observed when the blood glucose level increases above the normal level in conditions like hyperglycaemia. Age-related diseases exhibit increased levels of glycation and its end products, further supporting the idea that sugars and their metabolites may act as damaging molecules especially when they accumulate in the cells and tissues (Basta et al., 2004; Suji & Sivakami, 2004; Ali et al., 2017). Major mechanisms by which AGEs cause damage to the biomolecules include aggregation, precipitation or through ROS formation (Ali et al., 2014).
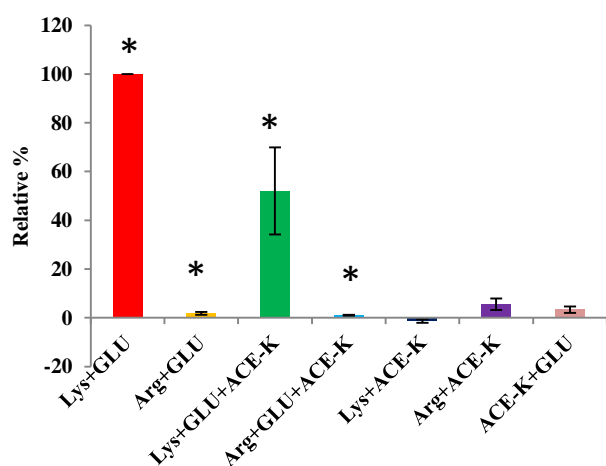

Figure 3. *Determination of carbonyl content of glycated amino acids. Arginine/Lysine (100 mg/mL) was incubated with glucose (100 mg/mL); Acesulfame-K (100 mg/mL) was incubated with sodium azide (3.0 mmol/L) at 37°C for five days. Amount of carbonyl content was estimated using the 2,4-dinitrophenylhydrazine method and absorbance was measured at 370 nm. Each value expressed as relative percentage considering Lys + GLU as 100.0%. Results are expressed as means $\pm$ SEM (n = 3). *, Statistical significance, p < 0.05. [ACE-K, acesulfame potassium; Arg, arginine; GLU, glucose; Lys, lysine].*

In the present study, the extent of glycation products generated in presence of two different amino acids, arginine and lysine was measured by established methods. Glucose was incubated with both the amino acids for five days and the amounts of early and advanced glycation products were measured. The role of Acesulfame-K in the differential prevention of glycation of both the systems was also analysed. Lysine and glucose system of glycation caused generation of more amounts of early and advanced glycation products as compared to the arginine-glucose system. The results presented in the manuscript deal with the interaction

between glucose and individual amino acids. The functional groups involved in the glycation process are carbonyl group of sugars and the amino group of amino acids (free or present in the proteins) (Ansari & Dash, 2013).
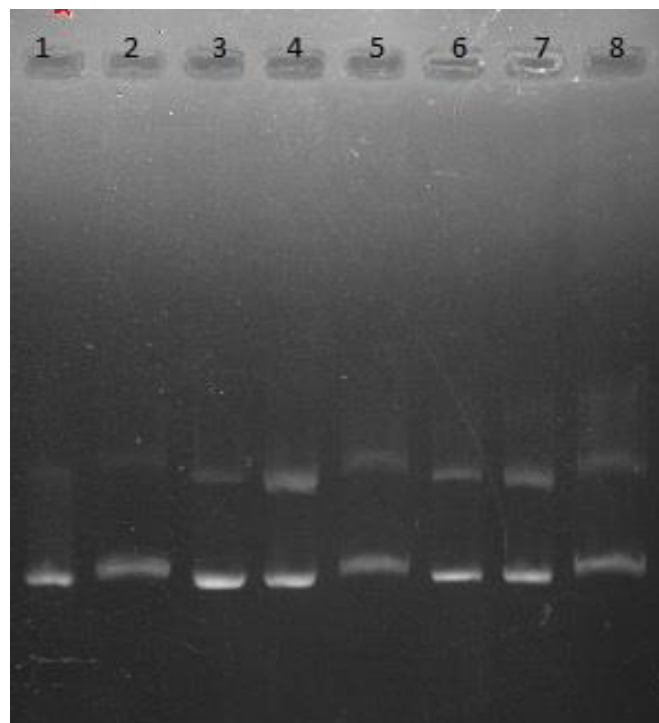

Figure 4: *Effect of acesulfame-K on DNA damage caused by glucose with Arginine/Lysine and ferric chloride.*

Lane descriptions:

Lane 1 - *pBR 322 alone*

Lane 2 - *pBR 322 + acesulfame-K (100 mg/mL)*

Lane3 - *pBR322 + lysine (100 mg/mL) + glucose (100 mg/mL)*

Lane4 - *pBR322 + lysine (100 mg/mL) + glucose (100 mg/mL) + FeCl₃ (100 µmol/L)*

Lane5 - *Lane 4 + Acesulfame-K (100 mg/mL)*

Lane6 - *pBR322 + arginine (100 mg/mL) + glucose (100 mg/mL)*

Lane 7 - *pBR322 + arginine (100 mg/mL) + glucose (100 mg/mL) + FeCl₃ (100 µmol/L)*

Lane 8 - *Lane 7+Acesulfame-K (100 mg/mL).*

As can be seen from Table 1 that amount of glycation parameters (browning, fructosamines and carbonyl content) is negligible when the glucose and amino acids (arginine and lysine) are incubated individually. On the other hand, the amount of glycation parameters increases several fold when glucose is incubated with either of the amino acids (Figures 1, 2, and 3). This indicates that glycation takes place only when both the reacting species (glucose and amino acid) are present in the reaction mixture. The presence of acesulfame-

K caused a significant decrease in the number of fructosamines and carbonyl content, total browning as well as protected DNA from glycoxidation. Acesulfame potassium may interfere with the formation of Schiff bases, Amadori products and AGEs. In an earlier report also it has been shown that Acesulfame prevents the formation of glycation products and DNA damage (Ali et al., 2017). However, the exact mechanism of action of Acesulfame potassium is not known and our group is working towards characterization of this preventive effect of Acesulfame potassium.

Glycation of DNA has shown to considerably alter the conformation of DNA macromolecule and it leads to strand breaks, depurination and mutations (Ahmad et al., 2011). The generation of glycation mediated ROS deprotonates the DNA and causes a conformational change from supercoiled to open circular and then to linear. Previous studies on the dynamics of DNA the stability showed that glycation leads to fragmentation and/or partial unwinding of the double helix (Mustafa et al. 2012). There was a significant damage of DNA induced by both the glycation systems in the presence of a metal ion. Acesulfame potassium prevented the glycation-induced damage of DNA. In an earlier report also it has been shown that Acesulfame prevents the formation of glycation products and DNA damage (Ali et al., 2017).

Conclusion

There are several factors which affect the generation of free radicals. These include the concentration and type of reacting molecules like sugars and amino acids. This study was designed to analyze the difference between the amino acids in the generation of glycation products in the presence of a common sugar, glucose. It can be concluded from the results presented that lysine is more reactive towards glucose as compared to arginine as indicated by the higher amount of glycation products generated in the lysine-glucose system. It can be also concluded that Acesulfame-K reduced the number of glycation products in both the systems as well as decreased the glycation-induced DNA damage. It can be established from the study that this artificial sweetener acts as a potent antiglycating agent.

Acknowledgement

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