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ARTICLE

In vitro study on glycation of plasma proteins with artificial sweeteners

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ABSTRACT Glycation is a non-enzymatic reaction between the carbonyl groups of sugars and the amino groups of proteins, nucleic acids and lipids. This process results in the formation of early glycation products, which rearrange to form more stable advanced glycation end products (AGEs). Glycation has been linked to a number of diseases such as, Alzheimer's, diabetes mellitus, cataract, Parkinson's, physiological aging, etc. Synthetic sugar substitutes are known as artificial sweeteners. Like sugar, they contain reactive groups, which can interact with amino groups of macromolecules inducing damage by glycation. In the present study, non-enzymatic interaction between commercially available aspartame, saccharin and sucralose-based artificial sweeteners and amino acid lysine was performed *in vitro*. Also, plasma proteins like bovine serum albumin, human serum albumin, immunoglobulin G, blood clotting factors VIII and IX were incubated with aspartame and sucralose-based sweeteners. The results indicate that glycation reaction also occurs between proteins and these artificial sweeteners like sugars. This is the first report indicating involvement of artificial sweeteners in glycation.
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KEY WORDS

artificial sweetener
glycation
Maillard reaction
plasma protein

Introduction

The non-enzymatic interaction between lysine residues in proteins and sugars is generally known as Maillard reaction or the browning reaction (Maillard 1912). It is a deleterious biochemical process, in which the carbonyl group of sugars reacts with the amino group of proteins to form glycosylamines, which on rearrangement form stable Amadori products (Monnier and Carami 1981). This early reversible stage is followed by the formation of more stable and irreversible advanced glycation end products (AGEs). Glycation products are synthesized continuously even at normal glucose levels, however, their deleterious effects are more pronounced when the glucose level in the blood increases above normal (Melpomeni et al. 2003). Glycation reactions are implicated in the development of diseases, such as Alzheimer's disease, diabetes mellitus, atherosclerosis, dialysis related amyloidosis, as well as physiological aging (Thorpe and Bayens 1996). Among the amino groups, lysine is a preferred group for non-enzymatic sugar attachment and subsequent formation of AGEs (Arai et al. 1987). Glycation results in cross linking and aggregation of proteins and this structural alteration disrupts the normal function of these glycated proteins (Lo et al. 1994). The complex process of glycation alters the biological activities of macromolecules like proteins and DNA, thus disrupting their metabolic processes and inhibiting their specific functions (Ali and Sharma 2015). Non-enzymatic glycation of bovine serum albumin (BSA) by glucose and fructose has been reported by Suarez and co-workers (1989). Similarly, an elevated blood glucose concentration in diabetes is known to promote glycation of human serum albumin (HSA) (Anguizola et al. 2013). Glycation of immunoglobulin G (IgG) has been found to increase in diabetic patients and it is of major value as it affects the overall immune competence (Yadav et al. 2013). However, there are no reports on glycation of blood clotting factors VIII and IX. Synthetic sugar substitutes are known as artificial sweeteners. Some of the most commonly used commercially available artificial sweeteners, Equal (aspartame-based), Splenda (sucralose-based) and Sweetex (saccharin-based) have been used in the current study. In comparison to sugar, they provide very less calories and hence they attract normal and overweight customers alike. Also, diabetic people have difficulty in regulating their blood sugar levels, so they limit their sugar intake by using sugar substitutes. Animal studies have convincingly proven that artificial sugar substitutes cause increased food intake and weight gain. This is because the sweet taste of sweeteners triggers an insulin response, causing storage of blood sugar in tissues. However, artificial sweeteners do not lead to hypoglycaemia and sugar craving (Swithers 2008).

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Aspartame, a low calorie sweetener is about 200 times sweeter than sucrose. On consumption, aspartame is broken down into its components - aspartic acid, phenylalanine and a small amount of methanol (Stegnik 1987; Prodolliet and Bruelhart 1993). Carcinogenicity of aspartame has been reported even at a daily dose of 20 mg/kg body weight, much less than the acceptable daily intake for humans (50 mg/kg body weight). Studies have shown that aspartame possesses antiinflammatory, antipyretic and analgesic action (La Buda et al. 2001; Sofferitti et al. 2006; Pradhan et al. 2011). In a study on patients with type 2 diabetes, it was shown that a sucrose meal and an aspartame-based meal induce a similar rise in glucose and insulin levels at baseline (Ferland et al. 2007). Hence, it would be damaging for diabetics to continue on consuming the sugarfree substitutes, considering they actually elevate blood glucose levels. Equal, a commercially available aspartame-based sweetener used in this study, contains 36 mg aspartame per 1 gram of sweetener with lactose and silicon dioxide as additives.

Sucralose is approximately 600 times sweeter than sugar and it is made by replacing three hydroxyl groups in sucrose with chlorine atoms. It is heat stable and it can therefore replace sugar in almost any food type. Sucralose belongs to a class of chemicals called organic chlorides, which is a cause of concern, as organic chlorides of some types are carcinogenic or toxic (Tandel et al. 2011). Commercially available sucralose-based sweetener Splenda comprises of sucralose with maltodextrin and dextrose as fillers. Splenda is known to reduce the beneficial bacteria in the gut and alter the microbial composition (Abou-Donia et al. 2008). Early analysis asserted that ingested sucralose passes unchanged through the gastro intestinal tract, however subsequent studies suggested that a part of it is metabolized. Also, reports suggest that cooking with sucralose at high temperature results in the generation of toxic compounds called chloropropanols (Schiffman and Rother 2013). Ingestion of sucralose has been shown to cause an increase in plasma glucose and insulin concentrations (Pepino et al. 2013). Taken together, these findings indicate that sucralose is not a biologically inert compound.

Saccharin, the first artificial sweetener was originally synthesized in 1879. It is 300 to 500 times as sweet as sugar and is often used to sweeten dietary beverages, foods, toothpastes, etc (Tandel et al. 2011). Investigations suggest that the consumption of saccharin containing products may result in obesity by interfering with fundamental physiological and homeostatic processes (Hampton 2008). Most countries permit the consumption of saccharin at restricted levels, whereas, some other countries have imposed a ban on saccharin products. Sweetex contains sodium saccharin with silicon dioxide and stearic acid in undetermined amounts.

The current study is based on the fact, that like sugars, artificial sweeteners contain reactive groups, which can interact with amino groups of macromolecules inducing damage by glycation. Moreover, some tabletop sweeteners contain additives like lactose and dextrose, which may enhance the damage due to glycation. Maillard reaction with commercially available artificial sweeteners Splenda and Sweetex was performed for observation of browning, followed by the measurement of Amadori products by periodate oxidation assay. The aspartame-based sweetener (Equal) was not considered for Maillard reaction as it involves boiling and aspartame is unstable at 100 °C. Splenda showed substantial browning in this preliminary assay, whereas the amount of browning in sample containing Sweetex was not considerable. *In vitro* glycation of the proteins bovine serum albumin (BSA), human serum albumin (HSA), immunoglobulin G (IgG) and blood clotting factors VIII and IX with Equal and Splenda was performed. The obtained results suggest that Equal and Splenda can glycate proteins possibly by the same reaction mechanism as sugars.

Materials and Methods

Materials

Bovine serum albumin, methylglyoxal and lysine were procured from Sigma Aldrich. Commercially available artificial sweeteners Equal, Splenda and Sweetex were used. All the other chemicals, which we used, were of high analytical grade.

Maillard reaction with artificial sweeteners

Lysine (0.1 g) was added to 10 ml of water and the pH was adjusted to 7.4. Test samples were prepared by adding either 0.1 g of Sweetex or Splenda to tubes containing 10 ml lysine. Tubes containing 0.1 g Sweetex or Splenda each in 10 ml distilled water and another tube containing 10 ml lysine were used as negative controls, while a tube containing methylglyoxal and lysine was used as a positive control. The solutions were heated at 100 °C for 3 h in a boiling water bath and were observed for browning. Absorbance was measured at 420 nm using a spectrophotometer.

Periodate oxidation assay

Glycation (Amadori product) was measured by the periodate method of Ahmed et al. (1986) with minor modifications. Maillard reaction samples (0.5 ml) were incubated with 0.1 ml of HCl (0.1 M) and 0.1 ml of $NaIO_4$ (0.05 M) for 30 min at room temperature. To terminate the oxidation, the samples were cooled on ice for 10 min and mixed with 0.1 ml pre-cooled $ZnSO_4$ (15%) in NaOH (0.7 M). The samples were vortexed throughout the addition and were centrifuged

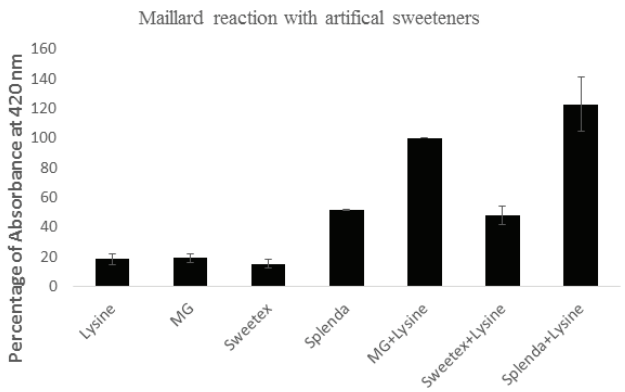

Figure 1. Measurement of Maillard reaction with artificial sweeteners.

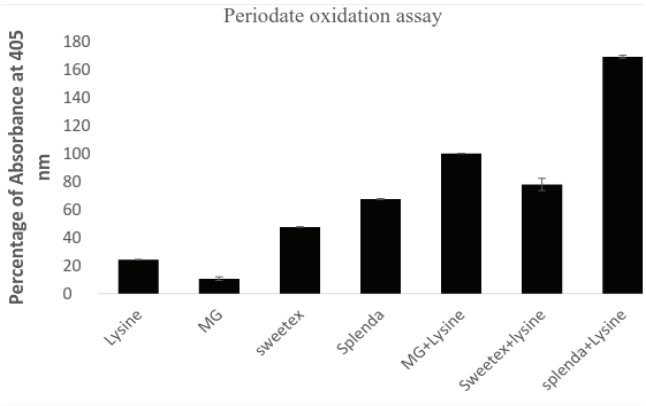

Figure 2. Periodate oxidation assay to determine the amount of glycosylamines formed.

for 10 min at 9000 g to remove precipitated zinc periodate. Supernatant (0.5 ml) was transferred to fresh test tubes. Formaldehyde detection reagent was freshly prepared by mixing 46 µl of acetylacetone in 10 ml of 3.3 M ammonium acetate. A volume of 1 ml of prepared reagent was added to each tube and glycosylamines were allowed to develop by incubation at 37 °C for 1 h. Absorbance was measured at 405 nm against a reagent blank using a spectrophotometer.

In vitro glycation of proteins with Equal and Splenda

Plasma proteins (bovine serum albumin, human serum albumin, immunoglobulin G, blood clotting factors VIII and IX) at a concentration of 10 mg/ml were incubated with and without glucose, Splenda and Equal, at a final concentration of 90 mg/ml for 7 days at 55 °C. All the incubations were carried out in 0.1 M potassium phosphate buffer (pH 7.4) containing 3 mM sodium azide to prevent bacterial contamination. Browning was measured at 420 nm using a spectrophotometer.

Results

Maillard reaction with artificial sweeteners

An aqueous solution of lysine (pH adjusted to 7) and artificial sweeteners (Sweetex and Splenda) was heated at 100 °C for 3 h. The solution with Splenda turned reddish brown, while the one with Sweetex turned yellow orange. The formation of Maillard product was measured at 420 nm. Figure 1 shows the amount of Maillard product formed in the lysine-artificial sweetener system, in comparison to the positive control containing methylglyoxal and lysine. It is apparent from the graph that Splenda produces more glycation product in comparison to methylglyoxal (120% approx.), whereas, the amount of glycation product produced by the Sweetex-lysine mixture is comparatively less.

Periodate oxidation assay

Periodate oxidation assay was performed to determine the amount of Amadori product produced in glycated samples. After the final incubation at 37 °C for 1 h, bright yellow colour was observed and absorbance was measured at 405 nm. Tubes containing Splenda/Sweetex and lysine were compared with positive control tubes containing methylglyoxal and lysine. As can be seen in the Figure 2, the amount of glycosylamines in the glycated sample containing Splenda was found to be more than that in the glycated samples containing methylglyoxal and lysine (160%). However, sample containing Sweetex produced less glycosylamines as compared to methylglyoxal/lysine (80%).

Glycation of bovine serum albumin

Incubation of bovine serum albumin with glucose, Equal or Splenda at 55 °C for 7 days resulted in browning and the intensity of colour increased with incubation time (Fig. 3). Both Equal and Splenda caused more browning of bovine serum albumin than glucose.

Glycation of human serum albumin

When human serum albumin was incubated with glucose, Equal or Splenda at 55 °C for 7 days, it also resulted in browning like bovine serum albumin. However, the glucose/human

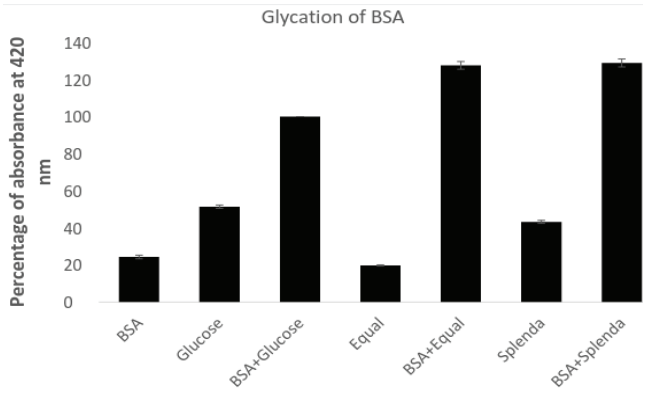

Figure 3. Glycation of bovine serum albumin (BSA) with glucose, Equal and Splenda.

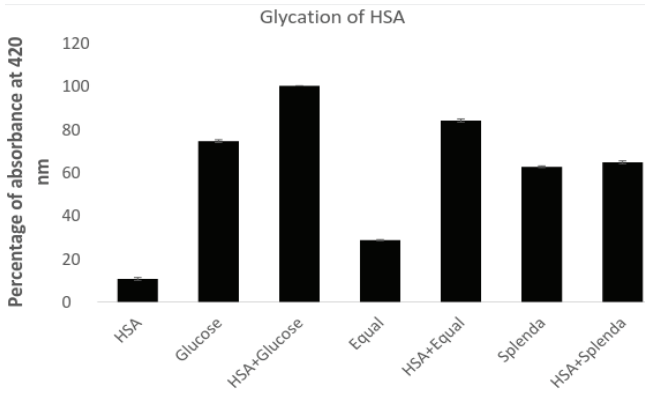

Figure 4. Glycation of human serum albumin (HSA) with glucose, Equal and Splenda.

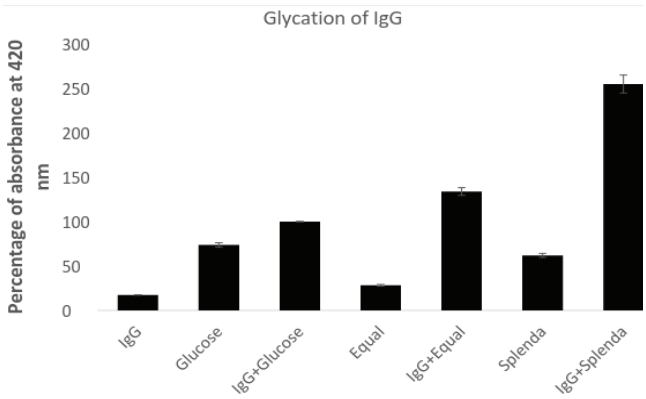

Figure 5. Glycation of immunoglobulin G (IgG) with glucose, Equal and Splenda.

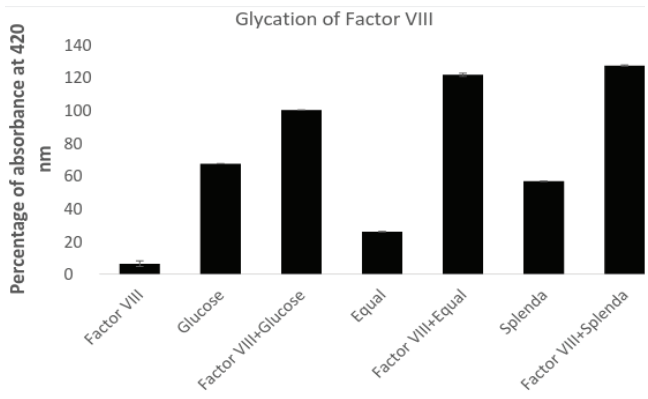

Figure 6. Glycation of blood clotting factor VIII with glucose, Equal and Splenda.

serum albumin produced intense browning as compared to Equal and Splenda (Fig. 4). It was also observed that Splenda caused least browning as compared to glucose and Equal.

Glycation of immunogloblulin G

An incubation of immunogloblulin G with glucose, Equal or Splenda at 55 °C for 7 days resulted in browning. The maximum browning was observed in the tube containing Splenda and immunoglobulin G (Fig. 5). Equal also lead to browning of immunoglobulin G more than glucose.

Glycation of blood clotting factor VIII

Both Equal and Splenda caused more intense browning (>120%) as compared to glucose when they were incubated with blood clotting factor VIII at 55 °C for 7 days (Fig. 6). However, the browning of factor VIII by Splenda was less as compared to immunoglobulin G.

Glycation of blood clotting factor IX

An incubation of blood clotting factor and sweeteners (Equal and Splenda) at 55 °C for 7 days caused less browning (approx. 80%) as compared to glucose (Fig. 7).

Disscussion

The formation and accumulation of glycation products are major factors in the development of diabetic complications, atherosclerosis, Alzheimer's disease and the aging process (Rahbar et al. 2003). Age-related diseases exhibit increased

levels of glycation end products, supporting the idea that sugars and their metabolites can damage macromolecules, especially on their accumulation in cells and tissues (Suji and Sivakami 2004). Although the mechanism of this damage was first described almost a century back by Maillard (1912), however, the exact events are yet to be figured out. Nonetheless, the major mechanisms, by which advanced glycation end products cause damage to the biomolecules, include cross linking, aggregation and precipitation.

In the present study, the formation of Maillard product (browning) was detected by boiling a mixtue of Splenda and Sweetex with lysine and browning was observed only on co-incubation of lysine and the artificial sweetener. Intense browning was observed on glycation of lysine with Splenda, however with Sweetex the browning was less significant. The formation of Amadori product was then confirmed by performing the periodate oxidation assay as it detects the presence of glycosylamines. Samples glycated with Splenda, showed the presence of Amadori product in amounts comparable to glycation samples containing methylglyoxal and lysine. On the other hand, the sample containing Sweetex produced less amounts of glycosylamines as compared to methylglyoxal/lysine.

The results presented in this study indicate, that Equal and Splenda can glycate proteins like bovine serum albumin, human serum albumin, immunogloublin G, blood clotting factors VIII and IX *in vitro*. Also, Equal is more reactive than glucose in glycating proteins. In the case of human serum albumin and factor IX, although Equal was found to be less reactive in comparison to glucose, the browning observed is substantial. Additional studies can be essential to determine if similar effects are observed *in vivo*.

Conclusion

Sugar substitutes and artificially sweetened products are in great demand as they mimic the sweet taste of sugar sans the calories. Besides its benefits, studies have convincingly proven that artificial sweeteners cause weight gain, cancer and many other health hazards (Tandel 2011). It can be concluded from the evidence, presented in this study that Equal and Splenda can potentially cause glycation of proteins and the intensity of damage caused to macromolecules is comparable to that caused by sugars. In some cases, the amounts of glycation products have been observed to be substantially more in the presence of artificial sweeteners (Equal and Splenda) than in comparison to glucose or methylglyoxal. Hence, the use of these sweeteners as a replacement of sugar may provide less calories, it could be the cause of a host of glycation related diseases in addition to artificial sweetener related disorders.

Glycation products can damage the molecular structure of proteins and hence disrupt their function. The current study shows that Equal can bring about glycation of human serum albumin, immunoglobulin G and blood clotting factors VIII and IX. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of proteins glycated by methylglyoxal has shown structural alteration of proteins (Ali et al. 2014). Similar studies are required to study the effect of glycation on the structure of the proteins glycated by artificial sweeteners as disruption of their function may have serious consequences.

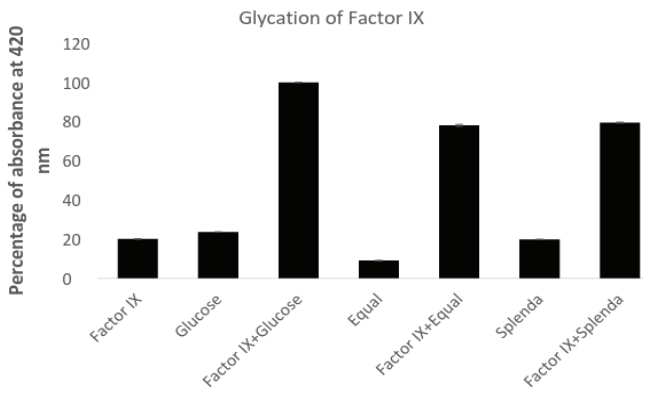

Figure 7. Glycation of blood clotting factor IX with glucose, Equal and Splenda.

Numerous studies on artificial sweeteners present conclusions ranging from 'safe only up to permissible levels of intake' to 'unsafe even if consumption is less than acceptable daily intake' (Tandel 2011). The scientific community is divided in their opinion on the safety aspect of artificial sweeteners. The findings in the current study suggest that it is necessary to investigate the health controversy over the perceived benefits of artificial sugar substitutes.

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