Effect of Aminoguanidine on Advanced Glycation End Products (AGEs) using Normal and Diabetic Plasma

[1]SAMINA KOUSAR*, [1]MUNIR AHMAD SHEIKH,
[1]MUHAMMAD ASGHER AND [2]MUHAMMAD SARWAR
[1]*Department of Chemistry and Biochemistry, University of Agriculture, Faisalabad, Pakistan.*
[2]*Institute of Animal Nutrition and Feed Technology, University of Agriculture, Faisalabad, Pakistan.*

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Summary: The world is facing an explosive increase in the incidence of diabetes mellitus and cost-effective complementary therapies are needed. Protein glycation takes place when elevated levels of circulating reducing sugars react with amino groups in proteins, forming complex, often unstable, irreversible and reactive compounds "AGEs", a process that may take weeks or months to accomplish. In this study, glycation and AGES inhibition by aminoguanidine (synthetic inhibitor of AGEs) weres investigated. Studies were performed by using normal and diabetic plasma with two incubation temperatures (37 °C & 50 °C). Samples were incubated for 5 weeks at both temperatures with varying concentrations of glucose and aminoguanidine. The results indicated that high temperature causes changes in protein structure that facilitate the AGEs formation. The increase in fluorescence was observed from 1st to 5th week of incubation that indicated increase in glycation. Aminoguanidine at 10 m*M* level showed profound results towards AGEs inhibition but in few cases results with 5 m*M* aminoguanidine are also comparable to 10 m*M*. AGEs level increased from 3rd to 5th week of incubation when measured by ELISA. ELISA was found to be sensitive and reliable method to check AGEs in diabetic and normal plasma as compared to fluorescence and aminoguanidine has the ability to decrease AGEs production.

Introduction

The non-enzymatic glycation of proteins, or the Maillard reaction, is a process which links chronic hyperglycemia to a series of pathophysiological changes considered important in the development of chronic complications of diabetes and different diseases [1]. The Maillard reaction or browning of proteins is subdivided into three main stages: early, intermediate, and late. In the early stage, glucose or other reducing sugars react with a free amino group of several molecules, including proteins, nucleic acids, and lipids, to form an unstable aldimine compound, the Schiff's base [2]. These propagators again react with free amino groups through oxidation, dehydration and cyclization reactions, forming yellow-brown, often fluorescent (some are non fluorescent), insoluble, irreversible compounds, usually called advanced glycation end products (AGEs). Candidate active AGEs compounds include *N*-(carboxymethyl)-L-lysine (CML) [3], pyrraline, pentosidine [4] and their cross-links [5].

An important feature of AGEs is their ability to form cross-links, a process which may lead to changes in cellular structure and integrity. Such alterations may have important impact on cellular barrier function [6]. Many cells in the body from tissues such as lung, liver, kidney or peripheral blood bear the receptor for advanced glycation end products (RAGE) that when bind to AGEs, contributes to aging and diabetes-related chronic inflammatory diseases such as atherosclerosis, asthma, arthritis, myocardial infarction, nephropathy, retinopathy or neuropathy [7].

There is need to explore different inhibitors to decrease the production of AGEs. For this purpose, different studies have been performed and inhibitors search is in progress. The first compound which has been extensively studied *in vitro* and *in vivo* to be a powerful inhibitor of AGEs formation is aminoguanidine (AG) [8]. Brownlee *et al.*, [8] selected AG because of its structure similarity to hydrazine-histidine, a compound known to reduce diabetes-induced vascular leakage. *i.e.* scavenging of dicarbonyls, thus preventing their conversion to AGEs. AG may limit the formation of AGEs [7]. AG prevents formation of reactive AGEs and their subsequent cross-linking with albumin that lead to a reduction in AGEs levels [9]. It has been proposed that AG may act as a glucose competitor for the same protein-to-protein bond that becomes the link for the formation and tissue accumulation of irreversible and

*To whom all correspondence should be addressed.

highly reactive AGEs [10]. The major aim of this study was to investigate AGEs inhibition by AG in normal and diabetic human plasma.

Results and Discussion

To study AGEs inhibition with AG, different combinations and concentrations were selected for experiments. Three concentrations (1, 5 and 10 m*M*) of AG were selected and experiments were carried out at two temperatures *i.e.* 37 °C and 50 °C at same time and samples were taken for analysis after 1st, 3rd and 5th week of incubation. Samples were dialysed to remove excess (or free) glucose before the estimation of AGEs by fluorescence and ELISA after protein estimation. Data values are mean of triplicates and S.D is shown in the form of error bars in each figure.

Fluorescence Measurement of Normal Human Plasma in the presence of Aminoguanidine (AG)

AGEs are diverse fluorescent and non-fluorescent compounds that are formed in complex reactions not yet fully understood, starting with the non-enzymatic reaction of reducing sugars or other carbonyl compounds with free amino groups of amino acid residues in proteins. Fluorescence intensity was significantly higher from 1st to 5th week of incubation and it was maximum with glucose 500 m*M* concentration (Fig. 1) at 37 °C. Glucose concentration 5.5 m*M* gives the minimum fluorescence in all weeks at the same temperature. All concentrations of AG produced different responses however; 10 m*M* give minimum fluorescence or maximum inhibition of AGEs with all glucose concentrations. It is clear from the results (Fig. 1) that 10 m*M* of AG has more ability to inhibit AGEs generation or production at physiological body temperature.

At 50 °C, maximum fluorescence was produced by 500 m*M* and minimum by 5.5 m*M* after 5th and 1st week of incubation respectively. AG concentration 5 m*M* and 1m*M* have less effect on fluorescence as compare to 10 m*M*. So 10 m*M* concentration of AG was dominant to inhibit fluorescence. Oxlund and Andreassen [11] studies are in line with our results as they investigated the alterations in the biophysical properties of connective tissues in diabetes mellitus that had been attributed to the nonenzymatic glycation of the collagens and the subsequent formation of browning products (fluorescence). AG may bind to carbonyl groups of

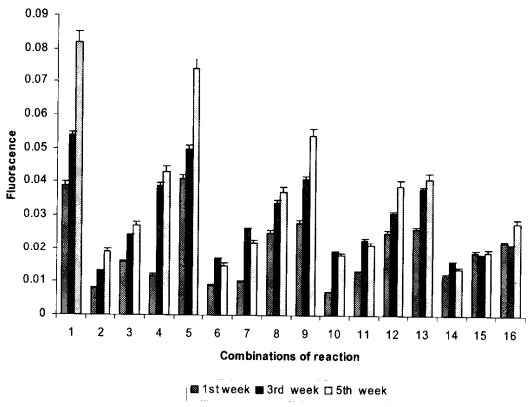

Fig. 1: Effect of aminoguanidine (AG) on fluorescence of normal human plasma incubated at 37 °C.

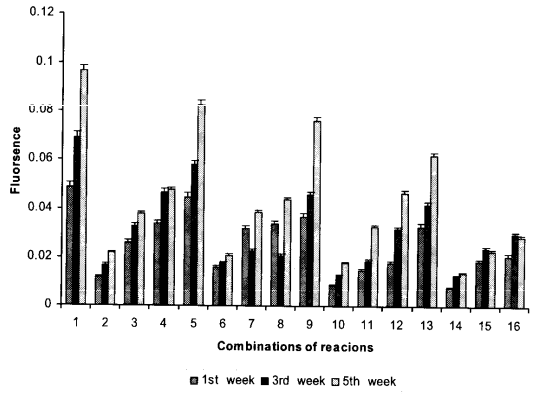

Fig. 2: Effect of aminoguanidine (AG) on fluorescence of normal human plasma incubated at 50 °C.

these nonenzymatic glycation products and thereby block the process and amount of fluorescent compounds in the tail tendon collagen of the diabetic rats.

Fluorescence Measurement of Diabetic Human Plasma in the Presence of Aminoguanidine (AG)

In diabetes mellitus, crosslinking of glucose increases due to persistent hyperglycemia which in turn increases fluorescence. When AG was used as an AGEs inhibitor with diabetic human plasma, fluorescence intensity was significantly higher in 5th week of incubation (Fig. 3). Glucose concentration of 500 m*M* showed maximum and 5.5 m*M* gave the minimum fluorescence at all weeks of incubation at 37 °C. All concentrations of AG produced good response

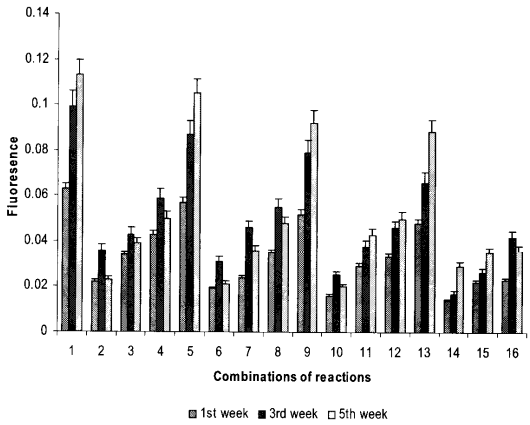

Fig. 3: Effect of aminoguanidine (AG) on fluorescence of diabetic human plasma incubated at 37 °C.

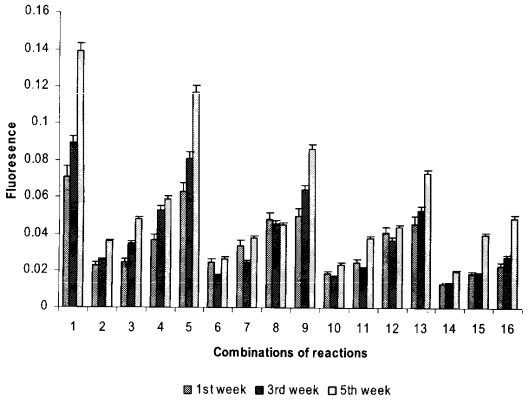

Fig. 4: Effect of aminoguanidine (AG) on fluorescence of diabetic human plasma incubated at 50 °C.

towards AGES inhibition and 10 m*M* concentration of AG give maximum inhibition with all glucose concentrations. 5m*M* of AG gave good response but only with 5.5 m*M* glucose concentration and 1m*M* of AG also showed least level of inhibition.

At 50 °C, maximum fluorescence was produced by 500 m*M* and minimum by 5.5 m*M* after 5th and 1st week of incubation respectively (Fig. 4). A 0.5 m*M* and 1m*M* AG concentration produced less effect as compare to 10 m*M* concentration. It was clear from figure [4], 10 m*M* concentration of AG was again dominant to inhibit fluorescence as more fluorescence indicates less ability of inhibitor (*e.g.* AG) to decrease AGEs production. Mayhan and Sharpe [12] used intravital fluorescent microscopy to examine basal macromolecular extravasation in non diabetic and diabetic hamsters treated with a topical application of AG (0.5 m*M*), and suggested that AG may be an important therapeutic approach for the treatment of diabetes-related vascular dysfunction. Lin *et al.,* [13] determined effects of AG as an inhibitor of AGEs and conclude that AG may prevent the fructose-derived changes in arterial stiffening, possibly through inhibition of the fructose-derived advanced glycation end product formation in Wistar rats.

Levels of AGEs in Normal and Diabetic Plasma Measured through ELISA in the presence of Aminoguanidine (AG)

ELISA ensures reproducibility, high sensitivity and efficient quantification of AGEs by taking the advantage of specific antibodies against AGEs. Less glycation was observed after 1st week and it went on increasing from 3rd to 5th week suggesting that AGEs production started after 1st week. In this study ELISA method was applied to measure AGEs of 3rd and 5th week incubated samples.

Results depicted in figure 5 showed that when AGES production and inhibition was monitored by AG at 37 °C after 3rd and 5th week of incubation with normal human plasma, maximum AGEs were given with 250 m*M* after 3rd and 5th week with values 0.048 and 0.071/ μg of plasma protein respectively. Maximum inhibition was given by 10 m*M* of AG with 500 m*M* and 5.5 m*M* glucose at both weeks, showing that 10 m*M* concentration of AG was more effective than other two concentrations.

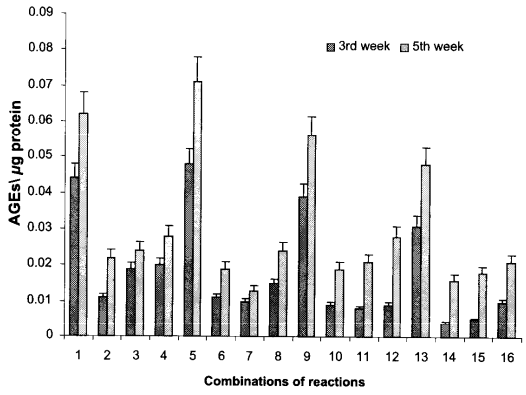

Fig. 5: Effect of aminoguanidine (AG) on AGEs level of normal human plasma at different weeks assayed by ELISA.

According to previous studies [14] different concentrations of AG along with other inhibitors were used and it was noted that 0.1 and 1.0 m*M* AG did not produce profound results in inhibition of post amadori AGES production however, when 10 m*M* AG was used for AGEs- BSA crosslink breaking showed inhibition. Our results are in agreement with other study [15], where AG was described as a possible drug to prevent diabetic complications by blocking the reactive carbonyl group of the Amadori product formed by non-enzymic glycosylation of proteins. AG can prevent glycation as well as the browning reactions, by decreasing the concentration of the active aldehyde form of the sugars.

When ELISA was used to measure AGEs inhibition by AG at 37 °C with diabetic human plasma (Fig. 6), it was clear that maximum AGEs were given by 500 m*M* of glucose (0.076 and 0.108/µg of plasma protein) after 3rd and 5th week respectively. Maximum inhibition was given by 10 m*M* AG while 5 *mM* concentration of AG also produced good response with all glucose concentrations at both weeks. 1 m*M* AG gave the least inhibition at both weeks as also seen with normal human plasma (Fig. 5). Our results are in line with previous finding [16], in which Maillard reaction between sugar and proteins was found to increase chemical modification and cross-linking of long-lived tissue proteins in diabetes. Glycoxidation products proceeded with a short lag phase suggested that autoxidative degradation of glucose was a major contributor to glycoxidation and cross-linking reactions. Chelators, sulfhydryl compounds, antioxidants, and AG inhibited formation of glycoxidation products, generation of fluorescence, and cross-linking of collagen without significant effect on the extent of glycation of the protein.

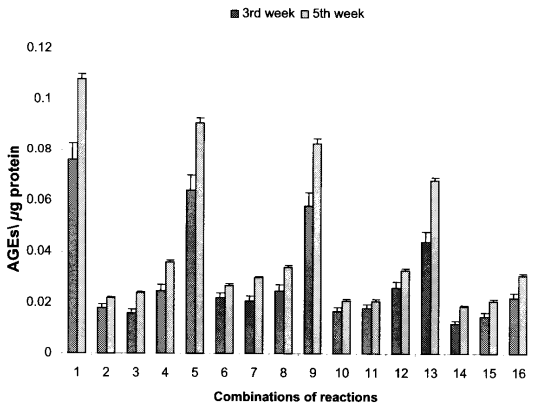

Fig. 6: Effect of aminoguanidine on AGEs level of diabetic human plasma at different weeks assayed by ELISA.

It is clear from the results, ELISA is more sensitive to detect AGEs as compared to fluorescence because in fluorescence nonenzymatic glycation of proteins and subsequent formation of browning products is measured. As all AGEs are not fluorescent so only fluorescent AGEs can be measured with this method while in ELISA, specific anitbodies against AGEs are used to measure their level in a given sample.

Experimental

Glycation and AGEs inhibition was monitored by synthetic inhibitor aminoguanidine (AG).

Four concentrations (G_1= 500 m*M*, G_2= 250 m*M*, G_3= 50 m*M* and G_4= 5.5 m*M*) of glucose were used, 5.5 m*M* is normal glucose concentration in blood while, 50 m*M* indicates hyperglycaemia, 250 m*M* concentration might be the concentration in diabetic patients with the onset of complications and 500 m*M* concentration was selected to check the degree of glycation and to check whether AGES production is effected by it or not. Normal and diabetic human plasma samples were diluted to the range with protein concentration up to 20 mg/mL Glycation assay, TBA was performed to measure degree of glycation of proteins for this test 20 mg/mL protein are required (data not given). Total proteins were estimated after dialysis before carrying out the experiments. Three concentrations of AG (I_1= 10 m*M*, I_2 = 5 m*M* and I_3 =1 m*M*) were used for experimental studies.

Selection of Conditions and Concentrations

To study the effect of AG with normal and diabetic human plasma sixteen of each combinations were made and all these were placed at 37 °C and 50 °C at same time for five weeks (Table-1). Plasma fractions were collected from diabetic patient's blood (type I) and stored at -20 °C. Normal plasma was pooled from blood samples of healthy male and female volunteers. Plasma of normal diabetic human were used for analysis as plasma has more proteins (clotting proteins) and if these proteins are glycated these can be measure by the same method. Samples

Table-1: Different combinations for plasma glycation inhibition.

S. No.	Combinations	S. No.	Combinations
	Temperature 37 °C/ 50 °C		Temperature 37 °C/ 50 °C
1.	B + G_1 + P	9.	B + G_3 + P
2.	B + G_1 +P +I_1	10.	B + G_3 +P+I_1
3.	B + G_1 + P+ I_2	11.	B + G_3+P +I_2
4.	B + G_1 + P+ I_3	12.	B + G_3 + P+ I_3
5.	B + G_2 + P	13.	B + G_4 + P
6.	B + G_2 +P+I_1	14.	B + G_4 +P+I_1
7.	B + G_2 +P +I_2	15.	B + G_4+P +I_2
8.	B + G_2 + P+ I_3	16.	B + G_4 + P+ I_3

after 1st, 3rd and 5th week of incubation were taken to perform experiments for glycation inhibition and AGEs level detection.

Glycation of Plasma (Preparation of Plasma- AGEs)

Plasma was incubated with all glucose concentrations in phosphate buffer saline (0.075*M* pH 7.4, 0.1 % sodium azide) at both 37 °C and 50 °C for 1-5 weeks simultaneously.

Glycation of Plasma with Aminoguanidine

Plasma in 0.075*M* PBS (pH 7.4, 0.1 % sodium azide) was incubated with all glucose concentrations and three AG concentrations. The reaction mixtures were incubated at 37 °C and 50 °C for 1-5 weeks at same time.

The plasma protein concentration in all combinations having protein was determined by Biuret method [17]. Samples were dialysed before AGEs measurement. Protein estimation was performed after dialysis. Glucose concentration was also measured before incubation of plasma samples and estimation of AGEs.

Advanced Glycation End Products Detection

The AGEs formation was assessed by their characteristic fluorescence emission spectra at 440 nm after excitation at 370 nm through spectrofluorometer and AGEs were determined by using ELISA. Blank samples were used to make the reading zero.

Enzyme Linked Immunosorbent Assay (ELISA)

ELISA was performed by using alkaline phosphatase enzyme and para nitrophenyl phosphate as a substrate. Anti-AGEs immunoglobulins (Antibodies) were purchased commercially from Sigma. AGE-BSA was prepared by using bovine serum albumin (BSA) following the procedure of Zhang *et al.*, [18] with slight changes according to laboratory conditions. Absorbance was measured at 405 nm, using microtiter ELISA plate reader.

Conclusion

Based on the above findings, it could be concluded that AG had the ability to inhibit not only glycation but also AGEs formation in diabetic and normal plasma. However, higher concentration of AG (10 m*M*) is more effective inhibitor than its lower concentrations. ELISA is a sensitive method and can be used to measure AGEs in given blood sample.

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