

Antiglycation activity of Thiamin-HCl and Benfotiamine in diabetic condition

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Abstract

Objective: To observe the antiglycation effect of water-soluble vitamin, thiamin-hydrochloride (B1) and its fat-soluble derivative benfotiamine and their comparison with two different glycation assays in diabetics.

Methods: Plasma of both the normal's and diabetic persons was taken for this experimental study which was conducted in the Department of Chemistry and Biochemistry, University of Agriculture, Faisalabad in 2008. Varying concentrations of both the glycation inhibitors and glucose were incubated for 5 weeks at 37°C. Thiobarbituric acid and periodate borohydride assays were used to measure the antiglycation activity.

Results: The increase in glycation was observed from 1st to 3rd week of incubation, while it decreased after the 5th week due to the formation of advanced glycation end products.

Conclusions: It was observed that 10mM concentration of benfotiamine and 5mM and 1mM concentrations of thiamine-hydrochloride produced fairly good response to decreased glycation. Comparison between two assays proved periodate borohydride to be more reliable and sensitive than thiobarbituric acid.

Keywords: Glycation, Diabetes, Maillard reaction, Thiamine-HCl, Benfotiamine, TBA, Periodate. (JPMA 62: 1033; 2012)

Introduction

Diabetes is a serious metabolic disorder which results in significant morbidity and mortality. The prevalence of diabetes mellitus is rapidly increasing. The World Health Organisation predicted that the number of adults aged ≥ 20 years with diabetes will increase from 135 million to 300 million in 2025.¹ Hyperglycaemia is considered the principal cause of diabetes complications like microvascular stress, cells injury and cell death.² The adverse effects of high plasma glucose depend upon the type of cells. Cells which express a high level of the glucose transporter 1 (GLUT1) are unable to regulate intracellular glucose concentrations and are thus very susceptible to hyperglycaemia-induced damage.³ Its detrimental effects are attributable to the formation of sugar-derived substances called Maillard products. The initial product of this reaction is called a Schiff base, which spontaneously re-arranges itself into an Amadori product, as is the case with haemoglobin A1c.⁴ These initial reactions are reversible depending on the concentration of the reactants. A series of subsequent reactions include dehydration, oxidation-reduction and other arrangements leading to the formation of advanced glycation end (AGE) products.⁵

Glycation damages the collagen and elastin throughout the body. Recycling of these proteins proceeds slowly and they

are out of reach of proteasomes and lysosomes. Cross-linked and glycated extracellular proteins (collagen) can cause pathologies that may lead to aging and diabetes.⁶

Living system has devised various defense mechanisms to protect the tissues against deleterious effects of glycation or AGEs. These include glyoxylase system (I and II) having oxaldehyde reductase and aldose reductase that catalyse the deglycation and detoxification of methylglyoxal (MG), the most common reactive intermediates of AGEs to D-lactate.⁷ Furthermore, a novel class of enzymes found in *Aspergillus* called amadoriases, was found to catalyse the deglycation of Amadori products. Now it is the need of the day to develop or isolate new compounds either from plants or synthetically to control diabetes and age accelerating diseases. Aminoguanidine (AG) is the first compound that has been extensively studied in vitro and in vivo to be a powerful glycation and AGE inhibitor.⁸ AG traps reactive carbonyl intermediates partially inhibiting carboxymethyl lysine (CML), carboxyethyl lysine (CEL), cross-linking and fluorescence in skin collagen.⁹ Among the side effects reported in patients treated with AG was pernicious-like anaemia¹⁰ and development of anti-nuclear antibodies.¹¹ Moreover, high rates of pancreatic and renal-neoplastic tumours were reported in diabetic rats treated with AG.¹² The main objective of this study was to investigate glycation inhibitory effect of thiamin-hydrochloride (HCl) and benfotiamine in

normal and diabetic human plasma and their comparison regarding antiglycation activity.

Materials and Methods

The experimental study was conducted in the Department of Chemistry and Biochemistry, University of Agriculture, Faisalabad in 2008. Four different concentrations of glucose — G1=500mM, G2=250mM, G3=50mM and G4=5.5mM — were prepared. Blood plasma samples were collected from Type II diabetic patients as well as from normal men or women. The samples were stored at -20°C till use. At the time of use, all plasma were pooled together. Thereafter, separately both in normal and diabetics, the required sample volume for experiments were obtained. Then all plasma samples were diluted to have protein concentration range up to 20mg/mL. Glycation level was assessed by two different methods. Total proteins were estimated by Biuret method¹³ before and after the dialysis of plasma samples. Three different concentrations - I1=10mM, I2=5mM and I3 =1mM - each for thiamine-HCl and benfotiamine were selected and used.

To study the effect of thiamine-HCl and benfotiamine, 16 combinations with normal (PN) and diabetic (PD) plasma were made; each with three replicates. All were placed simultaneously for five weeks at 37°C (Table -1).

The plasma was obtained from normal healthy volunteers and Type II diabetic patients with or without complications. Those with hypertension, cardiovascular diseases, thyroid disease or type I diabetes were excluded.

All plasma combinations with four concentrations of glucose were incubated for 1-5 weeks at 37°C.

Plasma samples after incubation were dialysed to remove free glucose after incubation as free glucose is the major hindrance in ascertaining the glycation level. Glucose was again estimated after dialysis by glucose oxidase method in order to confirm whether the concentration of glucose had decreased or not.

Enzymatic and non-enzymatic glycation was determined by thiobarbituric acid (TBA) colorimetric technique. This method is based on the reaction between fructose, amino acids and weak acid that will yield 5-hydroxymethyl furfural (HMF). For the determination of enzymatic glycation of plasma proteins, samples were reduced by using 0.1M sodium borohydride (NaBH₄) solution for the reduction of aldehyde or ketonic linkages. After reduction, non-enzymatic glycation was determined by the following formulae.^{14,15}

Non-enzymatic (NE) Glycation = (Collective (C) Glycation + Enzymatic (E) Glycation) - Enzymatic (E) Glycation

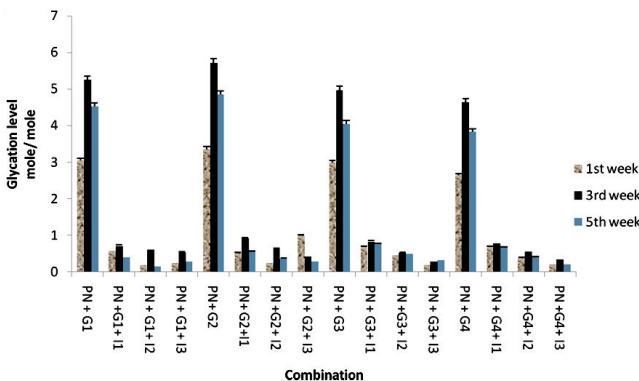
The periodate borohydride assay test was based on the

production of formaldehyde by periodate oxidation of cis-diol, aminol, ketol or ketoamine structures.^{16,17}

Statistically, the data was subjected to descriptive analysis for acquiring means along with standard deviation.

Results

The results obtained from thiamine-HCl in normal human plasma indicated that 250mM (G2) concentration of glucose had the maximum glycation level with value 5.711 mole/mole after 3rd week of incubation and it was minimum after the 1st week by 5.5mM (G4) with value 2.633 mole/mole (Figure-1). TBA measured that 5mM (I2) concentration of



Normal plasma protein (PN ? 20 mg/mL) was incubated with all glucose concentrations (G1= 500 mM, G2= 250 mM, G3= 50 mM & G4= 5.5 mM) and Thiamine- HCl as an inhibitor with three concentrations (I1= 10 mM, I2= 5 mM & I3 =1mM) in 0.075 M PBS (pH 7.4, 0.1% Sodium Azide) and reaction mixtures were incubated at 37 oC for 5 weeks at the same time. Samples were analysed after 1st, 3rd and 5th week and glycation level was measured in mole/mole (glucose/protein). *Values were the average of experiments carried out at n=3. TBA: Thiobarbituric Acid. HCL: Hydrochloride.

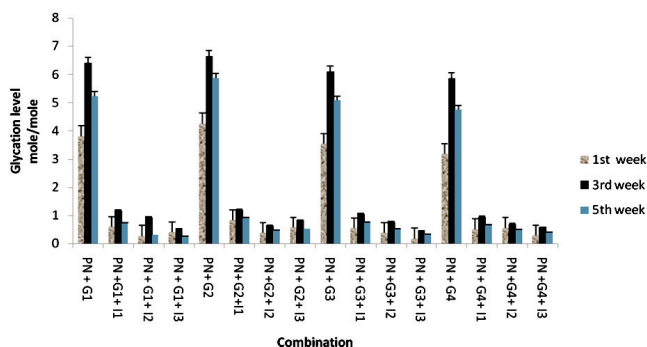
Figure-1: Effect of Thiamine-HCl on glycation level measured by TBA in normal human plasma.

Table: Different combinations for plasma glycation inhibition.

S No.	Combinations for Normal/ Diabetic Plasma
1	G1 + PN/D
2	G1 + PN/D +I1
3	G1 + PN/D + I2
4	G1 + PN/D + I3
5	G2 + PN/D
6	G2 + PN/D +I1
7	G2 + PN/D +I2
8	G2 + PN/D + I3
9	G3 + PN/D
10	G3 + PN/D +I1
11	G3 + PN/D +I2
12	G3 + PN/D + I3
13	G4 + PN/Ds
14	G4 + PN/D +I1
15	G4 + PN/D +I2
16	G4 + PN/D + I3

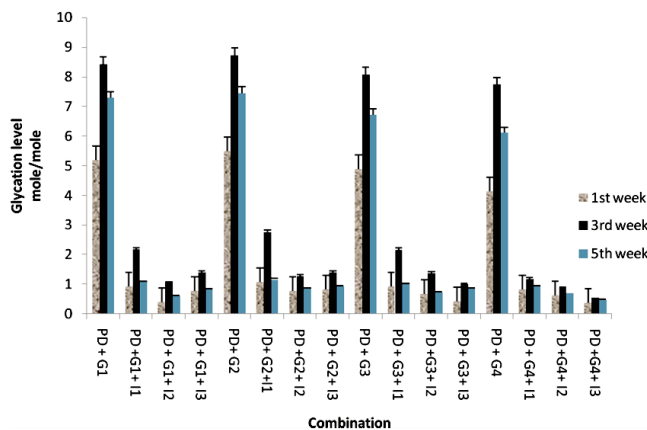
thiamin-HCl was more active in inhibiting glycation with glucose concentrations 500mM (G1) and 250mM (G2), while 1mM (I3) of thiamin showed maximum inhibition with 50mM (G3) and 5.5mM (G4). The 10mM (I1) concentration showed the least glycation inhibition.

With periodate borohydride assay, the maximum glycation response i.e. 6.645 mole/mole was observed after the 3rd week with G2 glucose concentration which had dropped to



Normal plasma protein (PN ~ 20 mg/mL) was incubated with all glucose concentrations (G1= 500 mM, G2= 250 mM, G3= 50 mM & G4= 5.5 mM) and Thiamine- HCl as an inhibitor with three concentrations (I1= 10 mM, I2= 5 mM & I3=1mM) in 0.075 M PBS (pH 7.4, 0.1% Sodium Azide) and reaction mixtures were incubated at 37 oC for 5 weeks at the same time. Samples were analysed after 1st, 3rd and 5th week and glycation level was measured in mole\ mole (glucose\ protein). *Values were the average of experiments carried out at n= 3. TBA: Thiobarbituric Acid. HCL: Hydrochloride.

Figure-2: Effect of Thiamine- HCl on glycation level measured by periodate borohydride assay in normal human plasma.

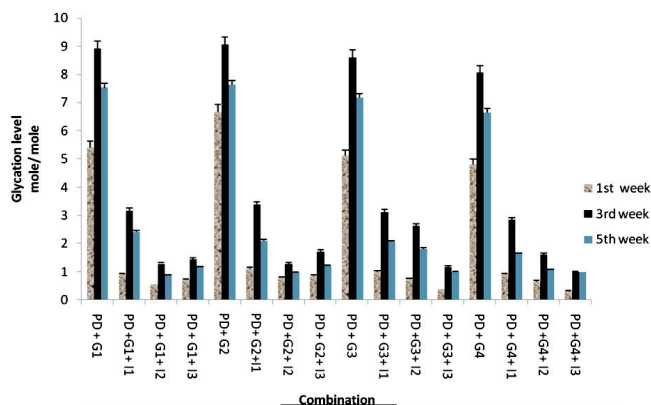


Diabetic plasma protein (PD ~ 20 mg/mL) was incubated with all glucose concentrations (G1= 500 mM, G2= 250 mM, G3= 50 mM & G4= 5.5 mM) and Thiamine- HCl as an inhibitor with three concentrations (I1= 10 mM, I2= 5 mM & I3=1mM) in 0.075 M PBS (pH 7.4, 0.1% Sodium Azide) and reaction mixtures were incubated at 37 oC for 5 weeks at the same time. Samples were analysed after 1st, 3rd and 5th week and glycation level was measured in mole\ mole (glucose\ protein). *Values were the average of experiments carried out at n= 3. TBA: Thiobarbituric Acid. HCL: Hydrochloride.

Figure-3: Effect of Thiamine-HCl on glycation level measured by TBA in diabetic human plasma.

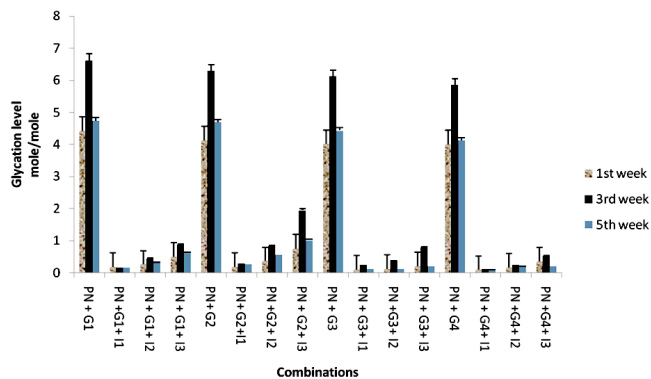
3.184 mole/mole with G4 in the 1st week of incubation (Figure-2). Different concentrations of thiamin showed comparable inhibitory responses: I2 produced maximum inhibition against G1 and G2 concentration of glucose. Likewise, I3 concentration of thiamin exhibited maximum inhibitory response against G3 and G4 glucose concentrations. While I1 of thiamine- HCl showed the least activity among the three concentrations used.

The diabetic human plasma which showed that G2



Diabetic plasma protein (PD ~ 20 mg/mL) was incubated with all glucose concentrations (G1= 500 mM, G2= 250 mM, G3= 50 mM & G4= 5.5 mM) and Thiamine- HCl as an inhibitor with three concentrations (I1= 10 mM, I2= 5 mM & I3=1mM) in 0.075 M PBS (pH 7.4, 0.1% Sodium Azide) and reaction mixtures were incubated at 37 oC for 5 weeks at the same time. Samples were analysed after 1st, 3rd and 5th week and glycation level was measured in mole\ mole (glucose\ protein). *Values were the average of experiments carried out at n= 3. TBA: Thiobarbituric Acid. HCL: Hydrochloride.

Figure-4: Effect of Thiamine- HCl on glycation level measured by periodate borohydride assay in diabetic human plasma.

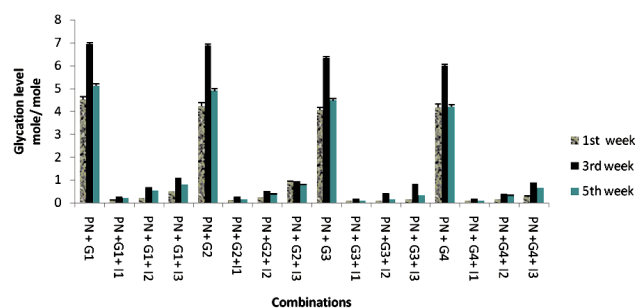


Normal plasma protein (PN ~ 20 mg/mL) was incubated with all glucose concentrations (G1= 500 mM, G2= 250 mM, G3= 50 mM & G4= 5.5 mM) and Benfotiamine as an inhibitor with three concentrations (I1= 10 mM, I2= 5 mM & I3=1mM) in 0.075 M PBS (pH 7.4, 0.1% Sodium Azide) and reaction mixtures were incubated at 37 oC for 5 weeks at the same time. Samples were analysed after 1st, 3rd and 5th week and glycation level was measured in mole\ mole (glucose\ protein). *Values were the average of experiments carried out at n= 3. TBA: Thiobarbituric Acid. HCL: Hydrochloride.

Figure-5: Effect of Benfotiamine on glycation level measured by TBA in normal human plasma.

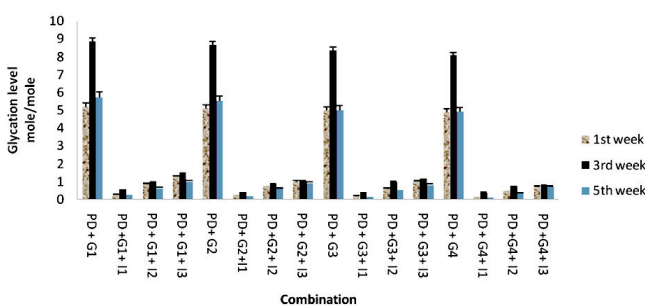
concentration of glucose produced maximum glycation (8.713mole/mole) after 3rd week of incubation with TBA test. Minimum glycation i.e. 4.137 mole/mole was observed after the 1st week of incubation with G4. Maximum inhibition was reflected by I2 with G1, G2 concentration of glucose, while I3 concentration of thiamin was overall efficient towards inhibition with G3 and G4 (Figure-3).

With periodate borohydride assay, maximum glycation i.e. 9.053 mole/mole was measured after the 3rd week with G2 concentration which dropped to minimum i.e. 4.818 mole/mole, with G4 concentration of glucose (Figure 4). Overall maximum inhibitory effect of Thiamine-HCl was seen by 5mM (I2) against G1 and G2, while I3 concentration of thiamine showed



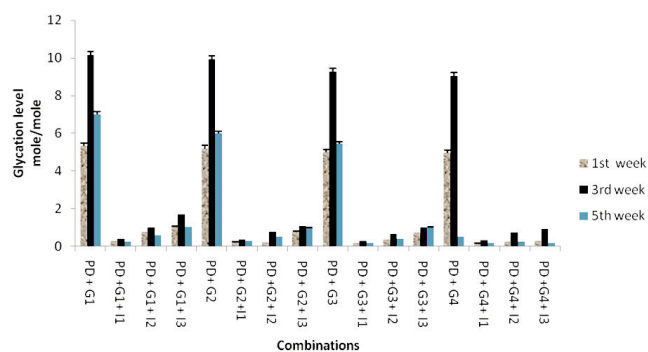
Normal plasma protein (Pn ~ 20 mg/mL) was incubated with all glucose concentrations (G1= 500 mM, G2= 250 mM, G3= 50 mM & G4= 5.5 mM) and Benfotiamine as an inhibitor with three concentrations (I1= 10 mM, I2= 5 mM & I3 =1mM) in 0.075 M PBS (pH 7.4, 0.1% Sodium Azide) and reaction mixtures were incubated at 37 °C for 5 weeks at the same time. Samples were analysed after 1st, 3rd and 5th week and glycation level was measured in mole\ mole (glucose\ protein). *Values were the average of experiments carried out at n= 3. TBA: Thiobarbituric Acid. HCL: Hydrochloride.

Figure-6: Effect of Benfotiamine on glycation level measured by Periodate borohydride assay in normal human plasma.



Diabetic plasma protein (PD ~ 20 mg/mL) was incubated with all glucose concentrations (G1= 500 mM, G2= 250 mM, G3= 50 mM & G4= 5.5 mM) and Benfotiamine as an inhibitor with three concentrations (I1= 10 mM, I2= 5 mM & I3 =1mM) in 0.075 M PBS (pH 7.4, 0.1% Sodium Azide) and reaction mixtures were incubated at 37 °C for 5 weeks at the same time. Samples were analysed after 1st, 3rd and 5th week and glycation level was measured in mole\ mole (glucose\ protein).r *Values were the average of experiments carried out at n= 3. TBA: Thiobarbituric Acid. HCL: Hydrochloride.

Figure-7: Effect of Benfotiamine on glycation level measured by TBA in diabetic human plasma.



Normal plasma protein (Pn ~ 20 mg/mL) was incubated with all glucose concentrations (G1= 500 mM, G2= 250 mM, G3= 50 mM & G4= 5.5 mM) and Benfotiamine as an inhibitor with three concentrations (I1= 10 mM, I2= 5 mM & I3 =1mM) in 0.075 M PBS (pH 7.4, 0.1% Sodium Azide) and reaction mixtures were incubated at 37 °C for 5 weeks at the same time. Samples were analysed after 1st, 3rd and 5th week and glycation level was measured in mole\ mole (glucose\ protein). *Values were the average of experiments carried out at n= 3. TBA: Thiobarbituric Acid. HCL: Hydrochloride.

Figure-8: Effect of Benfotiamine on glycation level measured by Periodate borohydride assay in diabetic human plasma.

maximum inhibitory response against G3 and G4 concentration of glucose.

The results obtained from benfotiamine as an inhibitor of glycation in normal human plasma showed that G1 concentration of glucose had maximum glycation level with value 6.616 mole/mole after the 3rd week of incubation. Glycation was seen minimum i.e. 4.007 mole/mole after the 1st week of incubation with G4 glucose concentration (Figure 5). TBA method showed that I1 concentration of benfotiamine was more active in inhibiting glycation, while I2 gave comparable response and I3 concentration exhibited the least glycation inhibition effect.

With periodate borohydride assay, maximum glycation response i.e. 6.933 mole/mole was observed with G1 after the 3rd week of incubation which dropped to 4.175 mole/mole with G4. Benfotiamine I1 concentration produced maximum inhibition as compared to others (Figure 6).

The effect of benfotiamine in diabetic human plasma which exhibited that G1 glucose concentration produced the maximum glycation (8.865 mole/mole) after the 3rd week of incubation with TBA test (Figure-7). Minimum glycation i.e. 4.879 mole/mole was observed after the 1st week of incubation with G4. I1 of benfotiamine was seen to be the most active concentration against glycation.

With Periodate borohydride assay, maximum glycation i.e. 10.151 mole/mole, was measured after the 3rd week of incubation with G1 which dropped to a minimum of 0.505 mole/mole with G4 after the 5th week of incubation (Figure-8). Different concentrations of benfotiamine showed comparable inhibitory responses, but, I1 produced fairly good response

compared to the other concentrations.

Discussions

In the present study, human blood plasma was used for analysis as it has more proteins which, on glycation, can be measured by the same glycation assay. Plasma samples were incubated at 37°C temperature for 5 weeks. TBA and Periodate borohydride methods were used to measure glycation and glycation inhibition.

The results depicted that maximum glycation was observed in G1 (500mM) and G2 (250mM) concentrations of glucose with benfotiamine and thiamin-HCl respectively.

It was observed from results that 5mM and 1mM concentrations of thiamin-HCl produced maximum inhibitory response with all glucose concentrations. These findings are supported by a previous study of non-enzymatic glycosylation of proteins and glycation inhibition by thiamine and other inhibitors.¹⁸ Our results are in conformity with a previous report¹⁹ claiming that thiamine pyrophosphate and pyridoxamine inhibit the formation of antigenic AGEs compared with aminoguanidine. Our findings are also corroborated with previous findings.²⁰ where high-dose therapy of thiamine and benfotiamine in streptozotocin (Stz)-induced diabetic rats was studied. It concluded that glyoxal and methyl glyoxal concentrations were more sensitive to benfotiamine than thiamine. Dose-dependent decrease was found for thiamine, whereas benfotiamine normalised CEL and CML at both (7 and 70mg/ kg) doses.

The results showed that maximum and more active glycation inhibition was observed by 10mM concentration of benfotiamine in both normal and diabetic conditions. It was also observed that 5mM concentration was also found active enough to produce glycation inhibitory response. Our results are compatible with previous reports²¹ which concluded that thiamine nitrate (water-soluble) and benfotiamine (lipidsoluble) have inhibitory effect on peripheral nerve function and the formation of AGEs in Stz-induced diabetic rats. Benfotiamine induced inhibition of AGEs and completely prevented diabetes-induced glycoxidation products (CML). These findings are contrary to a report which examined the effect of benfotiamine, on Stz. induced cerebral oxidative stress and 100mg/kg/day benfotiamine dose was given to rats for 14 days.²² It observed that diabetes-induced cerebral oxidative stress was alleviated without affecting levels of AGE and tumor necrosis factor (TNF)-alpha.

Protein glycation (glucosylation or glycosylation) by carbohydrates (browning) yield a heterogeneous class of compounds. These undergo a series of further re-arrangements, dehydration and condensation to form irreversible end products. They have now been implicated in the pathogenesis of different diseases like diabetes, renal failure and aging. There are some

potent inhibitors of glycation used to decrease protein glycation.²³ It was observed in the present study that glycation increased from 1st to 3rd week of incubation and slight decrease in glycation level was seen after the 5th week due to the formation of AGEs. It was also observed that both the concentrations of glucose G1 and G2 can cause maximum glycation.

Glycation assays TBA and periodate showed the same trend of glycation measurement, but periodate proved to be a more sensitive and effective method to measure the glycation level. Our findings are also in conformity with an earlier study where glycation level of membrane proteins from diabetics was found to be elevated compared to non-glycaemic control. It was concluded that periodate glycation assay for the quantitation of the Amadori products was the most convenient procedure.

Conclusions

Glucose concentrations 500mM and 250mM can produce maximum glycation, while 5mM and 1mM concentrations of thiamine-HCl and 10mM concentration of benfotiamine produced fairly good response to decrease glycation and both can be used as a protector against glycation in the human body. Periodate borohydride technique seems to be more effective and sensitive compared to the TBA test.

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