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Early metabolic changes in the gut leads to higher expression of intestinal alpha glucosidase and thereby causes enhanced postprandial spikes

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ABSTRACT

Aims: Prediabetes manifests several years earlier, before it progresses to diabetes. It is essential to track the earliest metabolic changes occurring in the prediabetic state and to understand the precise mechanism of how diabetes is initiated.

Main methods: Alpha glucosidase was isolated from rat intestine and assayed using maltose as substrate. *In vitro* glycation of the enzyme was studied using varying fructose content through measurement of fructosamine, general and specific fluorescence. *In vivo* experiments were carried out through feed of 4 g fructose per day. Protein expression was studied using western blot and mRNA expression using RT-PCR method.

Key findings: Fructose inhibits alpha glucosidase to the extent of 48.97% in 4 h at 2.5 M concentration. *In vivo* studies demonstrated an inhibition of 56.96% in three days. Activity was found to rise by seven days and normalized by 10 days. Protein expression was found to increase by 10.56 fold and SI mRNA by 41.84 fold on 10 days of fructose feed. Long term fructose feed for 60 days demonstrated increase in alpha glucosidase activity by 2.12 fold and increase in postprandial glucose spike.

Significance: Glycation of alpha glucosidase causes inhibition of the enzyme activity leading to compensation through higher protein expression. Long term fructose feed leads to more than two fold increase in enzyme activity causing postprandial spikes and ultimately manifesting as diabetes mellitus.

1. Introduction

Establishment of non-insulin dependent diabetes mellitus is a slow process that may take years to manifest. The number of people having prediabetes and diabetes is growing over time. More than 86 million US adults had prediabetes in 2014 and only 11% of those were aware of it [1]. Worldwide 425 million people were reported to have diabetes in 2017 wherein one in ten remains undiagnosed [2]. Prediabetes is an intermediate stage between normoglycemia and hyperglycemia, where fasting plasma glucose levels are between 100 and 120 mg/dl and postprandial glucose levels are between 140 and 199 mg/dl, commonly referred to as impaired fasting glucose (IFG) and impaired glucose tolerance (IGT). The glycated HbA1c value between 5.7% and 6.4% are considered as prediabetic while a value above 6.5% is taken as frank diabetes [3].

The external symptoms of polyuria and polydypsia as well as polyphagia are evident in the prediabetic state to a small extent and often go un-noticed. The speed of progression from prediabetes to diabetes may depend upon several factors that may include food habits, sedentary life style, lack of exercise, stress, *etc.* Prediabetes is associated with a risk of increased cardiovascular disorders and mortality [4], periodintitis, nephropathy and neuropathy [5–8]. According to American Diabetes Association about 70% of the prediabetics would be developing diabetes and what is more alarming is that majority are unaware that they are in the prediabetic state. Several enquiries into the changes that manifest in the prediabetic state have revealed that levels of amino acid and signalling lipid molecules to be reduced. Predominantly this included glycine and glutamine [9]. Prediabetes is also associated with higher insulin secretion that eventually leads to a drop in the beta cell function and finally reduced insulin production as seen in the diabetic patients [10].

Despite extensive metabolomic studies and a search for early markers that can demonstrate establishment of diabetes, the early metabolic changes that trigger diabetes still eludes us. The process of protein glycation is associated with secondary complications in diabetes [11]. This is due to the interaction of glucose with protein through Maillard

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reaction and Amadori rearrangement leading to formation of advanced glycation end products (AGEs). The glycated Hb1Ac is considered as a gold standard that can give a concrete idea about the glycemic control of the patient over a time period. It is often considered that this is a slow process often needing weeks and months to manifest. However, we have found that glycation of proteins can occur in a span of 2–4 h at 2.5 M fructose, the quantity of sugar found in most of the sweetened soft drinks (*e.g.* Mexican Coca cola) [12]. Fructose was found to be more effective glycating agent as compared to other sugars. The present study deals with the effect of fructose as a major protein glycating agent through *in vitro* and *in vivo* studies and its role in progression of prediabetes to diabetes.

It was hypothesised that high input of sugar would lead to glycation of the gut proteins and impact their functionality. Hence it was attempted to study the effect of glycation on alpha glucosidase, a major enzyme complex present in the duodenum that is responsible for breakdown of starch and other disaccharides into monosaccharides that are further absorbed by SGLT 1 and GLUT 2 transporter. Alpha glucosidase has been reported to be over expressed by almost 1.5 times higher than normal in diabetics [13] however the cause for such an increase is not known. Alpha glucosidase present in brush border of small intestine and breaks α 1–4 bond. Both sucrase-isomaltase (SI) and maltase-glucoamylase (MGAM) contains two catalytic subunits; at N and C terminals. SI is more abundant than MGAM; but MGAM has higher hydrolytic activity [14]. The present study attempts to evaluate the effect of protein glycation on intestinal alpha glucosidase and its impact on progression of prediabetes to diabetes. It is also a matter of interest to study whether usage of protein glycation inhibitors can prevent such changes.

2. Material and methods

2.1. In vitro study of alpha glucosidase

2.1.1. Isolation of rat intestinal alpha glucosidase

Enzyme from rat intestine was isolated using method described by Yasuda et al., in 2003 [15] with some modifications. Male Wistar rats weighing 180–200 g fasted for 20 h were sacrificed by cervical dislocation and region of small intestine was collected. Using ice cold saline and 50 mM sodium phosphate buffer (pH 7.4); the intestine was cleaned and mucosal layer was collected by everting the intestine and scrapping the luminal surface using glass slide. The mucosal scrapping were homogenized in 0.1 M sodium phosphate buffer (pH 7.4) containing 0.1% triton X-100 and centrifugation at 3000 rpm for 15 min was carried out. Cold butanol was added to supernatant in 1:1 proportion and centrifuged at 10,000 rpm for 30 min at 4 °C. The aqueous layer obtained was dialyzed against the same buffer. Dialyzed enzyme was used as a crude alpha glucosidase enzyme. The protein content was estimated by Lowry method [16].

2.1.2. Determination of enzyme activity and calculation of % inhibition

Enzyme activity was determined by incubating enzyme with maltose (25 mM) in 0.2 M sodium phosphate buffer (pH 7.4) for 30 min at 37 °C. The enzyme reaction was stopped by keeping the reaction tubes in boiling water bath for 10 min. Glucose released was measured by glucose-oxidase method using commercially available kit from Pathozyme diagnostics, India. For specific activity determination similar enzyme isolation protocol was used with some modification; enzyme was isolated from 2 cm duodenum region in 5 ml phosphate buffer. Enzyme activity expressed in terms of amount (μg) of glucose produced per mg of protein. Further the effect of different parameters on enzyme activity was calculated in terms of % inhibition.

$$\%Inhibition = \frac{Control - Test}{Control} \times 100$$

2.1.3. Effect of monosaccharides

Initially reaction mixtures containing dialyzed enzyme (10 mg/ml), in 50 mM phosphate buffer (pH 7.4), 0.01% sodium azide and 50 mM various monosaccharides (glucose, galactose, fructose, xylose and ribose) [17] were incubated for 72 h at 37 °C. After incubation, unbound excess sugars were removed by overnight dialysis using 12 KD dialysis bag against same buffer. Reaction mixture without any monosaccharide is referred to as control. Enzyme activity and % inhibition was calculated.

2.1.4. Glycation of alpha glucosidase by fructose

The same procedure described above was repeated using varying concentrations of fructose only (25 mM, 50 mM, 100 mM, 150 mM and 200 mM). To study the effect higher concentrations of fructose (as present in HFCS) [18], the isolated enzyme was treated with higher concentrations of fructose *viz.* 1.0 M, 1.5 M, 2.0 M and 2.5 M. Aliquots from treated enzyme samples were removed at different time intervals such as 1 h, 2 h, 4 h, 5 h and 6 h to observe the effect in short time interval. Standard anti-glycating agent aminoguanidine (AMG) [19] at a concentration of 2 mM was also used and served as positive control. After completion of incubation time, the treated enzyme processed with same protocol as described above. Enzyme activities were determined for all samples and % inhibition was calculated.

2.1.4.1. Determination of fructosamine. Fructosamine is an early product of protein glycation reaction [20]. The method described by Johnsons [21] with slight modifications was used for estimation of fructosamine. Aliquots of treated alpha glucosidase was incubated with 300 μM NBT in 100 mM carbonate buffer (pH 10.35) at 37 °C for 30 min and the absorbance was read at 530 nm on UV–Visible spectrophotometer. Extinction coefficient of 12,640/M/cm for monoformazan was used for expressing fructosamine as nM/mg of protein [22].

2.1.4.2. AGEs measurements. Aliquots were also checked for AGEs fluorescence using Cary Eclipse fluorescence spectrofluorometer from Agilent technologies Inc., USA. The presence of total AGEs was characterised by a typical fluorescence with excitation and emission maxima at 370 nm and 440 nm respectively [23]. Measurement of fluorescence intensity of pentosidine (AGE product) was characterised by typical fluorescence with excitation and emission maxima at 335 nm and 385 nm, respectively [24]. The results were expressed as AU/mg of protein.

2.2. In vivo alpha glucosidase study

2.2.1. Experimental design

All *in vivo* studies were carried out on various groups of experimental male Wistar rats (*Rattus norvegicus*). Each group contained six rats weighing approximately 180–200 g. The animal experiments have been carried out as per the Ethical Committee Guidelines after due submission of the animal protocols and approval. The animal experiments were carried out in the University animal house that is registered under the ethical committee certification (Reg. No. 233/CPCSEA). The model animals were allowed free access of food (Amrut, Pune) and, water *ad libitum*.

Groups of experimental rats as below

Group I: Control

Group II: Fructose fed rats (4 g/day for 3 days)

Group III: Fructose fed rats (4 g/day for 3 days) treated with AMG (oral dose of 20 mg/Kg body weight twice a day for 3 days)

Group IV: Fructose fed rats (4 g/day for 10 days)

Group V: Fructose fed rats (4 g/day for 10 days) treated with AMG (oral dose of 20 mg/Kg body weight twice a day for 10 days)

Group VI: Fructose fed rats (4 g/day for 30 days)

Group VII: Fructose fed rats (4 g/day for 10 days) treated with AMG

(oral dose of 20 mg/Kg body weight twice a day for 30 days)

Group VIII: Fructose fed rats (4 g/day for 45 days)

Group IX: Fructose fed rats (4 g/day for 10 days) treated with AMG (oral dose of 20 mg/Kg body weight twice a day for 45 days)

Group X: Fructose fed rats (4 g/day for 60 days)

Group XI: Fructose fed rats (4 g/day for 10 days) treated with AMG (oral dose of 20 mg/Kg body weight twice a day for 60 days)

2.2.2. Oral maltose tolerance test (OMTT)

OMTT was carried out to assess alpha glucosidase activity *in vivo*. Male rats were fasted overnight (approx. 16–20 h). Blood sample was withdrawn from tail and initial blood glucose level was measured by using Accu-Chek Active glucometer. Maltose load of 2 mg/g of body weight in saline was given orally to each rat. After oral administration of maltose, blood glucose levels were measured at the intervals of 30 min, 60 min and 120 min [25].

2.2.3. Quantification of AGEs of alpha glucosidase in fructose fed rats

Advanced glycation end products were measured using spectrofluorometer. Enzyme isolation protocol are previously described. The isolated enzyme samples were then used for total and specific AGEs fluorescence measurement (protocol is previously mentioned).

2.2.4. Quantification of protein expression by Western blotting

SDS-PAGE was carried out to separate crude enzyme. Polyacrylamide gel was cast using the concentration of 4% for stacking and 12% for resolving gel. 30 µg crude sample was loaded in each well. Separated proteins were transferred to the polyvinylidene difluoride (PVDF) membrane with semi dry blotter available from Bio-Rad Laboratories Inc., USA. The PVDF membrane was blocked using 5% dried skimmed milk powder prepared in phosphate-buffer saline. After overnight blocking at 4 °C, membrane was incubated at 37 °C for 1 h after which a treatment of anti-sucrase-isomaltase (SI) polyclonal antibody (1:1000 dilution) was given and incubated at room temperature for 3 h. After the incubation, SI antibody was removed and the membrane was washed for five min each; twice with phosphate buffer saline tween (PBST) and once with PBS. Then the membrane was incubated with HRP conjugated anti rabbit secondary antibody for 1 h at room temperature and washed for ten min each; twice with PBST and once with PBS. Transferred proteins were then incubated with Clarity™ ECL Blotting Substrate commercially available from Bio-Rad LaboratoriesInc, USA. Chemiluminescence was detected using Gel Documentation system (Syngene Bioimaging, UK). For quantification and normalizing the data, actin was used as a loading control. The treated PVDF membrane was stripped in stripping buffer to remove bound antibodies and the membrane was blocked overnight in 5% skimmed milk powder as described above. Antibodies specific to actin were used at dilution of 1:5000. The HRP conjugated secondary antibody treatment and detection protocol was followed as described above. Image Studio Lite Software (LI-COR Inc. US.) was used for densitometric quantification.

2.2.5. Alpha glucosidase mRNA expression study

For assessing the mRNA expression changes after treatment of fructose and AMG, all five groups of rats were analyzed for SI mRNA expression by RT-PCR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as control housekeeping gene. Total RNA of small intestinal tissues of each rat was extracted by SV Total RNA isolation kit (Promega, USA). DNA free RNA was quantified by Qubit RNA BR Assay kit (Thermo-Fisher Scientific). Reverse transcription was carried out to obtain cDNA using AccuScript high fidelity 1st strand cDNA synthesis kit from Agilent Genomics with 1.0 µg template RNA and 0.75 µl oligo dT for 16 µl reaction mixture. The primers used were as described in Table 1 For PCR reaction, 1.0 µg of the cDNA was added to a PCR reaction mixture consisting of 10× PCR buffer (5.0 µl), 50 mM $MgCl_2$ (0.75 µl), 10 mM dNTPs (1.0 µl), 10 pM of paired primers (1.0 µl each), 0.5 µl of Taq polymerase (Raq 5000 enzyme) and distilled water to make total volume of 25 µl. The PCR cycles were run in Arimax real time PCR thermocycler from Agilent Technologies, Inc., USA.

2.3. Statistical analysis

All data obtained were expressed as mean ± SD. Statistical analysis was performed using one-way analysis of variance (ANOVA). P-values at an α level less than 0.05 were considered to be statistically significant.

3. Results

3.1. Fructose significantly reduces alpha glucosiadse activity

To study the effect of dietary sugars on the duodenal alpha glucosidase activity, the freshly prepared enzyme was incubated with the sugars and its activity evaluated. Of the three hexoses and two pentoses studied, fructose is found to impact the alpha glucosidase activity by 34.55%. This is followed by ribose at 9.65% as compared to the control. Glucose galactose and xylose do not seem to impact the alpha glucosidase activity at 50 mM concentration. Further, increasing the fructose concentration to 200 mM concentration was found to reduce the alpha glucosidase activity by over 61.63%.

3.2. Impact of fructose at higher content from 1 to 6 h

In view of the high sugar content in sweetened soft drinks to the extent of 12–15 g per 100 ml which is consumed often on a regular basis, its effect on protein glycation was studied. It is evident that at 2.5 M (4 g) concentration of fructose even at as low as 1 h 37.88% inhibition of the enzyme is observed which rises to almost 48.97% in about 4 h. It is interesting to note that almost 33.68% inhibition is seen at about 4 h with as low as 1.5 M fructose (Fig. 1C).

3.3. Protein glycation inhibitor prevents fructose induced inhibition of alpha glucosidase

If glycation of alpha glucosidase leads to loss in the enzyme activity it is imperative that use of a protein glycation inhibitor should prevent the loss in activity. In view of this, aminoguanidine a routinely used protein glycation inhibitor was used at 2 mM concentration. It can be observed that barely 26.56% inhibition of the enzyme is evident at 4 h in presence of aminoguanidine as against more than 49.79% inhibition in the absence of the inhibitor (Fig. 2A). Likewise fructosamine formation was also found to be reduced by 28.06% in presence of the inhibitor (Fig. 2B). An analysis of total and specific AGEs formation (Fig. 2C and D, respectively) reveals decrease in AGEs formation in presence of inhibitor. Total and specific AGEs fluorescence was found to be reduced by 31.19 AU/mg of protein and 29.23 AU/mg of protein respectively in presence of the inhibitor.

3.4. Can similar changes be seen in the in vivo situation?

In view of the above findings it was attempted to study the *in vivo* effect of high fructose feed on alpha glucosidase activity. With a feed of 4 g per day the animals were sacrificed and the enzyme activity determined. In a span of three days the alpha glucosidase activity reduced by 56.96% (Table 2) and use of protein glycation inhibitor prevented such changes (reduction by just 6.54%). Further the glycation pattern shows an increase in the general AGEs as well as specific AGEs and the same is prevented with use of aminoguanidine (Table 2). On continuing this experiment further we found that by 7 days the inhibition is only to the extent of 27.77% and further by 10 days the activity was even higher than the normal values. It is interesting to note that the extent of glycation increases with time. Use of the glycation inhibitor is able to

Table 1
Primer sequence.

Gene	Accession number	Forward primer	Reverse primer
SI (Rat)	XM_346624	5′-GGAGGTTACATTCTACCATGTCAAG-3′ (395 bp)	5′-CCAGGTGATTTGTATTGGTTCATCA-3′ (395 bp)
GAPDH (Rat)	XM_216453	5′- GGTGCTGAGTATGTCGTGGAG-3′ (338 bp)	5′- ATGCAGGGATGATGTTCTGG -3′ (338 bp)

prevent such changes.

3.5. Effect of fructose feeding on SI protein expression

Western blot results (Fig. 3A and C), demonstrated that amount of SI proteins expressed in all fructose fed rat groups was higher than control rats. 10 days fructose fed rats showed 10.56 fold higher SI protein level than control, implying higher protein expression. SI protein levels were also increased within three day by 2.48 fold than control. Administration of AMG to fructose fed rats showed decreased (3.44 and 1.27 fold rise) protein expression than in absence of AMG (10.56 and 2.48 fold increase).

3.6. Effect of fructose feeding on SI mRNA expression

In view of the increased protein expression mRNA levels were measured. Threshold cycle value (CT value) for a particular set of sample is an indicative of the scale of expression of mRNA in RT-PCR. As per protein expression, the mRNA levels were also higher in all fructose fed rats. 10 days fructose fed rats showed highest mRNA expression among all groups (41.84 fold higher than control). While, AMG treatment showed 19.67 times higher mRNA level of SI mRNA levels. SI mRNA expression in rats fed with fructose for three days, with and without AMG was observed to be 1.39 and 21.87 fold higher respectively, than the control rats.

3.7. Effect of fructose feed on postprandial blood glucose

The effect of glycation on alpha glucosidase which inhibits the enzyme by three days is evident in Table 2 from a normal postprandial spike of 145 mg/dl the level reduced to 120 mg/dl by the third day. In tune with the increase in the enzyme activity by 10 days the oral maltose test also shows an increase in the 30 min postprandial blood glucose level. The level of the blood glucose is slightly higher at two hours

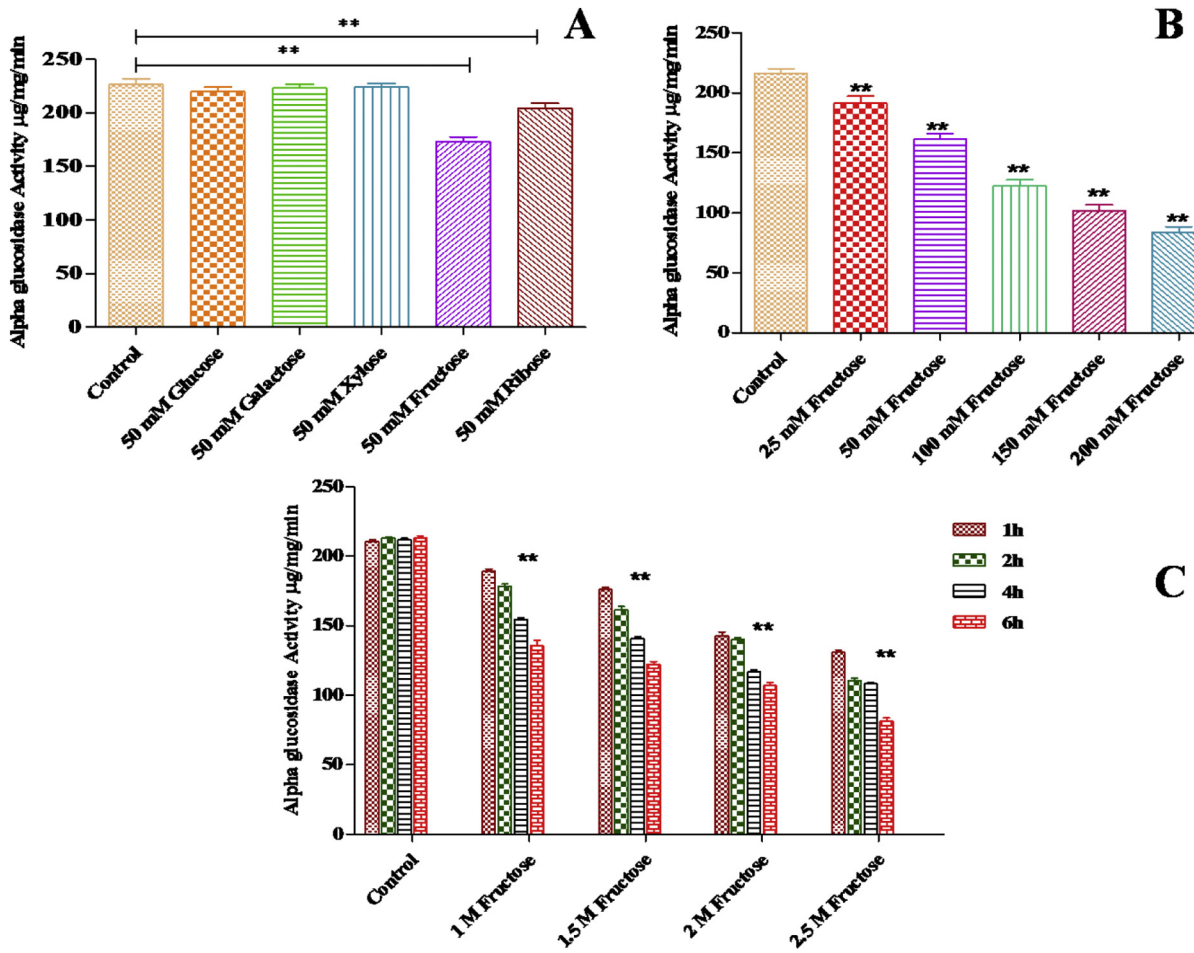

Fig. 1. *In vitro* effect of reducing monosaccharides on alpha glucosidase activity: A] Dietary sugars like glucose, galactose, xylose, fructose and ribose at 50 mM concentrations were incubated with alpha glucosidase for 72 h and activity was measured. B] Alpha glucosidase was incubated with 25 mM, 50 mM, 100 mM, 150 mM and 200 mM fructose up to 72 h and activity was measured. C] Fructose at higher concentration (resembling concentration as in HFCS) was incubated with alpha glucosidase for 6 h and activity is measured. The results were expressed as mean ± S.D. (n = 6). *P* value less than 0.05 is considered as statistically significant.

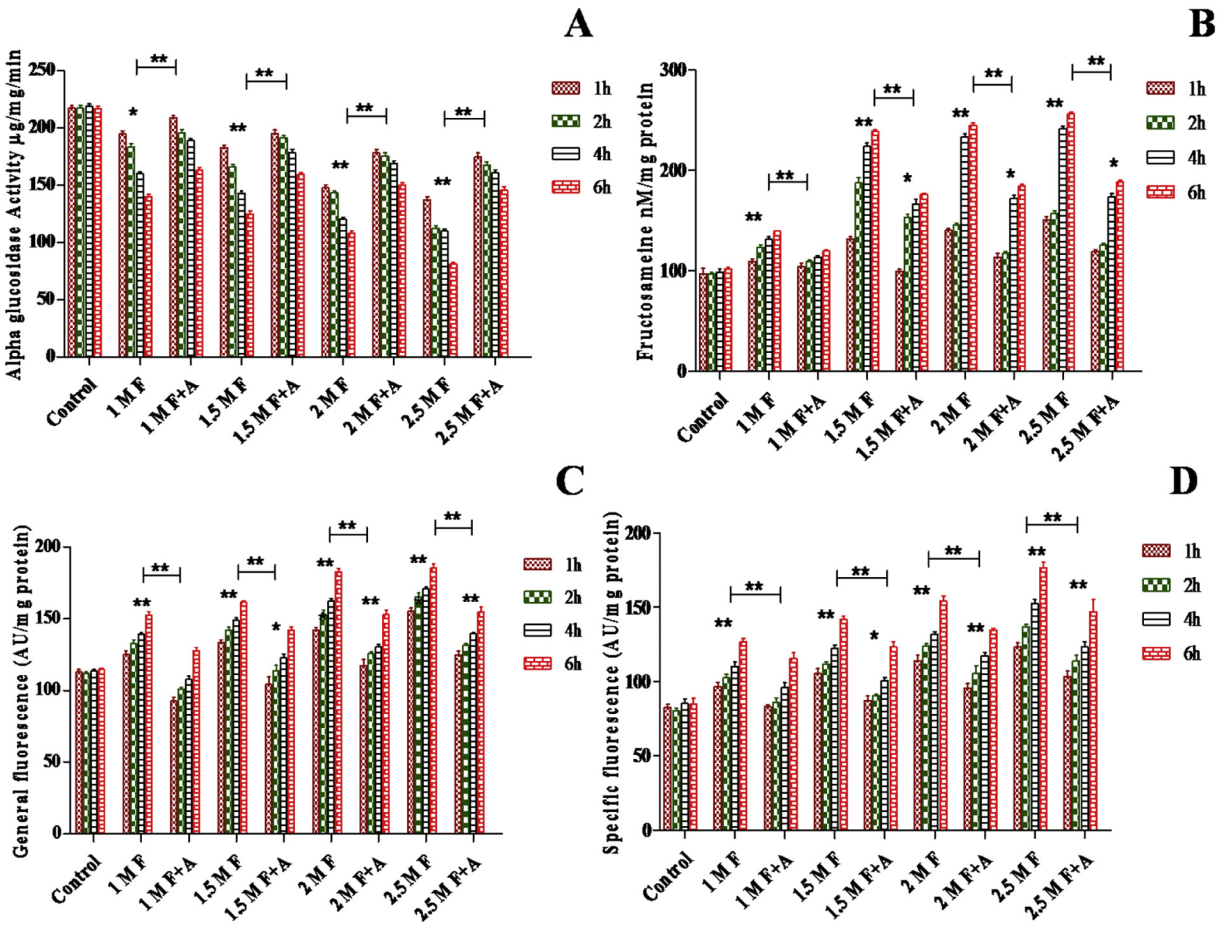

Fig. 2. Change in alpha glucosidase activity and glycation parameters after treatment of high fructose and aminoguanidine: A] Alpha glucosidase was incubated with high fructose concentration along with AMG for 6 h and activity is measured. B] Fructosamine measurement after fructose medicated glycation of alpha glucosidase C] General fluorescence D] Specific fluorescence. Fructosamine and AGEs formed after incubation of alpha glucosidase with fructose in presence and in absence of AMG was measured and expressed as nM/mg of protein and AU/mg of protein respectively. The results were expressed as mean ± S.D. (n = 6). P value less than 0.05 is considered as statistically significant.

as compared to the fasting values.

However prolonged fructose feed for 45 days and 60 days showed significant increase in postprandial spikes to the extent of 150 mg/dl and 158 mg/dl, respectively. It is also important to note that enzyme activity was recovered by 10 days and was in the range up to 30 days. Thereafter however there was a rise in enzyme activity to the extent of more than two fold by 60 days. Both specific and general fluorescence were found to be continuously increasing.

4. Discussion

Despite years of research to answer the question how diabetes is

Table 2
Effect of high fructose diet on OMTT, alpha glucosidase activity and AGEs fluorescence: High fructose diet (resembling concentration as in HFCS) was given for different time periods and OMTT was carried out; enzyme activity and fluorescence were also measured. BGL = mg/dl, activity is expressed as µg/mg/min and fluorescence as AU/mg of protein. The results were expressed as mean ± S.D. (n = 6).

	0 min	30 min	60 min	120 min	Activity	General fluorescence	Specific fluorescence
Control	78 ± 5	144 ± 3	121 ± 4	82 ± 5	249.50 ± 6.15	145.88 ± 2.14	104.54 ± 3.71
3 D F	87 ± 5	120 ± 2	107 ± 6	90 ± 5	107.37 ± 6.15	176.54 ± 4.01	165.21 ± 2.16
3 D F + A	77 ± 5	141 ± 4	126 ± 4	83 ± 7	233.16 ± 3.92	120.54 ± 3.28	126.54 ± 2.03
7 D F	80 ± 5	141 ± 5	121 ± 4	86 ± 3	180.21 ± 6.54	188.66 ± 2.08	187.66 ± 3.51
7 D F + A	81 ± 4	143 ± 6	117 ± 4	84 ± 7	220.74 ± 2.54	152.33 ± 2.51	152.33 ± 2.51
10 D F	72 ± 5	152 ± 5	133 ± 5	87 ± 5	273.70 ± 3.66	281.11 ± 1.84	214.88 ± 2.21
10 D F + A	83 ± 4	144 ± 4	123 ± 2	89 ± 5	247.70 ± 4.35	145.88 ± 3.09	165.21 ± 2.59
30 D F	100 ± 5	144 ± 4	120 ± 3	102 ± 5	310.84 ± 4.06	303.00 ± 6.02	251.33 ± 3.21
30 D F + A	93 ± 5	140 ± 4	110 ± 5	97 ± 2	269.60 ± 3.05	170.66 ± 6.02	187.33 ± 5.03
45 D F	110 ± 4	150 ± 4	132 ± 5	122 ± 4	383.94 ± 3.15	317.66 ± 8.73	275.66 ± 4.96
45 D F + A	104 ± 4	140 ± 5	120 ± 4	90 ± 4	316.77 ± 5.15	219.66 ± 7.50	206.00 ± 5.29
60 D F	112 ± 3	158 ± 5	145 ± 4	133 ± 5	530.75 ± 4.83	369.00 ± 9.16	302.66 ± 6.42
60 D F + A	107 ± 2	142 ± 3	122 ± 2	102 ± 3	404.98 ± 4.15	255.33 ± 5.50	224.33 ± 6.02

High fructose diet (resembling concentration as in HFCS) was given for different time periods and OMTT was carried out; enzyme activity and fluorescence were also measured. BGL = mg/dl, activity is expressed as µg/mg/min and fluorescence as AU/mg of protein. The results were expressed as mean ± S.D. (n = 6).

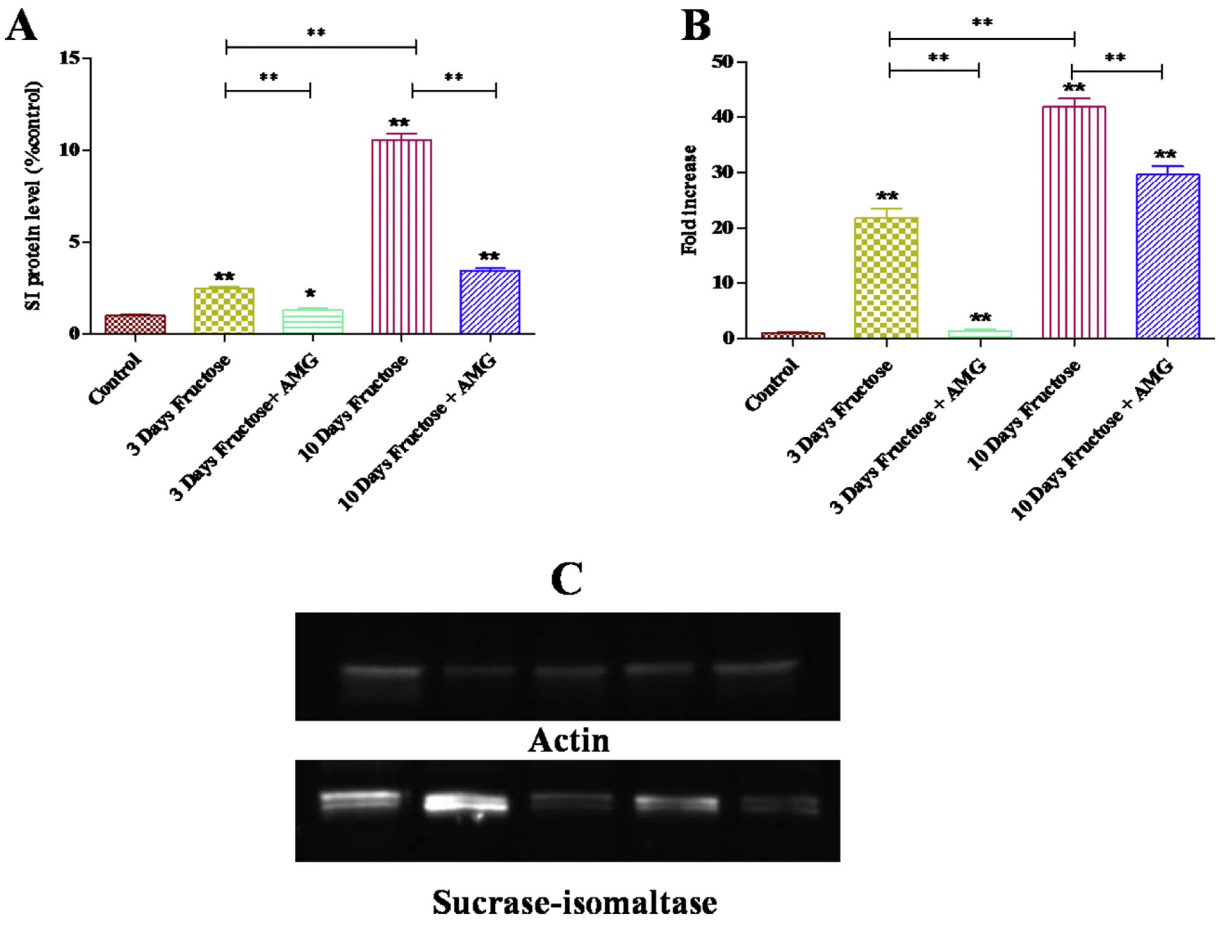

Fig. 3. Effect of high fructose feeding with AMG on SI complex protein (A, and C: Lane details (from left): Lane 1-AMG treated fructose fed for 10 days; lane 2-Fructose fed rats for 10 days; lane 3-AMG treated fructose fed for 3 days; lane 4-Fructose fed rats for 3 days; lane 5-Control) and mRNA expression (B): Comparison of quantitative protein and mRNA expression of alpha glucosidase in the control rats, 3 days and 10 days fructose fed rats with and without AMG was done by western blotting and RT-PCR respectively. The results were expressed as mean ± S.D. (n = 3). P value less than 0.05 is considered as statistically significant.

initiated? – there have been no conclusive answers. Several theories have been advanced but none have been able to satisfactorily prove the process. The present study is the first of its kind that distinctively gives a clear proof on what are the earliest changes occurring in prediabetes and how prediabetes can proceed to frank diabetes. We have been able to conclusively demonstrate that excess intake of carbohydrates especially sugar at the level present in sweetened drinks [12] can lead to glycation of intestinal and gut proteins impacting their structure and function. It is earlier reported that protein glycation causes inactivation of different enzymes including SOD, catalase, and esterase [26].

In the present study we have chosen alpha glucosiadase, as a probe: an enzyme involved in carbohydrate metabolism that breaks down carbohydrates and disaccharides into mono saccharides that can then be transported through SGLT 1 and GLUT 2 transporters into the blood stream. There have been several studies demonstrating the increase in the expression of the protein by as high as 1.5 fold as well as increase in the mRNA content in diabetics. An earlier study showed an increase in total activity, specific activity and expression of sucrase-isomaltase in all regions of small intestine of Streptozotocin-induced diabetic rats [27]. Force feeding of sucrose to rats showed increased activity of SI and its mRNA levels, while such changes were not observed in rats fed with carbohydrate free diets. This increase in SI activity was correlated with rise in SI mRNA levels [28]. Abnormal increase in mRNA encoding SI complex was also observed in 48 week old Otsuka Long-Evans Tokushima fatty (OLETF) rats [29].

Diet containing higher amount of high-medium-chain triglycerol and carbohydrate also raises SI mRNA levels in rats. Force feeding of high sucrose diet (40% energy as sucrose) causes increased SI mRNA levels within 12 h. Two to three fold increase in SI mRNA levels was observed in rats fed with high-carbohydrate diet than rats fed with low-carbohydrate diet. This change was persisting at least for 7 days [30]. It has also been suggested that deficiency of insulin leads to lower expression of the disaccharidase activity [31].

However, the basic cause for an increase in the alpha glucosidase activity was not known. It can be observed from Fig. 1C that fructose a component of sweetened drinks can cause inhibition of the enzyme to the extent of 34.68% at 1.5 M concentration in just four hours. It is common understanding that glycation of protein takes a long time to manifest. However our results prove that glycation can occur within hours and that too with sugar content commonly consumed in the form of sweetened drink.

We proceeded with *in vivo* studies to observe whether this phenomenon is also seen in the whole animal. We observed a reduction in the alpha glucosidase activity by three days of fructose feed (4 g/day) however to our surprise the activity was observed to rise by 7 days and further it was slightly even higher than control by ten days of fructose feed. Study of protein expression and mRNA demonstrated a 10.56 fold increase in the protein expression and a 41.84 fold increase in the mRNA expression. In other words, the reduction in activity of alpha glucosidase was offset by adaptation by the system through higher production of the protein. However the activity of the enzyme does not correlate to the amount of protein produced (Table 2 and Fig. 3). It can be observed from Table 2 that glycation of the enzyme continues and so also inhibition of the enzyme activity.

Further, with a view of studying how these changes occurring in the very early stages *i.e.* up to ten days manifest with time, we continued with the fructose feed for 60 days. It can be observed in the OMTT that the postprandial spike is not so strong even up to 30 days, but by 45 days there is a distinct rise in the 30 mins postprandial spike and by 60 days it can be termed as frank diabetes with an increase in the spike from 144 mg/dl to 158 mg/dl and a postprandial rise in blood sugar from 87 mg/dl to 133 mg/dl. The activity of the enzyme has also increased more than twofold from 249.50 AU in control to 573.75 AU by 60 days. In other words the process of protein glycation impacting the activity of alpha glucosidase eventually leads to higher and higher expression of the enzyme that can rapidly breakdown the disaccharides and result in postprandial spikes. These postprandial spikes then trigger hyperglycemia, that further leads to protein glycation of cellular and extracellular proteins eventually establishing as secondary complications in diabetes. It also essential to be understood that regular consumption of excess sweetened drink or even fruit juice would eventually cause protein glycation as these are rich in fructose. That protein glycation is the root cause of this process is confirmed through use of aminoguanidine an inhibitor of protein glycation that can effectively prevent these changes and stop progression of pre diabetes to diabetes.

5. Conclusion

The present study conclusively proves that glycation of proteins can occur in as short span as even one hour at higher concentration of sugars, fructose being the best glycating agent. Glycation of alpha glucosidase inhibits the enzyme which leads to compensation by the system through higher production of the mRNA and protein. Eventually this manifests in higher expression and activity of the enzyme leading to postprandial spikes. Hence, in a first report we demonstrate clearly how higher glycemic food leads to glycation of gut proteins and manifests as the earliest changes in the gut –the prediabetes stage, and if such glycemic feed continues, proceed to frank diabetes mellitus. The study also makes an important contribution in demonstrating how use of protein glycation inhibitors can prevent progression of prediabetes to diabetes.

Abbreviations

RT-PCR	reverse transcriptase polymerase chain reaction
SI	sucrase-isomaltase
IFG	impaired fasting glucose
IGT	impaired glucose tolerance
HbA1c	glycated Hb
ADA	american diabetes association
AGEs	advanced glycation end products
MGAM	maltase-glucoamylase
KD	kilo dalton
HFCS	high fructose corn syrup
AMG	aminoguanidine
NBT	nitro blue tetrazolium
OMTT	oral maltose tolerance test
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
PVDF	polyvinylidene difluoride
PBS	phosphate buffer saline
PBST	phosphate buffer saline with twin
ECL	enhanced chemiluminescense
GADPH	Glyceraldehyde-3-phosphate dehydrogenase
CT	threshold cycle
SOD	superoxide dismutase
SGLT 1	sodium glucose transporter 1
GLUT 2	glucose transporter 2
BGL	Blood glucose level

Declaration of interest

The authors have no conflicts of interest to declare.

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