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Original Article

GLYCATION INHIBITORS AND PROBIOTICS CAN AMELIORATE THE CHANGES CAUSED BY HIGH FRUCTOSE FEED

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ABSTRACT

Objective: To evaluate the use of protein glycation inhibitors and probiotics to ameliorate secondary complications in diabetes and to improve gut microbiota respectively in high fructose fed Wistar rat.

Methods: The study was conducted on male Wistar rats for 7 d. Blood glucose levels in oral glucose tolerance test (OGTT) were measured using glucometer, serum parameters were analyzed using commercial kits, antioxidant status was evaluated by measuring superoxide dismutase (SOD) and catalase (CAT) levels, total reactive oxygen species were estimated using a fluorescent 2', 7'-dichlorofluorescin diacetate (DCF-DA) dye, and tissue fluorescence of liver, kidney and intestine were measured using a spectrofluorimeter.

Results: OGTT pattern shows significant increase in blood glucose of fructose fed rats i.e. 154 mg/dl while, in aminoguanidine (AMG) treated and gut microbiota modulated animals it is 137 and 119 mg/dl resp. after 30 min on glucose administration. Marked reduction was found in SOD 6.37 and 11.25 U/mg of protein and catalase 186 and 65.5 U/mg of protein in liver and kidney of fructose fed animals when compared to fructose+AMG and fructose+EUGI. There is 5-6 fold significant increase in general and specific tissue fluorescence of liver and kidney, and 2.2 fold increase in liver reactive oxygen species was observed in fructose fed group as compare to control animals. Significantly higher glycation was found in intestine of fructose fed animals (general fluorescence 2.1 and specific fluorescence 3.1 AU/mg), more than that of diabetic control rats (general fluorescence 0.9 and specific fluorescence 1.6 AU/mg), represented an evidence for adverse impact of excess fructose on healthy gut.

Conclusion: The use of protein glycation inhibitor and use of pre and probiotics significantly improved the serum parameters and would prevent progression to secondary complications.

Keywords: Fructose, Glycation, Glycation inhibitors, Probiotics, Diabetes mellitus

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INTRODUCTION

Diabetes mellitus is known to initiate several changes in various tissues of the body. Chronic hyperglycemia can eventually lead to secondary complications of diabetes such as nephropathy, neuropathy, retinopathy and cardio vascular diseases (CVD). The underlying causes involve glycation of proteins, oxidative stress, and involvement of immunological response and eventual destruction of tissues through multifactorial events. Protein glycation is known to cause changes in the protein structure such as alpha beta transitions, changes in the charge on the protein and electrophoretic mobility of the protein [1]. This is initiated through Amadori rearrangement, and formation of Advanced Glycation Products (AGEs) through Maillard reactions. The AGEs influence the functionality of the proteins and changes their clearance pattern. It is likely that continued impact of food with high glycemic index and sugars can initiate such changes.

Throughout the life ecological aspect shapes the microbial diversity of an individual and the mutualistic symbiosis in between them, majorly contributes a steady microbiota [2-4]. While, such changes in the gut may influence the gut microbial flora. Earlier reports have demonstrated a change in gut microflora in diabetics [5-7].

The use of probiotics has been suggested to influence this distribution and promote healthy outcomes. The probiotic bacteria like *Lactobacillus acidophilus, Lactobacillus rhamnosus, Bifidobacterium longum, Bifidobacterium bifidum, Streptococcus thermophiles* and Yeast *Saccharomyces boulardii* are found to be beneficial in preventing growth of pathogenic organisms, reducing inflammation and allergies, helping control over dysbiotic bacterial overgrowth and reduction of intestinal permeability. *Bifidobacterium* and *Lactobacillus* may help to ward off pathogens like *Salmonella* and *Clostridium botulinum* [8]. There have been several reports in implicating the microbial flora in the pathogenesis of obesity [9,10], diabetes [10, 11] and chronic kidney disease [12].

The present study attempted to evaluate the effect of fructose feed on protein glycation in the gut and other tissues and study of the use of protein glycation inhibitors can prevent such processes. It was also intended to study, if the use of probiotics can prevent the gut microbial changes and help to stop progression of diabetes.

MATERIALS AND METHODS

Chemicals and materials

Chemicals and reagents used were of analytical grade and purchased from local authorized distributors. Amino guanidine carbonate salt (AMG) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Blood glucose level was determined by using ACCU-CHEK kit (Roche Diagnostics, Manheim, Germany).

The Prebiotic and probiotic combination sachet (EUGI™) manufactured by WALLACE Pharmaceuticals Pvt. Ltd., Mumbai was obtained locally.

Experimental animals

Male Wistar rats weighing about 190-200 g were used in the experiment. All the animals were maintained under laboratory conditions and were allowed free access to food (Amruth, Pune) and water *ad libitum*. Animal experiments were carried out as per the guidelines of animal ethical committee of the Institute and CPCSEA (Registration no. 233/CPCSEA).

***In vivo* experimental design**

The animals (n=4) were divided into five groups viz.

Group I: Control (without fructose feed)

Group II: Diabetic control: Diabetes was induced by giving a single dose of streptozotocin [40 mg/kg of body weight (BW)] through intraperitoneal route, in 0.1 M citrate buffer of pH 4.5, prepared freshly and animals were monitored for a period of 14 d to confirm diabetes.

Group III: Non-diabetic rats with fructose feed (10 g/d)

Group IV: Non-diabetic rats with fructose feed (10 g/d)+7 mg/kg BW AMG twice in a day.

Group V: Non-diabetic rats with fructose feed (10 g/d)+50 mg prebiotic and probiotic combination (EUGI) [Fructo oligo saccharides (300 mg), *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Bifidobacterium longum*, *Bifidobacterium bifidum*, *Streptococcus thermophilus* (0.24 billion of each) and *Saccharomyces boulardii* (0.05 billion) = 1 g] twice in a day.

The effect of doses was tested for 7 d of treatment. After the end of experiment, all the animals were fasted for 24 h and sacrificed to measure the biochemical parameters.

Oral glucose tolerance test

For performing OGTT, the rats were fasted overnight with free access to water ad libitum. Initial blood glucose level of each rat was checked. All the rats were fed orally with a glucose load of 3 mg/g BW. Blood samples were withdrawn from the tail vain at the time intervals of 0 min, 30 min, 60 min and 120 min after glucose administration and blood glucose was measured using ACCU-CHECK glucometer.

Determination of serum parameters

Blood glucose level was determined by using ACCU-CHECK glucometer. Total triglycerides and high-density lipoprotein (HDL) levels were measured by using commercially available kits (Span Diagnostics, Surat, Gujrat, India). Serum creatinine and total cholesterol were measured by diagnostic kit (Creast Biosynthesis, Goa, India; Biolab Diagnostics, Boisar, India). Very low-density lipoprotein (VLDL) levels were calculated from total cholesterol and HDL cholesterol values.

Determination of antioxidant status of liver and kidney

Kidney and liver were homogenized in 100 mmol of Tris–HCl buffer pH 7.4 using Teflon homogenizer. The homogenate was centrifuged at 9000 rpm for 20 min at 4 °C and supernatant was used for the estimation of superoxide dismutase (SOD) and catalase (CAT). Protein content was estimated by using Bradford method [13].

Superoxide dismutase was estimated using the method described by Mishra and Fridovich (1972) [14]. In brief, 0.1 ml of supernatant was added to 3.5 ml buffer (phosphate buffer 50 mmol, pH 7.4), 0.3 ml of 10 mmol ethylene diamine tetra acetate (EDTA), 1.2 ml of 130 mmol methionine and 0.6 ml of 150 µM nitro blue tetrazolium (NBT) in 100 mmol sodium carbonate buffer, pH 10.35. Similarly prepared a reaction mixture in which supernatant was replaced by equal volume of distilled water which served as blank and was placed at dark. The reaction was started by addition of 0.4 ml riboflavin (60 µM). The tubes were radiated in front of 18W fluorescent bulb for 30 min and the reaction was stopped by placing the tubes in dark. The absorbance was read at 560 nm. Unit of SOD is described as the amount of enzyme required for inhibition of 50% oxidation riboflavin.

Catalase activity was determined from liver and kidney as per method described by Beers and Sizer (1952) [15]. The change in optical density at 240 nm per unit time was taken as a measure of catalase activity. Phosphate buffer (100 mmol, 7.4 pH) 2.1 ml was taken into a cuvette and 50 µl of homogenate was added in the reaction mixture. Reaction was started by addition of 0.5 ml freshly prepared 30 mmol H_2O_2 solution. The rate of decomposition of H_2O_2 was measured immediately by using UV-Visible spectrophotometer at 240 nm. The activity of catalase was expressed in units per mg protein calculated by following formula,

$$\text{Enzyme Activity (U/mg protein)} = \frac{\Delta A \times 1000 \times \text{Total reaction Vol.}}{\text{Extinction Coefficient} \times \text{Vol. of enzyme}}$$

Where, Extinction coefficient = 43.6/M/cm

Measurement of total reactive oxygen species (ROS) in liver

The ROS were measured using a fluorescent 2', 7'-dichlorofluorescin diacetate (DCF-DA) dye [16]. The 100 mg of liver tissue was chopped and incubated with 1 ml of 10 mmol DCF-DA for 30 min at 37 °C. After incubation the samples were sonicated for 15 seconds with 3 strokes and centrifuged for 4000 rpm for 5 min at 4 °C and the fluorescence of supernatant was measured using spectrofluorimeter (Cary Eclipse Fluorescence Spectrophotometer, USA) at 488 nm excitation wavelength and 530 nm emission wavelength. DCF-DA is a non-polar compound that readily diffuses into cell, where it is hydrolyzed to non-fluorescent polar derivative DCFH and thereby trapped within the cells. In the presence of ROS, DCFH is oxidized to the highly fluorescent 2', 7'-dichlorofluorescein (DCF). The level of DCF fluorescence reflects the concentration of ROS.

Measurement of tissue fluorescence-liver, kidney and intestine

After sacrifice the rat's liver, kidney and intestine were washed with saline, minced and defatted with the mixture of chloroform and methanol (2:1 v/v) overnight. The tissues were homogenized in 0.1 N NaOH and centrifuged at 10,000 rpm for 30 min at 4 °C temperature. The amount of total (370/440 nm) and specific (335/385 nm) AGEs from the supernatant was measured using a spectrofluorimeter. The fluorescence intensities of the samples were measured and represented in terms of arbitrary units (AU) per mg of protein [17].

Statistical analysis

All the values were analysed by one-way analysis of variance (ANOVA). Obtained results were expressed as mean±SD for four rats in each group (n=4). p-value<0.05 were considered as significant.

RESULTS

Effect of treatment on oral glucose tolerant test (OGTT)

The effect of fructose feed was studied on the oral glucose tolerant test after 7 d of treatment. The rats fed with only fructose showed an increase in the blood glucose level. The OGTT pattern shows that, in fructose fed animals the blood glucose level significantly increased up to 154 mg/dl at 30 min while rats feed with fructose+AMG and fructose+EUGI shows the blood glucose level 137 mg/dl and 119 mg/dl respectively (fig. 1).

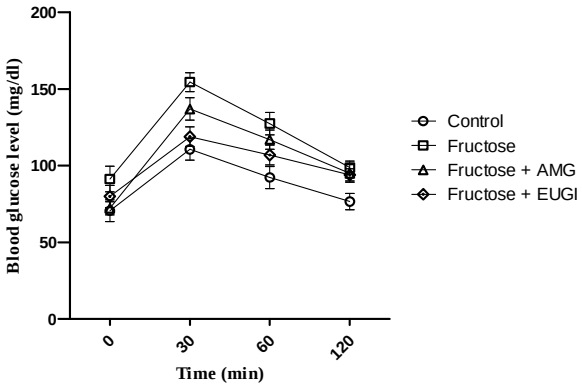

Fig. 1: Oral glucose tolerance test of experimental animals. Values were represented as mean ± SD (n=4.

Body weight and serum parameter studies

Animals fed with only fructose progressively gained more weight (250 g) than other animals and also demonstrated an increase in the fasting blood glucose level (95 mg/dl). While in fructose+AMG fed

animals the blood glucose level was 71 mg/dl as similar that of control (67.2 mg/dl). The AMG and EUGI treated animal shows significant reduction in serum lipid profile as compared to the fructose fed group (table 1).

Serum creatinine has been found to be a reliable indicator of kidney function. In fructose feed animals the serum creatinine level found slightly increased (0.725 mg/dl) than that of control (0.575 mg/dl), fructose+AMG (0.575 mg/dl) and EUGI treated animals (0.550 mg/dl) (table 1).

Urine volume

Urine volume and rate of excretion was significantly increased in fructose feed animal group as compared to other treatment groups (table 1).

Table 1: Effect of *in vivo* treatment on body weight, blood and urine parameters in experimental animals

S. No.	Tissue/Sample	Parameter	Control	Diabetic control	Non-Diabetic Groups		
		Fructose dose	**No fructose**	**No fructose**	**Fructose**	**Fructose+AMG**	**Fructose+EUGI**
1		Weight (g)	230.3±7.789	228.2±6.321	250.5±4.893	245.6±6.498	249.3±3.951
2	**Blood/Serum**	A) Initial Blood glucose (mg/dl)	66.0±3.91	202.0±14.31	66.5±4.43	65.25±4.42	64.75±2.50
		B). Blood glucose (mg/dl)	67.25±4.92	203.3±17.15	95.00±4.16	71.00±8.83*	79.00±4.69*
		C) Lipid profile test (mg %)					
		i] Total Cholesterol	58.0±4.32	130.5±9.67	83.00±2.58	68.00±4.32*	72.75±5.70
		ii] S. Triglycerides	59.0±6.83	147.0±14.65	167.0±10.13	85.0±12.91*	75.50±5.00*
		iii] S. HDL Cholesterol	41.50±2.64	39.00±2.58	37.00±2.58	30.75±2.21*	27.25±2.21*
		iv] VLDL Cholesterol	11.00±2.58	29.50±3.41	32.50±3.41	18.00±1.63*	13.25±2.21*
		D) Urea (mg/dl)	23.25±3.59	64.0±9.76	30.25±1.70	23.5±3.10*	24.0±1.82*
		E) Creatinine (mg/dl)	0.575±0.05	2.60±0.216	0.725±0.05	0.575±0.05*	0.550±0.05*
3	**Urine**	Volume (ml/day)	14.50±2.08	32.75±5.25	20.25±2.38	15.75±1.70*	16.00±1.63*

Each value expressed as means±SD, (n=4). *values significant at P<0.05 as against fructose fed.

Effect on antioxidant status and tissue fluorescence

The *in vivo* antioxidant status was evaluated in terms of liver and kidney SOD and catalase enzyme activity. In fructose fed animals liver and kidney SOD activities were reduced significantly (6.37 and 11.25 U/mg of protein respectively) as compared to control group. While in fructose+AMG treated group the values are nearly equal to that of control group. Similarly, the liver and kidney catalase activities in fructose fed group are significantly decreased (186 and 65.5 U/mg of protein) as that of control group. In fructose+AMG treated animals the liver and kidney catalase values are 204 and 95.75 U/mg of protein respectively. While EUGI treated animals the liver and kidney SOD values are 7.2 and 14.53 U/mg and catalase values are 200 and 83.75 U/mg of protein respectively (table 2).

There is 5-6 fold increase in general and specific fluorescence of fructose fed animals as compared to control animals. In control and fructose+AMG fed animals it was 2.86 and 4.42 AU/mg respectively. The tissue fluorescence of all the tissues increased significantly in fructose feed animals than of the control, fructose+AMG and EUGI treated animals (table 2).

Fluorescence of intestine

There is 5 and 4 fold increase in general and specific fluorescence of intestine (duodenum) in fructose feed animals than that of control group (0.426 and 0.807 AU/mg resp.). In AMG treated animals these values are 0.511 and 0.924 AU/mg as similar with control group. EUGI treatment found efficiently reducing the rate of protein glycation (0.902 and 2.147 AU/mg resp.) (table 2).

Liver reactive oxygen species (ROS)

Fructose feed animals showed a 2.2 fold increase in generation of ROS in liver as compared to that of control (1.47 AU/mg of tissue). While in AMG and EUGI treated animals it is 2.8 and 2.96 AU/mg of tissue respectively (table 2).

Table 2: Status of antioxidant parameters and tissue fluorescence in experimental animals

S. No.	Tissue/Sample	Parameter	Control	Diabetic control	Non-Diabetic Groups		
					Fructose	**Fructose+AMG**	**Fructose+EUGI**
1	**Liver**	a) SOD (U/mg of protein)	9.000±0.432	4.000±0.816	6.375±0.464	8.275±0.377*	7.225±0.434
		b) CAT (U/mg of protein)	223.8±8.098	123.3±9.069	186.3±4.787	204.0±6.325*	200.8±6.500*
		Fluorescence i] General (AU/mg)	0.235±0.026	8.240±0.796	4.463±0.613	1.009±0.154*	1.611±0.282*
		ii] Specific (AU/mg)	0.842±0.065	13.69±0.871	4.319±0.272	1.872±0.119*	3.018±0.095*
		ROS (488/520) AU/mg of Tissue	1.477±0.193	4.017±0.189	3.207±0.098	2.805±0.103	2.965±0.034
2	**Kidney**	c) SOD (U/mg of protein)	19.00±2.94	7.425±0.512	11.25±1.708	15.55±1.215*	14.53±1.176*
		d) CAT (U/mg of protein)	112.2±5.901	44.75±4.787	65.5±4.435	95.75±3.775*	83.75±6.449*
		Fluorescence i] General (AU/mg)	1.20±0.184	6.651±0.429	4.663±0.423	1.50±0.225*	2.73±0.354*
		ii] Specific (AU/mg)	2.86±0.393	15.66±2.336	17.57±1.284	4.429±1.028*	12.66±1.732*
3	**Intestine (Duodenum)**	Fluorescence i] General (AU/mg)	0.426±0.033	0.938±0.100	2.180±0.330	0.511±0.070*	0.902±0.058*
		ii] Specific (AU/mg)	0.807±0.102	1.640±0.235	3.100±0.331	0.924±0.110*	2.147±0.116*

Each value expressed as means±SD, (n=4). *values significant at P<0.05 as against fructose fed.

DISCUSSION

Earlier attempts in our lab have demonstrated that fructose is more efficient in glycation of protein than glucose hence it has chosen for the experiments. Further whether these changes can be prohibited leading to positive outcomes is evaluated. High fructose feed have influenced drastic changes in the whole body within a span of 7 d. It was observed that there is extensive glycation of various tissues and a strong

oxidative stress is initiated. These changes can snowball into various metabolic disorders. In a study conducted by Prakash *et al.* in 2011 [18] demonstrated that high fructose feeding in rat leads to impaired glucose tolerance and insulin resistance. It has been reported that, modulating composition of gut flora with prebiotics improved gut permeability, reduced metabolic endotoxemia, lowered inflammation, and alleviated glucose intolerance [19, 20]. Recent clinical study suggesting that gut microbiota modulation with novel probiotics prevents and efficiently controls type II diabetes along with its complications [21]. In support to these work we have found that, an oral glucose tolerance test in the various treated groups also demonstrates prevention in a shift towards a diabetic curve as seen in fructose fed rats and reduction in postprandial spikes is one of the major strategies to prevent establishment of diabetes through the use of probiotics and protein glycation inhibitors.

It is surprising to note that fructose feed for seven days led to an increase in fasting blood glucose level and influences the lipid profile of the animals (table 1). Use of aminoguanidine, a protein glycation inhibitor prevents such changes to a greater extent. Likewise the probiotics also seem to have a beneficial effect. It is significant that a fructose feed of 10 g/d influenced such chronic changes in serum creatinine and increased urine excretion. Oxidative stress helps to accelerate pathological conditions in diabetes [22-24] and promotes the rate of protein glycation [25, 26]. So, we attempted to study the effect of probiotics and glycation inhibitor AMG on ROS formation, antioxidant status and tissue fluorescence. The oxidative stress is also found to increase in the tissues such as the liver and kidney and is found to be reduced on treatment. The extent of protein glycation has been evaluated through study of general and specific fluorescence. The increase in tissue fluorescence indicates the extent of glycation. The general and specific fluorescence of liver and kidney increased significantly in fructose fed animals. Hyperglycemia causes glucose auto-oxidation, protein glycation, protein kinase C activation and lipid peroxidation which further results in generation of reactive oxygen species [27, 28]. Fructose feeding significantly increased rate of ROS generation in liver as compared to that of control animals. The glycated intestinal proteins presented an evidence for adverse effect of excess fructose feeding on healthy gut, promoting the prediabetic state.

CONCLUSION

In conclusion, this preliminary study throws light on the impact of high fructose feed on glycation of proteins and probable changes on such glycation. Fructose feed can greatly influence changes in the whole body in just 7 d implying that such repeated insults can eventually lead to establishment of diabetes. It is interesting to note that while aminoguanidine can inhibit protein glycation significantly, use of probiotics also demonstrate a similar effect points to the importance of gut microbiota in promoting positive changes. Thus the present study raises the possibility of prevention of establishment of diabetes and its progression through use of protein glycation inhibitors and prebiotics and probiotics.

ABBREVIATIONS

Oral glucose tolerance test-OGTT, aminoguanidine-AMG, super-oxide dismutase-SOD, catalase-CAT, cardio vascular diseases-CVD, advanced Glycation Products-AGEs, body weight-BW, prebiotic and probiotic combination-EUGI, high-density lipoprotein-HDL, very low-density lipoprotein-VLDL, ethylene diamine tetra acetate-EDTA, nitro blue tetrazolium-NBT, reactive oxygen species-ROS, 2', 7'-dichlorofluorescin diacetate-DCF-DA, 2', 7'-dichlorofluorescein-DCF, arbitrary units-AU, standard deviation-SD.

AUTHORS CONTRIBUTIONS

RSP and AUA have designed the research work, RSP, ADJ, MLN and LNB have performed the experiments. All authors equally contributed to drafting the paper. All authors have read and approved the final manuscript.

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CONFLICT OF INTERESTS

All authors have none to declare

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