Non-enzymatic glycosylation reaction contributes to a rise of blood glucose in alloxan-induced diabetic rats

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Abstract
Non-enzymatic glycosylation reaction, which proceeded at an accelerated rate in diabetes, directly caused sharp diminution of total haemoglobin due to glycosylated proteins including haemoglobin digested by macrophages. The diminution contributed to hypoxia of tissue that repressed the enzymatic activities in the respiratory chain as well as in the tri-carboxylic acid cycle (TCA) and Embden-Meyerhof-Parnas (EMP) pathways. It was postulated that non-enzymatic glycosylation reaction accelerated the rise of blood glucose. The theory was further proven by the hypoglycaemic activities of the extract from *Tremella aurantialba* broth (TBE). TBE inhibited the formation of advanced glycosylation end-products (AGEs) *in vitro* (IC_{50} =1.7 mg/ml) and *in vivo*. TBE, when given *in vitro*, increased the concentration of total haemoglobin and supply of oxygen, enhanced respiration of tissue, decreased the levels of NADH, speeded up catabolism of glucose and finally generated significant anti-hyperglycaemic and hypoglycaemic effect on alloxan-induced diabetic rats. In addition it elevated plasma insulin level. Oral administration of TBE (100 mg/Kg bw) once a day for 4 weeks resulted in significant reduction in the plasma levels of glucose, fructosamine and the ratio of glycosylated haemoglobin to total haemoglobin. Moreover, oral administration of TBE increased the total haemoglobin and plasma insulin levels and enhanced some key enzymatic activities of EMP, TCA pathways and respiratory chain in the blood of diabetic rats compared with control. (Int J Diabetes Metab 15: 52-59, 2007)

Key words: *non-enzymatic glycosylation reaction, glycosylated haemoglobin, total haemoglobin, respiration chain, EMP pathway, TCA pathway, Tremella aurantialba*

Introduction

According to the projections of the World Health Organisation the prevalence of diabetes is likely to increase by 35%.[1] Currently there are over 150 million diabetics worldwide and this is likely to increase to 300 million or more by the year 2025.[1,2] Therefore, it is necessary to look for new targets of therapy and new resources to manage this health problem. Diabetes mellitus (DM) is a chronic disease caused by inherited and/or acquired deficiency in the production and/or the effectiveness of the insulin produced. Such a deficiency results in elevated blood glucose levels which cause an accelerated development of diabetic complications such as retinopathy, nephropathy, neuropathy and cardiovascular diseases. Diabetic complications usually arise as a result of non-enzymatic protein glycation, which leads to the formation of heterogeneous, toxic and antigenic advanced glycation end products (AGEs). Hence, numerous studies have focused on the analysis of the implicated glycated proteins or the AGE-modified circulating proteins, their physiology, and various plants and animal products that inhibit non-enzymatic protein glycation. Very little is known about the increase in blood glucose resulting from non-enzymatic glycosylation reaction. An understanding of this process could help in the development and the therapy of diabetic mellitus and its complications.

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Higher Basidiomycetes mushrooms are unlimited sources of anticancer and immunostimulating polysaccharides.[3,4] *Tremella aurantialba* is used for food pharmaceutical product. The polysaccharides from its body and mycelia have many medical functions including relieving cough and reducing phlegm, decreasing blood glucose, curing cardiovascular and cerebral diseases, enhancing haemopoietic function and increasing body's immunity.[5-10] Previous studies have shown that the polysaccharides from mycelia possess hypocholesterolaemic and antihyperglycaemic activities, cure diabetes and its complications as well as control blood fat and blood pressure.[7,8]

The aim of this study was to examine Hb (O_2 carrier) and the enzyme activities of the respiratory chain and catabolic pathways of glucose in normal and diabetic rat. This study was also set to expound the hypothesis that non-enzymatic glycosylation reaction can contribute to the rise of blood glucose in diabetic rats. Finally, the hypothesis was tested with the anti-hyperglycaemic and hypoglycaemic effect of the extract of *T. aurantialba* broth (TBE), an *in vitro* and *in vivo* non-enzymatic glycosylation reaction inhibitor.

Materials and Methods

Preparation of crude extract of Tremella aurantialba broth (TBE)

Submerged incubation and fermentation: A strain of *T. aurantialba* JSU-05, kindly donated by Prof. Weikjing Qu, East China Normal University was used in this study. The stock culture was maintained on potato dextrose agar (PDA) slants and subcultured for six months. Slants were incubated

at 27 °C for 7 days and then stored at 4 °C. *T. aurantialba* UJS-05 was initially grown at 25 °C for 7 days on solid seed medium containing 15 g bran and 20 ml water and then transferred into the seed culture medium. During the culturing process the flask was shaken once per day from the third to the fifth day.

5 g of solid culture mentioned above was added to 100 ml seed medium containing 2% sucrose, 1 % corn powder and 0.5 % peptone. The seed culture experiments were performed at 27°C with a rotary shaker (Shanghai Pharmaceutical Industrial Academe, China) at 150 rpm for 2 days. The fermentation medium was inoculated with 10% (v/v) of the seed culture and then cultivated at 27 °C in 250 ml flasks containing 100 ml of mushroom complete medium containing 4 % sucrose, 0.4 % peptone, 1 % corn powder, 1 % bran, 0.15 % KH_2PO_4, and 0.075 % $MgSO_4$.

Preparation of crude extract: The fermented broth was adjusted to pH 2.5 with HCl and then centrifuged at 5000 rpm. The supernatant (3000 ml) was applied to the AB-8 macropores resin column (26×300 mm) balanced with 70 % ethanol dipping, 2 % NaOH solution and 500 ml and 2 % HCl solution. After the sorption process, stepwise elution was conducted with a discontinuous gradient of water, 20 %, 40 %, 60 %, 80 % and 100 % ethanol solution at 3 ml/min. The extraction of *T. aurantialba* broth (TBE) was obtained by collection and lyophilization of the 40 % EtOH fraction. The TBE sample was stored at 4 °C until analyzed.

Preparation of the polysaccharides from T. aurantialba mycelia (TMP)

Polyaccharides from *T. aurantialba* mycelia was prepared according to the hot water extraction method.[7,8] Mycelia, obtained by filtration of culture broths for isolation of water-soluble endo-polysaccharide were washed 3 times with distilled water and then dried at 80 °C to constant weight. Dry mycelia (200 mesh) were degreased with ethanol at 80 °C for 12 h in a water bath (mycelium/ethanol ratio: 1:10). The residues collected by centrifugation (4500×g for 15 min) and dried at 80 °C were extracted three times with distilled water for 4 h in a boiling water-bath (residue/distilled water ratio: 1:10), and then centrifuged at 4500×g for 15 min. Supernatants were collected and concentrated to 50 ml under reduced pressure by a rotary evaporator. The concentrated solution was deproteinated by mixing with the same volume of Sevage reagent, chloroform: n-butanol (4:1, v/v), then spinned for 5 min and centrifuged at 3000×g for 10 min, all for eight times. The obtained solution was precipitated with four volumes of 95% alcohol solution (v/v) and the precipitate was washed with alcohol, acetone and ether, respectively, each twice. This crude solution of *T. aurantialba* polysaccharide was named TMP.

Analyses of contents of protein, reducing sugar and polysaccharides in TBE and TMP

The level of polysaccharides was equal to gross sugar content minus reducing sugar content. The carbohydrate contents of the TBE and TMP were determined spectrophotometrically at 500 nm using the anthrone sulfate method.[11] The reducing sugar contents were determined as 3.5-dinitro-salicylic acid method [12] and the protein concentration was determined by Biuret reaction.[13]

Effect of TBE and TMP on non-enzymatic glycosylation of proteins in vitro

The inhibitory effect of TBE and TMP on non-enzymatic glycosylation proteins *in vitro* was estimated according to the method described by Wu *et al*.[14] Triplicate samples were measured and mean value was given. An inhibition ratio was calculated according to the following equation:

$$I = \frac{X - Y}{X} \times 100\%$$

Where I is inhibition ratio; X the intensity of fluorescence of blank and Y the intensity of fluorescence of sample.

Induction of diabetes in rats

Male Sprague Dawley rats of body weight between 180-200 g were purchase from the Central Animal House, Chinese Academy of Sciences (Nanjing, China). The animals were housed in steel cages at 22 ± 2 °C and light/dark (12/12 h) controlled room. All the rats were fed a standard palletized chow diet and water was freely available. Diabetes was induced by intraperitoneal injection of alloxan at a dose of 160 mg/kg body weight. The alloxan solution was freshly prepared, kept on ice and injected immediately. Ten rats were selected for the non-diabetic group and only injected with the citrate buffer. Five days later, blood glucose was measured in tail vein blood with the glucose assay kit (Jiancheng Biological Insititute, Nanjing, Jiangsu). The rats were confirmed diabetic when their blood glucose levels exceeded 13 mmol/L. Diabetic rats were randomly divided into four groups. Group 5 consisted of normal control rats.

Group 1 (10 rats): Alloxan-treated diabetic rats administered (i.p.) equivalent volume distilled water as control.

Group 2 (10 rats): Alloxan-treated diabetic rats which were supplemented with TBE (i.p.) at dose of 100mg/Kg body weight.

Group 3 (10 rats): Alloxan-treated diabetic rats supplied with TMP (i.p.) at dose of 100mg/Kg body weight.

Group 4 (10 rats): Alloxan-treated diabetic rats treated with metformin (i.p.) at dose of 100 mg Kg^{-1} body weight) in distilled water.

Group 5 (10 rats): Normal untreated rats, they were given distilled water.

The blood glucose levels were checked periodically for 28 days to confirm diabetes. After 28 days of treatment, all rats were fasted for 6-8 h and blood was collected into potassium oxalate and sodium fluoride tubes for analysis of the blood glucose, haemoglobin, glycosylated haemoglobin and fructosamine. Plasma was separated for determining the level of insulin. Liver and kidney tissues were excised immediately and were stored at -70 °C until required. Procedures involving animals and their care were conducted

in conformity with the institutional guidelines of School of Jiangsu Medicine University (Jiangsu, China).

Analytical methods

Determination of blood glucose, insulin, fructosamine, Hb and GHb in the serum of diabetic rats

Blood glucose concentration was estimated by using a colorimetric assay kit (Rongsheng-biotech, Shanghai, China) according to the manufacturer's protocol. Plasma insulin was assayed using an enzyme-linked immunosorbent assay (ELISA) kit (Boehringer Mannheim, Germany). Hb was estimated using the cyanmethemoglobin method described by Drabkin and Austin.[15] GHb was estimated according to the method of Sudhakar and Pattabiraman[16] with modifications according to Bannon et al.[17] Fructosamine level was determined according the method described by Xu et al.[18]

Determination of hexokinase activities and pyruvate dehydrogenase in liver and kidney of diabetic rats

The whole liver tissue was homogenized in 0.1mol/l Tris-HCl buffer (pH 7.4). The particle-free homogenate was used for the following analysis: protein was estimated by the method of Lowry *et al*,[19] using bovine serum albumin as the standard. Hexokinase activity was measured with respect to the amount of glucose utilized after the addition of ATP[20] and pyruvate dehydrogenase were assayed with the method described by Seymour and John.[21]

Determination of enzyme activities related with electron transport chain and tri-carboxylic acid cycle pathway in liver and kidney's mitochondria

Mitochondria were isolated from fresh liver and kidney tissues.[22] The purity of the mitochondria was assessed by estimating the activity of isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, succinate dehydrogenase, malate dehydrogenase, NADH-dehydrogenase, and cytochrome-C-oxidase. These enzymatic activities were used for the following analysis: The activity of isocitrate dehydrogenase was assayed by the method of King.[23] α-ketoglutarate activity was assayed according to the colorimetric determination of ferrocyanide produced by the decarboxylation of α-ketoglutarate with ferricyanide as electron acceptor,[24] and succinate dehydrogenase activity was assayed by the method of Slater and Bonner,[25] in which the rate of reduction of potassium ferricyanide was measured in the presence of potassium cyanide. The activity of malate dehydrogenase was estimated by the method of Mehler *et al.*[26] The method of Minakami *et al*[27] was followed for the determination of reduced nicotinamide adenine dinucleotide (NADH) - dehydrogenase activity. The activity of cytochrome-C-oxidase was assayed by the method of Warton and Tzagoloff.[28] All the readings were recorded using UV-2100 spectrophotometer.

Statistical analysis

The data for various biochemical parameters were analyzed using analysis of variance (ANOVA) and the group means were compared by Duncan's Multiple Range Test (DMRT). Results were expressed as mean ±S.D. from ≥ ten rats in each group. P-values < 0.05 were considered significant.

Results and Discussion

Anti-hyperglycermic and hypoglycermic effect of TBE, TMP and metformin.

TBE and TMP (the polysaccharides from *T. aurantialba* mycelia) contained neither protein nor reduced sugar. But 16.7% non-reduced sugar existed in TBE and 47.6% non-reduced sugar was observed in TMP (data not shown). The result proved that the main compositions of TBE were not similar to those of TMP. Table 1 demonstrated the effect of treatment with TBE, TMP and metformin on glucose levels for 4 weeks. In contrast with the normal group, administration (ip) of alloxan (160 mg/Kg body weight) led to 4.5-fold elevation of fasting blood glucose levels, which was maintained over a period of 4 weeks. Comparing with the control group, the blood glucose levels of experimental groups were significantly decreased (p<0.01) after the administration of drugs from the first week onwards. Although the decrease in glucose was maximum (41.4%) by the end of the second week in the group receiving TBE, no significant difference was observed between experimental groups (p>0.05).

Fig 1 showed a significant difference in the way that TBE, TMP and metformin facilitated secretion of insulin. In contrast with normal rat, the level of blood insulin in diabetic rat fell after the administration of alloxan. After treatment with 3 drugs, the level of insulin increased. However, effect of TBE on secretion of insulin was weaker than that of TMP and metformin (p<0.001).

Inhibitory effect of TBE, TMP and metformin on non-enzymatic glycosylation reaction

The TBE has shown to inhibit activities on the non-enzyme glycosylation reaction of protein i*n vitro* (Fig 2), which might produce the advanced glycosylation end-products (AGEs) implicated in the increase of cross-linking of collagen *in vivo*, thickening of basement membranes and loss of vascular compliance in diabetes[29] and atherosclerosis.[30] The concentration needed for 50% inhibition of AGEs (IC_{50}) was 1.7 mg/ml *in vitro*. However, the TMP did not show any inhibitory activity when the

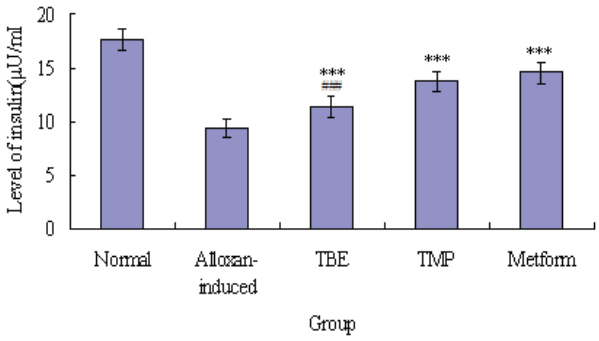

Figure 1: Effect of TBE, TMP and metformin on the secretion of insulin. Data shown are means ± SE representative. In contrast with the group with alloxan-treated: * P <0.05, ** P <0.01, *** P <0.001; In contrast with the group with TMP: $^{\#}P$ <0.05, $^{\#\#}P$ <0.01, $^{\#\#\#}P$ <0.001

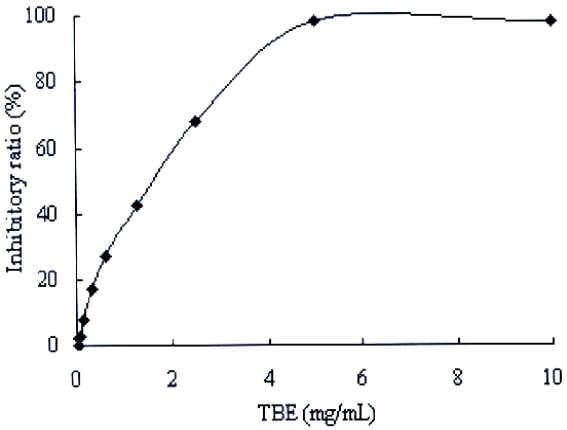

Figure 2: Effect of different concentration TBE on non-enzymatic glycosylation reaction in vitro

concentration of TMP reached 100 mg/ml. The inhibitory ratio of TBE used in subsequent tests was 76.8% at the concentration of 5 mg/ml.

Fructosamines are stable complexes of carbohydrates and proteins that are produced by an irreversible, non-enzymatic glycosylation of protein.[31] Fructosamine was an index of glycosylation reaction *in vivo*. Table 2 presented the levels of fructosamine, total Hb and GHb in the plasma of normal and experimental rats. There was a significant reduction in total Hb (37.4%) ($p < 0.001$). However, fructosamine and GHb levels rose significantly in diabetic rats compared to control. According to Qing *et al*,[32] Watala *et al*,[33] Ashokkumar and Leelavinothan[34] and Pari and Amarnath,[35] total Hb was significantly decreased and the level of GHb was significant elevated in diabetic patients and alloxan-induced diabetic rats. The administration of TBE brought the level of total Hb, fructosamine and GHb more towards near normal than the administration of TMP and metformin (Table 2). This can be explained by the fact that TBE was stronger in inhibition of non-enzymatic glycosylation than TMP and metformin *in vivo*.

Inhibitory effect of TBE, TMP and metformin on some key enzymes with metabolism of glucose and respiratory chain

Table 3 depicted the activities of hexokinase and pyruvate dehydrogenase in the liver and kidney of normal and experimental rats. A significant decrease of these enzymatic activities was observed in liver and kidney of diabetic rats ($P<0.05$). This is comparable with the findings of Pari and Amarnath,[35] Ngozi and Cynthia[36] and John and Seymour.[37] Oral administration of all tested drugs could reverse the decreased levels (Table 3). Similar results were observed on the effect of metformin on carbohydrate metabolic enzymes in neonatal streptozotocin diabetic rats[38] and on the effect of a polysaccharide (TAP) from the fruiting bodies of *Tremella aurantia* on glucose metabolism in rat liver.[9]

Table 4 shows the activities of some enzymes in liver and kidney related with TCA pathway, such as isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, succinate dehydrogenase and malate dehydrogenase in normal and experimental rats. A highly significant decline ($P < 0.001$) in the activities of mitochondrial enzymes was observed in diabetic rats in contrast with normal. Panneerselvam and Govindaswamy[39] and Brignone *et al*[40] obtained similar result in diabetic rats. After therapy with experimental drugs, these enzymatic activities were significantly enhanced compared with diabetic rats. Experimental drugs could reverse the above changes in a significant manner when compared to diabetic rats (Table 4). Especially, these enzymatic activities in TBE group were more near to normal value. Table 5 shows the activities of NADH-dehydrogenase and Cytochrome-C-oxidase, implicated in the electron transport chain in normal and experimental rats. These enzymatic activities were significantly depressed in diabetic rats in contrast with normal rats ($P < 0.01$). Kristal et al obtained a similar result in streptozotocin-induced diabetic rats.[41]

All experimental drugs significantly ($P<0.01$) reduced the difference and reversion of TBE was strongest among the experimental drugs (Table 5).

Non-enzymatic glycosylation reaction accelerates rise of blood sugar in alloxan-induced diabetic rats

Non-enzymatic glycosylation is the deleterious binding of sugars to protein that is commonly observed in diabetes and aging.[42,43] The definition of glycosylation comprises a series of reactions, such as the binding of the acyclic form of sugar to specific amino acids in protein to form a Schiff base, rearrangement of the Schiff base to form an Amadori product (so-called early products), and crosslinking and subsequent degradation of proteins to form advanced glycosylation end-products.[42,43] In diabetes, the non-enzymatic glycosylation reaction has been stepped up due to high concentration of glucose in the blood (Table 2). Some glycosylated proteins are digested by macrophages, lysosome-containing cells or extracellular proteolytic systems.[44,45] Similarly, non-enzymatic glycosylation reaction occurs in the protein in plasma containing Hb. After glycosylation, Hb is digested by macrophages and its derived cells, and total Hb levels decreased significantly (Table 2). Hb carries O_2 from lung and releases CO_2 to lung. The decrease in total Hb level induces tissue hypoxia. Simultaneously the GHb may impair Hb's functioning as an oxygen carrier.[46,47] In addition, glycosylation reaction increases the cross-linking of protein in plasma and blood viscosity. Moreover, sediments of degradation product from glycosylated proteins thicken basement membranes and reduce the lumina of blood vessel and decreases blood flow.

Hypoxia inhibits enzymes participating in the electron transport chain (respiratory chain) such as NADH-dehydrogenase and Cytochrome-C-oxidase (Table 5). Since NADH is the rate limiting factor of isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, succinate dehydrogenase, hexokinase and pyruvate dehydrogenase,[47] the accumulation of NADH leads to feedback repression on the activities of these enzymes, which further repress the tri-carboxylic acid (TCA) and the Embden-Meyerhof-Parnas (EMP) pathways. The TCA and EMP cycles are closely related to glucose metabolism (Table 3, 4), and finally

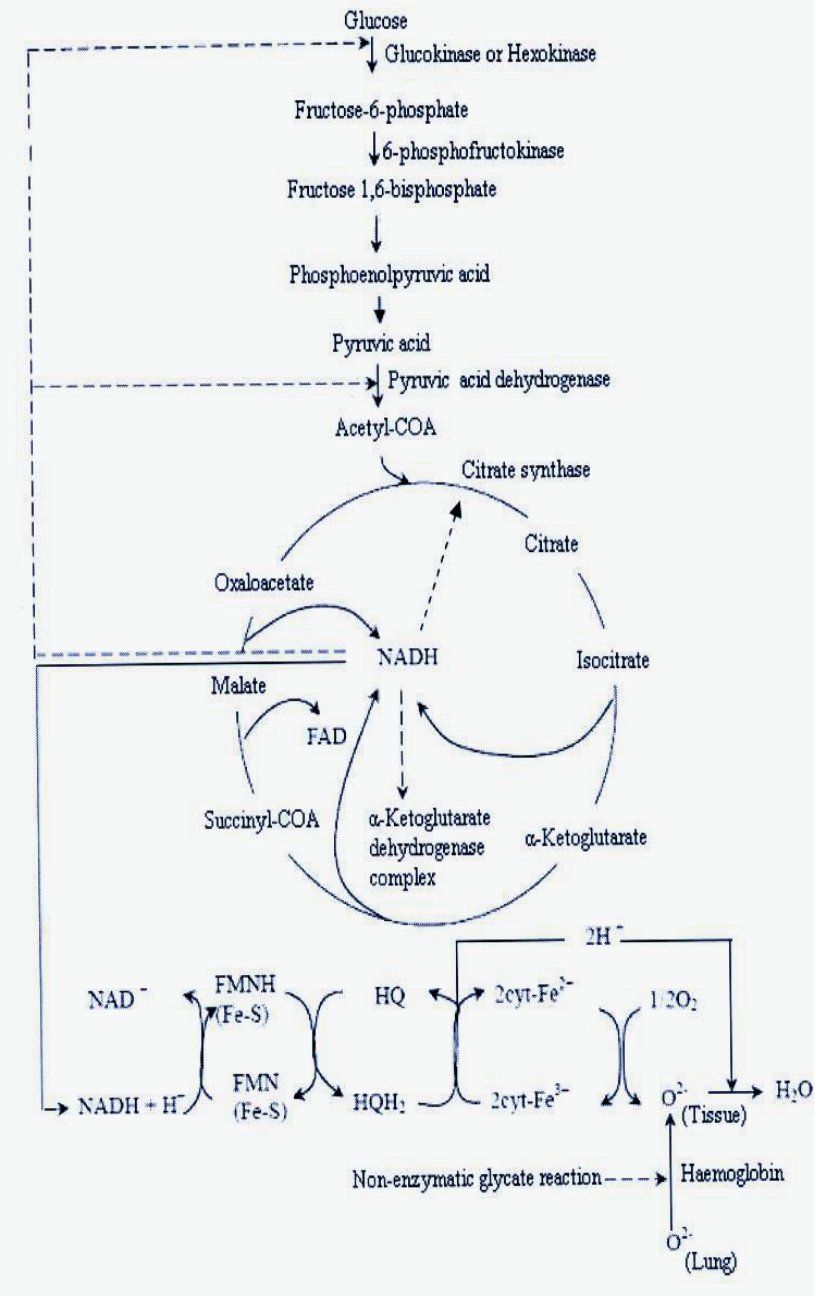

Figure 3: Effect of non-enzymatic glycated reaction on metabolism of carbohydrate. Solid line: metabolic route; dash line: feedback repression

Table1: Effect of TBE on blood glucose in normal and diabetic rats

Group	Blood glucose (mmol/ l)				
	Initial	7 days	14 days	21 days	28 days
Normal	5.871±0.68	5.83±0.75	5.62±0.48	6.02±0.67	6.11±0.54
Alloxan-induced	22.34±6.24	23.71±6.11	24.63±7.02	25.79±5.12	27.05±3.84
TBE	22.58±5.63	13.71±6.22***	13.24±6.75**	14.83±8.76**	15.48±8.52**
TMP	22.76±5.60	15.80±5.21**	16.89±5.34*	18.21±3.82**	20.29±3.09***
Metformin	22.68±5.29	15.49±7.03**	15.87±6.87**	16.38±7.01**	17.88±6.89**

Data shown are means ± SE. TBE, TMP and metformin reduced blood glucose level significantly compared to alloxan-induced diabetic rats: *P <0.05, **P <0.01, *** P <0.001

Table 2: Change in the levels of fructosamine, Hb, glycosylated haemoglobin in normal and experimental rats

Group	Fructosamine (mmol/l)	Total haemoglobin (g/dl)	Glycosylated haemoglobin (%)
Normal	2.221±0.214	14.75±1.40	3.22±0.17
Alloxan-induced	3.427±0.973	9.24±0.56	8.23±0.57
TBE	2.315±0.314**	14.69±0.81***$^{###}$	3.21±0.48***$^{###}$
TMP	2.707±0.291*	10.72±0.67***	6.87±0.36***
Metformin	3.062±0.365	12.56±0.65***$^{###}$	5.22±0.66***$^{###}$

Data shown are means ± SE. TBE, TMP and metformin reduced Hb and glycosylated haemoglobin level significantly compared to alloxan-induced diabetic rats. Fructosamine level was significantly lower in TBE and TMP-treated rats compared to alloxan-induced diabetic rats. * P <0.05, ** P<0.01, *** P <0.001In contrast with the group with TMP: $^{\#}$ P <0.05, $^{\#\#}$ P <0.01, $^{\#\#\#}$ P <0.001

Table 3: Hexokinase and pyruvate dehydrogenase activities in liver and kidney of normal and experimental rats

Group	Hexokinase		Pyruvate dehydrogenase	
	Liver	Kidney	Liver	Kidney
Normal	163.30±10.21	117.62±9.87	99.87±11.25	74.35±7.38
Alloxan-induced	87.83±9.43	57.05±9.34	67.54±10.24	35.12±6.24
TBE	158.16±12.34***$^{###}$	97.34±8.26***$^{##}$	90.25±9.71***$^{##}$	69.85±9.80***$^{###}$
TMP	137.15±8.76***	86.51±7.54***	78.26±8.30***	48.24±10.06**
Metformin	146.34±10.67***	89.43±9.17***	83.72±13.01***	53.12±5.13***

Data shown are means ± SE (n = 10 per group). Hexokinase (µmol of glucose phosphorylated/min/g protein); Pyruvate dehydrogenase (µmol of NADH /min/mg protein). TBE and TMP caused significant increases in hexokinase and pyruvate dehydrogenase activities compared to control. * P <0.05, ** P <0.01, *** P <0.001. In contrast with the group with TMP: # P <0.05, ## P <0.01, ### P <0.001

Table 4: Activities of enzymes related to the TCA pathway in liver and kidney of normal and experimental rats

Parameters		Normal	Alloxan-induced	TBE	TMP	Metformin
Isocitrate dehydrogenase	Liver	796.3±64.6	534.2±56.3	776.5±58.6***$^{###}$	598.3±67.2	601.3±56.9*
	Kidney	695.1±57.8	512.3±58.7	643.2±60.3***	603.2±59.9**	576.5±56.1*
α-ketoglutarate dehydrogenase	Liver	172.3±16.8	102.3±11.8	168.3±15.7***$^{###}$	132.5±14.8**	143.9±13.9***
	Kidney	57.2±6.45	32.1±3.48	51.2±5.73***$^{###}$	38.7±6.01**	37.2±4.91*
Succinate dehydrogenase	Liver	25.6±3.1	13.5±1.29	21.3±2.12***$^{###}$	16.2±1.87**	15.8±2.19*
	Kidney	13.4±0.96	8.6±0.76	12.8±0.87***$^{###}$	9.7±1.03**	9.1±0.64
Malate dehydrogenase	Liver	378.4±41.1	257.6±21.2	356.2±35.6***$^{##}$	297.3±33.7**	263.2±43.5
	Kidney	273.4±24.6	207.6±23.6	254.6±26.3***$^{##}$	223.1±21.9	205.4±24.7

Isocitrate dehydrogenase (nmoles of α-ketoglutarate liberated/h/mg protein); α-ketoglutarate dehydrogenase (µmol of potassium ferrocyanide liberated/h/mg protein); succinate dehydrogenase (µmol of succinate/min/mg protein) and malate dehydrogenase (µmol of NADH /min/mg protein) activities in TCA pathway of liver and kidney cells. TBE and TMP caused significant changes in enzyme activities compared to control. * P <0.05, ** P <0.01, *** P <0.001; # P <0.05, ## P <0.01, ### P <0.001 (TBE compared to TMP). Data shown are means ± SE (n = 10 per group).

Table 5: Activities of enzymes related to the respiratory chain in liver and kidney of normal and experimental rats

Group	NADH-dehydrogenase		Cytochrome-C-oxidase	
	Liver	Kidney	Liver	Kidney
Normal	28.6±2.74	20.7±3.21	6.92±0.64	6.45±0.67
Alloxan- induced	15.3±1.82	13.2±1.32	3.26±0.43	3.43±0.31
TBE	27.5±2.22***$^{###}$	19.7±1.91***$^{###}$	6.54±0.57***$^{###}$	6.23±0.59***$^{###}$
TMP	19.3±2.83**	14.9±1.83*	4.23±0.49***	4.16±0.43**
Metformin	18.7±2.09	17.3±3.09	3.89±0.61	4.27±0.37

NADH-dehydrogenase (µmol of NADH oxidized/min/mg protein) and cytochrome-C-oxidase (O.D×10min/mg protein) activities in the liver and kidney of normal and diabetic rats. TBE compared to alloxan-treated: * P <0.05, ** P <0.01, *** P <0.001； TBE compared to TMP: # P <0.05, ## P <0.01, ### P <0.001. Data shown are means ± SE.

causes the rise of blood glucose (Table 2). Although levels of NADH and oxygen tension of some tissues have not being determined due to limitation of tested condition, it has been reported that oxygen consumption of some tissues are significantly lower in diabetic patients.[40,49,50] Noda *et al.* created a model by applying 0.4 g/ml ethidium bromide

(EtBr) to the murine pancreatic β-cell line βHC9 and proved that reduced expression of the mitochondrial electron transport system causes NADH accumulation in β-cells, thereby impeding the TCA cycle and facilitates anaerobic glucose metabolism.[51]

TBE, TMP and metformin could facilitate pancreas to secrete insulin, but the insulin level of blood in the group treated with TBE was significant lower than that of other experimental groups. Taking into account that lower blood glucose usually required higher level of insulin, TBE might decrease blood glucose of diabetic rats by other routes coupled with facilitating secretion of insulin. In fact, TBE has been shown to inhibit non-enzymatic glycosylation reaction *in vitro*. Similarly, TBE inhibits non-enzymatic glycosylation reaction *in vivo*, to boost the level of total Hb in blood, increase supply of O_2 to tissue, reverse the respiratory effect to near normal status, decrease the NADH levels, relieve the repression of NADH on these enzymes of TCA pathway and EMP pathway near to normal levels (Table 3 and Table 4), enhance the metabolism of glucose and decrease the blood glucose of diabetic rats (Table 1).

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