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Research paper

Antihyperglycemic effect of fraxetin on hepatic key enzymes of carbohydrate metabolism in streptozotocin-induced diabetic rats

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ABSTRACT

Epidemiological studies have demonstrated that the diabetes mellitus is a serious health burden for both governments and healthcare providers. The present study was hypothesized to evaluate the antihyperglycemic potential of fraxetin by determining the activities of key enzymes of carbohydrate metabolism in streptozotocin (STZ) – induced diabetic rats. Diabetes was induced in male albino Wistar rats by intraperitoneal administration of STZ (40 mg/kg b.w). Fraxetin was administered to diabetic rats intra gastrically at 20, 40, 80 mg/kg b.w for 30 days. The dose 80 mg/kg b.w, significantly reduced the levels of blood glucose and glycosylated hemoglobin (HbA1c) and increased plasma insulin level. The altered activities of the key enzymes of carbohydrate metabolism such as glucokinase, glucose-6-phosphate dehydrogenase, glucose-6-phosphatase, fructose-1,6-bisphosphatase and hepatic enzymes (aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP)) in the liver tissues of diabetic rats were significantly reverted to near normal levels by the administration of fraxetin. Further, fraxetin administration to diabetic rats improved body weight and hepatic glycogen content demonstrated its antihyperglycemic potential. The present findings suggest that fraxetin may be useful in the treatment of diabetes even though clinical studies to evaluate this possibility may be warranted.

1. Introduction

Diabetes mellitus is a disease caused due to abnormality in carbohydrate metabolism and it is mainly linked with low blood insulin level or insensitivity of target organs to insulin. Impaired postprandial insulin secretion because of functional defects and the loss of surviving pancreatic β-cells leads to a subsequent decline in insulin sensitivity [1]. Defects in carbohydrate metabolic machinery and consistent efforts of the physiological system to correct the metabolic imbalance pose an over exertion on the endocrine system leading to the disruption of endocrine control. Deterioration of endocrine control exacerbates the metabolic disturbances by altering carbohydrate metabolic enzymes which leads to hyperglycemia [2].

Globally prevalence of diabetes mellitus is an upward trend. The International Diabetes Foundation estimates that 366 million adults aged 20–79 were affected by diabetes worldwide in 2011 and this figure is expected to rise 552 million by the year 2030 [3] with most of the increase coming from developing countries. India is home to the largest number of people with diabetes in the world, 40.9 million diabetic cases in 2007, and these numbers are predicted to 69.9 million by the year 2025 [4]. Liver is one of the chief storage organ for glucose reserve in the body and plays a crucial role in blood glucose homeostasis, because it consents to amass the superfluous blood glucose and to demobilize it in hypoglycemic states. It has been reported that the diabetic condition decrease the activities of enzymes in the glycolytic and pentose phosphate pathways, while increasing the activities of gluconeogenic and glycogenolytic pathways [5]. Liver cells have been used as an *in vitro* model for evaluating or screening for antihyperglycemic effect of plant or it constituents [6].

The modern antidiabetic synthetic drug in use for long-term therapy has been associated with various toxicities owing to which the developmental process in antidiabetic drug discovery has shifted its focus toward natural plant sources which are having minimal or no side effects [7]. Some natural products have the ability to lower blood glucose and it should be safer than allopathic drugs if utilization over a prolonged period. Among the natural products, coumarin compounds are attracting much interest because of their beneficial effects on diabetes and its late complications such as retinopathy [8,9]. They are found in many plants and natural food products, such as citrus fruits, tomatoes, vegetables and green tea. Fraxetin (7,8-dihydroxy-6-methoxy coumarin), a coumarin derivative, has been reported to possess antioxidative,

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anti-inflammatory, antiviral, antitumor and neuroprotective effects [10–13]. It is extracted from Chinese herbs, *Cortex Fraxini*, the chloroform fraction yields 11.1% of fraxetin [14].

The present study investigates the role of fraxetin on glucose utilization pathways and on hepatic glucose production since both of them contributes significantly to plasma glucose levels. For this, the activities of key enzymes of carbohydrate metabolism are measured in streptozotocin (STZ)-induced diabetic rats.

2. Materials and methods

2.1. Chemicals

Fraxetin and STZ were purchased from Sigma Chemical Co (St. Louis, Mo. USA). All other chemicals and solvents were of analytical grade and purchased from Himedia Laboratories Pvt. Ltd, Mumbai, India.

2.2. Animals

Male albino Wistar strain rats, weighing about 180–220 g were procured from Central Animal House, Department of Experimental Medicine, Rajah Muthiah Medical College and Hospital, Annamalai University. Rats were housed in clean, sterile, polypropylene cages under standard vivarium conditions (12 h light/dark cycle) with free access to standard chow (Hindustan Lever Ltd., Bangalore, India) and water. The animals were acclimatized to the laboratory condition for 2-week prior to the start of experiment. The experimental protocol was designed in accordance with the ethical norms approved by Ministry of Social Justices and Empowerment, Government of India and Institutional Animal Ethics Committee Guidelines (Reg. No. 856/2012).

2.3. Induction of diabetes

Diabetes was induced in overnight fasted experimental rats by a single intraperitoneal injection of STZ (40 mg/kg b.w) dissolved in freshly prepared citrate buffer (0.1 M, pH 4.5) [15]. STZ injected animals were allowed to drink 20% glucose solution overnight to overcome the initial drug-induced hypoglycemic mortality. Control rats were injected with same volume of citrate buffer alone. After 96 h, plasma glucose was determined and those rats with fasting blood glucose greater than 250 mg/dl were used in the present study.

2.4. Experimental design

The animals were randomly divided into six groups of six animals in each group (24 diabetic surviving and 12 normal). Previous finding in our laboratory, esculetin, a coumarin derivative like fraxetin shows antidiabetic effect at a dose of 40 mg/kg b.w [16]. Based on this report, fraxetin 20 mg, 40 mg and 80 mg doses were selected for dose fixation study. Fraxetin dissolved in vehicle solution of 1.0% dimethylsulfoxide (DMSO) and administered to experimental rats.

Group I	Normal control (vehicle treated)
Group II	Normal rats received fraxetin (80 mg/kg b.w) dissolved in 1 ml of 1.0% DMSO intra gastrically for 30 days
Group III	Diabetic control
Group IV	Diabetic rats received fraxetin (20 mg/kg b.w) dissolved in 1 ml of 1.0% DMSO intra gastrically for 30 days
Group V	Diabetic rats received fraxetin (40 mg/kg b.w) dissolved in 1 ml of 1.0% DMSO intra gastrically for 30 days
Group VI	Diabetic rats received fraxetin (80 mg/kg b.w) dissolved in 1 ml of 1.0% DMSO intra gastrically for 30 days

The initial and final body weight of the rats in each group was recorded. At the end of the experimental period, the animals were fasted overnight, anesthetized using ketamine hydrochloride (24 mg/kg b.w, intramuscular injection), and sacrificed by cervical decapitation. Blood samples collected in dry test tubes were allowed to coagulate at ambient temperature for 30 min centrifugation at 2000× *g* for 10 min used for the estimation of serum ALT, AST and ALP. Blood samples were collected in tubes containing potassium oxalate and sodium fluoride (3:1) mixture used for the estimation of plasma glucose and insulin. Hemoglobin (Hb) and glycosylated hemoglobin (HbA1c) levels were estimated in whole blood samples. Liver was immediately dissected, washed in ice-cold saline to remove the blood.

2.5. Biochemical analysis

Plasma glucose was estimated using a commercial kit (Sigma Diagnostics Pvt. Ltd., Baroda, India) by the method of Trinder [17]. Plasma insulin was assayed by ELISA kit (Boeheringer-Manneheim Kit, Manneheim, Germany). Hemoglobin and glycosylated hemoglobin were estimated by diagnostic kit (Agappe Diagnostic Pvt. Ltd., India) [18]. The AST, ALT and ALP were assayed using commercially available diagnostic kits (Sigma diagnostics (I) Pvt. Ltd., Baroda, India). A portion of the liver tissue was dissected out, washed with ice-cold saline and homogenized in 0.1 M Tris–HCl buffer (pH 7.4) for the assay of key enzymes of carbohydrate metabolism. The homogenate was centrifuged at 2000 g for 5 min or to remove the debris and the supernatant was used as source for the evaluation of enzymes of carbohydrate metabolism. The estimation of protein was carried out by the method of Lowry et al. [19].

Glucokinase was assayed by the method of Brandstrup et al. [20]. The reaction mixture in a total volume of 5.3 ml contained the following: 1 ml of glucose (0.005 M) solution, 0.5 ml of adinosine triphosphate (0.072 M) solution, 0.1 ml of magnesium chloride (0.05 M) solution, 0.4 ml of potassium dihydrogen phosphate (0.0125 M), 0.4 ml of potassium chloride (0.1 M), 0.4 ml of sodium fluoride (0.5 M) and 2.5 ml of Tris–HCl buffer (0.01 M, pH 8.0). The mixture was pre-incubated at 37 °C for 5 min. The reaction was initiated by the addition of 2 ml of tissue homogenate. 1 ml of the reaction mixture was immediately transferred to the tubes containing 1 ml of 10% trichloroethanoic acid that was considered as zero time. A second aliquot was removed and deproteinised after 30 min incubation at 37 °C. The protein precipitate was removed by centrifugation and the residual glucose in the supernatant was estimated by the method of Trinder [17].

Glucose 6-phosphate dehydrogenase was measured by the method of Ells and Kirkman [21]. The incubation mixture contained 1 ml of Tris–HCl buffer (0.05 M, pH 7.5), 0.1 ml of magnesium chloride, 0.1 ml of $NADP^+$, 0.5 ml of phenazine methosulphate, 0.4 ml of 2,6-dichlorophenol indo phenol dye solution and 0.5 ml of liver homogenate. The contents were incubated at 37 °C for 10 min. The reaction was initiated by the addition of 0.5 ml of glucose 6-phosphate. The absorbance was read spectrophotometrically at 640 nm against water blank at 1 min intervals for 3–5 min.

Glucose-6-phosphatase was assayed by the method of Koide and Oda [22]. Incubation mixture contained 0.7 ml of citrate buffer (0.1 M, pH 6.5), 0.3 ml of substrate (0.01 M) and 0.3 ml of tissue homogenate. The reaction mixture was incubated at 37 °C for 1 h. Addition of 1 ml of 10% trichloroethanoic acid to the reaction tubes terminated the reaction of the enzyme. The suspension was centrifuged and the phosphorus content of the supernatant was estimated by the method of Fiske and Subbarow [23]. The supernatant was made up to known volume. To this, 1 ml of ammonium molybdate was added followed by 0.4 ml of amino naphthol

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