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MODULATORY EFFECTS OF ANDROGRAPHOLIDE ON ATTENUATING THE KEY ENZYMES ACTIVITIES OF CARBOHYDRATE METABOLISM AND GLYCOGEN CONTENT IN STREPTOZOTOCIN INDUCED DIABETIC RATS

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ABSTRACT

Objectives: *Andrographis paniculata* and its active compound andrographolide are ancient herbal medicine recommended by the World Health Organization. In this study, the effect of andrographolide on changes in carbohydrate metabolic enzymes and glycogen content in muscle and kidneys of streptozotocin-induced diabetic rats were evaluated. ***Methods:*** Diabetes was induced into male albino Wistar rats by intraperitoneal administration of streptozotocin (STZ). The andrographolide at different doses (15,30and 60 mg/kg b.w) was administered orally to normal and STZ-diabetic rats for 45 days. ***Results:*** STZ intoxication leads to a significant increase ($P < 0.05$) in blood glucose and a decrease in insulin levels. The carbohydrate metabolic enzymes and glycogen content were also altered. The daily oral administration of andrographolide at different doses (15,30and 60 mg/kg b.w) to diabetic rats for 45 days; resulted a significant ($p < 0.05$) decline in blood glucose level and a significant increase in plasma insulin level. The altered activities of carbohydrate metabolic key enzymes in muscle and kidneys of diabetic rats were significantly ($p < 0.05$) reverted to near normal level by the administration of andrographolide. The obtained results were compared with glibenclamide, a standard oral hypoglycemic drug. ***Conclusions:*** Thus, the modulatory effects of andrographolide on attenuating these enzymes activities afford a promise for

persistent use for the treatment of diabetes in the future even though clinical studies to evaluate this possibility may be warranted.

KEYWORDS: Diabetes; andrographolide; *Andrographis paniculata*; Streptozotocin.

INTRODUCTION

Diabetes mellitus (DM) is a heterogeneous syndrome characterized by hyperglycemia resulting from impaired insulin secretion, action or both,[1] it is the most significant chronic disease and the cause of death in modern society.[2] According to the International Diabetes Federation (IDF), the global prevalence of diabetes is predicted to grow from 366 million in 2011 to 552 million by 2030.[3] Therefore, the human population worldwide appears to be in the midst of an epidemic of diabetes. The occurrence of diabetes in urbanized countries is rapidly increasing and this pandemic disease is one of the most persistent causes of death in developed countries. According to recent studies in India, the number of diabetic are 77 million and these numbers are projected to increase to 101 million by the year 2030 and the most prevalence will be among the urban population.[4]

Diabetes mellitus (DM) is defined as a state in which homeostasis of carbohydrate and lipid metabolism is improperly regulated by insulin.[5] Defects in carbohydrate metabolizing machinery and consistent efforts of the physiological systems to correct the imbalance in carbohydrate metabolism place an overexertion on the endocrine system, which leads to the deterioration of endocrine control. Continuing deterioration of endocrine control exacerbates the metabolic disturbances and leads primarily to hyperglycemia. For the maintenance of normoglycemic status, there must be a coordinated regulation of several metabolic pathways including gluconeogensis and glycolysis.[6] This presents a moving therapeutic target that requires different agents to address the different features of the disease.[7]

Diabetes mellitus and its associated complications are still an important medical and social problem in spite of the use of many oral hypoglycemic agents such as sulphonylureas and biguanides. Though, insulin and oral hypoglycemic agents are available to manage diabetes, none has been shown to modify the course of diabetic complications.[8] Since the synthetic drugs have detrimental side effects or contraindications, the World Health Organization (WHO) has recommended the assessment of traditional plant or phytochemicals for the management of DM. Thus there is an imperative need for novel therapeutic agents that can overcome the demerits of existing therapeutic modalities.[9,10]

Medicinal plants and its constituents continue to form a common platform for the discovery of new chemical entities in the modern drug discovery program. Numerous medicinal plants and their formulations are used for controlling hyperglycemia in traditional system of medicine.[11] *Andrographis paniculata* is a widely grown plant in India, Egypt, and Middle East countries. Its applications in the ayurvedic system of medicine are well documented and have been shown to possess a hypocholesterolemic effects in rats and dogs.[12,13] The extract of the seeds is also useful for the treatment of diabetes in Ayurvedic (Indian), Unani (Arabic) and Chinese system of medicine.[14] Andrographolide, a naturally occurring an alkaloid found abundantly in *Andrographis paniculata*. There are also reports showing that andrographolide-rich food source such as *Andrographis paniculata* exhibit insulin-like antihyperglycemic effect in the STZ-induced diabetic rats[15, 16] and also reverses the changes in the levels of glucose metabolism in liver.[17] There are no reports pertaining to the effect of andrographolide on carbohydrate metabolic enzymes in tissues other than the liver of diabetic animals. In the view of these facts, the present study was designed to evaluate the effects of andrographolide on carbohydrate metabolic enzymes in serum, muscle and kidneys of STZ-induced diabetic rats.

MATERIALS AND METHODS

Animals

Male Wistar rats of body weight 150-180g were obtained from National Center for Laboratory Animal Sciences (NCLAS), National Institute of Nutrition (NIN), India. The animals were maintained in the Department of Zoology, Osmania University, Hyderabad, India and fed on standard pellet diet and water *adlibitum*. The protocol of this study was approved by Institutional ethical committee of Osmania University, Hyderabad, India.

Chemicals

Andrographolide was procured from Sigma-Aldrich. Streptozotocin was purchased from Himedia, Bangalore, India. All other chemicals used were of analytical grade.

Induction of Diabetes mellitus

Diabetes mellitus was induced in overnight fasted adult Wistar strain albino male rats weighing 150–180g by a single intraperitoneal injection of 35 mg/kg streptozotocin (STZ). Streptozotocin was dissolved in citrate buffer (pH 4.5). Hyperglycemia was confirmed by the elevated glucose levels (above 250 mg/dl) in plasma determined at 72 hrs and then on day 7 after injection.

Experimental design

After the successful induction of experimental diabetes, the rats were divided into six groups each comprising a minimum of six rats.

Group 1: Control rats.

Group 2: Diabetes mellitus control rats.

Group 3: Diabetes mellitus rats administered with andrographolide (1.5 mg/ kg b.w/rat) in vehicle solution orally for 45 days using an intragastric tube.

Group 4: Diabetes mellitus rats administered with andrographolide (3.0 mg/kg b.w/rat) in vehicle solution orally for 45 days using an intragastric tube.

Group 5: Diabetes mellitus rats administered with andrographolide (6.0 mg/kg b.w/rat) in vehicle solution orally for 45 days using an intragastric tube.

Group 6: Diabetic rats administered with glibenclamide (600 µg/ kg b.w/rat) in aqueous solution orally for 45 days using an intragastric tube.

These doses of andrographolide were determined from a preliminary dose-response study evaluating the effects of 30 mg, 60 mg and 120 mg doses on fasting serum glucose in diabetic rats.

Body weight and blood glucose level measurements were conducted periodically. At the end of the experimental period, the rats were fasted overnight, anaesthetized and sacrificed by cervical decapitation. The blood was collected with or without EDTA for plasma or serum separation respectively.

Biochemical estimations

Blood glucose estimation

Blood glucose was estimated by Sasaki et al.[18] 10 µl of plasma (or) serum was added to 1.0 ml of working enzyme reagent, mixed well and incubated at 37°C for 15 min. The colour developed was read at 505 nm against blank containing distilled water instead of the sample. A standard was also processed similarly. The level of glucose is expressed as mg dl^{-1}.

Assayof insulin

A plasma level of insulin was determined using kits from Bio-Merieux, RCS, Lyon, France. 25 µl of the plasma was dispensed in microwells coated with anti-insulin antibody. To this, 100 µl of the enzyme conjugate was dispensed into each well, mixed for 5 sec and incubated at 25°C for 30 min. The wells were rinsed five times with washing buffer. Then, 100 µl of

solution A and then 100 µl of solution B were dispensed into each well. This was incubated for 15 min at room temperature. The reaction was stopped by adding 50 µl of 2 N HCl to each well and read at 450 nm. The values are expressed as µU ml^{-1}.

Tissue homogenate preparation

Kidney and muscle tissues (250 mg) were sliced into pieces and homogenised with 0.1 mol/L Tris-HCl buffer, pH 7.4, in cold condition to give 20% homogenate (w/v). The homogenate were centrifuged at 3000 rpm for 10 min at 0 °C in cold centrifuge. The supernatant was separated and used for various biochemical estimations.

Assay of hexokinase

Tissue hexokinase was assayed by the method of Lapeir and Rodnick.[19] To 1 ml of glucose add 0.5 ml of adenosine triphosphate (ATP), 0.1 ml of magnesium chloride, 0.4 ml of potassium dihydrogen phosphate, 0.4 ml of potassium chloride, 0.4 ml of sodium fluoride and 2.5 ml of Tris-HCl buffer and this 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% TCA 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.

Assay of glucose -6- phosphatase

G-6-Pase was measured by the method of Koide and Oda.[20] To 0.7 ml of citrate buffer (0.1 mol/l, pH 6.5) add 0.3 ml of substrate (0.01 mol/l) and 0.3 ml of tissue homogenate. The reaction mixture was incubated at 37°C for 1 h. The reaction of the enzyme was arrested by adding 1 ml of 10% TCA. The suspension was centrifuged. The supernatant was made up to a known volume. To this, 1 ml of ammonium molybdate was added followed by 0.4 ml of amino naphthol sulphonic acid (ANSA). The blue colour developed after 20 min was read at 680 nm.

Assay of Fructose-1,6-bisphosphatase

Fructose-1,6-bisphosphatase activity was measured by Gancedo and Gancedo.[21] The assay mixture in a final volume of 2 ml contained 1.2 ml of Tris-HCl buffer (0.1 mol/l, pH 7.0), 0.1 ml of substrate, 0.25 ml of magnesium chloride, 0.1 ml of potassium chloride solution, 0.25 ml of ethylene diamine tetra acetic acid (EDTA) solution and 0.1 ml of enzyme homogenate.

The incubation was carried out at 37°C for 5 min. The reaction of the enzyme was arrested by the addition of 10% TCA. The suspension was centrifuged and the supernatant was made up to a known volume. To this, 1 ml of ammonium molybdate was added followed by 0.4 ml of ANSA. The blue colour developed after 20 min was read at 680 nm.

Estimation of glycogen

Tissue glycogen was estimated by the method of Ong and Khoo.[22] The alkali extract of the tissue was prepared by digesting 50 mg of fresh tissue with 3 ml of 30% potassium hydroxide solution in boiling water bath for 15 min. The tubes were cooled and mixed with 5 ml of absolute alcohol. A drop of 1 mol/l ammonium acetate was added to precipitate glycogen and left in the freezer overnight for complete precipitation. Glycogen was collected by centrifugation at 2000 × *g* for 20 min. The precipitate was dissolved in distilled water with the aid of heating and again the glycogen was re-precipitated with alcohol and ammonium acetate and then centrifuged. The final precipitate was dissolved in saturated ammonium chloride solution and 4 ml of anthrone reagent was added by cooling the tubes in an ice bath. The tubes were shaken well, covered with marble caps and heated in a boiling water bath for 20 min. After cooling, the absorbance was read at 640 nm against reagent blank treated in a similar manner.

Statistical analysis

All the results were expressed as the Mean ± S.D.for six animals in each group. All the grouped data were statistically evaluated with SPSS\16.0 software. Hypothesis testing methods included one way analysis of variance (ANOVA) followed by least significant difference (LSD) test; significance level at $p< 0.05, 0.01, 0.001$ were considered to indicate statistical significance.

RESULTS

Fig. 1 presented the levels of plasma glucose and insulin in control and treated animals. The levels of plasma glucose were significantly ($p < 0.05$) increased whereas plasma insulin levels were significantly ($p < 0.05$) decreased in diabetic control rats. By supplementing with andrographolide (all doses), a significant ($p < 0.05$) decrease in plasma glucose level and significant ($p < 0.05$) increase in insulin levels were observed by the end of the experimental period. Andrographlide at a dose of 6.0 mg/kg b.w showed a highly significant effect than 3.0 and 1.5 mg/kg b.w. Based on these data, the effective dose was fixed at 6.0 mg/kg b.w and used for further analysis.

Fig. 2 showed the oral glucose tolerance test of the control and treated animals. An elevated blood glucose level (maximum value at 60 min) in the control rats was observed after glucose load and declined to near basal levels at 120 min, whereas, in STZ induced diabetic rats, the peak increase in blood glucose level was observed even after 60 min and remained high over the next 60 min. Supplementation with andrographolide as well as glibenclamide to diabetic rats elicited a significant decrease in blood glucose level at 60 min when compared with untreated diabetic rats.

Table 1 summarized the levels of hexokinase and fructose1-6-bis phosphatase in the control and experimental groups of rats. A significant ($P < 0.001$) decrease in hexokinase level and concomitant increase in fructose1-6- bis phosphatase level was observed in serum, muscle and kidneys of STZ-diabetic rats and it was normalized after the treatment with andrographolide and glibenclamide.

The activity of glucose 6-phosphatase in serum and kidney is significantly ($P < 0.001$) increased in diabetic rats. Oral administration of andrographolide and glibenclamide to diabetic rats for 45 days, significantly ($P < 0.001$) decreased the activity of glucose 6-phosphatase in serum and kidneys of experimental diabetic animals (Table 2).

The concentration of glycogen in kidney and muscle in the experimental rats was showed in Table 3. A reduced glycogen level in muscle and concomitant increased level in kidneys were found in the diabetic state. Diabetic rats treated with andrographolide significantly ($P < 0.05$) brought back the contents of glycogen in the tissues to near normal.

Table-1: Effect of andrographolide on hexokinase and fructose-1.6-bis phosphatase in control and experimental rats.

Groups	Hexokinase			Fructose-1,6-bis phosphataase		
	Serum	Muscle	Kidney	Serum	Muscle	Kidney
Normal Control	18.4±1.4	0.67±0.07	0.31±0.001	46.9±2.1	22.1±2.1	31.6±1.5
STZ-Diabetic Control	9.2±2.1 *ŧ	0.31±0.01 *ŧ	0.22±0.002 *ŧ	69.2±4.3 *ŧ	36.5±2.12 *ŧ	61.2±3.4 *ŧ
STZ-Diabetic +glibenciamide 600µg/kg b.w	14.5±0.91 ŧŧ	0.59±0.02 ŧŧ	0.32±0.004 ŧŧ	40.1±2.1 ŧŧ	26.3±2.03 ŧŧ	41.2±2.1 ŧŧ
STZ-Diabetic + Andrographolide 6.0 mg/kg b.w	17.1±1.1 ŧŧ	0.57±0.02 ŧŧ	0.35±0.001 ŧŧ	39.2±3.3 ŧŧ	24.3±1.91 ŧŧ	36.7±3.3 ŧŧ

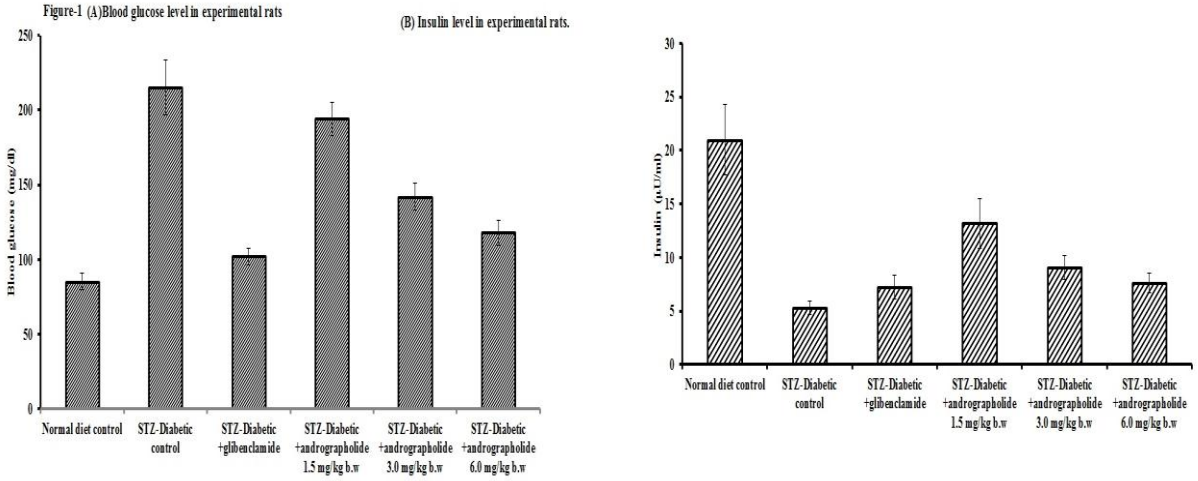
Values are mean±SD, n=6. Hexokinase in µmol glucose phosphorylated per minute per mg protein; Fructose 1,6-bis phosphatase in µmol Pi liberated per minute per mg protein. ŧŧ
***significantly different from control, ŧP<0.001, ŧsignificantly different from diaabetic control**

Table-2: Effect of andrographolide on glucose 6-phosphatase in control and experimental rats.

Groups	Glucose 6-Phosphatase	
	Serum	kidney
Normal Control	89.4±1.4	10.6±1.97
STZ-Diabetic Control	166.2±6.1 *†	31.4±3.21 *†
STZ-Diabetic +glibenciamide 600µg/kg b.w	109.5±8.91 ††	18.5±6.14 ††
STZ-Diabetic + Andrographolide 6.0 mg/kg b.w	97.1±4.1 ††	15.7±9.02 ††

Values are mean±SD, n=6. Glucose 6-phosphatase in µmol Pi liberated per minute per mg protein. †Ŧ

***significantly different from control, †P<0.001, Ŧsignificantly different from diaabetic control.**



DISCUSSION

Streptozotocin- induced hyperglycemia in animals is considered to be a good model for the preliminary screening of agents active against diabetes and is widely used.[23] It reliably produces many of the signs and symptoms of chronic human diabetes.[24] Streptozotocin is a potent DNA methylating agent and acts as a nitric oxide donor in pancreatic cells. ß-cells are particularly susceptible to damage by STZ, a cytotoxic agent.[25] As a result, the expression and secretion of insulin is declined notably there by leading to hyperglycemia, a clinical hallmark of diabetes. From the results obtained, it is evident that diabetic rats had much higher glucose level than of control rats. Some substances have shown antidiabetic effect by influencing ß -cell to stimulate insulin secretion and restore insulin sensitivity.[26] The present

data indicated that andrographolide significantly decreased blood glucose levels in treated diabetic rats as compared with control diabetic rats. This may be due to the insulin release effect of andrographolide on peripheral tissues, either by promoting glucose uptake and metabolism, or by inhibiting hepatic gluconeogenesis.[27]

Hexokinase, one of the key enzyme and also the first regulatory enzyme of glycolytic pathway in the catabolism of glucose, converts glucose into glucose-6-phosphate.[28] The activity of hexokinase which brings about the first phosphorylation step of glucose metabolism was found to be significantly reduced in the serum, muscle and kidneys of diabetic rats. A relative deficiency of insulin caused a decrease in the activity of this enzyme in experimental diabetes. Lowered activity of hexokinase can result in decreased glycolysis and thus decreased utilization of glucose for energy production.[29] Oral administration of andrographolide to STZ diabetic rats increased the activity of hexokinase and this may be due to increased uptake of glucose and ultimately increased glycolysis.

Glucose-6-phosphatase, an important enzyme in the homeostasis of blood glucose, plays a key role in the regulation of blood glucose levels by catalyzing the hydrolysis of glucose-6-phosphate in the common terminal step of the gluconeogenic and glycogenolytic pathways.[30] This enzyme is present in liver and kidney and this is an important regulatory enzyme involved in the release of glucose from the liver and kidneys.[31] In the present study, the activity of glucose-6- phosphatase significantly increased in serum and kidneys. This may be due to the action of streptozotocin that has been shown to increase the expression of glucose-6-phosphatase mRNA, which contributes to the increased activity of this enzyme in diabetes mellitus.[32] The increased activity of this enzyme lowered the concentration of glucose 6-phosphate and inhibited glycogen synthesis which in turn caused elevated levels of blood glucose.[33] Streptozotocin-diabetic rats when administered with andrographolide positively modulated the activities of this enzyme and this might probably due to an increase in the levels of insulin.

Fructose 1,6-bisphosphatase is one of the key enzyme of the gluconeogenic pathway, catalyze the rate limiting step of fructose-1,6-bisphosphate to fructose-6-phosphate and this step is necessary to achieve a reversal of glycolysis.[34] It is present in liver, muscle and kidneys.[35] This enzyme catalyzes one of the irreversible steps in gluconeogenesis and serves as a site for the regulation of these process.[36] The activities of fructose1-6-bisphosphatase are increased in the serum, muscle and kidneys of diabetic animals. This is due to the deficiency of insulin

that leads to the state of hyperglycemia.[37] andrographolide administered orally to STZ-induced diabetic rats significantly reduced the activities of fructose 1,6-bisphosphatase. This might be due to the modulatory effect of andrographolide against the activities of this enzyme, either through regulation by cyclic AMP or by metabolic activation or inhibition of glycolysis and gluconeogenesis. This is in line with previous report.[17]

Glycogen, a branched polymer and primary intracellular storable form of glucose residues synthesized by the enzyme glycogen synthase. Its quantity in various tissues is a direct manifestation of insulin activity as insulin supports intracellular glycogen deposition by stimulating glycogen synthase and inhibiting glycogen phosphorylase.[38] Glycogen deposition from glucose is impaired in diabetic animals[39] in proportion to the severity of insulin deficiency.[40] Streptozotocin-induced diabetic animals tends to show decreased glycogen content in liver and increased glycogen content in kidneys.[41] The decrease of glycogen in liver and muscle could be due to a state of insulin deficiency as they depend on insulin for influx of glucose.[42] In contrast, kidney glycogen content is increased in the present study and this is due to the entry of glucose in a hyperglycemic state as renal tissue is independent of insulin action.[43] This has been postulated to cause increased intra-renal glycogen deposition which leads to glycosylation of basement membrane collagen in the kidney.[44] A rise in renal glycogen content has been reported previously by other researchers.[41] Treatment with andrographolide could positively alter the glycogen content in the diabetic tissues by increasing insulin[17] and consequently decreasing blood glucose levels.

In conclusion, andrographolide possesses antidiabetic activity by stimulating the insulin production from the existing ß -cells of pancreas. Increased insulin secretion after treatment with andrographolide positively altered the deranged carbohydrate metabolism in the diabetic rats by decreasing gluconeogenesis and increasing glycolysis, ultimately decreasing hyperglycemia.

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Declaration of conflicting interests

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