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Original Article

EFFECT OF *CENTELLA ASIATICA* (LINN) LEAVES ON SELECTIVE MITOCHONDRIAL AND CYTOSOLIC ENZYMES IN STREPTOZOTOCIN INDUCED DIABETIC RATS

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ABSTRACT

Objective: The objective of the study is to evaluate the therapeutic effect of *Centella asiatica* leaves by assaying the activities of selective mitochondrial and cytosolic enzymes in the streptozotocin induced diabetic rats.

Methods: The rats were divided into five groups of 6 rats in each group. The methanol extract of *Centella asiatica* leaves (CALEt) was orally administered at a dose of 300 mg/kg body weight (b. w). The activities of both mitochondrial and cytosolic enzymes *viz* succinate dehydrogenase (SDH), malate dehydrogenase (MDH), glutamate dehydrogenase (GDH), lactate dehydrogenase (LDH) and glucose 6 phosphate dehydrogenase (G6PDH) were measured in the liver and kidney of the experimental rats.

Results: Diabetic rats showed a significant ($p<0.001$) reduction in the activities of mitochondrial enzymes such as SDH, MDH and GDH. Furthermore, the cytosolic enzyme G6PDH activity was significantly ($p<0.001$) decreased along with a significant increase in the LDH activity in diabetic rats. The daily oral treatment of CALEt to diabetic rats for 30 d reversed the above changes in a significant ($p<0.001$) manner.

Conclusion: The results obtained in this study indicated that methanol extract of *Centella asiatica* leaves could restore the altered activities of mitochondrial and cytosolic enzymes, thereby suggesting its position in the energy production. Our data suggest that *Centella asiatica* leaves should be further explored for its role in the treatment of diabetes mellitus.

Keywords: *Centella asiatica*, Streptozotocin, Diabetes mellitus, Mitochondrial enzymes, Cytosolic enzymes.

INTRODUCTION

Diabetes mellitus is a well-known chronic disease caused by hereditary or acquired deficiency in the production of insulin by the pancreas or by the unutilization of the insulin produced and such deficiency results in increased concentrations of glucose in the blood, that damages many of the body's systems, particularly the blood vessels and the nerves. It is a leading cause of death and disability worldwide; its universal prevalence was about 8% in 2011 and was predicted to increase to 10% by 2030 [1, 2]. Nearly 80% of people with diabetes live in low-and middle-income countries [3].

Mitochondria play a part in regulation of diverse physiological functions by providing energy for the majority of intracellular processes necessary for vital functions. Mitochondria are one of the key cell organelle in diabetes research because of its central role as a regulator of energy balance [4]. Magnetic resonance spectroscopy studies on human suggest that more subtle defects in mitochondrial function might take part in the pathogenesis of insulin resistance in diabetes, which is the most widespread metabolic disease in the world [5].

Mitochondrial function is usually required for normal glucose stimulated insulin release from pancreatic β cells. Albertoni *et al.* [6] reported that hyper glycemic condition had been connected with oxidative stress due to the altered mitochondrial metabolism. Reduced mitochondrial capacity in patients with type 2 diabetes was previously reported by Toledo *et al.* [7]. Recently, it has been reported that mitochondrial damage associated with β cell dysfunction is suspected to contribute to the development of diabetes [8]. TCA cycle plays an important role in diabetes mellitus because the activity of Kreb's cycle enzymes acts as indicators of glucose utilization. Glucose-6-phosphate dehydrogenase (G6PDH) is present in the cytosol and is a key enzyme of a pentose phosphate pathway that provides energy to a number of cells (notably erythrocyte) and maintains the NADPH (Nicotinamide adenine dinucleotide phosphate) level [9]. It is important in preventing the complications of diabetes mellitus [10]. There is a massive understanding among cytosolic and mitochondrial enzymes to sustain the complimentary conditions to regulate various biological functions.

Plants and herbs are mines of a huge number of bioactive phyto chemicals that might serve as lead for the expansion of effective, harmless and economical novel drugs [11]. Herbal formulation alone or in combination with oral hypoglycemic agents now and then produces good curative responses in some challenging cases where modern medicines alone have failed [12]. *Centella asiatica* is used in conventional and alternative treatment due to the broad range of pharmacological activities coupled with the secondary metabolites [13]. Traditionally, *Centella asiatica* was used for various diseases, including wound healing, bronchitis, asthma, diabetes, urethritis, liver complaints, allergy, cancer, diuretic, and hypertension also to improve mental ability [14]. Recently, we reported the anti-diabetic activity of *Centella asiatica* leaves in streptozotocin induced diabetic rats [15]. However, several aspects of the basic system of action of *Centella asiatica* still remain unclear up to now. This study was aimed to evaluate the effect of CALEt on SDH, MDH, GDH, G6PDH and LDH activities and also to find out whether the CALEt can control the physiological parameters and stabilize the altered Cori and TCA cycle in streptozotocin induced diabetic rats.

MATERIALS AND METHODS

Plant material

Fresh leaves of *Centella asiatica* were collected from Talakona forest, Chittoor District, Andhra Pradesh, India during November 2009. The taxonomic identification of *Centella asiatica* (L) plant was confirmed by a senior Botanist, Mr. Madhava Chetty, Department of Botany, S. V. University, and a voucher specimen was deposited in the S. V. University herbarium, Tirupati, Andhra Pradesh, India.

Animals

Wistar strain male albino rats, aged 3 mo and weighing 160±20 g, were purchased from the authorized dealer (M/S Raghavendra Enterprises, Bangalore, India). The rats were housed in hygienic polypropylene cages having 6 rats per cage and maintained under temperature controlled room (25±2 °C) with a photo-period of 12 h lights and 12 h dark cycle, humidity 50 ± 10%. The rats were fed with

water and a standard rat pellet diet ad libitum (purchased from Sai Durga Agencies, Bangalore, India). The experiments were carried out according to the guidelines of the Committee for the Purpose of Control and Supervision on Experiments on Animals, Government of India (CPCSEA, 2003) and approved by the Institutional Animal Ethical Committee at Sri Venkateswara University, Tirupati, India (Resolution No. 10/(i)/a/CPCSEA/IACE/SVU/PSR-MRA/10.06. 2010).

Chemicals

All the chemicals used in the present study were Analytical Grade (AR) and were obtained from Sigma (St. Louis, MO, USA), Fisher (Pitrsburg, PA, USA), BDH Chemicals (England), Merck (Mumbai, India), Ranbaxy (New Delhi, India), and SISCO Research Laboratories (India).

Plant extract preparation

Fresh leaves of *Centella asiatica* were washed thoroughly, shade dried for 3 w and milled into the fine powder using a mechanical grinder. The powdered material (2 kg) was soaked in 6 L methanol for 48 h and filtered. The extracted material was filtered through whatman number 1(0.45 μm Ref. HAWP04700, Bedford, MA, USA) filter paper. The above filtrate was collected and evaporated in a rotary evaporator (Rotavapor, R-210, Buchi Labortechnik, AG, Flawil, Switzerland) at 40-50 °C under reduced pressure. The yield (9.86 % w/w) was dried in vaccum desiccator and greenish material obtained was stored in the refrigerator at 4 °C until use.

Induction of experimental diabetes mellitus

The animals were fasted overnight, and diabetes was induced by a single intra-peritoneal injection of a freshly prepared streptozotocin (STZ) solution (50 mg/kg b. w [16, 17] dissolved in 0.1 M cold citrate buffer, P^H 4.5). After injection, rats had free access to food and water. The rats were allowed to drink 15% glucose solution for 72 h to counter hypoglycemic shock. The rats were considered as diabetic if their blood-glucose levels were more than 250 mg/dl on day 3 after STZ injection. The blood glucose levels were measured by using one touch-glucometer (Johnson & Johnson). After diabetes confirmation, rats were allowed for 7 d to acclimatize to diabetic condition, and rats with hyperglycemia (blood-glucose>250 mg/dl) were chosen for the study. Treatment was started on day 8 after STZ injection, which was also considered as the first day of treatment and continued further until end of the study period.

Experimental design

Grouping of animals

Rats of the same age group (3 mo old) were divided into 5 groups, six rats in each group:

Group I (Normal controls)-This group of rats received distilled water for equivalent handling.

Group II (Normal rats treated with CALEt)-This group of rats received CALEt daily for a period of 30 d at a dose of 300 mg/kg b.w.

Group III (Diabetic untreated rats)-In this group, diabetic rats are untreated for a period of 30 d, which served as diabetic controls.

Group IV (Diabetic rats treated with CALEt)-After the diabetic confirmation test, this group of rats received CALEt for a period of 30 d, at a dose of 300 mg/kg b.w.

Group V (Diabetic rats treated with Glibenclamide)-In this group, diabetic rats were treated with the standard anti-diabetic drug, glibenclamide 5 mg/kg b. w [18] for the same period as in the group IV. This group was maintained for better comparison of the protective effect of *Centella asiatica* against diabetic induced complications.

The dose of 300 mg/kg was selected based on the results of our previous study [15] where we found that 300 mg/kg b. w dose is the effective dose with perceptible useful effect or maximum dose beyond which no additional beneficial effect is seen. After completion of 30 d of treatment, the animals were sacrificed by cervical dislocation then the liver and kidney tissues were excised at 4^{o}C. The tissues were cleaned with ice-cold saline and immersed in liquid nitrogen. The tissues were stored immediately at-80^{o}C for further biochemical analysis.

Biochemical analysis

Activities of selected mitochondrial enzymes, including SDH and MDH were assayed by the modified version of Nachlas *et al.* [19]. The GDH activity was assayed by the method of Lee and Lardy [20]. Cytosolic enzymes G6PDH activity was assayed by the method of Bergmayer, and Bruns [21] and LDH activity was assayed by the method of Prameelamma and Swami [22].

Statistical analysis

The mean, standard deviation (SD) and probability test (Analysis of variance-ANOVA) were carried out according to Steel and Torrie [23] using BASIC programming techniques on SPSS PC for different parameters. The p-value of more than 0.05 was considered as not significant.

RESULTS

Table 1 and table 2 illustrate the effect of CALEt on the activities of SDH, MDH GDH, LDH and G6PDH in the liver and kidney of control and experimental rats. The mitochondrial enzyme (SDH, MDH GDH) activities were significantly (P<0.001) decreased in the diabetic control rats compared to the normal control rats. There were no significant changes in the activity of these enzymes in control rats treated with CALEt alone. Oral administration of CALEt to the diabetic rats significantly increased the enzyme activities compared with that of the diabetic untreated (control) rats. The enzymes in CALEt treated diabetic rats were similar with that of glibenclamide-induced augmentation.

Table 1: Effect of CALEt on liver cytosolic & mitochondrial enzyme activities in the control and experimental group of rats after 30 d treatment

Parameter/groups	Group I	Group II	Group III	Group IV	Group V
G-6-PDH(µ moles of formazan formed/mg protein/h)	5.24±0.14^a	5.62±0.07^b	2.75±0.11^c	4.82±0.16^d	4.46±0.07^e
LDH(µ moles of formazan formed/mg protein/h)	3.68±0.06^a	3.42±0.09^b	5.12±0.08^c	4.21±0.13^d	4.27±0.10^d
SDH(µ moles of formazan formed/mg protein/h)	8.95±0.09^a	9.22±0.09^b	5.11±0.07^c	8.15±0.11^d	7.35±0.14^e
MDH(µ moles of formazan formed/mg protein/h)	7.75±0.09^a	8.22±0.07^b	4.11±0.05^c	7.45±0.11^d	6.95±0.12^e
GDH(µ moles of formazan formed/mg protein/h)	8.37±0.15^a	9.12±0.08^b	5.77±0.08^c	7.64±0.07^d	7.19±0.05^e

Values are given as mean±SD of six individuals; Mean values that do not share same superscript differ significantly from each other at P<0.05

Table 2: Effect of CALEt on kidney cytosolic & mitochondrial enzyme activities in the control and experimental group of rats after 30 d treatment

Parameter/groups	Group I	Group II	Group III	Group IV	Group V
G-6-PDH(µ moles of formazan formed/mg protein/h)	3.71±0.17^a	3.96±0.05^b	2.43±0.13^c	3.35±0.11^d	3.22±0.09^d
LDH(µ moles of formazan formed/mg protein/h)	2.6±0.10^a	2.4±0.12^b	4.07±0.11^c	3.01±0.09^d	3.21±0.12^e
SDH(µ moles of formazan formed/mg protein/h)	6.69±0.03^a	6.95±0.1^b	4.3±0.15^c	6.36±0.07^d	5.87±0.07^e
MDH(µ moles of formazan formed/mg protein/h)	5.58±0.03^a	5.85±0.1^b	3.3±0.12^c	5.31±0.02^d	4.97±0.04^e
GDH(µ moles of formazan formed/mg protein/h)	6.64±0.11^a	6.97±0.13^b	4.79±0.12^c	6.07±0.17^d	5.77±0.11^e

Values are given as mean±S. D of six individuals; Mean values that do not share the same superscript differ significantly from each other at P<0.05

The cytosolic enzyme G6PDH activity was significantly ($p<0.001$) decreased along with the significant increase in the LDH activity in diabetic rats. Similar to the glibenclamide treatment, daily oral administration of CALEt to diabetic rats for 30 d reversed the altered G6PDH and LDH activities in a significant ($p<0.001$) manner. There were no significant changes in the activity of G6PDH and LDH in control rats treated with CALEt alone.

DISCUSSION

Streptozotocin (*N*-nitro derivative of glucosamine) is a naturally-occurring broad spectrum antibiotic and cytotoxic chemical that is principally toxic to the pancreatic β cells in mammals [24]. Induction of observational diabetes in the rats by streptozotocin is very convenient and easy to use [25]. Streptozotocin injection leads to the degeneration of β cells of islets of langerhans [26]. It has been reported that in the rats, a dose ranging from 25 to 100 mg/kg b. w STZ injected intravenously was doing well in inducing a dose-dependent hyperglycemia [24]. It is known that diabetic complications and increased oxidative stress are tissue-specific, moreover, accompanied via altered glucose transport, mitochondrial oxygen metabolism and energy production [27]. It was demonstrated that STZ inhibits the Krebs cycle and substantially decreases oxygen consumption by mitochondria [28, 29].

It is considerable evidence that diabetes is associated with tissue damage through oxidative stress [30], and that this extends to liver, skeletal muscle and heart tissues of STZ induced diabetic rodents [31, 32]. In the mitochondria of diabetic rats decreased oxygen utilization, and respiratory percentage was observed [33]. Similarly, decreased activities of citric acid cycle enzymes were observed by Sener *et al.* [34]. Recent metabolomic studies in models of type 1 diabetes reported down regulation of key TCA cycle and mitochondrial proteins [35], and also enzyme activities' [36].

In the current study the activities of the mitochondrial marker enzymes SDH and MDH levels were significantly ($P<0.001$) dropped in the liver and kidney of STZ induced diabetic rats. Decreased SDH activity in diabetic condition affects succinate-fumarate conversion, which indicates depressed oxidative metabolism in mitochondria. Malate dehydrogenase plays an important role in the citric acid cycle by providing oxaloacetate for the formation of citrate with acetyl-CoA and further generates malate, which can nourish the cytosolic gluconeogenic pathway [37]. The decreased levels of MDH enzyme in diabetic rats suggest decreased utilization of malate. Decreased levels of SDH and MDH enzymes in diabetic rats were previously reported in several studies [38, 39]. The noticeable finding in this study is that the decreased SDH and MDH activities were ameliorated by CALEt treatment in diabetic rats. Increased SDH and MDH activities in CALEt treated diabetic rats indicate better utilization of energy yielding intermediates by the TCA cycle. In this study, improved mitochondrial marker enzymes in CALEt treated diabetic rats were similar with that of glibenclamide-induced augmentation.

GDH is a mitochondrial matrix enzyme. It catalyzes the oxidative deamination of glutamate to α-ketoglutarate in a limited number of tissues in humans, including the liver, the kidney, the brain, and the pancreatic islets. In the pancreatic β cell, glucose stimulates insulin secretion by increasing the ATP:ADP ratio. An increased intracellular ATP and ADP ratio closes the ATP-sensitive potassium channel leading to the depolarization of cell membrane. On depolarization voltage gated calcium channels open, and increases the cytosolic calcium, which leads to release of insulin from stored granules. Amino acids stimulate insulin secretion through this pathway by increasing oxidation of glutamate through GDH because an essential amino acid abundant leucine is an allosteric activator of GDH enzyme activity [40]. In this study, the activity of GDH significantly decreased in the STZ induced diabetic rats. Ramudu *et al.* [41] and Rajeswar Reddy *et al.* [40] have reported the decreased GDH activity in the STZ induced diabetic rats. The decrease in GDH activity may be due to interference in energy metabolism, impairment of glutamate and activation of lipid peroxidation [42]. However, diabetic rats treated with CALEt for 30 d resulted in significant marked recovery of the GDH activity, which was almost similar with glibenclamide treatment. The increased activity might be due to synchronization of energy metabolism and elevation of glutamate in the cells by CALEt administration.

Lactose Dehydrogenase (LDH) is a cytosolic enzyme and present in a wide variety of organisms. Usually when oxygen is deficient or in short supply LDH converts pyruvate to lactate and in the liver during the Cori cycle, it performs the reverse reaction [43]. According to Ainscow *et al.* [44] over-expression of LDH activity may be directly responsible for an insulin secretory defect in some forms of diabetes. Increased LDH activity in diabetic rats has also been reported by various researchers [45, 46]. Similarly, in this study an increase in the activity of LDH was observed in the diabetic rats. During diabetic condition, extreme accumulation of pyruvate may result in higher LDH activity. Excessive pyruvate is converted into lactate for which LDH is needed; therefore, the activity of LDH may be increased due to less insulin availability in diabetes [47]. However, diabetic rats treated with CALEt for 30 d resulted in significant marked recovery of LDH activity. The confined lactate dehydrogenase activity by CALEt was parallel with the antidiabetic drug glibenclamide treatment.

The extra mitochondrial enzyme, G6PDH is a housekeeping enzyme that catalyzes the rate limiting step of the hexose monophosphate shunt (HMP). It converts glucose-6-phosphate to 6-phosphogluconate and generates the NADPH that is mandatory for the maintenance of reduced glutathione and reductive biosynthesis. G6PDH is the principle source of NADPH, which is of central importance to cellular redox regulation. The entire antioxidant system will be disturbed, if any change occurs in the G6PDH activity, which in turn alters NADPH levels, and makes tissues incredibly vulnerable to the oxidative damage [48]. In the present investigation, decreased G6PDH activity was noticed in the diabetic rats. Earlier, some of the investigators reported the reduced activity of G6PDH in type-2 diabetic individuals [49]. Similar to our findings, previous studies also demonstrated lower G6PDH activity in diabetic tissues [50, 51]. A decrease in the activity of glucose 6-phosphate dehydrogenase may equally slow down the pentose phosphate pathway in diabetic conditions [52]. The G6PDH activity was restored to normal level after treatment with CALEt for 30 d. The restored G6PDH activity by CALEt treatment confirmed the protective role of CALEt against diabetes complications, since CALEt administration showed a similar upturn with standard anti-diabetic drug glibenclamide.

CONCLUSION

The results obtained in this research indicate that methanol extract of *Centella asiatica* leaves could be effective in preventing the diabetes induced disturbances in mitochondrial and cytosolic enzymes. This was shown by improved activities of the enzymes in diabetic rats. However, further pharmacological and biochemical investigations are considered necessary to find out the active constituent and its system of action to understand the bioactive and ameliorative potential of the plant.

CONFLICT OF INTERESTS

Declared None

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