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INFLUENCE OF *CARDIOSPERMUM HALICACABUM* LEAF EXTRACT ON MEMBRANE-BOUND ATPases IN STREPTOZOTOCIN-INDUCED DIABETIC RATS

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Abstract

Background: *Cardiospermum halicacabum* L. proved to have anti-inflammatory, antihyperglycemic, antioxidant, antiglycation, analgesic and antipyretic activities. It also has been used in Ayurveda and folk medicine for the treatment of rheumatism, fever and earache.
Objective: The present study was aimed to evaluate the effect of *Cardiospermum halicacabum* leaf extract (CHE) on membrane-bound ATPases in streptozotocin (STZ)-induced diabetic rats.
Methods: Diabetes was induced in male albino Wistar rats by intraperitonial administration of STZ (40 mg/kg BW). CHE (200 mg/kg BW) or glibenclamide (600 µg/kg BW) was administered orally once daily for 45 days to normal and STZ-induced diabetic rats.
Results: The activities of membrane-bound ATPases such as total ATPase, Na^+/K^+-ATPase, Mg^{2+}-ATPase and Ca^{2+}-ATPase were significantly decreased in erythrocytes and tissues of STZ-induced diabetic rats. Oral administration of CHE to diabetic rats significantly increased the activities of these enzymes towards near normalcy.
Conclusions: Thus, the present study indicates that the beneficial role of membrane-bound ATPases in STZ-induced diabetic rats. The antihyperglycemic, antioxidant and antihyperlipidemic properties of CHE could be helpful to maintain the activities of membrane-bound ATPases in STZ-induced diabetic rats showing the membrane stabilizing property of extract.

Keywords: Streptozotocin, blood glucose, diabetes, membrane-bound ATPases, *Cardiospermum halicacabum*

Introduction

Diabetes mellitus a major impact on cardiovascular diseases morbidity and mortality and recently account for 80% of all diabetic deaths (WHO, 2004). A numerous studies have been reported concerning impaired cardiac function in diabetes (Norton et al., 1996; Cai et al., 2002; Price et al., 2003). The membrane-bound enzymes play an important role for development of diabetic vascular complications (Kiziltunc et al., 1997; Jain et al., 2000). Peroxidation of membrane phospholipids has been suspected a major mechanism of oxidant injury which leads to membrane damage and subsequently to cellular dysfunctions (Halliwell and Gutteridge, 1986; Vercesi et al., 1997). The increased free radicals generation during diabetes has deteriorated membrane structure and decreased membrane fluidity (Mecocci et al., 1997).

Cell membranes require optimal fluidity to maintain homeostasis and metabolism (Limaye and Sivakami, 2003). Membrane-bound ATPases such as Na^+/K^+-ATPase, Ca^{2+}-ATPase and Mg^{2+}-ATPase are responsible for transport of sodium/potassium, magnesium and calcium across the cell membranes at the expense of ATPase by hydrolysis. The abnormalities in Na^+/K^+-ATPase, Ca^{2+}-ATPase and Mg^{2+}-ATPase activity well documented in cardiac dysfunction in diabetes (Kiziltunc et al., 1997; Jain et al., 2000).

Plants or plant derived compounds have been used as a major source of drug for treatment of diabetes in worldwide. *Cardiospermum halicacabum* Linn. (Sapindaceae) is an annual or sometimes perennial climber, commonly found as a weed throughout India. The tender, young shoots are used as a vegetable, fodder, diuretic, stomachic, and rubefacient. It is used in rheumatism, lumbago, nervous diseases, and as a demulcent in orchitis and in dropsy. In Sri Lanka, it is used for the treatment of skeletal fractures. The juice of the herb is used to cure ear-ache and to reduce hardened tumours. It exhibits significant analgesic, anti-inflammatory and vaso-depressant activity, which is transient in nature. *In vitro* studies have revealed its antispasmodic and curative actions confirming the use of the herb in Ayurvedic medicine (Anonymous, 1992). Previous study has reported the anti-inflammatory activity of ethanolic extract against LPS-induced inflammatory responses in RAW264.7 cells (Sheeba and Asha, 2009). Experimental pharmacological studies have shown the analgesic and vasodepressant activities (Gopalakrishnan et al., 1976), antipyretic activity against yeast-induced pyrexia in rats (Asha and Pushpangadan, 1999), antimalarial (Waako et al., 2005), antioxidant activity (Kumaran and Karunakaran, 2006), suppressing the production of TNF-alpha and nitric oxide in human peripheral blood mononuclear cells (Venkatesh Babu and Krishnakumari, 2006; Thabrew et al., 2004) and anti-ulcer activity against ethanol induced gastric ulcer in rats (Sheeba and Asha, 2006). In our earlier studies have been reported that the extract of *Cardiospermum halicacabum* to possesses antihyperglycemic, antioxidants potential and prevents the abnormal protein glycation against STZ-induced diabetic rats (Veeramani et al., 2008; Veeramani et al., 2010; Veeramani et al., 2012).

No detailed investigation has been carried out to define the effect of CHE on membrane bound enzymes against STZ-induced diabetic rats. Hence, the current work planned to investigate the effect of CHE on membrane-bound ATPases in STZ-induced diabetic rats.

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Materials and methods

Animals

Male albino Wistar rats (weighing 180-200 g, 9 weeks old) were procured from the Central Animal House, Department of Experimental Medicine, Rajah Muthiah Medical College and Hospital, Annamalai University, and maintained in an airconditioned room [(25±1) °C] with a 12 h light/12 h dark cycle. Feed and water were provided *ad libitum*. The study was conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH, 1985) and the experimental study was approved by the Ethical Committee of Rajah Muthiah Medical College and Hospital (Reg No.160/1999/CPCSEA), Annamalai University, Annamalainagar.

Chemicals

Streptozotocin was obtained from Sigma-Aldrich Company (St. Louis, Missouri, USA). All other chemicals used were of analytical grade obtained from E. Merck, Mumbai and HIMEDIA, Mumbai, India.

Experimental induction of diabetes

The animals were made diabetic by an intraperitoneal injection of streptozotocin (STZ, 40 mg/kg BW, between 8:00 AM to 9:00 AM) in a freshly prepared citrate buffer (0.1M, pH 4.5) after an overnight fast. STZ injected animals were given 20% glucose solution for 24 h to prevent initial drug-induced hypoglycaemic mortality. The animals exhibited massive glycosuria (determined by Benedict's qualitative test, Benedict 1911) and hyperglycaemia within a few days. Diabetes was confirmed by measuring the fasting blood glucose concentration 96 h after induction. Albino rats with a blood glucose level above 220 mg/dL were considered diabetic and were used in this experiment.

Plant material

Leaves of *Cardiospermum halicacabum* (voucher No. AU-7032) were collected from the local areas, Jeyankondam, Ariyalur district, Tamil Nadu, India. The plant was botanically identified and authenticated in the Department of Botany, Annamalai University, Annamalainagar, Chidambaram, Tamil Nadu, India and a voucher specimen was deposited at the herbarium of botany.

Preparation of plant extract

The plant leaf was shade dried at room temperature [(32±2) °C] and the dried leaf was ground into fine powder using a pulverizer. The powered part was sieved and kept in deep freezer until use. 100 g of dry fine powder was suspended in 300 mL of ethanol for 72 h. The extract was filtered using a muslin cloth and concentrated at [(40±5) °C].

Experimental design

The animals were randomly divided into five groups of six animals each. In our earlier study, the extract was suspended in 2% gum acacia vehicle solution and fed by intubation at three different doses such as 50, 100 and 200 mg/kg BW. The dose of 200 mg exhibited maximum reduction of blood glucose when compared to the other two doses in STZ-induced diabetic rats (Veeramani et al., 2008). The active dose of 200 mg was used in this study.

Group I: Normal (2% gum acacia)
Group II: Normal + CHE (200 mg/kg BW.) in 2% gum acacia
Group III: Diabetic control
Group IV: Diabetic + CHE (200 mg/kg BW.) in 2% gum acacia
Group V: Diabetic + glibenclamide (600 µg/kg BW) in 2% gum acacia

After 45 days, the animals were anaesthetized using ketamine (24 mg/kg BW, intramuscular injection), and sacrificed by cervical dislocation. Between 8:00 am and 9:00 am blood sample and tissues were collected for the estimation of membrane bound enzymes activities.

Biochemical estimations

Estimation of total protein

Total protein was estimated by the method of Lowry et al. (1951). The standard ranging from 0.2-1.0 ml containing 20-100 µg of protein respectively was taken in different test tubes. The volume in each test tube was made up to 1 ml with distilled water and 1ml of water was taken as blank. Alkaline copper reagent (5 ml) was added to each tube and mixed thoroughly. The test tubes were allowed to stand at room temperature for 10 min. Folins ciocalteau reagent (0.5 ml) was added to each tube rapidly and mixed thoroughly. After incubation at room temperature the colour developed was read against blank at 680 nm and 0.1 ml of the sample was treated similarly. The level of total protein was expressed as mg/g of wet tissue.

Estimation of total ATPases

Total ATPase was assayed by the method of Evans (1969). To 1.5 ml of buffer, 0.1 ml of each NaCl, KC1, $MgCl^2$, $CaCl^2$, and ATP solutions were added. Then 0.1 ml of sample was added. The tubes were incubated at 37°C for 20 min. The reaction was arrested by addition 1 ml of 10% TCA. The tubes were centrifuged and the phosphorous content in the supernatant was determined by Fiske & Subbarow method

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(1925) using commercial diagnostic kit (Qualigens Diagnostics, India). The supernatant and aliquots of standards were made upto 5.0 ml with water. To these tubes, 1.0 ml ammonium molybdate followed by 0.5 ml ANSA reagent were added and mixed. The amount of phosphorous liberated was read at 620 nm after 20 min against a reagent blank in a colorimeter. The activity of total ATPases was expressed as µmoles of phosphorous liberated/h/mg protein.

Estimation of Na^+/K^+-ATPase

Na^+/K^+-ATPase was assayed according to the method of Bonting (1970). To 1 ml of tris buffer, 0.2 ml of each of MgSO4, KC1, NaCl, EDTA were added and equilibrated at 37°C for 10 min and the enzyme reaction was initiated by the addition of 0.1 ml of sample. The assay medium was then incubated for 1 h at 37°C. The reaction was arrested by addition 1 ml of 10% TCA. The tubes were centrifuged and the phosphorous content in the supernatant was estimated according to the method of Fiske & Subbarow (1925) using commercial diagnostic kit (Qualigens Diagnostics, India). The activity of Na^+/K^+-ATPase was expressed as µmoles of phosphorous liberated/h/mg protein.

Estimation of Ca^{2+}-ATPase

Ca^{2+}-ATPase was assayed by the method of Hjerten and Pan (1983). The incubation mixture contained 0.1 ml each buffer, $CaCl_2$ATP and water. After equilibrating the tubes at 37°C the reaction was initiated by the addition of 0.1 ml of sample. The contents were incubated at 37°C for half an h. The reaction was arrested by addition 1 ml of cold 10% TCA. The tubes were centrifuged and phosphorous content in the supernatant was estimated by Fiske & Subbarow method (1925) using commercial diagnostic kit (Qualigens Diagnostics, India). The activity of Ca^{2+}-ATPase was expressed as µmoles of phosphorous liberated/h/mg protein.

Estimation of Mg^{2+}-ATPase

The activity of Mg^{2+}-ATPase was assayed by the method of Ohinishi et al. (1982). The incubation mixture contained 0.1 ml of 375 mM Tris-HCl buffer (pH 7.6), 0.1 ml of 25 mM $MgCl^2$, 0.1 ml of 10 mM ATP, 0.1 ml water and 0.1 ml of sample. The contents were incubated for 15 min at 37◦C and the reaction was arrested by adding 0.5 ml of 10% TCA. The tubes were centrifuged and the phosphorous content in the supernatant was determined by Fiske & Subbarow method (1925) using commercial diagnostic kit (Qualigens Diagnostics, India). The activity of Mg^{2+}-ATPase was expressed as µmoles of phosphorous liberated/h/mg protein.

Statistical analysis

Values are given as means ± S.D. for six rats in each group. Data were analyzed by one-way analysis of variance followed by Duncan's Multiple Range Test (DMRT) using SPSS version 10 (SPSS, Chicago, IL). The limit of statistical significance was set at $P \leq 0.05$.

Results

Effect of CHE on total ATPase and Na^+/K^+-ATPase

Tables 1 and 2 represent the activities of total ATPase and Na^+/K^+-ATPase in erythrocytes and tissues (liver, kidney and heart) of normal and STZ-induced diabetic rats. In diabetic rats, the activities of total ATPase and Na^+/K^+-ATPase were shown to decrease significantly ($p<0.05$) as compared to control rats. Administration of CHE and glibenclamide to diabetic rats significantly ($p<0.05$) increased the activities of total ATPase and Na^+/K^+-ATPase to towards normal control rats.

Effect of CHE on Ca^{2+}-ATPase and Mg^{2+}-ATPase

Tables 3 and 4 represent the activities of Ca^{2+}-ATPase and Mg^{2+}-ATPase in erythrocytes and tissues (liver, kidney and heart) of normal and STZ-induced diabetic rats. In diabetic rats the activities of Ca^{2+}-ATPase and Mg^{2+}-ATPase were decreased significantly ($p<0.05$) and administration of CHE and glibenclamide to diabetic rats the above enzymes activities were increased significantly ($p<0.05$) to towards normal control rats.

Discussion

We have previously demonstrated that the *cardiospermum halicacabum* leaf extract (CHE) is having antihyperglycaemic, antioxidant, antiglycation and antihyperlipidemic activity on STZ-induced diabetic rats (Veeramani et al., 2008; Veeramani et al., 2010; Veeramani et al., 2012). The antihyperglycemic effect of CHE may be attributed to activation of glucose uptake, inhibition of intestinal glucose transporter and decreasing the expression of genes that control gluconeogenesis (Veeramani et al., 2008; Veeramani et al., 2010).

ATPases of cardiac cells play a significant role in the contraction and relaxation cycles of cardiac muscle by maintaining normal ion levels (Ca^{2+}, Na^+, K^+, and Mg^{2+}) within the myocytes. Alterations in the properties of these ion pumps may affect cardiac function. The abnormalities in

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Na^+/K^+-ATPase and Ca^{2+}-ATPase are well documented in cardiac dysfunction in diabetes (Dhalla et al., 1998; Pekiner et al., 2002; Jain and Lim, 2000). In the present study the activity of Na^+/K^+-ATPase was decreased significantly in STZ-induced diabetic rats. Thus, decreased activity of Na^+/K^+-ATPase could be due to enhanced lipid peroxidation by free radicals on STZ induction, since Na^+/K^+-ATPase is a 'SH' group containing enzyme and is lipid dependent (Ithayarasi and Devi, 1997). This decreased activity of Na^+/K^+-ATPase can lead to a decrease in sodium efflux, thereby altering membrane permeability (Finotti and Palatini, 1986). Hence, the present study we observed that the decrease activity of Na^+/K^+-ATPase may be considered as an index of cardiovascular complications induced by diabetes.

Table 1: Effect of *Cardiospermum halicacabum* leaf extract (CHE) on total ATPases in the erythrocytes and tissues of normal and STZ-induced diabetic rats.

Groups	Erythrocytes (U^a/mg protein)	Liver (U^a/mg protein)	Kidney (U^a/mg protein)	Heart (U^a/mg protein)
Normal control	3.07 ± 0.24^a	2.94 ± 0.23^a	1.62 ± 0.15^a	1.73 ± 0.16^a
Diabetic control	0.85 ± 0.06^b	1.03 ± 0.08^b	0.84 ± 0.06^b	0.63 ± 0.05^b
Normal + CHE (200 mg/kg BW)	3.10 ± 0.23^a	2.90 ± 0.20^a	1.65 ± 0.10^a	1.75 ± 0.12^a
Diabetic + CHE (200 mg/kg BW)	2.81 ± 0.21^c	2.55 ± 0.18^c	1.49 ± 0.09^c	$1.64 \pm 0.10^{c,a}$
Diabetic + glibenclamide (600 µg/kg BW)	$2.90 \pm 0.18^{c,d}$	2.70 ± 0.26^d	1.57 ± 0.14^d	1.70 ± 0.14^a

Values are given as means ± S.D from six rats in each group.; Values not sharing a common superscript vertically differ significantly at $p< 0.05$ (DMRT).; a-µmol of Pi liberated per hour.

Table 2: Effect of CHE on $Na^{+/}$ K^+-ATPase in the erythrocytes and tissues of normal and STZ-induced diabetic rats.

Groups	Erythrocytes (U^a/mg protein)	Liver (U^a/mg protein)	Kidney (U^a/mg protein)	Heart (U^a/mg protein)
Normal control	0.96 ± 0.08^a	0.94 ± 0.07^a	0.54 ± 0.04^a	0.59 ± 0.04^a
Diabetic control	0.48 ± 0.02^b	0.35 ± 0.02^b	0.31 ± 0.02^b	0.32 ± 0.03^b
Normal + CHE (200 mg/kg BW)	0.99 ± 0.06^a	0.95 ± 0.06^a	0.53 ± 0.03^a	0.62 ± 0.06^a
Diabetic + CHE (200 mg/kg BW)	0.86 ± 0.06^c	0.83 ± 0.08^c	0.46 ± 0.04^c	$0.54 \pm 0.04^{a,c}$
Diabetic + glibenclamide (600 µg/kg BW)	$0.90 \pm 0.07^{c,a}$	$0.89 \pm 0.08^{c,a}$	$0.50 \pm 0.05^{c,a}$	0.56 ± 0.05^a

Values are given as means ± S.D from six rats in each group.
Values not sharing a common superscript vertically differ significantly at $p< 0.05$ (DMRT).
a-µmol of Pi liberated per hour.

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Table 3: Effect of CHE on Ca^{2+}-ATPase in the erythrocytes and tissues of normal and STZ-induced diabetic rats.

Groups	Erythrocytes (U^a/mg protein)	Liver (U^a/mg protein)	Kidney (U^a/mg protein)	Heart (U^a/mg protein)
Normal control	$0.55 \pm 0.04^{a,c}$	0.59 ± 0.03^{a}	0.40 ± 0.03^{a}	0.35 ± 0.02^{a}
Diabetic control	0.39 ± 0.03^{b}	0.32 ± 0.02^{b}	0.19 ± 0.01^{b}	0.17 ± 0.02^{b}
Normal + CHE (200 mg/kg BW)	0.57 ± 0.05^{a}	0.63 ± 0.05^{a}	0.43 ± 0.03^{a}	0.36 ± 0.03^{a}
Diabetic + CHE (200 mg/kg BW)	0.49 ± 0.04^{c}	0.51 ± 0.04^{c}	0.35 ± 0.02^{c}	0.30 ± 0.03^{c}
Diabetic + glibenclamide (600 µg/kg BW)	0.51 ± 0.03^{c}	$0.55 \pm 0.05^{a,c}$	$0.37 \pm 0.03^{a,c}$	$0.33 \pm 0.02^{a,c}$

Values are given as means ± S.D from six rats in each group.; Values not sharing a common superscript vertically differ significantly at $p< 0.05$ (DMRT).; a-µmol of Pi liberated per hour .

Table 4: Effect of CHE on Mg^{2+}-ATPase in the erythrocytes and tissues of normal and STZ-induced diabetic rats.

Groups	Erythrocytes (U^a/mg protein)	Liver (U^a/mg protein)	Kidney (U^a/mg protein)	Heart (U^a/mg protein)
Normal control	0.46 ± 0.03^{a}	0.60 ± 0.06^{a}	0.40 ± 0.04^{a}	0.52 ± 0.03^{a}
Diabetic control	0.26 ± 0.02^{b}	0.30 ± 0.03^{b}	0.20 ± 0.02^{b}	0.19 ± 0.01^{b}
Normal + CHE (200 mg/kg BW)	0.49 ± 0.04^{a}	0.63 ± 0.04^{a}	0.38 ± 0.03^{a}	0.55 ± 0.04^{a}
Diabetic + CHE (200 mg/kg BW)	0.39 ± 0.03^{c}	0.56 ± 0.05^{a}	0.35 ± 0.02^{a}	0.44 ± 0.02^{c}
Diabetic + glibenclamide (600 µg/kg BW)	$0.42 \pm 0.03^{a,c}$	0.58 ± 0.03^{a}	0.37 ± 0.03^{a}	$0.48 \pm 0.03^{a,c}$

Values are given as means ± S.D from six rats in each group.
Values not sharing a common superscript vertically differ significantly at $p< 0.05$ (DMRT).
a-µmol of Pi liberated per hour.

Impaired calcium homeostasis has been reported in diabetic cardiomyopathy and other complications of diabetes mellitus (Golfman et al., 1996; Hattori et al., 2000). The Ca^{2+}-ATPase is the major active calcium transport protein responsible for the maintenance of normal intracellular calcium levels in a variety of cell types. Abnormal Ca^{2+}-ATPase activity and intracellular calcium levels has reported as important mechanisms responsible for the cardiac dysfunction exhibited by diabetic animals (Hattori et al., 2000). Diabetes-induced hyperlipidemia has been altered the membrane phospholipids and fatty acids and shown to depress membrane-bound enzyme activities, which influence intracellular calcium metabolism resulting in cardiac dysfunction (Kuwahara et al., 1997). The increased ROS formations during diabetes have been caused

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intracellular organelles in membranes which lead to decrease in cardiac Ca^{2+}-ATPase activity (Ziegelhoffer et al., 1997). Thus, the hyperlipidemia and oxidative stress seem to be the major contributing factors associated with abnormal calcium homeostasis in diabetic animals. Mg^{2+}-ATPase is involved in energy requiring processes in the cell whereas Ca^{2+}-ATPase is responsible for the signal transduction pathways and membrane fluidity. The intracellular concentration of calcium regulates the activities of Mg^{2+}-ATPase and Na^{+}/K^{+}-ATPase. The inhibition of these transport systems in the cell may result in a sustained increase in cytosolic Ca^{2+} concentrations producing over stimulation of cellular processes leading ultimately to cell death (Boutilier, 2001). In the present study, Ca^{2+}-ATPase and Mg^{2+}-ATPase activity were depressed in diabetic rats which may be increased hyperlipidemia and oxidative stress by diabetic rats.

Plants or plant derived compounds have been shown to influence membrane characteristics such as fluidity, stability and susceptibility to membrane oxidative damage (Jain and Lim, 2001; Babu and Sabitha, 2006). Previous investigation has been suggested that lipid peroxidation and glycosylation of proteins can cause reduction in the activities of enzymes and alteration in the structure and function of membranes (Flecha et al., 1990). Antioxidants has been shown to prevents lipid peroxidation, protein glycation and inhibition of Na^{+}/K^{+}-ATPase and/or Ca^{2+}-ATPase activity caused by hyperglycemia (Jain and Lim, 2001; Babu and Sabitha, 2006). In the present study, administration of CHE to diabetic rats significantly increased the activities of total ATPase, Na^{+}/K^{+}-ATPase, Ca^{2+}-ATPase and Mg^{2+}-ATPase in erythrocyte and tissues when compared with diabetic control rats. The potential hypolipidemic, antioxidant and antiglycating effects of CHE are well documented (Veeramani et al., 2008; Veeramani et al., 2010) in our previous reports. These activities may improve the fluidity of membrane, ameliorate calcium homeostasis and to protect cardiac dysfunctions associated in diabetes.

Conclusion

In conclusion, the result of the present study indicates that CHE is potent inhibitor of cardiac dysfunction and improve the membrane fluidity on STZ-induced diabetic rats as evidenced by increased activity of membrane-bound ATPases such as total ATPase, Na^{+}/K^{+}-ATPase, ca^{2+}-ATPase and Mg^{2+}-ATPase. The antihyperglycemic, antioxidant and antihyperlipidemic properties of CHE (Veeramani et al., 2008; Veeramani et al., 2010), could be helpful to maintain the levels of membrane-bound ATPases in STZ-induced diabetic rats showing the membrane stabilizing property of extract.

Conflict of interest: The authors have no conflict of interest to declare.

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