Beneficial effects of levo-carnitine on lipid metabolism and cardiac function in neonatal streptozotocin rat model of diabetes

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Abstract
Levo-carnitine (L-carnitine) facilitates the transport of long chain fatty acyl co-enzyme A (CoA) across the mitochondrial membrane for eventual oxidation and energy production. Carnitine deficiency results in free fatty acid accumulation and contributes to cardiovascular complications in experimental diabetes. In this condition, oral carnitine supplementation provides cardio-protection by its various metabolic effects. The beneficial effects of six weeks treatment with L-carnitine (600 mg/kg day orally) were studied in neonatal streptozotocin (STZ) diabetic rats. STZ (90 mg/kg) was administered to five day old rats and after fourteen weeks of STZ administration, the rats showed hyperglycemia, hyperinsulinemia, hypercholesterolemia, hypertriglyceridemia and associated cardiac defects like hypertension and bradycardia. L-carnitine treatment for six weeks after induction of diabetes and associated complications significantly lowered cholesterol and triglyceride levels and normalized blood pressure and heart rate; however the treatment was not found to produce beneficial effects against hyperglycemia and hyperinsulinemia in diabetic rats. The L-carnitine treatment may have fulfilled the carnitine deficiency and have improved the lipid metabolism and subsequently the cardiac function in diabetic rats. The study suggest that long term treatment with L-carnitine not only prevents but also partially reverses the diabetes-associated lipid metabolism and cardiac function abnormalities in neonatal STZ diabetic rats.

Keywords: L-carnitine; Diabetes mellitus; Lipid metabolism

Introduction

Diabetes mellitus is a syndrome characterized by altered metabolism of lipids, carbohydrates, proteins and an increased risk of cardiovascular diseases. Several investigators have observed heart dysfunctions in both diabetic patients and experimental animal models of chronic diabetes.[1] One of the cardiac defects in diabetes is the inability of the diabetic heart to utilize glucose as an energy substrate[2] and hence its exclusive dependence on fatty acids for energy production.[3] Extensive studies have shown that different membrane systems such as sarcolemma, sarcoplasmic reticulum and mitochondria as well as myofibrillar proteins are altered in the diabetic heart.[4] The depression of sarcoplasmic reticular calcium pump is associated with an increased concentration of long chain fatty acyl derivatives.[5,6] Changes in phospholipids composition and increase in the cholesterol content in sarcolemma have also been reported in diabetic cardiomyopathy.[7] The carbohydrate and lipid metabolism abnormalities and free fatty acid accumulation is found to be an important contributor of these cardiac defects in diabetes.[5,6]

L-carnitine is an amino acid found in all living tissues and plays a vital role in fatty acid metabolism as well as in regulating many bodily functions.[8,9] It is a water-soluble nutrient mainly present in non-vegetarian diet like beef and pork. It is synthesized endogenously in the liver[10] and plays an important role in fatty acid metabolism. It facilitates the transport of long chain fatty acyl co-enzyme (CoA) across the mitochondrial membrane for eventual oxidation and energy production.[11,12]

Heart and skeletal muscle normally cover most of their energy needs by fatty acid oxidation.[13,14] More than 90% of the total carnitine in the body is found in skeletal muscle and heart. 95% of the carnitine in normal cardiac muscle is located in cytosol and 95% of CoA is in mitochondrial matrix.[15] These distributions in normal hearts are likely to cause activated fatty acids to be funneled toward oxidation rather than toward lipid synthesis. Diabetes is a condition associated with myocardial carnitine deficiency and this has been demonstrated in chemically induced diabetic rats.[16-21] Myocardial carnitine deficiency is one of the causes of cardiac defects in experimental diabetes[21-23] and L-carnitine supplementation is beneficial for the treatment and prevention of diabetes associated metabolic and cardiac defects.[20,24-32] In all these studies, two major beneficial effects of L-carnitine have been observed; one is the improvement in lipid metabolism and second is improvement in glucose metabolism. However, majority of these experimental studies were carried out using STZ induced type 1 diabetes in Wistar rats in which diabetes was induced by cytotoxic actions of STZ on beta cells of pancreas. The beneficial effects of acute short term treatment with large dose of L-carnitine have been demonstrated in diabetes with mild cardiac defects. Studies

Received on: 02/07/07
Accepted on: 26 /10/07

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demonstrating the effects of long term supplementation with L-carnitine in diabetes with metabolic and profound cardiac defects are still in demand. Whether treatment with L-carnitine produces any long term beneficial effect even after well developed metabolic and cardiac defects in diabetes is still a question. As L-carnitine could serve as a cardio-protective agent in diabetes, studies on neonatal STZ diabetic rat model which more closely resemble the human type 2 diabetes with associated cardiac defects would be more interesting. Hence, we have carried out an experimental study to investigate the beneficial effects of long term treatment with L-carnitine in neonatal STZ diabetic rats.

Materials and Methods

Animals

Neonatal STZ diabetic rat model using Sprague Dawley (SD) strain has been demonstrated in the past to have plasma glucose values of 275 mg/dL or higher, marked basal hyperglycemia (>200 mg/dL), defective glucose induced insulin secretion, poor response of heart and adipocytes to insulin *in vitro*, reduced rate of glucose disposal *in vivo*, hypertension, and reduced rates of contractility and relaxation of heart (bradycardia).[33-41] Hence we have selected this model for our study.

Healthy male SD rats at the time of their birth were selected and divided into four groups. Streptozotocin (90 mg/kg) dissolved in saline was given i.p. to five day old rats of groups 3 and 4 for induction of type 2 diabetes mellitus and associated complications. All rats were kept under care with the supervision of veterinarian and the mother rats had free access to their neonates for breast feeding till weaning (4 weeks). Fourteen weeks after injection of STZ to animals of groups 3 and 4, fasting blood glucose levels were determined. Oral glucose tolerance test on these animals was performed to check diabetic state, and heart rate and blood pressure were checked by the tail cuff method using the Harvard Blood Pressure Monitor. Animals with glucose intolerance, fasting blood glucose more than 140 mg/dL, hypertension and bradycardia having weight 250-300 gm were confirmed for the study in diabetic groups (group 3 and 4). Non-STZ / healthy SD rats weighing 250-300 gm were used for the study in control groups (group 1 and 2). L-carnitine (600 mg/kg day) dissolved in saline was administered orally for 6 weeks to treatment groups (group 2 and 4) while control groups (group 1 and 3) received only saline. Group 1 and 3 served as control for corresponding treatment groups 2 and 4 respectively. Thus, grouping of animals (n=7) was as follows:

Group 1. : Saline treated control rats
Group 2. : L-carnitine treated control rats
Group 3. : Saline treated Diabetic rats
Group 4. : L-carnitine treated Diabetic rats

Study rats were housed in individual cages under natural light and dark cycles at a temperature of 28 ± 4ºC. Standardized pellet diet was given to the rats during the study and water bottles were filled twice each day. All animals had free access to food and water. The rats were weighed once each week.

The current investigation conformed to the Guideline of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Animal Welfare Board, Government of India, for the Care and Use of Laboratory Animals.[42] The study protocol was approved by the Institutional Animal Ethics Committee of the institute (L. M. College of Pharmacy, Ahmedabad, Gujarat, India).

Heart rate and blood pressure measurement and collection of blood samples

After six weeks treatment, heart rate and blood pressure of all animals were recorded by the tail cuff method using the Harvard Blood Pressure Monitor. Blood samples were collected from the tail vein of lightly anesthetized overnight fasted animals from all the groups. Serum was separated for the estimation of glucose, insulin, triglyceride and total cholesterol.

Estimation of serum glucose

Serum glucose levels were determined by the glucose oxidase method using Bayer Diagnostics reagent kit (Ahmedabad, India). The analysis method is based on a principle that the glucose oxidase enzyme when added to serum sample and incubated at 37°C for 15 minutes, converts glucose to gluconic acid and hydrogen peroxide. The hydrogen peroxide is converted to water and oxygen by the enzyme peroxidase. 4-aminophenazone, an oxygen acceptor, takes up the oxygen and together with phenol forms a pink colored chromogen. The optical density (OD) at 510 nm of the color formed is directly proportional to the glucose level. By comparing OD of standard and blank with the test, the serum glucose was estimated in our study.[43-45]

Estimation of serum insulin

Serum insulin was assayed by the radioimmunoassay (RIA) method with RIA kit supplied by Board of Radiation and Isotope Technology, Bhabha Atomic Research Centre (Navi Mumbai, India). The analysis method is based on the principle that the insulin in serum when added to a tube containing a fixed amount of antibody and a fixed amount of the radio-labeled insulin (I^{125}- labeled), competes with the radio labeled insulin for the antibody. The amount of radio-labeled insulin bound to the antibody is inversely proportional to the amount of insulin in serum. The bound radio-labeled insulin can be measured by gamma scintillation counter. In our study, a standard plot of number of gamma scintillation count vs amount of the standard insulin was constructed and from the plot, the amount in the test sample was derived from the number of count of the test sample.[46-47]

Estimation of serum total cholesterol

Total serum cholesterol was determined by enzymatic method with a Bayer Diagnostics reagent kit (Ahmedabad, India). The analysis method is based on the principle that the total cholesterol ester of serum can be converted into cholesenone and produce hydrogen peroxide by the repeated action of cholesterol esterase and cholesterol oxidase. When this hydrogen peroxide reacts with phenol and 4-aminoantipyrine in presence of enzyme peroxidase, it gives purple-red colored Quinone. The concentration of cholesterol in serum sample is directly proportional to the OD of this purple-red color which can be measured at 500

Table 1: Effect of diabetes and L-carnitine on fasting serum glucose, insulin, cholesterol and triglyceride levels of experimental rats.

	Control groups		Diabetic groups	
	Untreated (n=7)	Carnitine treated (n=7)	Untreated (n=7)	Carnitine treated (n=7)
Fasting glucose (mg/dl)	76.12 ± 10.61	67.53 ± 6.53	139.38 ± 13.76 [a]	138.95 ± 30.85 [a]
Fasting Insulin (μU/ml)	19.83 ± 8.25	22.6 ± 9.42	36.87 ± 9.42 [a]	38 ± 10.32 [a]
Total serum cholesterol (mg/dl)	88.39 ± 10.21	74.71 ± 9.15	114.98 ± 14.1 [a]	77.57 ± 7.38 [b]
Serum triglyceride (mg/dl)	65.8 ± 7.94	56.27 ± 8.2	95.63 ± 8.46 [a]	69.13 ± 9.26 [b]

Results are expressed as mean ± SD for n=7 animals, [a] Significantly higher *(P < 0.05)* than untreated control group, [b] Significantly lower *(P < 0.05)* than untreated diabetic group

Table 2: Effect of diabetes and L-carnitine on blood pressure and heart rate of experimental rats.

	Control		Diabetic	
	Untreated (n=7)	Carnitine treated (n=7)	Untreated (n=7)	Carnitine treated (n=7)
Blood pressure (mmHg)	88.66 ± 8.2	80 ± 12.44	165 ± 8.73 [a]	95 ± 8.47 [b]
Heart rate (min^{-1})	361.12 ± 3.97	354 ± 4.76	318.4 ± 6.08 [a]	354 ± 5.56 [b]

Results are expressed as mean ± SD for n=7 animals, [a] Significantly higher *(P < 0.05)* than untreated control group, [b] Significantly different *(P < 0.05)* than untreated diabetic groups

nm. By comparing OD of standard and blank with the test, the total serum cholesterol was estimated in our study.[48]

Estimation of serum triglyceride

Total serum triglyceride was determined by enzymatic method with a Bayer Diagnostics reagent kit (Ahmedabad, India). The method is based on a principle that when the serum triglyceride reacts with lipoprotein lipase, it produces glycerol which forms dehydroxyacertone phosphate and hydrogen peroxide by reaction with glycerol kinase and glycerol 3-phosphate oxidase. This hydrogen peroxide further reacts with 4-aminoantipyrine and ADPS (n-ethyl-n-sulfopropyl-n-anisidine) to give red colored complex. The OD of purple-red color formed during the reaction is directly proportional to the triglyceride concentration of serum and can be measured at 546 nm. By comparing OD of standard and blank with the test, the serum triglyceride was estimated in our study.[49]

Euthanasia of study animals

After completion of study, the rats were submitted to euthanasia with carbon dioxide (CO_2). The CO_2 chamber is primed with CO_2 before placing the rat inside for euthanasia. Then the animal is placed in the chamber and the flow rate was adjusted to displace at least 20% of the chamber volume per minute to prevent CO_2 settlement at the bottom. The flow is continued until the rate loses consciousness and stops breathing completely. It continued for a brief period following the respiratory arrest to assure the demise of the animal. The process was repeated for all animals separately.[50,51]

Statistical analysis

When there are only two groups of observations, the statistical analysis usually employed for comparison of the two groups is a Student t-test. But in a study having more than two groups, like our study, it is inappropriate to simply compare each pair using a t-test because of the problem of multiple testing. The correct way is to use a one-way analysis of variance (ANOVA) to evaluate whether there is any evidence that the means of the results differ and the Tukey multiple comparison test is used to investigate which of the means are different. The results of Tukey multiple comparison are presented as a matrix showing the result for each possible pair, either as a p-value or as a confidence interval.[52,53] Many statistical software packages are available providing the Tukey multiple comparison test as an option when conducting a one-way ANOVA, we have used 'Primer of Biostatistics Version 4.0' (McGraw Hill) for our statistical analysis. P < .05 was taken as the level of statistical significance.

Results

Type II diabetes with metabolic complications like hyperglycemia, hyperinsulinemia, hypercholesterolemia, hypertriglyceridemia and related cardiovascular complications like hypertension and bradycardia resulted after fourteen weeks of injection of STZ to neonatal animals (Table 1 and 2). Treatment with L-carnitine did not influence the fasting blood glucose and insulin levels in both control as well as diabetic rats (Table 1). L-carnitine treatment did not affect serum cholesterol and triglyceride levels in control but significantly lowered the same in the diabetic rats (Table 1). Furthermore, diabetic rats showed higher blood pressure and reduced heart rate. L-carnitine treatment significantly normalized the blood pressure and heart rate in diabetic rats (Table 2). Induction of diabetes resulted in increase in body weight and increase in food and water intake in diabetic rats (Table 3). Treatment with L-carnitine did not influence body weight, food and water intake either in control or in diabetic rats (Table 3).

Discussion

Glucose and fatty acids are the primary sources of energy production in the heart. Energy produced from fatty acid oxidation is comparatively more than the energy produced from glucose oxidation; and hence fatty acid oxidation is the

Table 3: Effect of diabetes and L-carnitine on general features of experimental rats.

	Control groups		Diabetic groups	
	Untreated (n=7)	Carnitine treated (n=7)	Untreated (n=7)	Carnitine treated (n=7)
Body wt (g)	257 ± 15	247 ± 18	319 ± 19 [a]	288 ± 40
Food intake (g/24 hrs)	7 ± 2.32	7 ± 2.1	13 ± 4.2 [a]	12 ± 4
Water intake (g/24 hrs)	5 ± 3.53	5 ± 5.01	12 ± 4.87 [a]	9 ± 3.89

Results are expressed as mean ± SD for n=7 animals, [a] Significantly higher *($P < 0.05$)* than untreated control group

primary source of ATP production in aerobic heart.[13,14] It is a well known fact that fatty acid oxidation takes place in the mitochondria and requires the presence of both CoA and carnitine.[12] Adequate carnitine levels are required for normal fatty acid metabolism in the heart muscle. Lack of sufficient carnitine to transport fatty acyl groups of acyl-CoA into the mitochondria is responsible for the accumulation of free fatty acid and related intermediates, such as long-chain fatty acyl-CoA and acyl carnitine and the reduction of ATP.[54] The amphiphilic properties of these intermediates especially acyl carnitine may facilitate their incorporation into membranes,[55] with consequent perturbations in membrane proteins of sarcolemma and sarcoplasmic reticulum and depression of ATP-dependent calcium transport.[6] Lopaschuk et al.[5] have shown that treatment with carnitine results in protection against the accumulation of long-chain acyl carnitine in sarcoplasmic reticulum membrane of diabetic rats. Pieper et al.[56] have shown attenuation of increased long-chain acyl CoA and loss of ATP in isolated working heart from diabetic rats. These beneficial effects of L-carnitine were due to lowering of myocardial acyl CoA level.[24] Furthermore, in a recent study of Malone et al.,[17] 12 of the 32 diabetic rats that died between 10 to 18 weeks of STZ administration had much lower serum free carnitine. Even among the surviving diabetic rats which were not receiving carnitine continued to have reduced heart rate than normal control animals. But the carnitine receiving rats were found to have improvement in the heart rate after 2 weeks and normal heart rate by 10 weeks of STZ administration in their study. Thus protective effects of L-carnitine against the diabetes induced cardiac defects have been well demonstrated but the effects in diabetes after establishment of profound cardiac defects have not been studied well enough.

In the present study, rats treated with STZ displayed many of the features observed with uncontrolled diabetes mellitus, including hyperglycemia, hyperinsulinemia, hyperlipidemia (hypercholesterolemia, hypertriglyceridemia), hypertension and bradycardia. These symptoms were perhaps due to impaired glucose and lipid metabolism with reduced rates of contractility and relaxation of heart, which were in accordance with the previous reports.[33-41] The accepted mechanisms of cardiac abnormalities (reduced heart rate and elevated blood pressure) in STZ-diabetic rats were through the adverse effects of long-chain acyl derivatives[6,54,55] and diabetic neuropathy.[57-59]

L-carnitine treatment at the dose of 600 mg/kg day did not decrease the fasting serum glucose and insulin levels. Thus, in contrast with the reports of Paulson et al.[24] and Rodrigues et al.,[20] the L-carnitine treatment in our study failed to decrease serum glucose level. Paulson et al.[24] used inbred strains of rats and Rodrigues et al.[20] used a very high dose (3 gm/kg day) of L-carnitine in type 1 diabetes model of Wistar rats. Hence, the difference between the results of our study and of their studies may be due to the difference in the diabetic model, dose of L-carnitine and/or rat strain used. Further studies in type 2 diabetes models using higher dose of L-carnitine are required to draw the conclusion on blood glucose lowering effects of L-carnitine.

As L-carnitine plays an important role in the transport of long chain fatty acids across the mitochondrial membrane,[12] it also influences lipid metabolism. Carnitine deficiency is associated with various cardiac defects[6,54] including diabetic cardiomyopathy.[22] However the cardiac defects associated with decreased carnitine stores can be alleviated by the administration of L-carnitine in the presence of diabetes[20,24-32] or without diabetes.[60,61] In the present study, the diabetic rats exhibited hypertension and bradycardia with increased cholesterol and triglycerides levels. Long term L-carnitine supplementation normalized the blood pressure and heart rate with significant reduction in cholesterol and triglycerides levels in diabetic rats. The reversal of diabetes induced bradycardia observed in our study was in accordance with studies of Malone et al.[62] The reduction of blood pressure and improvement in heart rate may suggest some beneficial effects of L-carnitine against various cardiac insults intracellularly and/or effects against diabetic neuropathy. The significant reduction in cholesterol and triglyceride levels by L-carnitine treatment, clearly shows that an improvement in lipid metabolism occurred. L-carnitine has a potential to increase myocardial energetics and to maintain myocardial levels of ATP in ischemia;[63] hence improvement in cardiac function and lipid metabolism observed in our study if taken together, may suggest that L-carnitine would have attenuated the accumulation of long-chain acyl derivatives by improving their transport which would have in-turn improved the supply of mitochondrial acyl CoA and would have improved the myocardial energetics and working. All these beneficial effects would be as a result of fulfillment of carnitine deficiency of diabetic heart. Overall, it may be suggested that long term supplementation with L-carnitine partially reverses the cardiac defects in experimental type 2 diabetic rats.

In conclusion, L-carnitine treatment in experimentally induced type 2 diabetes improved the lipid metabolism and

normalized the heart rate and blood pressure by subsequent improvement in myocardial energetics. L-carnitine treatment is beneficial in diabetes especially with carnitine deficiency and lipid metabolism abnormalities. It would be interesting to further investigate the cellular mechanisms of beneficial effects of L-carnitine on lipid metabolism and cardiac function in type 2 diabetic rats.

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