Effect of a novel insulinotropic agent, succinic acid monoethyl ester, on lipids and lipoproteins levels in rats with streptozotocin-nicotinamide-induced type 2 diabetes

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In the present study, the effect of succinic acid monoethyl ester (EMS) on the pattern of lipids and lipoproteins in streptozotocin-nicotinamide induced type 2 diabetes was investigated. Type 2 diabetes was induced in male Wistar rats by single intraperitoneal injection (i.p.) of 45 mg/kg streptozotocin, 15 min after the i.p administration of 110 mg/kg body weight of nicotinamide. The carboxylic nutrient EMS was administered intraperitonially at a dose of 8 μmol/g body weight for 30 days. At the end of experimental period, the effect of EMS on plasma glucose, insulin, thiobarbituric acid reactive substances (TBARS) and hydroperoxide (HP) and serum triglycerides (TG), phospholipids (PL), free fatty acids (FFA), total cholesterol (TC), very low density lipoprotein-cholesterol (VLDL-C) and low density lipoprotein-cholesterol (LDL-C), high-density lipoprotein-cholesterol (HDL-C) and the percentage of antiatherogenic index (AAI) (ratio of HDL-C to total cholesterol) were studied. Administration of EMS to diabetic rats resulted in a significant reduction in the elevated levels of plasma glucose, TBARS and hydroperoxides as well as TG, PL, FFA, TC, VLDL-C and LDC-C levels. The decreased plasma insulin and serum HDL-C and percentage of AAI in diabetic rats were also reversed towards near normal. The effect produced by EMS was compared with metformin, a reference drug. The results indicates that the administration of EMS and metformin to nicotinamide-streptozotocin diabetic rats normalized plasma glucose, insulin concentrations and caused marked improvement in altered lipids, lipoprotein and lipid peroxidation markers during diabetes. Our results show the antihyperlipidemic properties of EMS and metformin in addition to its antidiabetic action. Moreover, the antihyperlipidemic effect could represent a protective mechanism against the development of atherosclerosis.

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1. Introduction

Diabetes mellitus (DM) is associated with an increased risk of thrombotic, atherosclerotic and cardiovascular disease. Hyperlipidemia is metabolic complication of both clinical and experimental diabetes (Gandhi 2001). Low-density lipoprotein in diabetic patients leads to abnormal metabolism and is associated with increase in very low-density lipoprotein (VLDL) secretion and impaired VLDL catabolism. Ultimately this leads to atherosclerotic plaque (Howard 1987). A number of known factors for coronary artery disease such as hypertension, obesity and dyslipidemia

Keywords. Antiatherogenic index; cholesterol; lipoproteins; phospholipids; triglycerides; type 2 diabetes

Abbreviations used: AAI, Antiotherogenic index; CHD, coronary heart disease; DM; diabetes mellitus; EMS, succinic acid monoethyl ester; FFA, free falty acids; HDL-C, high density lipoprotein-cholesterol; LDL-C, low density lipoprotein-cholesterol; NIDDM, non-insulin dependent diabetes mellitus; PL, phospholipids; TBARS, thiobarbituric acid reactive substance; VLDL-C, very low density lipoprotein-cholesterol.

are more common in diabetics than in the general population. The World Health Organization (WHO) predicts that the number of cases worldwide for diabetes, now as of 171 million, will touch 366 million or more by the year 2030 (Wild *et al* 2004). Patients with DM are more likely to develop microvascular and macrovascular complications than the non diabetic population (Baynes 1991). Dyslipidemia is a frequent complication of DM and is characterized by low levels of high density lipoprotein-cholesterol (HDL-C) and high levels of low density lipoprotein-cholesterol (LDL-C) and triglyceride (TG). Several groups of hypoglycemic drugs are currently available to treat DM.

Treatment of hyperlipidemia in diabetes involves improving glycemic control, exercise and the use of lipid lowering diets, drugs and hypoglycemic agents. (Betteridge 1997; Miller *et al* 2001). Mendez and Balderas (2001) showed that non-glucidic nutrient such as L-arginine administration tended to normalize the glycemia, hyperlipidemia, and lipid peroxidation, which indicates non-glucidic nutrients exerted an inhibitory effect on lipid peroxidation and also improve the lipid profile, that may be relevant in preventing diabetic complications.

Esters of succinic acid are new potent insulin secretagogues (Picton *et al* 2001) and have been proposed as novel antidiabetic agent for type 2 diabetes. Succinic acid ester protects pancreatic cell against streptozotocin-induced DM (Malaisse and Akkan 1993). A carboxylic nutrient such as succinic acid monoethyl ester (EMS) (figure1). has the similar role as glucose-induced proinsulin synthesis and insulin release (Alarcon and Wicksteed 2002; Zawalich and Zawalich 1992). EMS has been reported to possess insulinotropic (Ladriere *et al* 1998), insulin release and blood glucose lowering effect (Juan *et al* 1998).

Metformin is an oral hypoglycemic agent belonging to biguanides. It is used as an orally active antihyperglycemic drug in the treatment of human type 2 DM (Bailey *et al* 1996). Metformin enhances the sensitivity of both hepatic and peripheral tissues to insulin. The drug also inhibits gluconeogensis in the liver. Metformin has a favourable impact on lipid profile, decreasing plasma TG and low-density lipoprotein-cholesterol levels (Bailey *et al* 1996; Setter *et al* 2003). Previous studies in our laboratory have shown that EMS exerts antihyperglycemic action and antiperoxidative effect in type 2 diabetic rats (Pari and Saravanan 2005; Saravanan and Pari 2006).

The objective of this investigation was to ascertain the scientific basis for the use of EMS in the management of diabetes, and to test the effect of this compound on altered serum lipids, lipoproteins and lipid peroxidation markers using streptozotocin-nicotinamide-induced type 2 diabetic rats.

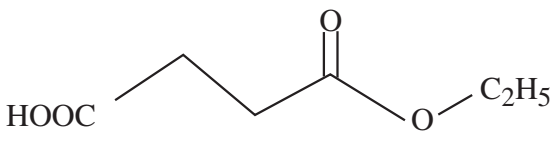

Figure 1. Succinic acid monoethyl ester.

2. Materials and methods

2.1 *Drug and chemicals*

Succinic acid monoethyl ester (EMS), and all other biochemicals and chemicals used in this experiment were purchased from Sigma Chemical Company Inc., St Louis, MO, USA. The chemicals used were of analytical grade.

2.2 *Animals*

Healthy male albino Wistar rats (200-220 g body wt.) obtained from Central Animal House, Rajah Muthiah Medical College, Annamalai University were used in the present study. The rats were fed on pellet diet (Hindustan Lever Limited, Mumbai) and water *ad libitum*. The rats were maintained according to guidelines of the National Institute of Nutrition, Indian Council of Medical Research, Hyderabad, and the study approved by the ethical committee (Vide No: 285, 2005), Annamalai University.

2.3 *Experimental induction of type 2 diabetes in rats*

Non-Insulin dependent diabetes mellitus (NIDDM) was induced in overnight fasted rats by a single intraperitonial injection (i.p.) of 45 mg/kg streptozotocin, 15 min after the i.p. administration of 110 mg/kg body wt. of nicotinamide (Masiello *et al* 1998). Streptozotocin (STZ) was dissolved in citrate buffer (pH 4.5) and nicotinamide was dissolved in normal saline. Hyperglycemia was confirmed by the elevated glucose levels in plasma, determined at 72 h and then on day 7 after injection. The rats with permanent NIDDM (180–200 mg glucose/dl) were used for the study.

2.4 *Experimental procedure*

In the experiment, a total of 30 rats (18 diabetic surviving rats, 12 control rats). The rats were divided into five groups of six rats each.

Group 1. Control rats.
Group 2. Control rats administered intraperitoneally with EMS 8 μmol/g body wt. daily for 30 days.
Group 3. Diabetic rats.
Group 4. Diabetic rats administered intraperitoneally with EMS 8 μmol/g body wt. daily for 30 days (Pari and Saravanan 2005).

Group 5. Diabetic rats given metformin 25 mg/kg body wt. (Yanardag *et al* 2005) in 1 ml of saline daily for 30 days.

At the end of experimental period, the rats were deprived of food overnight and blood was collected in a tube containing potassium oxalate and sodium fluoride for the estimation of plasma glucose and insulin.

2.5 *Analytical methods*

2.5a *Determination of plasma glucose and insulin:* Plasma glucose was estimated spectrophotometrically using commercial diagnostic kits [Sigma Diagnostics (I) Pvt Ltd, Baroda, India] (Trinder 1969). Plasma insulin was estimated by using enzyme linked immunosorbent assay (ELISA) kit (Roche diagnostics, Germany).

2.5b *Determination of lipid peroxidation:* Thiobarbituric acid reactive substances (TBARS) was estimated by the method of Fraga *et al* (1988). Hydroperoxide was determined by the method of Jiang *et al* (1992).

2.5c *Determination of lipid profiles*: Phospholipids was estimated by the method of Zilversmit and Davis (1950). The free fatty acids and triglycerides were estimated by the method of Falholt *et al* (1968) and Foster and Dunn (1973) respectively.

2.5d *Determination of cholesterol in the lipoprotein fractions:* HDL-C fraction was separated by the precipitation techniques of Burnstein *et al* (1970) and the cholesterol content was determined by the method of Zlatkis *et al* (1953).

To 1 ml of serum added 0.18 ml of heparin-manganese chloride reagent and mixed. The solution was allowed to stand at 4°C for 30 min and then centrifuged in a refrigerated centrifuge at 1800 *g* for 30 min. The supernatant represented the HDL-C fraction. An aliquot of supernatant was used for cholesterol estimation. The values were expressed as mg/dl.

VLDL-cholesterol was calculated using the following equation (Friedward *et al* 1972):

VLDL-C = TG/5, the values were expressed as mg/dl. LDL-C was calculated using the following equation: LDL-C = Total cholesterol – (HDL-C + VLDL-C), The values were expressed as mg/dl. The antiatherogenic index (AAI) was calculated according to the method of Guido and Joseph (1992). AAI was calculated from the data using the formula:

$$AAI = \frac{\text{HDL-C x 100}}{\text{TC-HDL-C}}$$

The values were expressed as a percentage.

2.6 *Statistical analysis*

The data for various biochemical parameters were analysed using analysis of variance (ANOVA) and the group means were compared by Duncan's Multiple Range Test (DMRT). Values were considered statistically significant when $P<0.05$ (Duncan, 1957).

3. Results

3.1 *Effect of EMS on plasma glucose and insulin*

Figure 2 demonstrates the level of plasma glucose and insulin in control and experimental animals. The level of plasma glucose was significantly increased whereas the plasma insulin was significantly decreased in diabetic rats. Administration of EMS and metformin to diabetic rats resulted in a significant decrease in level of plasma glucose and significant increase in the levels of insulin. Our previous study also showed similar results upon treatment with EMS in diabetic rats (Pari and Saravanan 2005). This confirms that EMS potentially improved the glycemic control during diabetes. The effect produced by EMS compared with metformin – a reference drug.

3.2 *Effect of EMS on plasma thiobarbituric acid reactive substances and hydroperoxide*

Figures 3 and 4 show the concentration of thiobarbituric acid reactive substances (TBARS) and hydroperoxides in

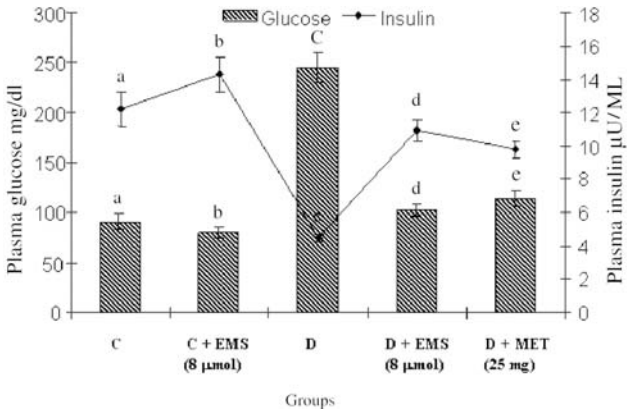

Figure 2. Changes in the levels of plasma glucose and insulin in control and experimental animals.
C, Control; D, diabetic rats; EMS, succinic acid monoethyl ester; MET; Metformin.
Values are given as mean ± SD for 6 rats in each group.
Letters (a-e) on each bars, means with different letters differ significantly at $P<0.05$ (DMRT).

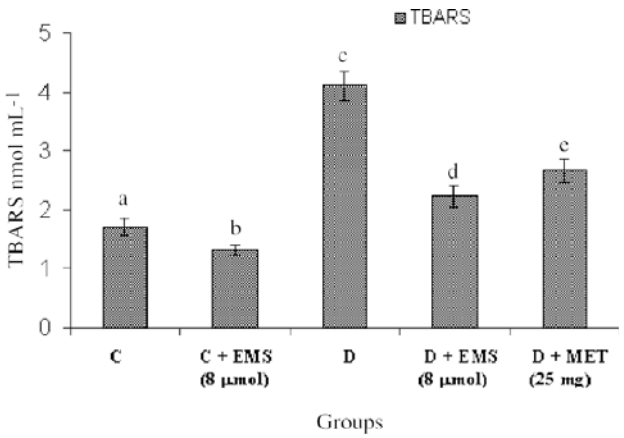

Figure 3. Changes in the levels of plasma tbars in control and experimental animals.
C, Control; D, diabetic rats, EMS, succinic acid monoethyl ester; MET, metformin.
Values are given as mean ± SD for 6 rats in each group.
Letters (a-e) on each bars, means , with different letters differ significantly at $P<0.05$ (DMRT).

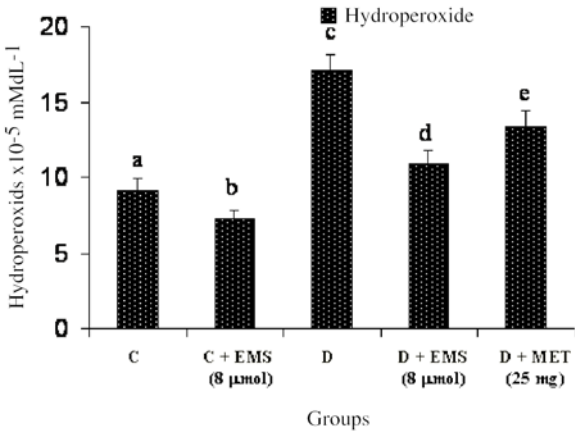

Figure 4. Changes in the levels of plasma hydroperoxide in control and experimental animals.
C, Control, D, diabetic rats, EMS, succinic acid monoethyl ester; MET, metformin.
Values are given as mean ± SD for 6 rats in each group.
Letters (a-e) on each bars, means with different letters differ significantly at $P<0.05$ (DMRT).

plasma of control and experimental animals. There was a significant elevation in plasma TBARS and hydroperoxides during diabetes when compared to the corresponding control group. Administration of EMS and metformin to diabetic rats significantly decreased the lipid peroxidative markers to near normal levels.

3.3 *Effect of EMS on serum lipid profiles and serum lipoproteins*

Table 1 illustrates the effect of EMS on the levels of serum, triglycerides, free fatty acids and phospholipids in control and experimental rats. The levels of all these lipids were significantly increased in diabetic rats whereas the administration of EMS and metformin to diabetic rats significantly decreased the levels of lipids.

Table 2 demonstrates the level of serum total cholesterol and lipoproteins in control and experimental rats. The level of total cholesterol, LDL-C and VLDL-C, were significantly increased whereas the level of HDL-C and the percentage of AAI (ratio of HDL to total cholesterol) were significantly decreased in diabetic rats. Administration of EMS and metformin to STZ-nicotinamide diabetic rats restored all these changes to near normal levels.

4. Discussion

Diabetes is associated with profound alterations in plasma lipid and lipoprotein profile and with an increased risk of coronary heart disease (Betteridge 1997). It is well documented that EMS triggers the proinsulin synthesis and insulin release as glucose-induced insulin synthesis and release (Maechler and Wollheim 2000) MacDonald and Fahien (1988) reported that initiation of insulin release by esters of succinate by mitochondrial metabolisms is sufficient to initiate and support insulin release from β cells (Ainscow *et al* 2000). Daily administration of EMS to diabetic rats for 30 days caused a statistically significant reduction in food and fluid intakes and an increase in the body weight. This could be the result of improved glycemic control produced by EMS (Pari and Saravanan 2005).

There is increasing evidence that lipid peroxidation plays an important role in the premature development of atherosclerosis (Steinberg 1990; Witztum 1994). Abnormally high levels of free radicals, lipid peroxidation and simultaneous decline in antioxidant defense mechanism can lead to damage of cellular organelles and enzymes. Elevated levels of lipid peroxidation in circulation of diabetic rats are one of the characteristic features of chronic diabetes (Feillet *et al* 1999). It has been shown that nonglucidic nutrients decrease lipid peroxidation and so they may protect against atherogenesis (Yanni *et al* 2003). Our study shows that administration of EMS and metformin tend to bring the peroxide back to near normal levels, which indicates that EMS may inhibit oxidative stress.

Hyperlipidemia is a recognized complication of DM characterized by elevated levels of cholesterol, triglycerides and phospholipids; and changes in lipoprotein composition (Segal *et al* 1984). The results of our present study clearly show that EMS has a lipid lowering effect on serum TG,

Table 1. Effect of EMS on serum total cholesterol and lipoproteins in control and experimental rats.

Groups	Cholesterol (mg/dl)	HDL-cholesterol (mg/dl)	LDL-cholesterol (mg/dl)	VLDL-cholesterol (mg/dl)	AAI (%)
Control rats	87.38 ± 3.71[a]	54.40 ± 4.62[a]	18.22 ± 1.55[a]	9.01 ± 0.80[a]	175.30 ± 15.01[a]
Control rats + EMS (8 μmol/g)	80.60 ± 7.94[a]	61.60± 4.47[a]	14.23 ± 1.03[a]	8.58 ± 0.62[a]	202.03 ± 14.67[b]
Diabetic rats	155.05 ± 9.30[b]	20.10 ± 1.20[b]	11 9.14 ± 7.13[b]	16.30 ± 1.07[b]	26.00 ± 1.55[c]
Diabetic rats + EMS (8 μmol/g)	126.40 ± 9.84[c]	46.80 ± 3.64[c]	51.80 ± 4.03[c]	13.14 ± 1.02[c]	92.00 ± 7.16[d]
Diabetic rats + metformin (25 mg/kg)	134.94 ±10.54[c]	37.62 ± 2.94[e]	72.10 ± 5.63[d]	14.87 ± 1.20[d]	76.83 ± 6.00[e]

Values are given as mean ± SD for 6 rats in each group.
[a-e] With in column, means with different letters differ significantly at $P<0.05$ (DMRT).

Table 2. Effect of EMS on serum triglycerides, free fatty acids and phospholipids in control and experimental rats.

Groups	Triglycerides (mg/dl)	Free fatty acids (mg/dl)	Phospholipids (mg/dl)
Control rats	52.08 ± 4.42[a]	76.12 ± 6.47[a]	107.20 ± 10.01[a]
Control rats + EMS (8 μmol/g)	48.46 ± 3.52[a]	62.60 ± 3.43[b]	89.03 ± 7.03[b]
Diabetic rats	85.03 ± 5.09[b]	135.04 ± 8.11[c]	160.35 ± 9.60[c]
Diabetic rats + EMS (8 μmol/g)	60.70 ± 5.02[c]	86.03 ± 7.10[d]	120.31 ± 9.37[d]
Diabetic rats + metformin (25 mg/kg)	74.07 ± 6.07[d]	97.51 ± 8.02[e]	128.05 ± 10.01[d]

Values are given as mean ± SD for 6 rats in each group.
[a-e] With in column, means with different letters differ significantly at $P<0.05$ (DMRT).

TC, VLDL, LDL, free fatty acids (FFA) and phospholipids (PL). There is a substantial evidence that lowering the total cholesterol (TC), particularly LDL level will lead to a reduction in the incidence of coronary heart disease (CHD), which is still a leading cause of death in diabetic patients.

Increased TG and reduced HDL-C levels are the key characteristics of dyslipidemia in type 2 diabetes (Lehto *et al* 1997). Hypertriglyceridemia in type 2 diabetes can result from an increased hepatic VLDL over production and impaired catabolism of triglyceride-rich particles. The function of lipoprotein lipase, key enzyme in removal and degradation of triglycerides from circulation is attenuated by both insulin deprivation and insulin resistance.

The abnormal high concentration of serum lipids in diabetes is mainly due to the increase in the mobilization of FFA from the peripheral depots, since the insulin inhibits the hormone sensitive lipase (Bopanna 1997). Serum-FFA concentration are a result of the balance between the release from lipolysis, neosynthesis and disposal and represent the major determinant of insulin effect on free fatty acid oxidation and non-oxidative metabolism (Bonadonna *et al* 1990).

Hypercholesterolemia and hypertriglyceridemia have been reported to occur in diabetic rats (Chakrabarti *et al* 2003) and significant increase observed in our experiment was in accordance to these studies. Furthermore, increase in circulatory VLDL and their associated triglycerides are largely due to defective clearance of these particles from circulation (Segal 1984). The increase and fall in the individual lipoprotein levels is a reflection of the total serum cholesterol levels: i.e. the levels of HDL-C, LDL-C and VLDL-C increase or decrease with levels of total serum cholesterol, and it is their ratio that determines the pathophysiology of lipoprotein metabolism. As there is a close relationship between elevated serum TC level and the occurrence of atherosclerosis, the ability of the EMS in selective reduction of TC through the reduction of VLDL and LDL components could be beneficial in preventing atherosclerotic conditions and thereby reduce the possibility of CHD in general.

Considering the effect of the EMS on serum HDL, our results clearly show that the level of this lipoprotein fraction increased with this treatment. However, in our observation an improvement of HDL level with a concomitant rise in the ratio of HDL to TC (expressed in terms of AAI) following non-glucidic nutrient treatment may be considered as an expression for a reliable risk assessment factor of diabetes and CHD.

EMS treatment increased the insulin output from pancreas in diabetic animals and insulin activates the enzyme lipoprotein lipase, which hydrolyses lipoprotein-bound TG (Goodman and Gilman 1985; Welihinda *et al* 1982). The strong antihyperlipidemic effect of EMS could also be through its control of hyperglycemia, as this is a major determinant of total and VLDL and TG concentration (Laakso 1995). Administration of EMS normalized these effects, possibly by controlling the hydrolysis of certain lipoproteins and their selective uptake and metabolism by different tissues (Laakso 1995). EMS may have insulinotropic action or synergistic effect on insulin activity.

5. Conclusion

In conclusion, non glucidic nutrient-EMS is a structurally new insulinotropic with intense antihyperglycemic in animal models of type 2 diabetes. Based on our above findings, daily administration EMS by type 2 diabetic subjects will be useful in the prevention and treatment of dyslipidemia associated with diabetes. In addition, it appears that EMS administration could also serve to improve lipid metabolism, as well as inhibit lipid peroxidation in diabetic rats. Future research and clinical trials in this area may lead to use EMS as a new type of therapeutic agent in the treatment of type 2 diabetes.

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