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Effect of dietary tocotrienols (Tocomin) and lovastatin on *ex vivo* and Cu^{++}-mediated *in vitro* susceptibility of LDL, sd-LDL and lb-LDL to oxidation in absence or presence of glucose in diabetic-hyperlipidemic rats

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ABSTRACT

Several studies indicate that increased atherogenecity of LDL during diabetes is associated with a preponderance of small dense (sd)-LDL subpopulation, that is more prone to oxidative modification than large buoyant (lb)-LDL. In this study, we describe the hypoglycemic, hypolipidemic and antioxidant properties of dietary tocotrienols (Tocomin) and Lovastatin supplementation in diabetic-hyperlipidemic rats. Diabetes in rats was induced by streptozotocin (6.0 mg/100 g body wt) and they were treated with 7.0 mg Tocomin or 2.0 mg Lovastatin for 23 weeks. At the end of the study, diabetic control rats had a significant increase in plasma glucose and blood HbA1, plasma TG, TC, VLDL-C, LDL-C, HDL-C, HDL_2-C, HDL_3-C and non-HDL-C. Tocomin and Lovastatin mediated a substantial decline in glucose and HbA1 to near normal levels, Our ex vivo results demonstrate that even in normal animals, relative to lb-LDL, the in vivo oxidizability of sd-LDL, measured as base line diene conjugation (BDC) was higher by 2.90-fold, which was further increased to 3.3-fold due to diabetes. Similarly, in comparison to a lag phase value of 53 min for lb-LDL in normal rats, sd-LDL lag phase was reduced to only 15.0 min, which was further shortened to 9.6 min in diabetic rats, indicating a substantially enhanced Cu^{++}-catalyzed sd-LDL oxidizability. In conclusion, although multiple therapeutic benefits of Tocomin and Lovastatin are comparable, considering the host of side effects exhibited by Lovastatin, daily intake of dietary tocotrienols will be useful in the prevention and treatment of diabetes, diabetes linked hyperlipidemia with and without CHD and atherosclerosis.

Keywords: Small dense LDL; Tocotrienols; Lovastatin; Large buoyant LDL; BDC; diabetic-hyperlipidemic rats.

INTRODUCTION

The prevalence of diabetes mellitus (DM) for all age group worldwide is quite high. In India the total number of subjects with DM is projected to increase from 31.7 million in 2000 to 79.4 million in 2030 [1]. Several studies have established that in both men and women DM is a major

independent risk factor for cardiovascular disease (CVD) [2-4]. Hyperglycemia is the most important factor in the onset and progress of diabetic complications mainly by producing oxidative stress [5]. Altered cellular metabolism caused by hyperglycemia play an important role in increasing the risk of cardiovascular, renal, ophthalmic and neurological complications of DM [6]. Although blood glucose is known to be highly predictive of microvascular disease, the contribution of all the measured risk factors can explain no more than 25 % of the excess macrovascular coronary heart disease (CHD) associated with diabetes [7]. The excessive non-enzymatic glycosylation of proteins associated with markedly increased free radical production, stimulate formation of glycosylated hemoglobin and advanced glycosylation end products (AGEs), which cause extensive cellular and tissue damage, including vascular injury [8]. The dyslipidemic profile of diabetics includes increased levels of plasma TG, TC, VLDL-C, LDL-C and small dense (sd)-LDL-C, increased glycation of LDL and decreased plasma antiatherogenic HDL concentration [4]. Previous reports indicate that altered plasma lipoprotein profile in the excess atherosclerosis associated with DM may be most critical, because at any total cholesterol level, in comparison to non diabetic subjects, diabetic patients have 3-to 5-fold higher CHD mortality rates [9]. In addition, 80 % of all type 2 diabetics will die of an atherosclerotic event [3-4,10]. It is possible that increased atherogenecity of LDL during DM is associated with a preponderance of sd-LDL subpopulation, that is more prone to oxidative modification than large buoyant (lb)-LDL [11]. Lipoprotein profiles that are relatively rich in sd-LDL particles are associated with up to 3-fold greater risk of myocardial infarction (MI) than those mainly consist of lb-LDL particles [12]. Recently, Koba et al. [13] have reported that prognosis of CHD was closely linked not to the LDL particle size but to the concentration of highly atherogenic sd-LDL. To the best of our knowledge, no therapeutic interventions to specifically reduce the elevated levels of highly atherogenic sd-LDL in diabetic-hyperlipidemic patients or animals have been reported [11,13]. Furthermore, chronic diabetes may enhance oxidative stress not only through the increased production of ROS but also through weakening the antioxidant defense system. In this context antioxidant role of serum HDL-complexed arylesterase/paraoxonase enzyme in the protection of LDL as well as HDL from oxidative modification is noteworthy [14]. The involvement of increased oxidative stress in diabetes is supported by several data such as increased concentration of plasma lipid peroxidation products, namely, conjugated diene, lipid hydroperoxide and TBARS and decreased levels of antioxidants [15-16]. Otero et al. [17] have demonstrated that the consumption of vitamin E in normal human LDL subjected to Cu^{++}-induced oxidation was delayed by a glucose concentration frequently found in subjects with poorly controlled diabetes. However, high glucose concentration accelerated the LDL oxidation once LDL associated vitamin E was consumed, thus glucose may act as either antioxidant or prooxidant depending on cell concentration of vitamin E. Since DM is associated with hyperglycemia as well as hyperlipidemia, oral hypoglycemic agents along with insulin and hypolipidemic drugs usually statins are used for its treatment. However, insulin along with other hypoglycemic drugs commonly used in the treatment of hyperglycemia in diabetics are unable to restore normal pattern of glucose homeostasis on permanent basis. Furthermore, imperfect normalization of glucose metabolism by replacement insulin therapy may alter the concentrations and compositions of potentially atherogenic lipoproteins [18]. Moreover, these drugs are quite expensive and exert a host of side effects [19, 2-3].

Thus, there is need to seek newer and alternative approaches for effective therapy in the management of hyperglycemia along with dyslipidemia. The tocotrienol isomers (α-, β-, γ-, δ-) are naturally occurring analogues of tocopherol isomers (vitamin E), found mainly in rice bran and palm oil. Tocotrienols (T3s) differ from tocopherols (Ts) by possessing three double bonds in phytyl side chain. Unlike Ts, T3s have been shown to have an intrinsic hypocholesterolemic activity in animals and humans. Previously published reports indicate a strong hypolipidemic

effect of tocotrienol rich fraction (TRF) or purified tocotrienols (Tocomin) in various animal models as well as hyperlipidemic humans [20-21]; normolipidemic and hyperlipidemic rats and humans [22-25] and type 2 diabetic patients with hyperlipidemia [26]. A potent hypocholesterolemic, antiatherosclerotic and antioxidant effect of TRF/Tocomin in cholesterol/oxidized cholesterol-induced atherosclerotic rabbits have also been reported previously [27-28]. There is only one report [29], indicating some fasting blood glucose and HbA_1 lowering effect of dietary TRF, when fed to STZ-induced diabetic rats for 12 weeks. However, TRF mediated reduction in glucose from 556 to 376 mg/dl, and in HbA_1 from 13.1 to 10.0 mg/dl, indicate that these parameters were still in diabetic range [29]. However, a detailed coordinated investigation pertaining to combined hypoglycemic, hypolipidemic and anti-lipid/lipoprotein-peroxidative impact of tocotrienols in diabetic patients or diabetic animals has been lacking. Statins including Lovastatin are potent competitive inhibitors of HMG-CoA reductase and are highly effective lipid lowering agents [30,16]. They are universally marketed and used by both nondiabetic and diabetic-hyperlipidemic patients with and without CHD. Few scattered reports indicate the antioxidant properties of Lovastatin in hyperlipidemic-diabetic hamsters [16], atherosclerotic rabbits [31] and in patients with hypercholesterolemia [32]. However, hypoglycemic effect of Lovastatin was not observed in hyperlipidemic-diabetic hamsters [16].

We have investigated the hypoglycemic, hypolipidemic and antioxidant impacts of Tocomin and Lovastatin when fed to diabetic-hyperlipidemic rats for 14 weeks and compared with diabetic control rats. The efficacy of feeding 6.0 mg Tocomin or 0.50 mg Lovastatin/rat/day in preventing the increase in fasting plasma glucose, glycosylated HbA_1, TG, TC, VLDL-C, LDL-C, sd-LDL-C, lb-LDL-C, HDL-C, and its subfractions, HDL_2-C and HDL_3-C including non-HDL-C levels in diabetic-hyperlipidemic rats was investigated. In addition, quantification of cholesterol and apoB content in LDL and its subpopulation, sd-LDL and lb-LDL of diabetic-hyperlipidemic rats treated with Tocomin or Lovastatin has been done. In order to understand the mechanism(s) of lipid lowering actions of Tocomin and Lovastatin in these rats, we have measured the enzymatic activity of hepatic HMG-CoA reductase. The effect of long-term diabetes on plasma total antioxidants, arylesterase activity, plasma lipid peroxidation products, that is, conjugated diene, lipid hydroperoxide and TBARS were determined. Furthermore, therapeutic role of Tocomin and Lovastatin in the normalization of the above parameters was investigated. In addition, antioxidant impact of Tocomin and Lovastatin on base line levels of ex vivo diene conjugation and lag phase time of in vitro Cu^{++}-induced oxidation of LDL, sd-LDL and lb-LDL both in absence and the presence of glucose was undertaken.

EXPERIMENTAL SECTION

Chemicals

Twenty five percent palmvitae oil suspension of tocotrienols containing d-α-tocopherol and purified individual d-α-tocotrienol (80 %), d-γ- tocotrienol (90 %), d-δ-tocotrienol (60 %), and d-α-tocopherol (60 %) as well as RBD palm olein was supplied as a gift from CAROTECH BHD, Chemor, Malaysia. Tocomin^R suspension (250 mg/g) contained 6.4 % d-α-tocotrienol, 1 % d-β-tocotrienol, 10.2 % d-γ-tocotrienol, 3.2 % d-δ-tocotrienol and 5.7 % d-α-tocopherol. Cholesterol lowering drug, Lovastatin, was a gift from Saimira Innoform Pvt. Ltd., Chennai, India. All other chemicals and reagents used in this study were of analytical grade.

Animals/treatment

Male albino rats, weighing about 190-200 g were purchased from IVRI, Barely,UP, were conditioned to animal house environment prior to the experiment. The protocol of the study was

approved by the animal ethical committee of the J N Medical College. The rats were given pelleted rat chow and water ad libitum. In order to induce experimental diabetes, twenty five overnight fasted rats were injected with streptozotocin (STZ, freshly dissolved in 10 mM citrate buffer, pH 4.5, 6.0 mg/100 g body wt) intraperitonially [33]. Rats in normal control group were injected with buffer only. After 15 days, twenty two rats showed average plasma glucose level of 266 mg/dl. These rats were classified as diabetic and included in the present investigation. Tocomin and Lovastatin suspension in palmvitae oil was administered through gastric intubation in two divided doses (morning and evening) of 0.5 ml each /rat/day, containing 3.5 mg Tocomin or 1.0 mg Lovastatin.

Experimental design

In normal control group (N-C), eight rats were given 0.5 ml palmvitae oil through gastric intubation for 23 weeks. Eight rats in diabetic control group (D-C) were administered 0.5 ml palmvitae oil. In diabetic Tocomin treated group (D-TT), seven rats were given 7.0 mg (0.5 ml, morning and evening) of Tocomin, whereas, seven rats in diabetic Lovastatin treated group (D-LT) were fed 2.0 mg (0.5 ml morning and evening) for 23 weeks. At the end of the treatment, overnight fasted rats in each group were anaesthetized and blood drawn by cardiac puncture. The blood from each rat in a given group was collected using heparin as anticoagulant. Blood was mixed gently by inversion 2-3 times and immediately stored at 4˚C for 30 min. The samples were centrifuged at 2,500 rpm for 30 min. Plasma was aliquoted and either stored at 4˚C or frozen at -20˚C for future use.

Measurement of glucose, hemoglobin, glycosylated hemoglobin and lipids

Fasting plasma glucose levels were determined by an enzymatic method using a kit from Autospan, New Delhi. The quantitative determination of hemoglobin in blood was done according to the procedure as described in commercial kit from Ranbaxy. The quantitative determination of glycosylated hemoglobin (HbA_1) in erythrocytes hemolysate was done by the colorimetric method of Nayak and Pattabiraman [34]. Triglycerides in plasma samples were determined by the enzymatic method as per kit instructions. Plasma VLDL-C was determined by dividing plasma triglyceride values (mg/dl) by a factor of 5 as described by Friedewald et al. [35]. Plasma LDL was isolated by precipitation method as described by Wieland and Seidel [36]. Small dense (sd)-LDL and large buoyant (lb)-LDL fractions isolated from LDL according to the procedure described by Hirano et al. [11]. Isolation of HDL and its subfractions, HDL_2 and HDL_3 were done by dual-precipitation method as described by Patsch et al. [37]. Total cholesterol content in plasma, LDL, sd-LDL, lb-LDL, HDL, HDL_2 and HDL_3 subfractions were quantified as described by Annino and Giese [38]. Non-HDL-cholesterol concentrations were calculated as the difference between TC and HDL-C.

Measurement of plasma lipid peroxides and oxidation of LDL, sd-LDL and lb- LDL

The susceptibility of isolated LDL, sd-LDL and lb-LDL to oxidation was assessed by determining the lag phase of conjugated diene formation using the method of Esterbauer et al. [39]. The formation of conjugated diene was calculated by using an extinction coefficient of 2.52 $x10^4$ $M^{-1}cm^{-1}$and expressed as nmole malondialdehyde (MDA) equivalents per mg LDL, sd-LDL or lb-LDL protein. In experiments, where effect of glucose on in vitro oxidation of the above lipoproteins was assessed, 2.5 mg glucose per ml was added.

Protein estimation

The protein was determined by the method of Bradford [40], using bovine serum albumin as standard. Aliquots of plasma, LDL, sd-LDL, lb-LDL, HDL, HDL_2 and HDL_3 were first

precipitated with 10 % TCA. The protein pellets were dissolved in 0.5 N NaOH and suitable aliquots were used for protein determination.

Statistical evaluation

Statistical analysis of data was done by employing two-tailed Student t- test as described by Bennet and Franklin [41]. P-values less than 0.05 were considered significant.

DISCUSSION

The present study demonstrates that rats after 14 weeks of STZ administration became substantially hyperglycemic as well as hyperlipidemic. Daily treatment of these rats with 7.0 mg Tocomin (tocotrienols) or 2.0 mg Lovastatin for 23 weeks lowered the elevated fasting blood glucose and HbA_1 levels to near normal values. Nazaimoon and Khalid [29] have initially reported that feeding a diet supplemented with TRF (1g/kg) to STZ-induced diabetic rats for 12 weeks was associated with some reduction of blood glucose (32 %) and HbA_1 (24 %) levels. Although, in principle our results are in agreement with their findings but the hypoglycemic effect of TRF was minimal and both the glucose and HbA_1 levels after TRF treatment were fully within diabetic range. In another report [16], feeding of vitamin E (d-l-α-tocopheryl acetate), probucol or Lovastatin supplemented diet to hyperlipidemic-diabetic hamsters failed to reduce elevated fasting blood glucose. However, among other statins, only pravastatin has been shown to exhibit beneficial effects on glucose metabolism especially in the postprandial state in CAD patients with impaired glucose tolerance [42]. Our results imply that there is a significant association between improved glycemic control and supplementation of Tocomin or Lovastatin. Although a detailed investigation is needed to elucidate the possible mechanism(s) involved, it is intriguing to postulate that both Tocomin and Lovastatin, being potent antioxidants, may have effectively protected the β-cells from total damage by STZ and/or glucotoxicity. Thus, in the presence of residual functional islet cells, the blood glucose and HbA_1 levels in both the treated groups were reduced. Our results appear to be in agreement with an earlier study, where Vitamin E could also exert it's protective effect indirectly by reducing free radical mediated damage to islet of β-cells and thus improving insulin action [43]. Our data also suggest that the amount of total antioxidants present in the circulation or perhaps in the tissues during the chemical insult may be an important factor. In diabetic rats, plasma total antioxidants level was significantly reduced, which was substantially increased following treatment with Tocomin or Lovastatin. Therefore, similar to d-α-tocopherol, dietary tocotrienols or Lovastatin could also exert their protective effects indirectly by improving insulin action as antioxidants, leading to normalization of glycemic state.

As expected, treatment of diabetic-hyperlipidemic rats with Tocomin and Lovastatin significantly prevented the increase in plasma TG, TC, VLDL-C, LDL-C, HDL-C and its subfractions, HDL_2-C and HLD_3-C including atherogenic non-HDL-C levels. The cholesterol content of HDL_2-C, which is considered to be a strong predictor of presence and extent of CAD [44], was significantly and equally reduced in both the treated groups. However, Lovastatin, in comparison to Tocomin, was more effective in selectively reducing the atherogenic non-HDL-C, whereas, levels of plasma antiatherogenic HDL-C and HDL_3-C in Lovastatin treated diabetic rats were significantly higher than Tocomin treated group. The preferential increase in antiatherogenic HDL-C in Lovastatin treated rats is consistent with a similar increase in HDL-C levels of Lovastatin treated hyperlipidemic-diabetic hamsters [16]. In the present study, chronic diabetes was associated with a substantial increase in plasma HDL-C and its subfractions, HDL_2-C and HDL_3-C levels, which is consistent with other reports, where a significant increase in

HDL-C level in diabetic rats has been reported [45-46]. Since these diabetic rats are considered as essentially models of type 1 diabetes [47], an increased plasma HDL-C level has been reported in type 1 diabetic patients [48-49]. However, in contrast to our results, type 2 diabetics [4], as well as hyperlipidemic-diabetic hamsters [16] exhibited a decrease in plasma HDL-C.

Table 1.Effect of tocomin and lovastatin on plasma glucose, blood hemoglobin, glycosylated hemoglobin and total antioxidants

Group	Glucose* (mg/dl)	Hemoglobin† (g/dl)	HbA_1† (mg/dl)	Total antioxidants† (µmole/dl)
N-C	74.6±6.3	16.63±0.10	4.15±0.02	56.28±1.17
D-C	296.3±41.1 (+297.18 %)[a]	11.23±0.12 (-28.15 %)[b]	9.71±0.43 (+133.97 %)[a]	35.06±2.01 (-37.70 %)[a]
D-TT	108.7±23.2 (-63.31 %)[a]	15.62±0.18 (+39.09 %)[a]	5.38±0.09 (-44.59 %)[a]	68.19±2.03 (+94.49 %)[a]
D-LT	112.8±15.9 (-61.93 %)[a]	16.28±.03 (+44.96 %)[a]	4.98±0.04 (-48.71 %)[a]	61.70±2.18 (+75.98 %)[a]

** Data are mean ± S.D. from plasma of individual rats in each group.*
† Data are mean ± S.D. from pooled blood or plasma samples in each group.
N-C: normal control; D-C: diabetic control; D-TT: diabetic Tocomin treated; D-LT: diabetic Lovastatin treated.
[a] Significantly different from N-C and D-C at p<0.001.
[b] Significantly different from N-C at p<0.05.

Table 2. Impact of tocomin and lovastatin on plasma and lipoprotein lipids

Parameters	N-C	D-C	D-TT	D-LT
Triglycerides	88.7±8.1	160.1±1.3 (+80.49 %)[a]	115.2±2.6 (-28.4 %)[a]	103.6±3.1 (-35.29 %)[a]
Total cholesterol	134.7±12.0	240.6±2.8 (+78.61 %)[a]	171.3±1.3 (-28.80 %)[a]	162.5±3.4 (-32.46 %)[a]
VLDL-cholesterol	19.62±2.31*	35.71±019 (+82.00 %)[a]	24.36±0.13 (-31.78 %)[a]	21.63±0.42 (-39.42 %)[a]
LDL-cholesterol	96.28±2.961	162.21±3.11 (+68.47%)[a]	119.68±1.71 (-26.21%)[a]	110.66±3.21 (-31.77 %)[a]
HDL-cholesterol	35.41±0.11	72.18±1.1 (+103.84 %)[a]	41.56±2.18 (-42.42 %)[a]	44.82±0.28 (-37.90 %)[a]
HDL_2-cholesterol	11.19±0.23	23.82±0.46 (+112.86 %)[a]	13.66±0.92 (-42.65 %)[a]	13.16±0.26 (-44.75 %)[a]
HDL_3-cholesterol	23.69±0.73	43.29±0.19 (+82.73 %)[a]	29.17±0.63 (-32.61 %)[a]	33.56±0.39 (-22.47 %)[a]
Non-HDL-cholesterol	117.6±3.6	188.7±3.1 (+60.45 %)[a]	132.6±5.1 (-29.72 %)[a]	125.6±6.2 (-33.43 %)[a]

** Values are mean (mg/dl) ± S.D. from pooled plasma samples in each group.*
[a] Significantly different from N-C at p<0.001.
[a] Significantly different from D-C at p<0.001.

Consistent with published reports [11,13] that relative to lb-LDL, the concentration of more atherogenic sd-LDL was substantially increased in patients with diabetes, CHD alone or diabetes with CHD, in our study, sd-LDL-C and sd-LDL-apoB levels of diabetic-hyperlipidemic rats were increased by 210 % and 153 %, respectively. Thus, > 60 % of LDL-C and LDL-apoB were recognized in sd-LDL fraction of diabetic rats. Treatment of diabetic rats with Tocomin or Lovastatin significantly reduced both the cholesterol and apoB content of sd-LDL, as well as their percent share of LDL, close to normal control values. Based on these results one can conclude that cholesterol and apoB content of sd-LDL as well as their percent share of LDL have always increased or decreased in tandem, indicating a very good correlation between sd-LDL-C

and sd-LDL-apoB values. It is interesting to mention that sd-LDL is known to be generated from large triglyceride-rich VLDL particle, production of which is enhanced by insulin resistance during diabetes, thus resulting in an increased prevalence of sd-LDL [50-51]. Our results are consistent with these findings, showing a significant increase in the levels of plasma TG-rich VLDL-C (82 %), TG (80 %) and sd-LDL-C (210 %) in chronic diabetic rats.

Table 3.Effect of tocomin and lovastatin on cholesterol and apoB content of LDL sd-LDL and lb-LDL[*]

Parameters	N-C	D-C	D-TT	D-LT
LDL-C	94.62±5.32	167.29±2.12 (+76.80 %)[a]	121.16±2.10 (-27.61 %)[a]	109.38±2.36 (-34.86 %)[a]
LDL-apoB	138.42±6.22	153.61±2.13 (+10.97 %)[b]	139.268±2.51 (-9.06 %)[a]	128.56±1.11 (-16.30 %)[a]
Sd-LDL-C	33.43±2.18	103.78± 0.12 (+210.43 %)[a]	55.62±2.3 (-46.40 %)[a]	53.61± 0.86 (-48.34 %)[a]
% LDL-C	33.62±0.69	68.09±0.32 (+102.58 %)[a]	42.49±2.12 (-37.59 %)[a]	44.06±0.18 (-35.29 %)[a]
Sd-LDL-apoB	37.28±5.19	94.316±2.28 (+152.57 %)[a]	54.86±3.11 (-41.73 %)[a]	57.32±2.16 (-39.12 %)[a]
% LDL-apoB	29.21±2.39	66.18±0.46 (+126.56 %)[a]	42.38±0.76 (-35.96 %)[a]	49.06±0.38 (-25.86 %)[a]
Lb-LDL-C	66.22±2.11	68.41±0.11 (+3.30 %)[c]	73.21±0.67 (+6.85 %)[a]	62.48±0.33 (-8.66 %)[a]
% LDL-C	66.72±2.00	40.01±0.86 (-40.03 %)[a]	60.35±0.18 (+50.83 %)[a]	59.02±2.01 (+47.51 %)[a]
Lb-LDL-apoB	94.23±7.21	56.29±3.11 (-40.26 %)[a]	85.41±1.21 (+51.73 %)[a]	76.54±2.11 (+35.79 %)[a]
% LDL-apoB	72.31±1.21	40.28±2.01 (-44.29 %)[a]	63.46±2.10 (+57.54 %)[a]	61.67±1.89 (+53.10 %)[a]

[*] *Values are mean (mg/dl) ± S.D. from pooled plasma samples in each group.*
[a] *Significantly different from N-C and D-C at p<0.001.*
[b] *Significantly different from N-C at p<0.05.*
[c] *Not significant from N-C.*

Therefore, the greater atherogenic potential of sd-LDL, in comparison to lb-LDL, may explain the higher incidence of CHD in diabetic patients than in isolated hypercholesterolemia [11,13]. In addition, because of greater preponderance of sd-LDL, a moderately high LDL-C (between 130 and 160 mg/dl) in a type 2 diabetic patient is equivalent to a much higher LDL-C in terms of CHD risk for a nondiabetic subject [2,4]. Therefore, the primary target of therapy in type 2 diabetic patients should be the lowering of highly atherogenic sd-LDL subpopulation. However, no therapeutic interventions to reduce the elevated levels of sd-LDL-C in diabetic-hyperlipidemic patients or animals have been reported [11,13]. In the present study, which represents an initial demonstration, administration of dietary tocotrienols (Tocomin) or Lovastatin to diabetic rats was associated with a concomitant and significantly higher decline in the cholesterol and apoB concentrations of more atherogenic sd-LDL subspecies than LDL.

An increasing number of studies have pointed out a possible role of LDL peroxidation in the occurrence of atherogenic lesions, which are one of the most frequent complications of diabetes [52]. Our results show that due to substantial increase in oxidative stress along with the depletion of endogenous plasma antioxidants in diabetic-hyhyperlipidemic rats, the ex vivo base line diene conjugation (BDC) level of sd-LDL in comparison to lb-LDL BDC value, was higher by more than 2.90-fold, indicating a markedly enhanced susceptibility of sd-LDL to in vivo oxidation. Similarly, treatment of diabetic rats with Tocomin or Lovastatin reduced the ex vivo BDC levels

of sd-LDL, lb-LDL and LDL, with a maximum effect on sd-LDL. Consistent with ex vivo BDC levels of LDL, sd-LDL, and lb-LDL, susceptibility of these particles to Cu^{++}-induced oxidation, as measured by their lag time, was decreased in diabetic rats. It is important to mention that in comparison to a lag phase value of 95 min for LDL and 53 min for lb-LDL in normal rats, the lag phase of sd-LDL was only 15.0 min, indicating a substantially increased in vitro oxidative susceptibility to Cu^{++}-induced oxidation. In treated groups, both Tocomin and Lovastatin increased the resistance of sd-LDL to oxidative modification, as shown by an increase in lag time from a value of 9.6 min in D-C to 13.2 and 12.8 min respectively. In contrast to sd-LDL, the lag time of lb-LDL was reduced from 53 min in N-C to 41 min in D-C, which was fully restored to a normal value of 54 and 53 min in both Tocomin or Lovastatin treated rats. Consistent with known property of glucose that it may act either as LDL antioxidant or prooxidant depending on the vitamin E content of LDL [17], presence of glucose (2.5 mg/ml) during Cu^{++}-induced oxidation of LDL, sd-LDL and lb-LDL from normal rats, further reduced their lag phases. However, in diabetic rats, which were deficient in antioxidants, and had a high plasma glucose level, addition of glucose,

Table 4.Impact of tocomin and lovastatin on plasma lipid peroxidation products in diabetic-hyperlipidemic rats[*]

Group	Conjugated diene	Lipid hydroperoxide	TBARS
N-C	11.68±0.26	1.179±0.058	1.492±0.97
D-C	16.98±1.22 (+45.37 %)[a]	1.976±0.038 (+67.59 %)[a]	2.927±0.086 (+96.17 %)[a]
D-TT	14.06±0.61 (-17.19 %)[a]	1.489±0.069 (-24.64 %)[a]	2.198±0.063 (-24.90 %)[a]
D-LT	14.80±0.39 (-12.83 %)[b]	1.599±0.062 (-19.07 %)[a]	1.989±0.079 (-32.04 %)[a]

[*] Values are mean (µmole/dl) ± S.D. from pooled plasma samples in each group.
[a] Significantly different from N-C and D-C at p<0.001.
[b] Significantly different from D-C at p<0.05.

Table 5 Effect of tocomin and lovastatin on ex vivo and Cu^{++}-mediated in vitro susceptibility of LDL, sd-LDL and lb-LDL to oxidation in absence or presence of glucose in diabetic-hyperlipidemic rats

Group	LDL oxidation		LDL oxidation with glucose		Sd-LDL oxidation		Sd-LDL oxidation with glucose		Lb-LDL oxidation		Lb-LDL oxidation with glucose	
	BDC†	Lag phase‡	BDC	Lag phase	BDC	Lag phase	BDC	Lag phase	BDC	Lag phase	BDC	Lag phase
N-C	192	95	216	58	241	15.0	269	11.2	83	53	81	42
D-C	302 (+57.29%)[τ]	53	309 (+43.05%)[τ]	47	401 (+66.39%)[τ]	9.6	417 (+55.01%)[τ]	7.3	118 (+42.16%)[τ]	41	117 (+44.44%)[τ]	42
D-TT	275 (+43.22%)[τ]	59	276 (+27.77%)[τ]	52	316 (+31.12%)[τ]	13.2	332 (+23.42%)[τ]	12.7	109 (+31.32%)[τ]	54	108 (+33.33%)[τ]	44
D-LT	244 (+27.08%)[τ]	72	249 (+15.27)[τ]	61	295 (+22.40%)[τ]	12.8	314 (+16.72%)[τ]	13.1	105 (+26.50%)[τ]	53	1108 (+33.33%)[τ]	47

[*] Values are obtained from LDL, sd-LDL and lb-LDL oxidation in absence or presence of 2.5 mg/ml glucose.
† Base line diene conjugation values are expressed as nmole malondialdehyde (MDA) equivalents/mg protein.
‡ The lag phase is defined as the interval between the intercept of the tangent of the slope of the curve with the time expressed in minutes.

further mediated a prooxidant effect only on sd-LDL lag phase with no effect on lb-LDL and LDL. This preferential prooxidant effect of glucose on lag phase of sd-LDL from normal and diabetic rats is consistent with its enhanced susceptibility to both ex vivo and in vitro oxidation, relative to lb-LDL. In Tocomin or Lovastatin treated diabetic rats, due to the presence of high

plasma concentrations of antioxidants, tocotrienols (Tocomin)/Lovastatin, prooxidant effect of glucose was blocked and the lag phase time of sd-LDL was increased. A markedly increased in vivo and in vitro oxidizability of sd-LDL subpopulation in diabetic rats, suggests that it may be more prone to further oxidation in the vessel wall of diabetic subjects as reported by Chait et al. [53]. Tribble et al. [54] also reported that in subjects with either the lb-LDL or the sd-LDL phenotype, oxidative susceptibility increased, and antioxidant concentrations decreased, from IDL to lb-LDL to sd-LDL. Previously published reports indicate that difference in the oxidative susceptibility between sd-LDL and lb-LDL, as seen in the present study, is apparently due to their physical-chemical properties. In comparison to lb-LDL, sd-LDL has reduced content of antioxidants, free cholesterol, and increased amount of more oxidizable polyunsaturated fatty acids and preformed hydroperoxides [55-56]. It is likely that presence of extremely low antioxidant content in sd-LDL subspecies of diabetic rats was apparently responsible for the prooxidant effect of glucose on sd-LDL, which was partially blocked in Tocomin or Lovastatin treated diabetic rats. Consistent with the findings of Otero et al. [17] that glucose during Cu^{++}-mediated oxidation of LDL, may act either as antioxidant or prooxidant, depending on the LDL associated α-tocopherol content, our ex vivo and in vitro results also show similar dual effect of glucose on the oxidative modification of LDL subfractions, which may be dependent on their antioxidant, that is, tocotrienols (Tocomin) or Lovastatin content. An enhanced susceptibility to in vivo and in vitro oxidative modification of sd-LDL, relative to lb-LDL, which was significantly blocked in diabetic-hyperlipidemic rats treated with Tocomin or Lovastatin, represents an initial demonstration.

The combined results demonstrate that a strong hypoglycemic and hypolipidemic effect of dietary tocotrienols (Tocomin) and Lovastatin coupled with their potent antioxidative properties can provide additional benefits in the inhibition of oxidative stress, particularly in the resistance of highly atherogenic sd-LDL to oxidation and hence in the prevention and treatment of both type 1 and type 2 diabetes, diabetes linked hyperlipidemia with and without CHD and atherosclerosis. However, considering the host of side effects exhibited by Lovastatin, use of dietary tocotrienols (Tocomin) as a multitherapeutic agent should be preferred. In addition, daily use of Tocomin as a dietary supplement will be highly cost effective as well as a good source of vitamin E.

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