ISSN : 0974 - 7532 Volume 8 Issue 4

Research & Reviews in

BioSciences

Regular Paper

RRBS, 8(4), 2014 [133-151]

Effect of dietary tocotrienols and lovastatin on *ex vivo* and copper-mediated *in vitro* oxidative modification of LDL, LB-LDL and more atherogenic sd-LDL on rats, expose to cigarette smoke

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ABSTRACT

Several epidemiologic studies have established a strong and consistent link between cigarette smoking and increased cardiac morbidity and mortality. Other studies show that in addition to substantial increase in oxidative stress, certain other compounds of cigarette smoke, such as nicotine and carbon monoxide play a role in atherogenesis. In response to proatherogenic action of cigarette smoke. In this study, our results show a substantial increase of 292 % in sd-LDL and 82 % in sd-LDL-apoB of S-C rats, as compared to respective normal control values. Thus, >84 % of LDl-C and >58 % of LDL-apoB were recognized in sd-LDL subpopulation. In contrast to sd-LDL, percent share of cholesterol and apoB content of LDL was significantly reduced by 36 % and 27 % in lb-LDL, indicating a preferential shift of less atherogenic lb-LDL to more atherogenic sd-LDL subspecies in hyperlipidemic rats. Tocomin (Tocotrienols) or Lovastatin treatment significantly normalized (93-99 %) both the cholesterol and apoB concentrations of sd-LDL and lb-LDL, as well as their percent share of LDL, similar to their counterparts in N-C. We initially recommend daily supplementation of young smokers with dietary tocotrienols (Tocomin). In conclusion, based on Tocomin mediated multiple therapeutic benefits, described in the present study, daily intake of tocotrienols as a dietary supplement by novice/young/old moderate or heavy smokers as well as chronic smokers including passive smokers may be useful in the prevention and treatment of tobacco-induced dyslipidemia/hyperlipidemia and atherosclerosis. In addition, daily use of dietary tocotrienols will be efficacious, cost effective, and a good source of vitamin E.

KEYWORDS

Cigarette smoke;
Dietary tocotrienols (Tocomin);
Lovastatin;
LDL;
lb-LDL;
sd-LDL;
Oxidative stress;
Novice smokers;
Nicotine;
Hyperlipidemia and atherosclerosis.

INTRODUCTION

A World Health Organization (WHO) survey projects that at least one billion people could die this century because of tobacco-related illnesses compared to 100 million in the 20th century[101]. In the year 2006, WHO reports that 47 % of men and 12 % of women are smokers, and smoking is responsible for the death

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of about 3.5 million subjects every year, meaning about 10,000 death cases per day. In the year 2000, 4.83 million premature deaths in the world were attributable to smoking; 2.40 million in developing countries and 2.43 million in industrialized countries[100]. At present, approximately 5 million people die each year from tobacco-related illnesses, and if current trends continue, this Figure will rise to about 10 million by 2025, most of the increase being in the Third World, where the annual number of deaths from smoking is predicted to rise to 7 million (UNS, 2004). A recent most exhaustive study of smoking, jointly conducted by scientists from India, Canada and the UK indicate that in India, tobacco fatalities could touch one million a year by 2010- of which 70 per cent would be young people-as against the current Figure of 90,000[51].

Oxidative modification of LDL may be a key early step in the pathogenesis of atherosclerosis[30]. Evidence of oxidized LDL has been obtained from arterial walls of animal models of atherosclerosis and of CHD patients[41]. Cigarette smoke contains numerous free radical species that can contribute to the oxidation of LDL *in vitro*[27], although data are conflicting[25]. LDL isolated from smokers may be more susceptible to oxidation than LDL from nonsmokers[79] however, others did not find any difference in LDL oxidizability between smokers and nonsmokers[81]. Aqueous extracts of cigarette smoke can produce oxidative modification of LDL *in vitro*[52,109] and *in vivo*[103,104,106,107] in animal models. In addition, peroxynitrite generated in the extracts of cigarette smoke is involved in oxidative modification of apoB of LDL *in vitro*[103-105]. It is possible that increased atherogenecity of LDL in smokers is associated with a preponderance of small dense (sd-) LDL subpopulation, that is highly susceptible to oxidative modification than large buoyant (lb-) LDL[90-94] . Several studies have reported a 2- to 3-fold increase in CHD risk among patients with a predominance of sd-LDL particles[11,12]. Similarly, Quebec cardiovascular study has confirmed that a predominance of sd-LDL is a strong and independent predictor of CHD in the first seven years of follow-up[83]. Recently[53], have reported that the progression of CHD was closely linked not to the LDL particle size, but to the concentration of sd-LDL. Therefore, sd-LDL has been highlighted as a new strong and useful marker for the risk of CHD. In the only published report[57], have demonstrated that prevalence of sd-LDL is increased in smokers. In addition, prevalence as well as concentration of sd-LDL was significantly increased in type 2 diabetic hyperlipidemic subjects with CHD, as well as in subjects with various types of hyperlipidemia[48]. The involvement of increased oxidative stress in smokers is supported by an increased concentration of lipid peroxidation product, MDA, and decreased levels of antioxidants in the plasma of smokers[20,21,54,82]. However, several studies showed a nonsignificant increase in MDA in smokers vs. nonsmokers[66].

Oxidation takes place when naturally occurring antioxidant agents such as Vitamin E and β-carotenes that normally inhibit LDL oxidation do not occur. The term "antioxidant" refers to any molecule capable of stabilizing or deactivation of free radicals before they attack cells. The tocotrienols isomers (α-, β-, γ- and δ-) are naturally occurring analogues of tocopherol isomers (Vitamin E) found mainly in cereal grains and palm oil. Tocotrienols have been shown to have an intrinsic hypochlolesterolemic activity in animals and humans. The cholesterol lowering effect of tocotrienols was attributed mainly to their down regulation of HMG-CoA reductase-the rate-limiting enzyme of the cholesterol biosynthetic pathway. Palm oil represents one of the most abundant natural sources of tocotrienols. The distribution of vitamin E in palm oil is 30% tocopherols and 70% tocotrienols.

An encouraging development in the treatment and management of hyperlipidemia/dyslipidemia has been the introduction of a new class of fungal-derived compounds (statins) that are potent competitive inhibitors of HMG-CoA reductase, the rate controlling enzyme in the biosynthetic pathway for cholesterol. Statins have been widely used as the most potent class of drugs for the treatment of diabetes, CHD alone, diabetes with CHD, hypertriglyceridemia and combined hyperlipidemia[33,55,99]. Currently, statins including atorvastatin calcium (Lipitor) are quite effective lipid-lowering agents and universally marketed and used by diabetic and nondiabetic dyslipidemic patients with or without CHD. However, statins as well as fibrates and bile acid sequesterants have been reported to exert a host of side effects[10,42]. In contrast, dietary tocotrienols have no toxicity and provide an effective lipid lowering property in addition to their potent antioxidant activity. Recently, our lab has demonstrated a strong

hypoglycemic, antidiabetic, hypolipidemic and antioxidant effect of dietary tocotrienols (Tocomin) and lovastatin after a 14-week treatment of STZ-induced diabetic rats[3]. The tocotrienol isomers (α-, β-, γ- and δ-) are naturally occurring analogues of tocopherol isomers (Vitamin E) found mainly in cereal grains and palm oil. Tocotrienols differ from tocopherols by possessing three double bonds in the phytyl side chain. Unlike tocopherols, tocotrienols have been shown to have an intrinsic hypochlolesterolemic activity in animals and humans. The cholesterol lowering effect of tocotrienols was attributed mainly to their downregulation of HMG-CoA reductase-the rate-limiting enzyme of the cholesterol biosynthetic pathway. The tocopherol isomers, on the other hand, do not inhibit cholesterol synthesis and thus do not lower serum cholesterol[58,70]. Previously published reports indicate a strong hypolipidemic and antioxidant effect of tocotrienol rich fraction (TRF) or purified tocotrienols (Tocomin) in normolipidemic and hyperlipidemic rats[15,58,60], normolipidemic and genetically hyperlipidemic swines and chickens[72,73,77] hyperlipidemic rabbits[85] cholesterol/oxidized cholesterol-induced hyperlipidemic/atherosclerotic rabbits[110,111] and hyperlipidemic hamsters[78]. It has been earlier demonstrated that tocotrienols or TRF exhibits a strong hypolipidemic activity in normolipidemic and hyperlipidemic humans[14-16,58,71,74,76] and in a type II familial hypercholesterolemic patient with severe xanthomas[16]. Our laboratory has demonstrated a strong hypolipidemic, antidiabetic and antioxidant impact of tocotrienols in type 2 diabetic patients with hyperlipidemia[13,48], also reported that T_3 significantly decreased plasma lipid peroxidation in patients with hyperlipidemia and carotid stenosis with no change in their lipid and lipoprotein parameters. It has been established that LDL-C/HDL-C and HDL-C/TC ratios are good predictors for the presence and severity of CAD[31]. Experiments involving the effects of T_3 on apoB (LDL) and apoA-1 (HDL) and apoB/apoA-1 ratio have been reported in chickens, swine and humans. The apoB/apoA-1 ratio, considered being a better indicator than LDL-C/HDL-C ratio, for assessment of CHD, was reduced in T_3 treated subjects[74]. In addition, TRF treatment to hypercholesterolemic humans and type 2 diabetic patients significantly improved the HDL/LDL and HDL-C/TC ratios[13,75]. Desmethyl tocotrienol and didesmethyl tocotrienol also mediated a significant decrease in serum TG, TC, LDL-C, apoB, Lp(a), platelet factor 4 and thromboxane B_2 levels of hypercholesterolemic humans after a double blind, 12-week study[71]. A dose-dependent effect of tocotrienol rich fraction containing mixture of the novel desmethyl- and didesmethyl tocotrienols (TRF25) has been investigated in hypercholesterolemic humans[76]. The results showed that intake of a dose of 100 mg/day of TRF25 for 35 days caused maximum decrease in serum TC, LDL-C, apoB and TG levels when compared to baseline values[76]. The synergistic effect of TRF25 has been reported in hypercholesterolemic humans. Administration of TRF in combination with lovastatin to hypercholesterolemic humans for 35 days exerted a synergistic lipid lowering effect, when compared to values obtained from subjects given TRF25 or lovastatin alone. In hyperlipidemic patients with type 2 diabetes, low doses of pitavastatin (1 mg) and fenofibrate (100 mg) were both effective in decreasing sd-LDL-C concentration but via a different mechanisms: the former decreases total LDL including sd-LDL, while the latter decreased sd-LDL specifically[88]. Recently[56], demonstrates that, in diabetic patients with mixed dyslipidemia, combination therapy with simvastatin and fenofibrate had a greater positive effect on LDL cholesterol size profile, with a shift from more atherogenic sd-LDL-C to less atherogenic lb-LDL-C, than either fenofibrate or simvastatin monotherapy. However, no one to date has studied a detailed and investigation pertaining to effect of cigarette smoke on cholesterol dynamics, overall oxidative status including the indices of free radical mediated lipid/lipoprotein peroxidation and status of enzymatic and nonenzymatic antioxidant defense system in plasma and erythrocytes, particularly, *in vivo* and *in vitro* oxidizability of more atherogenic sd-LDL, less atherogenic lb-LDL, including LDL, and plasma HDL-associated arylesterase activity. On the other hand, Tocotrienols have been shown as anti-osteoporotic and antioxidant[1] properties. Osteoporosis is a metabolic bone disease affecting both men and women especially postmenopausal women. Osteoporosis has been associated with oxidative stress and therefore, the protective effects of antioxidants such as vitamin E were studied. Lately, there has been a growing interest in Tocotrienols, a potent vitamin E with anti-cholesterol[68], ant-cancer, anti-lipid peroxidation[67]

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and perhaps anti-osteoporotic[2].

We investigated the efficacy of antioxidant and hypolipidemic agents Tocomin (Tocotrienols) and Lovastatin by analyzing all the parameters in plasma and lipoprotein lipids, and differential *in vivo* and *in vitro* oxidizability of highly atherogenic sd-LDL, less atherogenic lb-LDL as well as LDL, as investigated in rats exposed to cigarette smoke, treated with and without Tocomin.

MATERIAL AND METHODS

Chemicals

1-Chloro 2, 4-Dinitrobenzene was purchased from Central drug house, Pvt. Ltd. (India). All other chemicals used for this study were of analytical grade and obtained from HIMEDIA (India), Sisco (India), Ashirwad (India), Sigma-Aldrich (USA), Miles (USA), Acros (USA) and Tocotrienols drug as well as RBD palm olein were supplied as a gift from CAROTECH BHD, Chemor, Malaysia. Cholesterol lowering drug, Lovastatin, was a gift from Saimira Innoform Pvt. Ltd., Chennai, India.

Estimation

Plasma triglyceride[95], Plasma Cholesterol, LDL and HDL[9], The method for the isolation of small dense (sd-) LDL and large buoyant (lb-) LDL from isolated LDL is based on the two-step procedure of[48], Plasma VLDL-C [38], Fractionation of Plasma lipoprotein such as LDL, sd-LDL, lb-LDL[102], HDL and its fractions- HDL_2, HDL_3, Blood Nicotine and plasma Carbonmonoxide saturation[98], Plasma FRAP[18], free radical scavenging activity, Nicotine content in blood samples was determined by the method of[98]. Carbon monoxide Saturation in blood samples was determined by the method of[98]. Triglycerides were determined by using enzymatic kit. The method uses a modified Trinder color reaction to produce a fast, linear, end point reaction[95]. Plasma very low density lipoprotein-cholesterol (VLDL-C) was calculated by dividing plasma triglycerides values (mg/dl) by a factor of 5 as described by[38]. Free fatty acid in plasma was estimated as described by[32]. The procedure of[37] was used for extracting free fatty acid from plasma lipids. *Ex vivo* and *in vitro* Cu^{++}-mediated LDL, sd-LDL, lb-LDL oxidation[34,35] were measured using standard kits by following known procedures

Experimental design

Healthy male albino rats, weighing about 150-180 g were purchased from Indian Veterinary Research Institute (IVRI), Bareilly (India), were maintained to animal house environmental condition prior to the experiment. For the present study, animals were divided into following 3 groups: NC (normal control), SC (smoke control), S-T_3T (smoke exposed Tocotrienols treated) and Smoke Exposed Lovastatin Treated (S-LT).

Diet/Drug/Exposure to cigarette smoke

The rats were given pelleted rat chow. Exposure to cigarette smoke was done in morning and evening by keeping two rats in bottomless metallic container (10×11×16 inch). Maintenance and treatment of all the animals was done in accordance with the principles of Institutional Animal Ethics Committee constituted as per the directions of the Committee for the J.N. Medical College, Aligarh Muslim University, India. For investigating the hypolipidemic and antioxidant effect of Tocomin and Lovastatin, rats were divided in the following groups as described below:

Normal control (N-C)

Seven rats were given 1.0 ml palmvitae oil through gastric intubation for four weeks.

Smoke exposed control (S-C)

Seven rats in this group were administrated 1.0 ml palmvitae oil though gastric intubation for four weeks.

Smoke exposed tocomin (Tocotrienols) treated (S-T_3T)

Seven rats in this group were given 6.0 mg Tocomin/ rat/day, through gastric intubation for four weeks.

Smoke exposed lovastatin treated (S-LT)

Seven rats in this group were given 0.50 mg Lovastatin/rat/day, through gastric intubation for four weeks.

Collection of blood and plasma

For the estimation of different parameters, overnight fasted rats in each group were anaesthetized and blood drawn from cardiac puncture, and were collected in heparinised tube. Plasma was separated from blood by centrifugation at 2500 rpm for 30 min.

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Statistical evaluation

Statistical analysis of data was done by employing two-tailed Student t-test as described by[17]. P value less than 0.02 were considered significant.

RESULT

Antioxidative activities of tocomin, α-tocotrienol, γ-tocotrienol, δ- tocotrienol and α-tocopherol

Antiradical activity or hydrogen donating ability of Tocomin, α-tocotrienol, γ- tocotrienol, δ-tocotrienol and α-tocopherol was measured by using DPPH, which reflects the antioxidative properties of these compounds. As shown in Figure 1, the half quenching concentrations (IC_{50}) were as follows: Tocomin, 50.85 μM; α-tocotrienol, 46.91 μM; γ-tocotrienol, 44.55 μM; δ-tocotrienol, 38.12 μM; and α-tocopherol, 70.75 μM. These findings indicate that as compared to α-tocopherol, γ-, α-and δ-tocotrienols were more efficient scavengers of peroxyl radicals by 34 %, 37 % and 46 %, respectively. Since Tocomin is a mixture of α-tocotrienol (25.6 %), γ-tocotrienol (40.8 %), δ-tocotrienol (12.8 %) and α-tocopherol (22.8 %), its efficiency as a scavenger of peroxyl radical was higher by 28 %, in comparison to α-tocopherol. The reduction in the quenching efficiency of Tocomin in comparison to α-, γ- and δ--tocotrienols is apparently due to the presence of 23 % α-tocopherol, which has a lowest peroxyl radical scavenging efficiency of 70.75μM.

Average body weight and diet consumption in each group of rats before and after 4 weeks of treatment

As shown in TABLE 1, the average body weight (g) of smoke exposed control rats (S-C), Tocomin treated (S-T_3T) and Lovastatin treated (S-LT) rats was 178, 184 and 179 (g), respectively, whereas for normal control (N-C) rats the average body weight was 177 g, whereas, the average body weight of N-C, S-T_3T and S-LT rats showed a significant gain of 31 %, 49 % and 22 % respectively after 4 weeks of treatment. On the other hand, average diet consumption/group/day (g) of S-C, S-T_3T and S-LT was 155, 172 and 172 (g), respectively, whereas, the average diet consumption of N-C, S-T_3T and S-LT rats showed a significant gain of 4 %, 17 % and 6 % respectively after 4 weeks of treatment. In addition, there was no significant change on average diet consumption of S-C rats, in comparison to their initial diet consumption (before treatment). These results demonstrate that in smoke exposed Tocomin

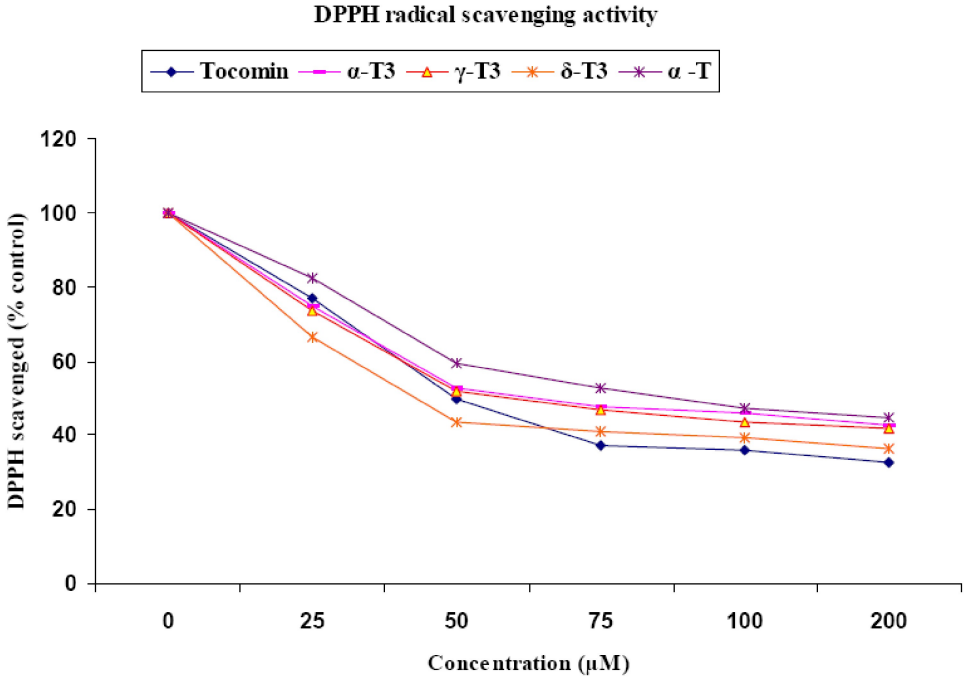

Figure 1 : Free radical scavenging activities of Tocomin, α-tocotrienol, γ-tocotrienol, δ-tocotrienol and α-tocopherol. The antioxidant activities of the above compounds at the indicated concentrations were carried out as described in methods. The assay is based on the reduction of 2, 2-diphenyl, 1-picrylhydrazyl (DPPH), which gives strong absorption maxima at 517nm. Values represent the mean of triplicate determinations. The average error in the data points in these assay were mean ± less than 3 %. The average absolute absorbance value of 100 % DPPH was 0.00786 ± 0.00029.

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treated rats the average diet consumption and the gain in body weight after 4 weeks was significantly higher than rats in N-C and S-LT groups.

Impacts of tocomin and lovastatin on hemoglobin, blood carbon monoxide saturation and blood nicotine in smoke exposed rats treated for 4 weeks

Data presented in TABLE 2, indicated the hemoglobin (Hb), blood carbon monoxide saturation (carboxyhemoglobin) and blood nicotine. Hemoglobin a level was significantly reduced by 20 %, in smoke exposed (S-C) rats, when compared to N-C value. However, a highly significant increase in Hb concentration was observed in smoke exposed rats treated with Tocomin (21 %) and Lovastatin (17 %) for 4 weeks when compared to S-C rats. Both Tocomin and Lovastatin administration to smoke exposed rats mediated an increase in Hb levels close to normal value. Whereas, blood carbon monoxide saturation and blood nicotine levels were increased from 6.1 (SCO %) and 1.2 µg/ml in N-C to 10.6 SCO % (73 %) and 4.6 µg/ml (299 %)\ respectively, in S-C rats. After 4 weeks of Tocomin and Lovastatin treatment blood carbon monoxide saturation and blood nicotine levels showed a significant reduction of 30 % and 70 % in S-T_3T respectively, whereas in S-LT, blood carbon monoxide saturation and blood nicotine levels were reduced by 26 %, and 50 % respectively, in comparison to values in S-C rats.

Effect on plasma lipids

As seen in TABLE 3, all the plasma lipids parameters were significantly increased in smoke exposed control (S-C) rats, when compared to N-C values. Total lipids (TL), triglycerides (TG), free fatty acids (FFA) and total cholesterol (TC) significantly increased from 393, 50, 123 and 82 mg/dl in N-C to 507, 101, 145 and 149 mg/dl, respectively, in S-C group. After 4 weeks of Tocomin treatment, levels of TL, TG, FFA and TC were significantly decreased by 9 %, 40 %, 11% and 29 %, respectively, when compared to corresponding S-C values. Whereas, in Lovastatin treated rats, TL, TG, FFA and TC levels were significantly reduced by 7 %, 40 %, 13 % and 35 % respectively, in comparison to corresponding values in S-C group. These results demonstrate that 4-week treatment of smoke exposed rats with 6 mg Tocomin or 0.50 mg Lovastatin mediated a similar and significant reduction in above lipid parameters.

Effect on plasma lipoprotein lipids

As seen in TABLE 4, plasma VLDL-C, LDL-C and non-HDL-cholesterol (non-HDL-C) levels were significantly increased from 10, 52 and 63 mg/dl in N-C to 21 mg/dl (105 %), 110 mg/dl (112 %) and 131 mg/dl (109 %) respectively, in S-C. After 4 weeks of Tocomin or Lovastatin treatment, both VLDL-C, LDL-C and non-HDL-C levels showed a significant reduction 41 %, 44 % and 43 %, respectively, in S-T_3T, whereas, in S-LT, VLDL-C, LDL-C and non-HDL-C were significantly reduced of 41 %, 48 % and 46 %, respectively, in comparison to corresponding values in S-C rats. HDL-C, HDL_2-C and HDL_3-C levels were decreased from 19, 6 and 13 mg/dl in N-C to 17 mg/dl

TABLE 1 : Average body weight and diet consumption in each group of rats before and after 4 weeks of Tocomin and lovastatin treatment.

Group	Average body weight/rat (g)		Average diet consumption/group/day (g)	
	Before treatment	After treatment	Before treatment	After treatment
N-C	177.14 ± 2.67*	232.85±15.63 (+31.44%)[a]	165±5.00	171.66±10.40 (+4.03%)
S-C	177.85±3.93*	210.12±18.14 (+18.14%)[a]	155±5.00	150±5.00 (-3.22%)
S-T_3T	184.28±7.86*	274.28±13.14 (+48.83%)[a]	171.66±7.63	200±10 (+16.50%)[a]
S-LT	179.28±6.07*	218.57±13.45 (+21.91%)[a]	171.66±7.63	181.66±2.88 (+5.82%)[a]

*Values are mean ± SD from 7 rats in each group. N-C, normal control; S-C, smoke exposed control; S-T_3T, fed 6 mg Tocomin/ rat/day and S-LT, given 0.50 mg Lovastatin/rat/day for 4 weeks. Significantly different from N-C at [a]p<0.001. Significantly different from S-C at [a]p<0.001.

TABLE 2 : Impacts of Tocomin and lovastatin on blood hemoglobin, carbon monoxide saturation and nicotine in cigarette smoke exposed rats after 4 weeks of treatment.

Group	Hemoglobin (g/dl)	Carbon monoxide Saturation (SCO %) or (Carboxyhemoglobin)	Nicotine (µg/ml)
N-C	14.38±0.264*	6.11±0.034	1.16±0.033
S-C	11.51±0.015* (-19.96%)[a]	10.58±0.271 (+73.15 %)[a]	4.63±0.042 (+299.13 %)[a]
S-T_3T	13.98±0.017* (+21.45%)[a]	7.37±0.023 (-30.34 %)[a]	1.37±0.043 (-70.41 %)[a]
S-LT	13.51±0.021* (+17.37%)[a]	7.86±0.064 (-25.75 %)[a]	2.33±0.035 (-49.67 %)[a]

*Values are mean ± SD from pooled blood of 7 rats in each group. N-C, normal control; S-C, smoke exposed control; S-T_3T, fed 6 mg Tocomin/ rat/ day and S-LT, given 0.50 mg Lovastatin/rat/day for 4 weeks. Significantly different from N-C at [a]p<0.001. Significantly different from S-C at [a]p<0.001.

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TABLE 3 : Impacts of Tocomin and lovastatin on plasma total lipid, triglycerides, free fatty acid and total cholesterol in cigarette smoke exposed rats after 4 weeks of treatment.

Group	Total lipid	Triglycerides	Free fatty acid	Total cholesterol
N-C	393.34±1.60*	50.08±0.831	123.41±0.432	81.79±2.81
S-C	506.97±2.25 (+28.89 %)[a]	101.29±2.67 (+102.25 %)[a]	145.19±0.513 (+17.64 %)[a]	148.81±4.36 (+81.94 %)[a]
S-T_3T	462.43±1.60*(-8.78 %)[b]	61.23±1.93 (-39.54 %)[a]	129.49±0.393 (-10.81%)[b]	104.92±2.85 (-29.49 %)[a]
S-LT	470.21±1.15*(-7.25 %)[b]	60.78±1.85 (-40.0 %)[a]	126.61±0.372 (-12.79 %)[a]	96.87±2.65 (-34.90 %)[a]

Values are mean (mg/dl) ± SD from pooled plasma of 7 rats in each group. N-C, normal control; S-C, smoke exposed control; S-T_3T, fed 6 mg Tocomin/rat/day and S-LT, given 0.50 mg Lovastatin/rat/day for 4 weeks. Significantly different from N-C at [a]p < 0.001. Significantly different from S-C at [a]p<0.001 and [b]p<0.05.

TABLE 4 : Impacts of Tocomin and lovastatin on plasma VLDL-C, LDL-C, HDL-C, HDL_2-C AND HDL_3-C subfractions and non-HDL-C in cigarette smoke exposed rats after 4 weeks of treatment.

Parameters	N-C	S-C	S-T_3T	S-LT
VLDL- C	10.01±0.056*	20.54±0.262* (+105.19 %)[a]	12.11±0.195* (-41.04 %)[a]	12.20±0.216* (-40.60 %)[a]
LDL-C	52.00±0.231	110.22±0.295 (+111.96 %)[a]	61.52±0.404 (-44.18 %)[a]	56.98±0.292 (-48.30 %)[a]
HDL- C	19.06±0.081	17.01±0.063 (-10.75 %)[a]	30.05±0.216 (+76.77%)[a]	25.99±0.196 (+52.79 %)[a]
HDL_2- C	6.06±0.015	4.00±0.011 (-33.99 %)[a]	12.00±0.031 (+200.0 %)[a]	9.00±0.024 (+ 125.00 %)[a]
HDL_3- C	12.98±0.052	12.36±0.063 (-4.77 %)[c]	17.98±0.081 (+45.46 %)[a]	16.95±0.073 (+37.13 %)[a]
†Non-HDL- C	62.73±3.63	130.80±4.79 (+108.51 %)[a]	74.87±3.25 (-42.75 %)[a]	70.88±3.83 (-45.81 %)[a]

For the calculation of non-HDL-C, TC and HDL-C data is taken from TABLE 2 and 3. *Values are mean (mg/dl) ± SD from pooled plasma of 7 rats in each group. N-C, normal control; S-C, smoke exposed control; S-T_3T, fed 6 mg Tocomin/rat/day and S-LT, given 0.50 mg Lovastatin/rat/day for 4 weeks. Significantly different from N-C at [a]p<0.001 and [c]p<0.02. Significantly different from S-C at [a]p<0.001.

(11 %), 4 mg/dl (34 %) and 12 mg/dl (5 %), respectively, in S-C values. After 4 weeks of Tocomin treatment (S-T_3T) HDL-C, HDL_2-C and HDL_3-C levels showed a significant increase of 77 %, 200 % and 45 %, respectively, when compared to corresponding values in S-C, whereas, in S-LT, HDL-C, HDL_2-C and HDL_3-C levels were increased by 53 %, 125 % and 37 %, respectively. These results demonstrate that both Tocomin and Lovastatin are equally effective in reducing VLDL-C and LDL-C levels. On the other hand, in comparison to N-C values, treatment of smoke exposed rats with Tocomin mediated a significantly higher increase in HDL-C, HDL_2-C and HDL_3-C concentration than the increase seen in Lovastatin treated rats.

Effect on plasma LDL, small dense LDL and large buoyant LDL subfractions

Data presented in TABLE 5, depict levels of plasma LDL-C, LDL-apoB, small dense (sd-) LDL-C, sd-LDL-apoB, large buoyant (lb-) LDL-C and lb-LDL-apoB. In normal rats, LDL-C and its apoB content were 52 and 121mg/dl, respectively, in N-C, the cholesterol and apoB content of sd-LDL subpopulation was 15 and 39 mg/dl, respectively, whereas, cholesterol and apoB concentrations associated with lb-LDL were 37 and 81 mg/dl. In comparison to control values, smoke exposed rats showed an increase in plasma LDL-C from 52 to 110 mg/dl (112 %) and LDL-apoB from 121 to 139 mg/dl (15 %), whereas sd-LDL-C was increased from 15 to 60 mg/dl (292 %) sd-LDL-apoB was increased from 39 to 71 mg/dl (82 %). In comparison to sd-LDL, the increase in lb-LDL-C of S-C rats was only 35 %, whereas, lb-LDL-apoB level was reduced by 16 % when compared to corresponding values in N-C. Following 4 weeks of Tocomin or Lovastatin treatment to smoke exposed rats, LDL-C levels were significantly reduced by 44 % and 48 %, respectively, and restored these values close to the N-C value. Consistent with a small increase in LDL-apoB

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content of S-C rats, after 4 weeks of Tocomin or Lovastatin treatment, the decline in LDL-apoB was also minimal. The cholesterol and apoB associated with sd-LDL decreased from 60 and 71 mg/dl to 19 mg/dl (68 %) and 40 mg/dl (44 %), respectively, in S-T_3T, whereas, sd-LDL-C and its apoB content in S-LT was reduced to 17 mg/dl (72 %) and 39 mg/dl (46 %), respectively. On the other hand, the cholesterol level of lb-LDL subfraction in both the treated groups was 43 and 40 mg/dl, which is close to a value of 37 mg/dl in N-C. As indicated above, apoB associated with lb-LDL fraction was reduced from an N-C value of 81 to 67 mg/dl in S-C, whereas, a significant increase to a similar value of 82 mg/dl (21%) indicating a full restoration of lb-LDL-apoB levels and similar to value in N-C.

In normolipidemic rats, the percent wise distribution of cholesterol and apoB from LDL particle to sd-LDL and lb-LDL fractions were 29 %, 32 %, 71 % and 67 %, respectively, in smoke exposed rats the percent sd-LDL-C and sd-LDL--apoB was significantly increased by 85 % and 59 %, when compared to the corresponding percent values from normal rats. Similarly, the percent lb-LDL-C and lb-LDL-apoB in S-C rats was increased by 36 % and 27 %, in comparison to corresponding percent values of N-C rats. The percent increase in sd-LDL-C and sd--LDL-apoB registered in smoke exposed rats, was significantly reduced by 38-45 % after 4 weeks of Tocomin or Lovastatin treatment. In contrast, percent lb-LDL-C and lb--LDL-apoB values in both the treated groups were significantly increased by 31-55 %, when compared to the corresponding percent values of smoke exposed rats. These results demonstrate that a homogeneous preparation of sd-LDL and lb-LDL can be obtained by employing heparin-Mg^{++}-mediated precipitation of purified plasma LDL. In addition, the results also show that the prevalence and concentration of sd-LDL, which is considered more atherogenic than LDL or lb-LDL was substantially increased in smoke exposed rats. In smoke exposed rats treated with Tocomin or Lovastatin for 4 weeks, the sd-LDL concentration was significantly reduced to a level similar to control value (N-C).

Impact on the ratios of LDL-C/HDL--C, HDL-C/TC, sd-LDL-C/HDL-C and lb-LDL-C/HDL-C

As shown in TABLE 6, LDL-C/HDL--C, HDL-C/TC, sd-LDL-C/HDL-C and lb-LDL-C/HDL-C ratios were calculated from the data presented in TABLE 2, 3 and 4. LDL-C/HDL-C ratio was significantly increased from 2.72 in N-C to 6.11 (125 %) in S-C group, when compared to ratio in N-C. After 4 weeks of treatment, the increase in LDL-C/HDL-C ratio was significantly prevented and decreased to 2.04 and 2.19 in S-T_3T and S-LT, respectively, which is close to normal control value. On the other hand, HDL-C/TC ratio was significantly decreased from 0.233 in N-C to 0.121 (48 %) in S-C group. Tocomin or Lovastatin treatment to these rats significantly prevented the increase in HDL-C/TC ratios and fully restored them to a ratio value similar to N-C. In N-C, when sd-LDL-C values were divided by HDL-C, a ratio of 0.802 was obtained, which is substantially lower than LDL-C/HDL-C ratio of 2.72. In smoke exposed rats, sd-LDL-C/HDL-C ratio was substantially increased to a ratio value of 3.32 (314 %), when compared to N-C value. As expected, sd-LDL-C/HDL-C ratio in Tocomin and Lovastatin treated rats were substantially reduced to a value of 0.632 (81 %) and 0.653 (80 %), respectively, these ratio values in S-T_3T and S-LT are significantly lower than a ratio of 0.802 in N-C. In comparison to sd--LDL-C/HDL-C, the ratio of lb-LDL-C/HDL-C in S-C rats was increased by 44 %, from an N-C value of 1.93 to 2.77. Consistent with these results, the lb-LDL-C/HDL-C ratios in treated groups where only partially (21-26 %) restored, in comparison to ratio value of N-C rats. These results indicate that sd--LDL-C/HDL-C ratio may be considered as a better and novel predictor for CAD risks than LDL-C/HDL-C ratios. In addition, the ratios related to sd--LDL-C and HDL-C in Tocomin or Lovastatin treated rats were positively modulated and restored similar to normal control value, indicating normalization of cholesterol levels associated with the above lipoproteins.

***Ex vivo* and Cu^{++}-mediated *in vitro* oxidation of LDL, Sd-LDL and Lb-LDL in smoke. Exposed rats after 4 weeks of tocomin and lovastatin treatment**

As depicted in TABLE 7, the *ex vivo* base line diene conjugation (BDC) levels of LDL, sd-LDL and lb-LDL in smoke exposed rats were increased by 60 %, 62 % and 15 %, respectively, in comparison to the

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TABLE 5 : Impacts of Tocomin and lovastatin on plasma LDL, small dense and large buoyant LDL subpopulation in cigarette smoke exposed rats after 4 weeks of treatment.

Parameters	N-C	S-C	S-T_3T	S-LT
LDL-C	52.00±0.231*	110.22±0.295* (+111.96 %)[a]	61.52±0.404* (-44.18 %)[a]	56.98±0.292* (-48.30 %)[a]
LDL- apoB	120.84±0.921	138.87±0.523 (+14.92 %)[a]	125.07±0.823 (-9.93 %)[b]	128.82±0.725 (-7.23 %)[c]
Sd-LDL-C	15.30±0.042	59.92±0.096 (+291.63 %)[a]	18.99±0.071 (-68.30 %)[a]	16.98±0.054 (-71.66 %)[a]
% LDL-C	29.40±0.321	54.36±1.26 (+84.89 %)[a]	30.86±0.624 (-43.23 %)[a]	29.75±0.462 (-45.27 %)[a]
Sd-LDL- apoB	39.07±0.814	71.25±1.021 (+82.36 %)[a]	40.03±0.716 (-43.81 %)[a]	38.52±1.190† (-45.93 %)[a]
% LDL-apoB	32.31±0.832	51.30±1.62 (+58.77 %)[a]	32.01±0.605 (-37.60 %)[a]	29.90±0.531 (-41.71 %)[a]
Lb-LDL-C	36.97±0.056	50.01±0.154 (+35.27 %)[a]	42.98±0.044 (-14.05 %)[a]	39.95±0.085 (-20.11 %)[a]
% LDL-C	71.03±1.213	45.35±0.962 (-36.15 %)[a]	69.81±0.943 (+53.93 %)[a]	70.10±1.129 (+54.57 %)[a]
Lb-LDL-apoB	80.54±1.191	67.65±1.120 (-16.00 %)[a]	82.17±1.10 (+21.46 %)[a]	82.10±0.624 (+21.35 %)[a]
% LDL-apoB	66.62±0.926	48.70±0.835 (-26.89 %)[a]	65.62±0.859 (+34.74 %)[a]	63.70±0.825 (+30.80 %)[a]

***Values are mean (mg/dl) ± SD from LDL, sd-LDL and lb-LDL subpopulation, isolated from pooled plasma of 7 rats in each group. N-C, normal control; S-C, smoke exposed control; S-T_3T, fed 6 mg Tocomin/ rat/ day and S-LT, given 0.50 mg Lovastatin/rat/day for 4 weeks. Significantly different from N-C at [a]p<0.001. Significantly different from S-C at [a]p<0.001, [b]p<0.01 and [c]p<0.02**

corresponding N-C values. Feeding of Tocomin to smoke exposed rats partially blocked the *in vivo* oxidation of LDL, sd-LDL and lb-LDL and reduced their BDC levels by 27 %, 31 % and 11 %, respectively. Similarly, after Lovastatin treatment (S-LT), BDC levels in LDL, sd-LDL and lb-LDL were reduced by 20 %, 19 % and 9 %, respectively, in comparison to the corresponding N-C values. As expected, the lag phase time of LDL oxidation was reduced from 95 min in N-C to 62 min in S-C, whereas, for lb-LDL oxidation, it was reduced from 54 min in N-C to 41 min in S-C. Treatment of smoke exposed rats with Tocomin or Lovastatin restored the lag phase time of LDL oxidation to 83 min and 71 min, respectively, whereas, lag phase time of lb-LDL oxidation in both the treated groups was restored to 50 min and 45 min, respectively. In comparison to lag phase time of LDL or lb-LDL oxidation in N-C, sd-LDL oxidation resulted in a strikingly lower lag phase time of 16.40 min, which was significantly reduced to 12 min in S-C. Following treatment with Tocomin or Lovastatin the lag phase time of sd-LDL oxidation was increased to 16 min and 14 min, respectively. These result demonstrate that relative to lb-LDL, both *ex vivo* and *in vitro* conjugated diene formation in sd-LDL were markedly increased in smoke exposed rats, resulting in a strikingly lower lag phase time, indicating a substantially enhanced susceptibility of sd-LDL to *in vivo* oxidation. Similarly, treatment of smoke exposed rats with dietary Tocotrienols (Tocomin) or Lovastatin mediated an antioxidant effect by preferentially and significantly blocking the *ex vivo* formation of conjugated diene of more atherogenic sd-LDL, when compared to lb-LDL or LDL.

DISCUSSION

Cigarette smoking is firmly established as a primary risk factor for atherosclerotic cardiovascular disease. Increased oxidative stress is one of the principal mechanisms by which it may exert its pathological influence. This study is the first to examine the effect of dietary tocotrienols supplementation on overall proatherogenic actions of cigarette smoke. The cigarette smoke induced extensive proatherogenic changes, that occurred in young smokers, were reflected on a variety of parameters, such as, blood nicotine, carboxyhemoglobin, plasma and lipoprotein lipids including cholesterol and plasma lipid peroxidation

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TABLE 6 : Effect of Tocomin and lovastatin on the ratios of LDL-C/HDL-C, HDL-C/TC, SD-LDL-C/HDL-C and lb-LDL-C/ HDL-C in cigarette smoke exposed rats after 4 weeks of treatment

Ratio[†]	Group			
	N-C	S-C	S-T_3T	S-LT
LDL-C/HDL-C	2.72±0.021[*]	6.11±0.036[*] (+124.63 %)[a]	2.04±0.026[*] (-66.61 %)[a]	2.19±0.021[*] (-64.15 %)[a]
HDL-C/TC	0.233±0.032	0.121±0.003 (-48.06 %)[b]	0.286±0.077 (+136.36 %)[b]	0.268±0.036 (+121.48 %)[d]
Sd-LDL-C/HDL-C	0.802±0.008	3.32±0.031 (+313.96 %)[a]	0.632±0.006 (-80.96 %)[a]	0.653±0.005 (-80.33 %)[a]
Lb-LDL-C/HDL-C	1.93±0.021	2.77±0.31 (+43.52 %)[a]	1.43±0.012 (-25.90 %)[a]	1.53±0.016 (-20.72 %)[a]

[†]For the calculation of ratios, data is taken from TABLE 2, 3 and 4. [*]Values are mean ± SD from pooled plasma of 7 rats in each group. N-C, normal control; S-C, smoke exposed control; S-T_3T, fed 6 mg Tocomin/ rat/ day and S-LT, given 0.50 mg Lovastatin/ rat/day for 4 weeks. Significantly different from N-C at [a]p<0.001 and [b]p<0.01. Significantly different from S-C at [a]p<0.001, [b]p<0.05 and [d]p<0.01.

TABLE 7 : *ex vivo* and copper-mediated *in vitro* oxidation of LDL, sd-LDL and lb-LDL in cigarette smoke exposed rats after 4 weeks of Tocomin and lovastatin treatment

Group	Conjugated diene[*]					
	LDL Oxidation		Sd-LDL Oxidation		Lb-LDL Oxidation	
	Basal	Lag phase[**]	Basal	Lag phase[**]	Basal	Lag phase[+]
N-C	180.55[+]	95	234.52	16.40	166.66	54
S-C	288.49[+] (+59.78%)[†]	62 (- 34.73%)[¶]	379.04 (+61.62%)[†]	12 (-26.82%)[¶]	192.22 (+15.33%)[†]	41 (-24.07%)[¶]
S-T_3T	210.63[+] (-26.98%)[††]	83 (+33.87%)[§]	260.00 (-31.40%)[††]	16 (+33.31%)[§]	170.23 (-11.44%)[††]	50 (+21.95%)[§]
S-LT	230.63[+] (-20.05%)[††]	71 (+14.51%)[§]	305.63 (-19.36%)[††]	14 (+16.67%)[§]	175.28 (-8.81 %)[††]	45 (+9.75%)[§]

The conjugated diene values are expressed as nmole malondialdehyde equivalents/mg protein. Basal conjugated diene represent the status of oxidized LDL, sd-LDL and lb-LDL *in vivo*. [**]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. [+]Values are obtained from LDL, sd-LDL and lb-LDL subpopulation, isolated from pooled plasma of 7 rats in each group. N-C, normal control; S-C, smoke exposed control; S-T_3T, fed 6 mg Tocomin/rat/day and S-LT, given 0.50 mg Lovastatin/rat/day for 4 weeks. [†]Percent increase with respect to basal value in N-C. [††]Percent decrease with respect to basal value in S-C. [¶]Percent decrease with respect to lag phase value in N-C. [§]Percent increase with respect to lag phase value in S-C.

products including *ex vivo* and *in vitro* oxidizability of LDL sd- LDL and lb-LDL. Treatment of smoke exposed rats with tocotrienols (Tocotrienols 6mg/day) for 4 weeks, significantly reduced the overall oxidative burden and effectively ameliorated the above altered parameters, thus, indicating a strong hypolipidemic/anti-atherogenic and antioxidant effect of Tocotrienols. Several studies show that in addition to increase in oxidative stress, certain other compounds of cigarette smoke, such as nicotine and carbon monoxide (CO) play a role in atherogenesis. Nicotine alone acutely increases endothelial dysfunction by means of impaired vascular reactivity[64]. It may lead to increased platelet adhesiveness[45]. Carbon monoxide constitutes 4% of cigarette smoke and directly leads to high levels of carboxyhemoglobin. Through sustained exposure to high levels of CO, chronic hypoxia ensues, leading to increased exercise-induced ischemia, ventricular dysfunction with CAD[4] . In addition, in one cross-sectional study in Britain, carboxyhemoglobin levels appeared to be better predictor of atherosclerotic disease than smoking histories[43]. Consistent with earlier reports[49]. It is now well established that tobacco smoking is an independent, modifiable risk factor for coronary, cerebral, and peripheral arterial diseases. Cigarette smoking increases the risk of myocardial infarction in both men and women[36]. The gas phase of cigarette smoke contains more than 10^{15} free radicals per puff, and the extent to which these compounds affect endothelial dysfunction and inflammation remains unclear[6]. Several studies show that in addition to increase in oxidative stress, certain other compounds

of cigarette smoke, such as nicotine and carbon monoxide (CO) play a role in atherogenesis. Nicotine alone acutely increases endothelial dysfunction by means of impaired vascular reactivity[64]. Coronary vasospasm caused by nicotine in cigarette smoke has been demonstrated in young women[23]. Nicotine also increase the diurnal secretion of cortisol[47], which may contribute to cardiac arrhythmias[61], and it may lead to increased platelet adhesiveness[45]. Carbon monoxide constitutes 4% of cigarette smoke and directly leads to high levels of carboxyhemoglobin. Through sustained exposure to high levels of CO, chronic hypoxia ensues, leading to increased exercise-induced ischemia, ventricular dysfunction, and arrhythmias in patients with CAD[4]. In addition, in one cross-sectional study in Britain, carboxyhemoglobin levels appeared to be better predictor of atherosclerotic disease than smoking histories[43]. Consistent with earlier reports[26,49], our results shows that, Hemoglobin level was significantly reduced by 20 %, in smoke exposed (S-C) rats, when compared to N-C value. However, a highly significant increase in Hb concentration was observed in smoke exposed rats treated with Tocomin (21 %) and Lovastatin (17 %) for 4 weeks when compared to S-C rats. Both Tocomin and Lovastatin administration to smoke exposed rats mediated an increase in Hb levels close to normal value. Whereas, blood carbon monoxide saturation and blood nicotine levels were increased from 6.1 (SCO %) and 1.2 µg/ml in N-C to 10.6 SCO % (73 %) and 4.6 µg/ml (299 %)\ respectively, in S-C rats. After 4 weeks of Tocomin and Lovastatin treatment blood carbon monoxide saturation and blood nicotine levels showed a significant reduction of 30 % and 70 % in S-T_3T respectively, whereas in S-LT, blood carbon monoxide saturation and blood nicotine levels were reduced by 26 %, and 50 % respectively, in comparison to values in S-C rats.

For many years, α-tocopherol (α-T) was generally considered the most potent chain breaking antioxidant in the vitamin E family [22]. However, several reports have shown tocotrienols (T_3s) to be more potent than tocopherols. Tocotrienols have been shown to exhibit greater free radical scavenging properties as cell membrane constituents than tocopherols[108]. They quench free radicals in cell membranes and protect them against lipid peroxidation. The higher antioxidant potency of α-T_3 as compared to α-T is attributed to the combined effects of three properties: it's higher recycling efficiency from chromanoxyl radical, its more uniform distribution in membrane bilayer, and its stronger disordering of membrane lipids which makes interaction of chromanols with lipid radicals more efficient. Since, Tocomin used in the present study is a mixture of α-T_3, γ-T_3, δ-T_3 and α-T; we have examined the efficacy of individual T_3s, α-T and Tocomin as a scavenger of peroxyl radical. Our result showed that, by using DPPH, the order of antiradical activity or hydrogen donating ability, expressed in terms of half quenching concentration (IC_{50}) was δ-T_3> α-T_3> γ-T_3> Tocomin> α-T. Our results are in agreement with earlier reports[72] indicating that in intact membranes, including LDL particles, tocotrienols may have a significantly greater antioxidant effect than α-T and they may provide greater protection against coronary artery disease (CAD). The possible mechanism for this superior efficacy of tocotrienols compared to α-T has been reported elsewhere[69]. The reduction in the free radical quenching efficiency of Tocomin (50.83 µM) in comparison to δ-T_3, α-T_3, γ-T_3 is apparently due to the presence of 23 % α-T, which has a lowest peroxyl radical scavenging efficiency of 70.75 µM. These results are consistent with a previous report indicating the presence of higher percentage (20 %) of α-T in tocotrienol rich fraction (TRF), reduces the antioxidant activity[72].

These results indicate a strong protective effect of dietary tocotrienols, which may help lower the risk of myocardial infarction in smokers. Oxidative stress mediated by free radicals present in the gas phase and tar of cigarettes has been hypothesized to be central to the pathogenesis of smoking-related atherosclerosis[40]. The exact toxic components of cigarette smoke and the mechanisms involved in cigarette smoke related cardiovascular dysfunction are largely unknown, but cigarette smoke increases inflammation, thrombosis, and oxidation of LDL-C. Recent experimental and clinical data support the hypothesis that cigarette smoke exposure increases oxidative stress as a potential mechanism for initiating cardiovascular dysfunction[6]. Our results indicate a modest and significant increase in plasma total lipid, TG, free fatty acid (FFA) and TC in smokers. The increase in plasma TG levels is apparently due to an increase in VLDL which can be the result of either increased VLDL production or decreased VLDL clearance. It is possible that massive free radical load in

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smokers may stimulate VLDL production by increasing adipose tissue lipolysis, increasing hepatic de novo fatty acid synthesis, and decreasing hepatic fatty acid oxidation, all of which provide fatty acid substrate for esterification into TG and assembly into VLDL particles in the liver as well as increase in plasma FFA. Tocomin effectively blocked the increase in the above lipid parameters and reversed them to a level similar to their respective control values of nonsmokers. As expected, all the plasma lipids parameters were significantly increased in smoke exposed control (S-C) rats, when compared to N-C values. Total lipids (TL), triglycerides (TG), free fatty acids (FFA) and total cholesterol (TC) significantly increased from 393, 50, 123 and 82 mg/dl in N-C to 507, 101,145 and 149 mg/dl, respectively, in S-C group. After 4 weeks of Tocomin treatment, levels of TL, TG, FFA and TC were significantly decreased by 9 %, 40 %, 11% and 29 %, respectively, when compared to corresponding S-C values. Whereas, in Lovastatin treated rats, TL, TG, FFA and TC levels were significantly reduced by 7 %, 40 %, 13 % and 35 % respectively, in comparison to corresponding values in S-C group. These results demonstrate that 4-week treatment of smoke exposed rats with 6 mg Tocomin or 0.50 mg Lovastatin mediated a similar and significant reduction in above lipid parameters. As seen in TABLE 4, plasma VLDL-C, LDL-C and non-HDL-cholesterol (non-HDL-C) levels were significantly increased from 10, 52 and 63 mg/dl in N-C to 21 mg/dl (105 %), 110 mg/dl (112 %) and 131 mg/dl (109 %) respectively, in S-C. After 4 weeks of Tocomin or Lovastatin treatment, both VLDL-C, LDL-C and non-HDL-C levels showed a significant reduction 41 %, 44 % and 43 %, respectively, in S-T_3T, whereas, in S-LT, VLDL-C, LDL-C and non-HDL-C were significantly reduced of 41 %, 48 % and 46 %, respectively, in comparison to corresponding values in S-C rats. HDL-C, HDL_2-C and HDL_3-C levels were decreased from 19, 6 and 13 mg/dl in N-C to 17 mg/dl (11 %), 4 mg/dl (34 %) and 12 mg/dl (5 %), respectively, in S-C values. After 4 weeks of Tocomin treatment (S-T_3T) HDL-C, HDL_2-C and HDL_3-C levels showed a significant increase of 77 %, 200 % and 45 %, respectively, when compared to corresponding values in S-C, whereas, in S-LT, HDL-C, HDL_2-C and HDL_3-C levels were increased by 53 %, 125 % and 37 %, respectively. These results demonstrate that both Tocomin and Lovastatin are equally effective in reducing VLDL-C and LDL-C levels. On the other hand, in comparison to N-C values, treatment of smoke exposed rats with Tocomin mediated a significantly higher increase in HDL-C, HDL_2-C and HDL_3-C concentration than the increase seen in Lovastatin treated rats. Data presented in TABLE 5, depict levels of plasma LDL-C, LDL-apoB, small dense (sd-) LDL-C, sd-LDL-apoB, large buoyant (lb-) LDL-C and lb-LDL-apoB. In normal rats, LDL-C and its apoB content were 52 and 121mg/dl, respectively, in N-C, the cholesterol and apoB content of sd-LDL subpopulation was 15 and 39 mg/dl, respectively, whereas, cholesterol and apoB concentrations associated with lb-LDL were 37 and 81 mg/dl. In comparison to control values, smoke exposed rats showed an increase in plasma LDL-C from 52 to 110 mg/dl (112 %) and LDL-apoB from 121 to 139 mg/dl (15 %), whereas sd-LDL-C was increased from 15 to 60 mg/dl (292 %) sd-LDL-apoB was increased from 39 to 71 mg/dl (82 %). In comparison to sd-LDL, the increase in lb-LDL-C of S-C rats was only 35 %, whereas, lb-LDL-apoB level was reduced by 16 % when compared to corresponding values in N-C. Following 4 weeks of Tocomin or Lovastatin treatment to smoke exposed rats, LDL-C levels were significantly reduced by 44 % and 48 %, respectively, and restored these values close to the N-C value. Consistent with a small increase in LDL-apoB content of S-C rats, after 4 weeks of Tocomin or Lovastatin treatment, the decline in LDL-apoB was also minimal. The cholesterol and apoB associated with sd-LDL decreased from 60 and 71 mg/dl to 19 mg/dl (68 %) and 40 mg/dl (44 %), respectively, in S-T_3T, whereas, sd-LDL-C and its apoB content in S-LT was reduced to 17 mg/dl (72 %) and 39 mg/dl (46 %), respectively. On the other hand, the cholesterol level of lb-LDL subfraction in both the treated groups was 43 and 40 mg/dl, which is close to a value of 37 mg/dl in N-C. As indicated above, apoB associated with lb-LDL fraction was reduced from an N-C value of 81 to 67 mg/dl in S-C, whereas, a significant increase to a similar value of 82 mg/dl (21%) indicating a full restoration of lb-LDL-apoB levels and similar to value in N-C.

In normolipidemic rats, the percent wise distribution of cholesterol and apoB from LDL particle to sd-LDL and lb-LDL fractions were 29 %, 32 %, 71 % and 67

%, respectively, in smoke exposed rats the percent sd-LDL-C and sd-LDL--apoB was significantly increased by 85 % and 59 %, when compared to the corresponding percent values from normal rats. Similarly, the percent lb-LDL-C and lb-LDL-apoB in S-C rats was increased by 36 % and 27 %, in comparison to corresponding percent values of N-C rats. The percent increase in sd-LDL-C and sd--LDL-apoB registered in smoke exposed rats, was significantly reduced by 38-45 % after 4 weeks of Tocomin or Lovastatin treatment. In contrast, percent lb-LDL-C and lb--LDL-apoB values in both the treated groups were significantly increased by 31-55 %, when compared to the corresponding percent values of smoke exposed rats. These results demonstrate that a homogeneous preparation of sd-LDL and lb-LDL can be obtained by employing heparin-Mg^{++}-mediated precipitation of purified plasma LDL. In addition, the results also show that the prevalence and concentration of sd-LDL, which is considered more atherogenic than LDL or lb-LDL was substantially increased in smoke exposed rats. In smoke exposed rats treated with Tocomin or Lovastatin for 4 weeks, the sd-LDL concentration was significantly reduced to a level similar to control value (N-C).

Therefore, tocotrienols may exert their cholesterol lowering effect in dyslipidemic smokers and hyperlipidemic rats exposed with cigarette smoke in a similar manner as previously reported for hyperlipidemic animals[58] and humans. Mechanism wise, as previously shown in HepG2 cells, as well as in normolipidemic and hyperlipidemic rats, tocotrienols reduce cholesterol synthesis by suppressing HMG-CoA reductase activity, which in turn is reduced by a decline in its protein mass[58,70]. The decline in protein mass may be achieved by inhibition of HMG-CoA reductase synthesis and/or enhanced degradation. Consistent with *in vivo* results in rats[58], γ-tocotrienol has been shown to mediate the suppression of enzymatic activity and protein mass of HMG-CoA reductase in HepG2 cells through decreased synthesis (57 % of control) and enhanced degradation (2.4-fold versus control) of the enzyme[70]. In addition, γ-tocotrienol was shown to upregulate LDL receptor in mammalian cells and may be implicated in part for the reduction of apoB-lipoprotein *in vivo*[70]. Thus, tocotrienols reduce cholesterol formation in mammalian cells by suppressing HMG-CoA reductase activity through two actions: decreasing the efficiency of translation of HMG-CoA reductase mRNA and increasing the controlled degradation of HMG-CoA reductase protein, posttranscriptionally[70]. In addition, another report indicates that γ-tocotrienol influences apoB secretion by both cotranslational and posttranslational processes involving a decreased rate of apoB translocation and accelerated degradation of apoB in HepG2 cells. This activity correlated with a decrease in free and esterified cholesterol[86]. Taken together, the information indicates an association between the suppression of hepatic cholesterol synthesis and apoB secretion, and the observed lowering of apoB and LDL-C levels in animal and human models[86]. However, elucidation of precise *in vivo* mechanism(s) of Tocomin-mediated inhibition of HMG-CoA reductase at molecular level remains to be investigated. It has previously been established that LDL-C/HDL-C and HDL-C/TC ratios are good predictors for the presence and severity of CAD[31]. LDL-C/HDL-C ratio was significantly increased from 2.72 in N-C to 6.11 (125 %) in S-C group, when compared to ratio in N-C. After 4 weeks of treatment, the increase in LDL-C/HDL-C ratio was significantly prevented and decreased to 2.04 and 2.19 in S-T_3T and S-LT, respectively, which is close to normal control value. On the other hand, HDL-C/TC ratio was significantly decreased from 0.233 in N-C to 0.121 (48 %) in S-C group. Tocomin or Lovastatin treatment to these rats significantly prevented the increase in HDL-C/TC ratios and fully restored them to a ratio value similar to N-C. In N-C, when sd-LDL-C values were divided by HDL-C, a ratio of 0.802 was obtained, which is substantially lower than LDL-C/HDL-C ratio of 2.72. In smoke exposed rats, sd-LDL-C/HDL-C ratio was substantially increased to a ratio value of 3.32 (314 %), when compared to N-C value. As expected, sd-LDL-C/HDL-C ratio in Tocomin and Lovastatin treated rats were substantially reduced to a value of 0.632 (81 %) and 0.653 (80 %), respectively, these ratio values in S-T_3T and S-LT are significantly lower than a ratio of 0.802 in N-C. In comparison to sd--LDL-C/HDL-C, the ratio of lb-LDL-C/HDL-C in S-C rats was increased by 44 %, from an N-C value of 1.93 to 2.77. Consistent with these results, the lb-LDL-C/HDL-C ratios in treated groups where only partially (21-26 %) restored, in comparison to ratio value of N-C rats. These results indicate that sd--LDL-C/HDL-C ratio may be

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considered as a better and novel predictor for CAD risks than LDL-C/HDL-C ratios. In addition, the ratios related to sd--LDL-C and HDL-C in Tocomin or Lovastatin treated rats were positively modulated and restored similar to normal control value, indicating normalization of cholesterol levels associated with the above lipoproteins.

As discussed earlier, severe hyperlipidemia in S-C rats was associated with an increase in LDL and appearance of high concentrations of sd-LDL subpopulation, which is shown to be more prone to oxidation than lb-LDL or LDL and hence more proatherogenic[24,28,29,90,92,94]. Our results demonstrate that in normal rats, relative to lb-LDL, *in vivo* oxidizability of sd-LDL, measured as *ex vivo* base line diene conjugation (BDC), was increased (29 %), while lag phase of its Cu^{++}-induced oxidation was reduced to 16.4 min, in comparison to a lag phase value of 54.0 min for lb-LDL and 95.0 min for LDL, indicating a 3.2- to 5.8-fold shortening in the lag phase of sd-LDL. This marked difference in the oxidizability between sd-LDL and lb-LDL of normal rats is in agreement with previous reports indicating an inherently reduced concentration of antioxidants, and free cholesterol, increased amount of more oxidizable polyunsaturated fatty acids including preformed hydroperoxides in sd-LDL[28,80,87,93]. During 4 weeks of sustained oxidative stress and depletion of endogenous antioxidants in hyperlipidemic S-C rats, the *in vivo* oxidizaibility of sd-LDL was further increased, resulting in a ~2-fold increase in *ex vivo* BDC level, in comparison to lb-LDL, while there was no significant difference in the percent decline in lag phase values of sd-LDL (-27 %) and lb-LDL (-24 %) in S-C, when compared to their values in N-C group. Both Tocomin and Lovastatin feeding significantly blocked the *in vivo* and *in vitro* oxidation of LDL density subfractions, as seen by a decrease in *ex vivo* BDC levels and an increase in their lag phase values. In Tocomin fed rats, the lag phase values of LDL, sd-LDL and lb-LDL were restored to 87 %, 98 % and 93 % of their corresponding normal control values, while in Lovastatin treated rats, their lag time values were restored to 75 %, 85 % and 83 % of N-C values, indicating a better antioxidative effect of dietary tocotrienols than Lovastatin. Based on an earlier initial study in young smokers, Fuller *et al*. (2000) recommended that the only way for young smokers to reduce oxidative damage to the vasculature by tobacco is to quit smoking rather than to take antioxidant vitamin E (d-α-tocopheryl acetate, 400 IU/day) or vitamin C (1000 mg/day) or combination of vitamins E & C supplements. Similarly, in another initial report[19], has demonstrated that young, novice cigarette smokers have lower blood antioxidant capacity and higher lipid peroxidation levels compared to nonsmokers, despite having similar dietary intake. Based on these results, Bloomer emphasized the importance of smoking abstinence to young, novice cigarette smokers. These reports clearly indicate that daily intake of routine dietary antioxidants by young, novice smokers, or heavy dose of antioxidant vitamin supplements by young smokers will not sufficiently alleviate the enormous *in vivo* oxidative stress/damage induced by cigarette smoke. However, since, dietary tocotrienols, because of their potent hypolipidemic/antiatherogenic and antioxidant actions, were able to substantially ameliorate/normalize all the altered parameters including atheroprotective function of HDL described in the thesis, we initially recommend daily supplementation of young smokers with dietary tocotrienols (Tocomin). In conclusion, based on Tocomin mediated multiple therapeutic benefits, described in the present study, daily intake of tocotrienols as a dietary supplement by novice/young/old moderate or heavy smokers as well as chronic smokers including passive smokers may be useful in the prevention and treatment of tobacco-induced dyslipidemia/hyperlipidemia and atherosclerosis. In addition, daily use of dietary tocotrienols will be efficacious, cost effective, and a good source of vitamin E

ACKNOWLEDGEMENTS

The authors like to acknowledge University Grant Commission (UGC), New Delhi (India), for financial support. This study was carried out at the Department of Biochemistry, J N Medical College and Hospital, Aligarh Muslim University, Aligarh, India. The authors like to thank Dr. Z. H. Beg, Er Shakir Khan, Nasir Khan for giving guidance from time to time and Dr. Asif Ali, Chairman, for providing facilities to carry out research work. The author also likes to thank W. H. Leong, vice president CAROTECH, Inc., for kindly providing Tocomin, as a gift.

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