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Original Article

Effects of antioxidant vitamins along with atorvastatin and atorvastatin–niacin combination on diet-induced hypercholesterolemia in rats

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Abstract: The present study investigated the effects of antioxidant vitamins along with atorvastatin and atorvastatin-niacin combination on diet-induced hypercholesterolemia in rats. High cholesterol diet produced a significant increase in the serum total cholesterol, LDL-C, VLDL-C, TG, atherogenic index and decrease in HDL-C and HDL/LDL ratio. The lipid peroxidation and oxidative stress were significantly high in the hyperlipidemic control group. Atorvastatin improved atherogenic index but not the HDL/LDL ratio whereas atorvastatin-niacin combination improved both atherogenic index and HDL/LDL ratio. However, both atorvastatin and atorvastatin-niacin did not affect antioxidant status significantly. Co-administration of vitamin-E and vitamin-C along with atorvastatin and atorvastatin-niacin have improved serum lipid profile, prevented lipid peroxidation and improved antioxidant status. Addition of β-carotene along with lipid lowering drugs did not show additional benefits on serum lipid profile, lipid peroxidation and antioxidant status. Atorvastatin, atorvastatin-niacin combination when added with anti-oxidant vitamins, increased reduced glutathione level but did not affect MDA level, SOD and catalase activity in the liver tissue. Administration of both vitamin-E and vitamin-C along with atorvastatin-niacin therapy produced a significant improvement in the lipid profile as well as antioxidant status. Addition of β-carotene along with atorvastatin-niacin-vitamin-E-vitamin-C combination improved lipid profile but improvement was not as marked as observed with atorvastatin-niacin-vitamin-E-vitamin-C combination. The same beneficial effects of atorvastatin-niacin combination on lipid profile were not observed when it was combined with anti-oxidant vitamins especially β-carotene. The pro-oxidant role of β-carotene may be responsible for this effect.

Keywords: hypercholesterolemia, lipid peroxidation, antioxidants, vitamin-E, vitamin-C, atorvastatin

Introduction

Hypercholesterolemia is one of the major risk factors for coronary artery disease (CAD) and atherosclerosis. It is the LDL that plays a crucial role in the atherogenesis [1]. It is the oxidative modification that imparts an atherogenecity to LDL [2]. Fatty streaks develop in response to specific phospholipids contained in LDL that become oxidized as a result of exposure to the oxidative waste of the artery wall cells [3, 4]. It has been shown that lipid peroxidation is involved in the oxidative modification of LDL [5, 6]. The lipid peroxidation starts only after the depletion of natural antioxidants such as vitamin-E, vitamin-C, β-carotene, etc. in the body [7]. This was supported by the fact that the low serum levels of antioxidant vitamins are associated with high risk of CAD [8, 9]. Antioxidant vitamins prevent lipid peroxidation both *in vivo* and *in vitro* [10, 11]. Numerous epidemiological evidences support the beneficial role of the dietary antioxidant vitamins [12-14]. However some studies have questioned the beneficial role of antioxidant vitamins [15, 16]. Atorvastatin-niacin therapy improves lipid profile but has no significant effects, if any, on antioxidants status. It has been observed that co-administration of antioxidants with atorvastatin-niacin therapy suppress the response of such

therapy to HDL-C [17]. Hence, the present work was undertaken with the aim to study the effects of anti-oxidant vitamins in combination with atorvastatin and atorvastatin-niacin on diet -induced hyperlipidemia in rats.

Material and methods

Animals

Healthy rats (Sprague-Dawley strain) of either sex weighing 180-220 g were divided into different groups each of six (**Table 1**). Normal group received standard pellet diet (Pranav agro industries, Vadodara, India). All other groups received a high cholesterol diet along with respective treatments. Animals were treated for seven days. Water was made available *ad libitum*. The study was approved by the institutional animal ethics committee established in accordance with committee for the purpose of supervision and control of experiments on animals (CPCSEA) [18].

Drugs administration

Atorvastatin (1.4 mg/kg, as suspension in 1% CMC), niacin (250 mg/kg, as solution in distilled water), vitamin-E (60 mg/kg, as solution in olive oil), vitamin-C (80 mg/kg, as solution in distilled water) and β-carotene (40 mg/kg, as solution in arachis oil) were administered orally by gavage, once in a day in the morning (9.00 to 10.00 a.m.) to respective groups for 7 days.

Diet-induced hyperlipidemia

Method of Blank [19] with modification was used to produce diet-induced hyperlipidemia. Briefly, normal group received standard chew diet and all other groups received high cholesterol diet consisting of – standard Pellet diet 92%, cholesterol 2.0 %, cholic acid 1 % and coconut oil 5% for seven days. The standard pellet diet (Pranav Agro Industries, Vadodara.) consisted of crude protein (22.06%), crude oil (4.04%), crude fiber (4.0%), Ash (10.0%) and sand silica (0.15%). The standard pellet diet supplies energy of 3620 Kcal/kg.

Reagents and chemicals

Cholesterol, sodium cholate, vitamin-E, vitamin-C, niacin were purchased from Kemphasol, Bombay; β-carotene was purchased from HiMedia Laboratory Ltd, Bombay. Atorvastatin was received as gift sample from Zydus research centre, Ahmedabad, India. All other chemicals and reagents were of analytical grade.

Table 1. Animal groups and respective treatments.

Animal groups	Treatments
Group 1	Normal (Vehicle only)
Group 2	Hyperlipidemic control
Group 3	Atorvastatin
Group 4	Atorvastatin + vitamin-E
Group 5	Atorvastatin + vitamin-C
Group 6	Atorvastatin + β-carotene
Group 7	Atorvastatin + vitamin-E + vitamin-C
Group 8	Atorvastatin + β-carotene + vitamin-C
Group 9	Atorvastatin + β-carotene + vitamin-E
Group 10	Atorvastatin + Niacin
Group 11	Atorvastatin + Niacin + vitamin-E
Group 12	Atorvastatin + Niacin + β-carotene
Group 13	Atorvastatin + Niacin + vitamin-E + vitamin-C
Group 14	Atorvastatin + Niacin + β-carotene + vitamin-E + vitamin-C

Blood collection and biochemical estimation

The animals were treated for 7 days. At the end of experimental period, the rats in each group were deprived of food overnight but not the water and sacrificed. The blood was collected by retro-orbital puncture technique and serum was separated. The serum total cholesterol (TC), triglyceride (TG) and high density lipoprotein cholesterol (HDL-C) were estimated using commercially available kits (Bayer diagnostic (P) Ltd. India). Very low density lipoprotein-cholesterol (VLDL-C) was calculated as TG/5. Low density lipoprotein-cholesterol (LDL-C) levels were calculated using Friedewald's formula [20]. The Atherogenic index was calculated using formula – Atherogenic Index (AI) = (VLDL-C + LDL-C)/ HDL-C. The liver tissues were collected, washed thoroughly in normal saline, bloated and preserved at -40°C for further analysis. The liver homogenates were prepared in tris-hydrochloride buffer. They were subjected to protein [21], malondialdehyde (MDA) [22], superoxide dismutase (SOD) [23], catalase [24], and reduced glutathione (GSH) [25] estimation.

Statistical analysis

All values were expressed as Mean ± SEM. The statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tuckey's multivarient test. The value of p less than 5% ($p < 0.05$) was considered statistically significant.

Results

There were no significant differences in food intake among various groups. There was significant increase in serum TC, TG, LDL-C, VLDL-C in hyperlipidemic control group compared to normal group. The HDL-C as well as HDL to LDL ratios was significantly decreased in hyperlipidemic control. This was evidenced by increased levels of atherogenic index. Atorvastatin significantly reduced serum TC, TG, LDL-C and VLDL-C levels compared with the hyperlipidemic control and the atherogenic index declined significantly. The combination of atorvastatin with vitamin-E and vitamin-C showed significant beneficial effects on serum lipid profile. Only this combination showed additional benefits as compared with statin alone. When β-carotene was added to this combination, the beneficial effects of vitamin-E and C disappeared (**Table 2**).

Compared to atorvastatin alone, the atorvastatin-niacin significantly improved serum lipid profile. When this combination was supplemented with vitamin-E and C, maximum improvement in serum lipid profile was observed. However, when β-carotene was added to this combination, no additional benefits were observed, in fact increase in the atherogenic index was found. The atorvastatin-niacin with β-carotene decreased the beneficial effects of atorvastatin-niacin on serum lipid profile (**Table 2**).

MDA is a marker of lipid-peroxidation. There was significant lipid-peroxidation in hyperlipidemic control group as indicated by increased MDA levels compared with normal group (**Table 3**). Atorvastatin alone and in combinations with different vitamins significantly reduced liver lipid-peroxidation. Atorvastatin–vitamin-E–vitamin-C combinations showed highest inhibition of lipid-peroxidation. Atorvastatin-niacin combination significantly reduced lipid peroxidation when compared with hyperlipidemic control group (**Table 3**). However, atorvastatin produced significant inhibition of lipid peroxidation compared with atorvastatin-niacin combination. Atorvastatin-niacin combination when combined with different anti-oxidant vitamins did not show any additional benefits. The atorvastatin-niacin-β-carotene has significantly reduced lipid peroxidation compared with both atorvastatin alone and atorvastatin-niacin.

SOD levels were significantly increased in hyperlipidemic control group as compared with normal group. Atorvastatin alone did not affect SOD levels. However, in combination with different vitamins it significantly reduced SOD levels in liver tissues (**Table 3**). Atorvastatin-Niacin did not decrease liver SOD activity and even showed increase. When atorvastatin-niacin was combined with different anti-oxidant vitamins, increase in SOD activity was observed instead of reduction. Highest increase in SOD activity was observed with atorvastatin-niacin-β-carotene-vitamin-E-vitamin-C (**Table 3**).

Catalase levels were significantly increased in hyperlipidemic control group when compared with normal group. Atorvastatin alone did not affect catalase activity. However, in combination with different anti-oxidant vitamins, atorvastatin significantly increased catalase activity. Highest improvement in catalase activity was achieved with atorvastatin-vitamin-E-vitamin-C

Table 2. Effects of various treatments on serum lipid profile of diet-induced hyperlipidemic rats.

Groups (n=6)	TC (mg/dl)	TG (mg/dl)	HDL-C (mg/dl)	LDL-C (mg/dl)	VLDL-C (mg/dl)	HDL/LDL Ratio	Atherogenic Index
Group 1	64.55 ± 2.99	57.49 ± 3.55	24.71 ± 1.39	28.33 ± 2.11	11.51 ± 0.71	0.87 ± 0.05	1.61 ± 0.20
Group 2	308.74 ± 30.62*	121.34 ± 16.93*	10.20 ± 1.70*	276.31 ± 29.77*	24.27 ± 3.50*	0.04 ± 0.01 *	29.47 ± 1.96*
Group 3	164.60 ± 9.89**	72.65 ± 4.12**	12.72 ± 1.33	135.50 ± 2.51**	14.53 ± 0.82**	0.09 ± 0.04	11.79 ± 0.25**
Group 4	132.32 ± 7.04**	94.06 ± 4.79	19.22 ± 2.10	89.29 ± 6.31**	15.80 ± 0.95	0.22 ± 0.02	5.47 ± 0.35**‡
Group 5	143.34 ± 6.78**	107.58 ± 14.33	17.65 ± 1.65	104.18 ± 6.62 **	21.51 ± 1.39	0.17 ± 0.02	7.12 ± 0.49**‡
Group 6	199.10 ± 20.92**	92.24 ± 17.17	10.51 ± 2.89	170.14 ± 24.00**	18.44 ± 3.34	0.06 ± 0.03	17.94 ± 0.95**
Group 7	62.45 ± 2.01**‡	67.05 ± 3.15**	25.98 ± 1.12**	23.06 ± 1.87**‡	13.41 ± 0.79**	1.13 ± 0.05**	1.40 ± 0.24**‡
Group 8	152.29 ± 16.33**	57.03 ± 9.03**	24.86 ± 3.46**	91.16 ± 12.60**	11.41 ± 1.86**	0.27 ± 0.11	4.13 ± 0.42**‡
Group 9	173.49 ± 14.13**	65.34 ± 6.25**	22.23 ± 4.40**	115.56 ± 43.57**	13.07 ± 1.25**	0.19 ± 0.39	5.79 ± 1.02**‡
Group 10	93.04 ± 5.38**	95.61 ± 1.43	16.17 ± 1.36	57.74 ± 6.44**	19.12 ± 0.22	0.28 ± 0.05**	4.75 ± 0.49**‡
Group 11	94.53 ± 1.96**	69.36 ± 3.74 **	23.07 ± 1.33**	57.06 ± 2.51**	14.40 ± 0.78**	0.40 ± 0.04**	3.10 ± 0.25**‡
Group 12	200.82 ± 41.42	94.91 ± 14.33	20.14 ± 5.74	161.69 ± 42.29 *	18.98 ± 0.87	0.12 ± 0.07	8.97 ± 0.75**
Group 13	63.53 ± 1.87**†	68.04 ± 3.93**	25.80 ± 1.02**	24.09 ± 1.47**	13.60 ± 0.78**	1.07 ± 0.08**	1.46 ± 0.22**‡
Group 14	94.86 ± 4.33**	71.69 ± 3.68**	19.28 ± 0.55	65.16 ± 3.57 **	14.35 ± 0.82**	0.30 ± 0.03	4.12 ± 0.80**‡

All values represent Mean ± SEM from six rats. Statistical analysis was carried out using One Way ANOVA followed by Tukey's test. The value of $P < 0.05$ was considered statistically significant. *: compare with control group, **: compared with the hyperlipidemic control group, ‡: compared with the atorvastatin, †: compared with all other groups.

Table 3. Effects of various treatments on the lipid peroxidation and the anti-oxidants status of hyperlipidemic rat livers.

Groups (n=6)	MDA (nmol/mg protein)	SOD (U/min/mg protein)	Catalase (U/min/mg protein)	GSH x 10^{-3} (µg/mg protein)
Group 1	1.85 ± 0.18	6.13 ± 0.67	167.20 ±14.00	933.00 ± 60.80
Group 2	45.70 ± 18.40*	13.98 ± 0.87*	3.60 ± 0.10*	357.60 ± 12.46*
Group 3	3.62 ± 0.08**	15.15 ± 0.46	4.50 ± 0.63	458.40 ± 8.21
Group 4	30.8 ± 4.30**	4.77 ± 0.22**‡	221.00 ± 18.00**‡	398.70 ± 27.10
Group 5	7.80 ± 0.79**	4.58 ± 0.25**‡	162.00 ± 12.00**‡	363.00 ± 16.80
Group 6	1.51 ± 0.20**	5.98 ± 0.59**‡	45.50 ± 5.82*‡*	718.20 ± 19.20**
Group 7	1.12 ± 0.06**	4.20 ± 0.21**‡	277.12 ± 21.14**	812.04 ± 22.48**‡
Group 8	1.21 ± 0.07**	6.48 ± 0.53**‡	147.40 ± 14.50**‡	1049.12 ± 59.77**‡
Group 9	1.47 ± 0.14**	7.57 ± 0.48**‡	116.90 ± 29.10**‡	1330.10 ± 82.20**‡
Group 10	183.1 ± 12.20**	25.87 ± 1.45	5.75 ± 0.49	480.60 ± 32.10
Group 11	176 ± 3.20**	23.41 ± 2.34	6.44 ± 0.63	640.00 ± 34.68**
Group 12	2.18 ± 0.51**‡	7.57 ± 0.47	49.70 ± 20.30**	975.40 ± 23.60**
Group 13	176 ± 30.00	76.47 ± 3.02	8.80 ± 0.29	935.30 ± 36.12**
Group 14	125.5 ± 30.00**‡	147.50 ± 9.43	17.94 ± 1.73**	365.30 ± 21.12

All values represent Mean ± SEM from six rats. Statistical analysis was carried out using One Way ANOVA followed by Tukey's test. The value of $P < 0.05$ was considered statistically significant. *: compared with normal group, **: compared with hyperlipidemic control group, ‡: compared with atorvastatin-niacin.

combination (**Table 3**). Atorvastatin-niacin did not increase catalase activity. When supplemented with β-carotene or vitamin-E-vitamin-C-β-carotene, atorvastatin-niacin combination significantly increased the catalase activity (**Table 3**).

Due to oxidative stress, there was significant decrease in reduced glutathione levels in hyperlipidemic control groups. Atorvastatin when given in combination with more than one vitamin significantly increased the reduced GSH levels. Highest increase in the GSH level was observed with atorvastatin-β-carotene-vitamin-E combination (**Table 3**). Atorvastatin-niacin did not affect liver reduced glutathione levels. However, when combined with vitamin-E, β-carotene, vitamin-C, it significantly increased GSH levels. When all the vitamins were given with atorvastatin-niacin, no additional improvements in GSH levels were observed (**Table 3**).

Discussion

Cholesterol homeostasis in the body is maintained by the balance between cholesterol biosynthesis, and its metabolism. The cholesterol biosynthesis is controlled by the rate limiting enzyme-HMG CoA Reductase. Atorvastatin blocks this enzyme and thereby prevents cholesterol biosynthesis. In the present study, the cholesterol lowering effects of atorvastatin (mainly LDL-C) can be attributed to HMG CoA reductase inhibition. Niacin by limiting lipolysis in adipose tissue, decreasing esterification of hepatic TG, and increasing the activity of lipoprotein lipase reduces serum TG and TC levels. The combination of atorvastatin-niacin caused and additive reduction in serum total cholesterol, LDL-C, TG, VLDL-C levels [26].

The inhibition of lipid peroxidation observed with atorvastatin might be secondary to lipid

lowering effect as well as its antioxidant effects [27]. However, the drug alone did not improve SOD, catalase activities, and GSH levels in the present study. The combinations of atorvastatin with other vitamins significantly inhibited lipid peroxidation due to their anti-oxidant properties [28, 29]. However, its combination with more than one vitamin did not show any additional inhibition in the lipid peroxidation. Atorvastatin alone did not affect SOD and catalase activities in liver tissues. The combination of atorvastatin with vitamin-E, vitamin-C, or β-carotene, improved liver SOD and catalase activities. Highest improvement was observed with vitamin-E-vitamin-C combination. Supplementation of β-carotene did not show any additional benefits. The GSH levels were significantly increased when atorvastatin was combined with more than one vitamin compared with its combination with individual vitamins. This might be attributed to their sparing effects. Vitamin–C has been found to increase intracellular glutathione levels by sparing effect [30].

The atorvastatin-niacin alone and in combination with vitamins inhibited lipid peroxidation. However, inhibitory effects on lipid peroxidation were not as beneficial as observed with atorvastatin-vitamins combinations. Atorvastatin –niacin –vitamins combinations showed high SOD activities and low catalase activities compared with atorvastatin-vitamins combinations. Since, SOD is a stress protein; its levels were increased during oxidative stress [31]. It may be likely that antioxidant vitamins in combination with Atorvastatin-niacin induce oxidative stress. Studies have shown that excessive vitamins in the body may act as pro-oxidant leading to oxidative stress [32].

In conclusion, antioxidant vitamins when given with Atorvastatin additionally improve lipid profile, inhibit lipid–peroxidation, and improve antioxidant status. More than one vitamin with Atorvastatin did not show any additional advantage. When Atorvastatin-niacin combination was used with vitamins, the beneficial effects on lipid profile, lipid peroxidation and anti-oxidant status were not as marked as with Atorvastatin–vitamins combinations. When more than two vitamins were used with Atorvastatin–niacin, it may lead to oxidative stress. Hence, vitamins in combination with lipid lowering drugs must be used with care.

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