



## EFFECT OF DIETARY TOCOTRIENOLS ON INFECTION AND INFLAMMATION INDUCED LIPOPROTEIN OXIDATION IN HAMSTERS

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### ABSTRACT

The tocotrienol isomers ( $\alpha$ -,  $\beta$ -,  $\gamma$ - and  $\delta$ -) are naturally occurring analogues of the Vitamin E family found mainly in cereal grains and palm oil, and are widely used as antioxidant and hypolipidemic agents. We have initially investigated the role of mixture of dietary  $\alpha$ -,  $\beta$ -,  $\gamma$ - and  $\delta$ -tocotrienols (Tocomin) in inhibiting the infection/inflammation induced lipoprotein oxidation in hamsters. Syrian hamsters were injected with bacterial lipopolysaccharide (LPS, 200  $\mu$ g), zymosan (20 mg), or turpentine (0.5 ml) to mimic acute infection, acute systemic inflammation, and acute localized inflammation, respectively. Tocomin (1.0 mg) was administered daily for 10 days before and 12 h after LPS or 24 h after turpentine or zymosan injection. Our results demonstrated that tocotrienols pretreatment significantly decreased the levels of plasma lipid peroxidation products, whereas plasma total antioxidant level was significantly increased. In addition, pretreatment with tocotrienols significantly reversed/restored the low density lipoprotein (LDL) and erythrocytes lipid peroxidation, including an increase in lag phase of copper-induced LDL oxidation. Moreover, tocotrienols mediated a significant amelioration in LDL-lysophosphatidylcholine (LPC) content as well as in arylesterase activity, indicating a potent antioxidant property of tocotrienols. In conclusion, our results indicate that the alleviation of inflammatory conditions and oxidative stress as well as inhibition of LDL oxidation are due to potent free radical scavenging properties of dietary tocotrienols and, thus, can be used as a dietary supplements in the prevention and treatment of systemic inflammatory process which might induce atherosclerosis.

**Keywords:** LDL Oxidation, Infection and Inflammation, Tocotrienols

### INTRODUCTION

Recent studies have suggested a link between atherosclerosis and infection and inflammation<sup>1</sup>. Atherosclerosis is a multifaceted disease process with several different well defined risks factors, such as hypercholesterolemia, smoking, hypertension and diabetes. Although some studies have suggested that specific infectious agents play a direct role in the vessel wall in the formation of atherosclerotic lesions<sup>2</sup>. Both infection and inflammation are accompanied by a systemic host response known as the acute-phase response (APR). Acute phase response represents a complex reaction of the host that is accompanied by alterations in lipid and lipoprotein metabolism that could be a mechanism for enhanced susceptibility to atherogenesis<sup>3</sup>. In animal models lipopolysaccharide (LPS) treatment rapidly increases serum triglyceride (TG), total cholesterol (TC) and LDL cholesterol levels, while exhibited a decrease in antiatherogenic high density lipoprotein (HDL) cholesterol levels<sup>1</sup>. The increase in serum and lipoprotein lipids in hamsters treated with LPS is apparently due to increase in hepatic 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase activity<sup>4</sup>.

A specialized feature of infection/inflammation is the production of plenty of damaging oxygen free radicals in the system, which leads to a condition of oxidative stress that enhances the peroxidation of lipids<sup>5</sup>. Oxidative modification of lipoproteins plays a central role in the pathogenesis of atherosclerosis<sup>6</sup>. Although lipid oxidation in the vessel wall is thought to occur as result of local deficiency of endogenous antioxidants or an excess of free metal ions, only limited data support these hypotheses<sup>7-8</sup>. Research has shown that human atherosclerotic plaques contain massive amounts of lipid peroxidation products, despite the presence of large quantities of  $\alpha$ -tocopherol and ascorbate<sup>9</sup>. In addition, vitamin E, despite its effect on reducing oxidative stress markers and lowering plasma TG levels, was not effective in inhibiting foam cell formation in the aortic arch of hyperlipidemic-diabetic hamster model<sup>10</sup>. Results of *ex vivo* experiment indicate that supplementation of lipid-soluble antioxidants to humans<sup>11</sup> and animals<sup>6</sup> increases the resistance of LDL to oxidation. An increase in serum conjugated diene and malondialdehyde (MDA) in LPS, zymosan or turpentine treated hamsters was previously reported<sup>12</sup>. In rats, LPS treatment significantly decreases plasma

total antioxidants capacity and increases MDA level, which were reduced by subsequent lipoic acid treatment<sup>13</sup>. An increase in erythrocytes MDA concentration was previously reported in LPS treated rats, which were significantly reduced following treatment with vitamin A, C, E or EGB761<sup>14-15</sup>.

Furthermore, circulating LDL is protected from oxidative stress by HDL-associated enzymes, particularly paraoxanase (PON), which destroys biologically active phospholipids<sup>16</sup>. The serum PON activity is decreased in rabbits after administration of croton oil and PON depleted HDL is unable to protect LDL from oxidation *in vitro*<sup>17</sup>. Because reactive oxygen is generated as part of host defense and paraoxanase protects LDL from oxidative stress, it may be postulated that APR may increase LDL oxidation *in vivo*<sup>12</sup>. Aslan *et al*<sup>18</sup> have reported a significant decrease in PON and arylesterase activities in *H. pylori* infected subjects, which seems to be related to decrease in HDL-C, and in part, to increased oxidative stress and inflammatory condition induced by *H. pylori* infection.

Increased use of natural products like fruits and vegetables may protect against free radical-mediated LDL oxidation and lipid peroxidation by providing dietary sources of antioxidants<sup>19</sup>, such as vitamin E. Vitamin E consists of four tocotrienol isomers ( $\alpha$ -,  $\beta$ -,  $\gamma$ -,  $\delta$ -) and four tocopherol isomers (vitamin E) which are found mainly in cereal grains, rice bran and palm oil and all of them are membrane soluble antioxidants. Published reports indicate that both *in vitro* and in intact membranes including LDL particles tocotrienols exert significantly greater protection against coronary artery disease (CAD)<sup>20-21</sup>. Dietary tocotrienols have been reported to exhibit a strong antioxidant property in hyperlipidemic animals<sup>22-23</sup> and humans<sup>24</sup>. Several investigators reported that tocotrienols have greater antioxidant activity than tocopherols, and protect more efficiently against some free radical-related diseases than does tocopherols<sup>25-9</sup>. However, none of the studies have reported the inhibitory effect of tocotrienols on infection and inflammation induced LDL oxidation and oxidative stress in hamsters. Therefore, in the present study, we have examined the role of Tocomin (tocotrienols) in attenuating the oxidative stress in three distinct models of infection and inflammation, which are produced by the administration of LPS, zymosan, or turpentine, that are well characterized inducers of APR.

## MATERIALS AND METHODS

### Chemicals

Twenty five percent palmvitae oil suspension of Tocomin (tocotrienols) containing 6.4 % d- $\alpha$ -tocotrienol, 1 % d- $\beta$ -tocotrienol, 10.2 % d- $\gamma$ -tocotrienol, 3.2 % d- $\delta$ -tocotrienol and 5.7 % d- $\alpha$ -tocopherol as well as RBD palm olein were supplied as a gift from CAROTECH BHD, Chemor, Malaysia. Lipopolysaccharides (*E. coli*, 055:B5) and zymosan A were purchased from Sigma-Aldrich (USA). Oil of turpentine was purchased from Vasco Drug Laboratories (India). All other chemicals and reagents used in this study were of analytical grade.

### Animal procedures

Male golden hamsters, weighing about 175-200 g were purchased from Central Drug Research Institute, Lucknow, India, were acclimatized for 7 days. The protocol of the study was approved by the animal ethical committee of the J. N. Medical College, A M University, India. The hamsters were given standard pellet rodent chow (Ashirwad Industries, India) and water *ad libitum*. For the induction of acute systemic infection, acute localized sterile inflammation or acute noninfectious systemic inflammation, hamsters were injected with LPS, turpentine oil or zymosan, respectively. Lipopolysaccharide, 200  $\mu$ g in 0.5 ml saline and zymosan, 20 mg in 0.5 ml warm (50°C) PBS, pH 7.0, were injected intraperitoneally, whereas, turpentine oil, 0.5 ml, was injected subcutaneously. Hamsters in control group were injected intraperitoneally with 0.5 ml saline only. Subsequently, food was withdrawn from both control and treated hamsters<sup>12</sup>. Tocomin (1 g) was prepared in ethanol and palmvitae oil (1:20).

In normal control group, five hamsters were given 1.0 ml palmvitae oil/hamster/day, through gastric intubation, 10 days before and 24 h after saline injection. Five hamsters in each LPS, turpentine or zymosan control group were administered 1.0 ml palmvitae oil/hamster/day, 10 days before and 12 h after LPS injection or 24 h after turpentine or zymosan injection. Five hamsters in each LPS, turpentine or zymosan Tocomin treated group were given total 10 mg (5 mg each in morning and evening) Tocomin/day, 10 days before and 12 h after LPS injection or 24 h after turpentine or zymosan injection. The dose of Tocomin was adjusted according to previous published reports<sup>24</sup>. At the end of experiment, hamsters in each group were anaesthetized and blood was collected by cardiac puncture in heparinized tubes for plasma separation.

### Measurement of plasma "total antioxidant power" (FRAP) and arylesterase activity

The method of Benzie and Strain<sup>33</sup> was used for measuring the ferric reducing ability of plasma, the FRAP assay, which estimate the "total antioxidant power". Plasma arylesterase activity was determined by the method of Ayub et al.<sup>34</sup> by using phenyl acetate as the substrate.

### Isolation of lipoprotein and measurement of lipoprotein oxidation

The precipitation method described by Wieland and Seidel<sup>35</sup> was used for the isolation of plasma LDL. The method of Patsch et al.<sup>36</sup> was used for the isolation of HDL. Lipid peroxidation products in serum were measured by several methods. Conjugated diene was measured according to the method of Corongiu et al.<sup>37</sup>. Quantification of lipid hydroperoxide was done according to the method Nourooz-Zadeh et al.<sup>38</sup>. Lipid peroxide contents, measured as thiobarbituric acid reactive substances (TBARS), were assayed by the method of Yagi<sup>39</sup>. Lysophosphatidyl choline (LPC) content in LDL was determined by Quinn et al.<sup>40</sup>. The LPC band on silica-gel plates was identified by comigration with standard, scraped, and assayed for phosphorus content as described by Bartlett<sup>41</sup>.

### Measurement of *ex vivo* and *in vitro* Cu<sup>++</sup>-mediated susceptibility of LDL to oxidation

The *ex vivo* and *in vitro* Cu<sup>++</sup>-mediated susceptibility of isolated LDL to oxidation was assessed by determining the lag phase of conjugated diene formation using the method of Esterbauer et al.<sup>42</sup>. Prior to oxidation studies LDL sample was dialyzed against 5 mM

phosphate buffer saline (PBS), pH 7.4, for 12 h. The incubation mixture contained 50  $\mu$ g of LDL in 1.0 ml of 10 mM PBS, pH 7.0. The MDA content in LDL was assayed by the method of Niehaus and Samuelsson<sup>43</sup>.

### Measurement of erythrocytes lipid peroxidation products and mda release from intact erythrocytes

The determination of MDA in erythrocytes was carried out according to the method of Stocks and Dormandy<sup>44</sup>. The procedure of Cynamon et al.<sup>45</sup> was employed for the determination of MDA release from intact erythrocytes. The MDA values in the samples were calculated by using a molar extinction coefficient of  $1.52 \times 10^2$  M<sup>-1</sup>cm<sup>-1</sup>.

### Protein estimation

The protein was determined by the method of Bradford<sup>46</sup> using bovine serum albumin as standard.

### Statistical evaluation

Results were expressed as mean  $\pm$  SD. Statistical analysis of data was done by employing two-tailed Student *t*-test as described by Bennet and Franklin<sup>47</sup>.

## RESULTS

### Impact of tocotrienols on plasma total antioxidants and lipid peroxidation products

We determined the effect of tocotrienol pretreatment on plasma total antioxidants and lipid peroxidation products. As seen in Fig.1, after 12 h of LPS or 24 h of turpentine or zymosan injection, plasma total antioxidants was significantly reduced from a control value of 75 to 30 (61 %), 50 (33 %) or 45 (40 %)  $\mu$ mole/dl, which was significantly increased to 61 (106 %), 70 (40 %) or 65 (44 %)  $\mu$ mole/dl in LPS, turpentine or zymosan Tocotrienols pretreated hamsters, respectively. As evident from the data presented in Fig. 2, Plasma conjugated diene, lipid hydroperoxide and MDA were significantly increased by 70-109 %, 77-145 %, and 79-172 %, respectively, in LPS, turpentine or zymosan stressed hamsters, with a maximum effect on LPS stressed hamsters, which were significantly reduced by 13-29 %, 29-36 % and 21-46 %, respectively after tocotrienols pretreatment. These results illustrated that not only LPS but other APR inducers, turpentine or zymosan, also increased the plasma lipid peroxidation products. Thus, the data depicted that tocotrienols pretreatment in stressed hamsters significantly restored the plasma total antioxidants level and blocked the increase in plasma conjugated diene, lipid hydroperoxide and MDA to a level close to normal value.

Normal control, fed 0.5 ml palmvitae oil/hamster/day, 10 days before and 24 h after saline injection; N-T, fed 10 mg Tocotrienols/hamster/day, 10 days before and 24 h after saline injection; L-C, T-C, Z-C, LPS, turpentine or zymosan stressed control; LPS Tocomin treated, fed 10 mg tocotrienols/hamster /day, 10 days before and 12 h after LPS injection; Turpentine or zymosan Tocomin treated, fed 10 mg tocotrienols/hamster/day, 10 days before and 24 h after turpentine or zymosan injection.

Since, infection and inflammatory diseases are characterized by excess generation of oxygen free radicals, which exhibit erythrocytes membrane lipid peroxidative damage *in vivo*, we have examined the cause of this membrane damage by quantifying an end product of fatty acid peroxidation, namely, MDA. As shown in Fig.3, MDA content of erythrocytes hemolysate was significantly increased in LPS, turpentine or zymosan group. Pretreatment of LPS, turpentine or zymosan stressed hamsters with tocotrienols significantly prevents the increase in MDA content and decreased it, in comparison to corresponding LPS, turpentine or zymosan value. Furthermore, erythrocytes from LPS, turpentine or zymosan stressed group showed a greater susceptibility to hydrogen peroxide-induced lipid peroxidation than those from normal control group. A significant increase of 109 %, 54 % or 73 % in the release of MDA in LPS, turpentine or zymosan was observed, when compared to normal value. Tocotrienols pretreatment to these stressed hamsters significantly blocked the increase in MDA release

and decreased it by 31 %, 20 % or 20 %, respectively. These results demonstrate that the induction of infection and inflammation in hamsters is associated with a significant increase in erythrocytes

membrane lipid peroxidation, which is significantly prevented by the administration of tocotrienols.

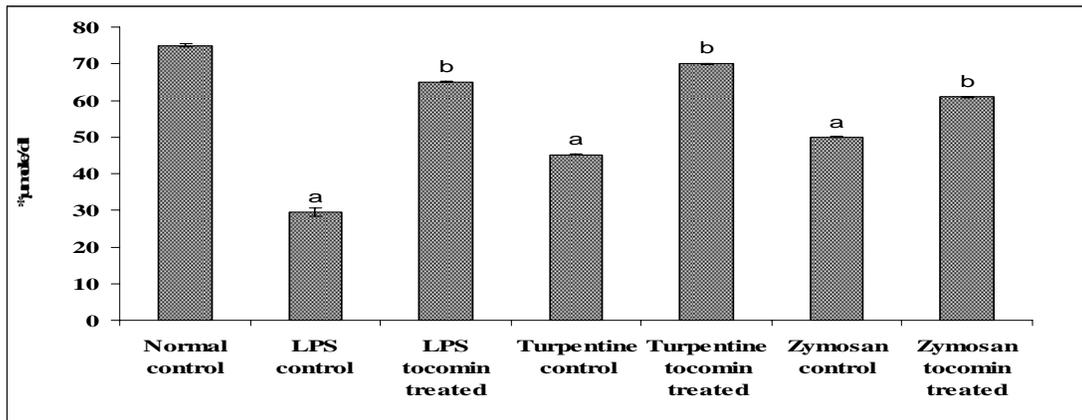


Fig. 1: Antioxidant impact of Tocomin pretreatment on plasma total antioxidants level in three groups of stressed hamsters

\*Values are mean ±SD from pooled plasma of 5 hamsters in each group. <sup>a</sup>Each value are significantly different from normal control at p<0.001. <sup>b</sup>Each value are significantly different from their respective controls at p<0.001.

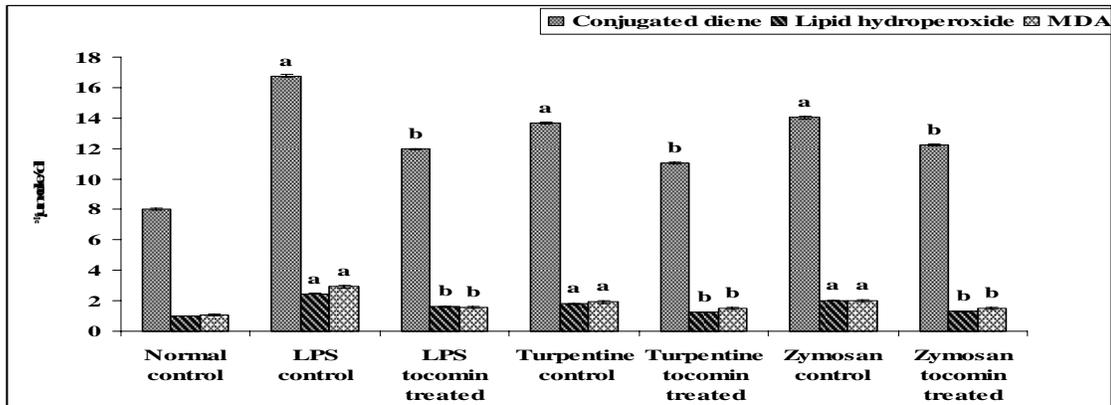


Fig. 2: Antilipoperoxidative effect of Tocomin pretreatment on plasma conjugated diene, lipid hydroperoxide and MDA level in three groups of stressed hamsters.

\*Values are mean ±SD from pooled plasma of 5 hamsters in each group. <sup>a</sup>Each value are significantly different from normal control at p<0.001. <sup>b</sup>Each value are significantly different from their respective controls at p<0.001.

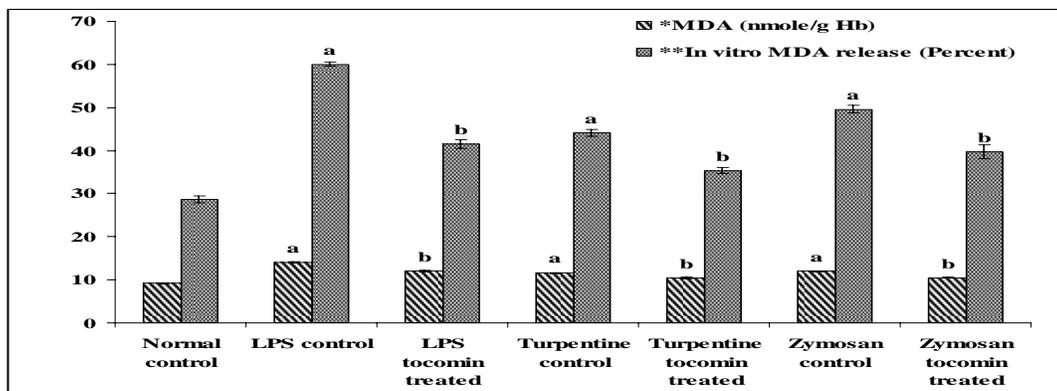


Fig. 3: Impact of Tocomin pretreatment on erythrocyte MDA and in vitro MDA release in three groups of stressed hamsters

\*Values are mean ± SD from pooled hemolyzate prepared from erythrocytes of 5 hamsters in each group. \*\*Values are mean ± SD from pooled intact erythrocytes of 5 hamsters in each group. <sup>a</sup>Each value are significantly different from normal control at p<0.001. <sup>b</sup>Each value are significantly different from their respective controls at p<0.001.

### Ex vivo and Cu<sup>++</sup>-mediated in vitro oxidation of LDL in LPS, turpentine or zymosan stressed hamsters pretreated with tocotrienols

As seen in Table 1, the *ex vivo* base line diene conjugation (BDC) levels of LDL in LPS, turpentine and zymosan stressed hamsters were increased by 1.8-, 1.4- and 1.6-fold, respectively, in comparison to the normal control value. Pretreatment of these stressed hamsters with tocotrienols partially blocked the *in vivo* oxidation of LDL and reduced their BDC levels by 1.2-, 1.2- and 1.3-fold, respectively, when compared to their respective controls. Maximal *in vitro* Cu<sup>++</sup>-mediated oxidation of LDL was achieved after 12 h of incubation. The maximal conjugated diene value of LDL in normal control group was substantially increased by 4.1-fold, when compared to corresponding basal values in normal control. Compared to corresponding maximal values in normal group, LDL associated maximal conjugated diene formation of LPS, turpentine or zymosan hamsters were increased by 2-, 1.4- and 1.8-fold, respectively. Due to tocotrienols pretreatment, the decrease in maximal conjugated diene values of LDL in all the three stressed groups were 1.5-, 1.4- and 1.6-fold, respectively, in comparison to corresponding values in LPS, turpentine or zymosan groups. As expected, the lag phase time of LDL oxidation was reduced from 90

min in normal group to 30, 60 and 50 min in LPS, turpentine and zymosan stressed hamsters. Pretreatment of LPS, turpentine or zymosan groups with tocotrienols restored the lag phase time of LDL oxidation and increased them to 60, 72 and 70 min, respectively.

The Table 1 also depicted that the *ex vivo* base line level of MDA in LDL was significantly increased by 2.7-, 1.8- and 2.3-fold, in LPS, turpentine and zymosan stressed hamsters, respectively, as compared to normal control group. Pretreatment with tocotrienols blocked the *in vivo* increase in MDA of LDL in all the three distinct groups of stressed hamsters and reduced their levels by 1.7-, 1.5- and 1.7-fold, respectively, when compared to corresponding basal values in LPS, turpentine and zymosan stressed hamsters. The maximal MDA content of LDL was significantly increased by 11-fold as compared to basal value in normal control group. In addition, the maximal MDA value in LPS, turpentine and zymosan group were also significantly increased by 2.3-, 1.4- and 2.0-fold, respectively, when compared to their respective controls. Pretreatment of LPS, turpentine and zymosan stressed hamsters with tocotrienols prevented the increase in maximal MDA content of LDL and reduced them by 1.6-, 1.3- and 1.8-fold, respectively, as compared to their corresponding maximal MDA values.

**Table 1: Ex vivo and Cu<sup>++</sup>-mediated in vitro susceptibility to LDL oxidation in three groups of stressed hamsters pretreated with Tocomin**

Group	Conjugated diene <sup>†</sup>		Lag Phase <sup>††</sup>	MDA <sup>†</sup>	
	Basal	Maximal		Basal	Maximal
Normal control	220.47 <sup>*</sup>	901.90 (4.1) <sup>§</sup>	90	**4.43±0.29	48.72±0.186 (11) <sup>§</sup>
LPS control	400.79 <sup>*</sup> (1.8) <sup>§</sup>	1803.8 (2) <sup>¶</sup>	30	**11.91±0.21 <sup>a</sup> (2.7) <sup>§</sup>	110.8±0.220 <sup>a</sup> (2.3) <sup>¶</sup>
LPS tocomin treated	325.63 <sup>*</sup> (1.2) <sup>#</sup>	1202.5 (1.5) <sup>  </sup>	60	**6.91±0.23 <sup>a</sup> (1.7) <sup>#</sup>	67.50±0.365 <sup>a</sup> (1.6) <sup>  </sup>
Turpentine control	300.63 <sup>*</sup> (1.4) <sup>§</sup>	1302.7 (1.4) <sup>¶</sup>	60	**7.74±0.186 <sup>a</sup> (1.8) <sup>§</sup>	69.41±0.122 <sup>a</sup> (1.4) <sup>¶</sup>
Turpentine tocomin treated	260.55 <sup>*</sup> (1.2) <sup>#</sup>	911.90 (1.4) <sup>  </sup>	72	**5.15±0.294 <sup>a</sup> (1.5) <sup>#</sup>	55.48±0.326 <sup>a</sup> (1.3) <sup>  </sup>
Zymosan control	350.71 <sup>*</sup> (1.6) <sup>§</sup>	1578.1 (1.8) <sup>¶</sup>	50	**9.95±0.29 <sup>a</sup> (2.3) <sup>§</sup>	99.18±0.156 <sup>a</sup> (2.0) <sup>¶</sup>
Zymosan tocomin treated	280.55 <sup>*</sup> (1.3) <sup>#</sup>	1000.2 (1.6) <sup>  </sup>	70	**5.91±0.292 <sup>a</sup> (1.7) <sup>#</sup>	54.62±0.165 <sup>a</sup> (1.8) <sup>  </sup>

<sup>†</sup>The conjugated diene and MDA values are expressed as nmole MDA equivalents/mg protein.

\*Values are obtained from LDL isolated from pooled plasma of 5 hamsters in each group. \*\*Values are mean ± SD from LDL isolated from pooled plasma of 5 hamsters in each group. <sup>††</sup>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.

<sup>§</sup>Fold increase with respect to basal value in normal control; <sup>#</sup>Fold decrease with respect to basal value from their respective controls; <sup>¶</sup>Fold increase with respect to maximal value in normal control; <sup>||</sup>Fold decrease with respect to maximal value from their respective controls.

Significantly different from normal control at <sup>a</sup>p < 0.001. Significantly different from LPS control at <sup>b</sup>p < 0.001. Significantly different from turpentine control at <sup>c</sup>p < 0.001. Significantly different from zymosan control at <sup>d</sup>p < 0.001.

The above results demonstrate a markedly enhanced *ex vivo* and Cu<sup>++</sup>-induced *in vitro* susceptibility of LDL to oxidative modification, which may contribute to the increased risk associated with atherogenesis. In addition, tocotrienols pretreatment to stressed hamsters, exhibited a potent antioxidant effect by preferentially blocking the *ex vivo* and *in vitro* formation of conjugated diene and

increased the lag phase time of atherogenic LDL. Moreover, these results also signify that the host response to infection and inflammation produces LDL that not only contains more oxidized lipids, but is also more susceptible to further oxidation.

#### Impact on LDL-LPC content

Lysophosphatidylcholine (LPC) is known to exert several proatherogenic effects and is a marker for oxidative modification of LDL. Therefore, we have investigated the possible inhibitory effect of tocotrienols on LDL-LPC content in LPS, turpentine or zymosan stressed hamsters. As shown in Fig.4, lysophosphatidylcholine content in LDL was substantially increased from 5.04 in normal control to 41 (712 %), 20 (295 %) and 31 (510 %) mg/dl in LPS, turpentine and zymosan stressed group.

Pretreatment of these stressed hamsters with tocotrienols decreased the LDL-LPC content by 38 %, 24 % and 34 %, when compared to their respective controls. These results indicate that induction of infection and inflammation in hamsters was associated with a marked increase in LDL-LPC content, which may be due to increase in plasma PAF-AH activity. These levels were significantly prevented by the administration of tocotrienols.

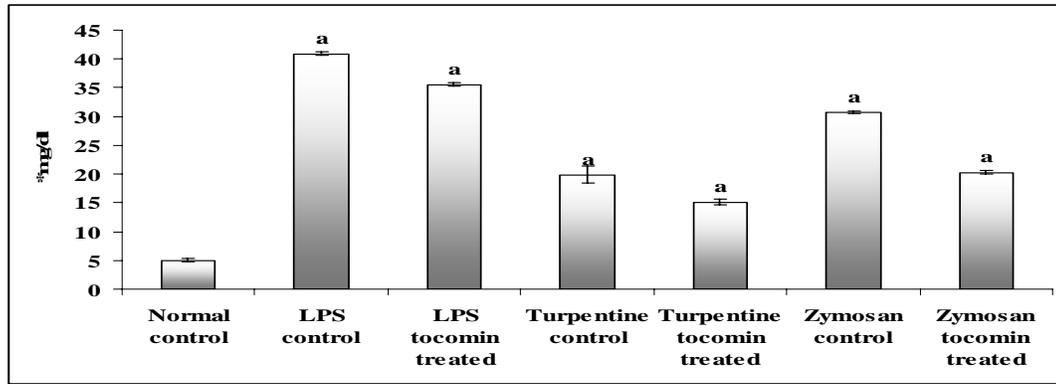


Fig. 4: Impact of Tocomin pretreatment on LDL-lysophosphatidylcholine content in three groups of stressed hamsters.

\*Values are mean (mg/dl)  $\pm$  SD from pooled plasma of 5 hamsters in each group. Significantly different from N-C at  $^*p < 0.001$ . Significantly different from L-C at  $^*p < 0.001$ . Significantly different from T-C at  $^*p < 0.001$ . Significantly different from Z-C at  $^*p < 0.001$ .

#### Regulatory effect of tocotrienols pretreatment on plasma/HDL-arylesterase activity

It is well known that enzyme arylesterase, also known as paraoxonase, is associated with plasma HDL. The antioxidant activity of HDL is believed to reside in its enzymes, particularly arylesterase. Several reports indicated that HDL-associated arylesterase enzyme protects against LDL oxidative modification. Therefore, we have also investigated the antioxidative impact of tocotrienols on enzymatic activity of arylesterase in isolated HDL fraction of LPS, turpentine or zymosan stressed hamsters. As shown in Fig.5, arylesterase activity of normal hamsters was reduced from a value of 705 units in plasma to 517 units (27 %) in HDL fraction,

isolated from plasma of normal hamsters by precipitation method. This decrease in HDL-associated arylesterase activity may be apparently due to decrease in percent recovery (73 %) of HDL particles, during their fractionation from plasma.

Arylesterase activity in plasma and HDL of LPS stressed hamsters was significantly reduced from 705 and 517 units in normal control to 468 (34 %) and 320 units (38 %) in LPS stressed hamsters. Treatment of LPS stressed hamsters with tocotrienols for 10 days before and 12 h after LPS injection significantly blocked the decrease in plasma and HDL arylesterase activity and increased them by 30 % and 31 %, respectively, when compared to corresponding LPS control values.

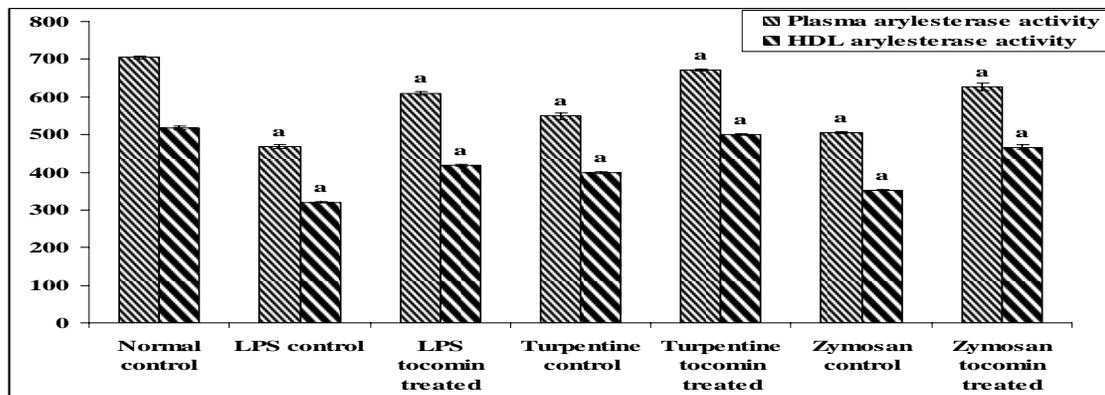


Fig. 5: Effect of Tocomin pretreatment on plasma arylesterase activity in three groups of stressed hamsters

\*Values are mean  $\pm$  SD from pooled plasma/HDL of 5 hamsters in each group.

Significantly different from N-C at  $^*p < 0.001$ . Significantly different from L-C at  $^*p < 0.001$ . Significantly different from T-C at  $^*p < 0.001$ . Significantly different from Z-C at  $^*p < 0.001$ .

After 24 h of turpentine and zymosan injection to hamsters, a significant decrease of 22 -28 % and 23 -32 % in plasma and HDL arylesterase activity was observed, when compared to corresponding normal values. Pretreatment of tocotrienols to turpentine and zymosan stressed hamsters elevated the arylesterase activity in plasma and HDL by 22 -24 % and 26 -33 %, respectively. These results indicate that due to an increase in oxidative stress, arylesterase activity in plasma and HDL fraction was significantly reduced. Pretreatment of stressed hamsters with tocotrienols significantly prevented this decrease in arylesterase activity and restored to a level 81 to 97 % of normal control values.

#### DISCUSSION

Plasma lipoprotein oxidation has been extensively studied and it has been shown that oxidative modification of plasma lipoproteins plays a crucial role in the pathogenesis of atherosclerosis<sup>8</sup>. Although lipid oxidation in the vessel wall is thought to occur as a result of a local deficiency of endogenous antioxidants or an excess of free metal ions, only limited data support these hypotheses. Because plasma contains several antioxidants<sup>11</sup> and lipoproteins with oxidative damage have been isolated from atherosclerotic lesions<sup>9</sup>, it is unclear whether oxidized lipoproteins originate in the arterial wall or are produced in the circulation and then enter the intimal space. Our data show that systemic oxidation of lipid/lipoprotein particles

occurs as a part of the host response to infection and inflammation. Plasma conjugated diene (which measure the initial phase of lipid peroxidation), lipid hydroperoxide (intermediate product of lipid peroxidation) and MDA (which measure the degradation phase of lipid peroxidation) was significantly increased in hamsters after LPS, turpentine or zymosan administration. A similar increase in serum conjugated diene and MDA has been reported in 3 models of stressed hamsters. However, protective effect of antioxidant vitamins was not investigated<sup>12</sup>. The increase in plasma lipid peroxidation products is associated with a significant decline in total antioxidants capacity of plasma. The former suggests increased production of oxidants while the latter indicates diminished antioxidant defense. Both the changes indicate an existence of profound oxidative stress. These results are in concordance with well known prooxidant properties of LPS, turpentine or zymosan. However, simultaneous administration of LPS and 5 mg Tocomin (tocotrienols) to hamsters, 12 h prior to killing, depicted that the increase in plasma total antioxidants was significantly lower (19 %) with no decrease in the levels of conjugated diene and lipid hydroperoxide except a slight decline in MDA, indicating that pretreatment of hamsters with dietary Tocomin, 10 days prior to LPS administration is essential (data not shown). Therefore, LPS-, turpentine- or zymosan-induced oxidative stress was not only attenuated but significantly reversed/restored in hamsters pretreated with Tocomin. Similar to plasma, MDA content of erythrocytes in LPS, turpentine or zymosan stressed hamsters were significantly increased. The intact erythrocytes isolated from 3 groups of stressed hamsters exhibited a further increase (>4-fold) in susceptibility to hydrogen peroxide induced MDA release, as compared to the respective basal MDA content. Pretreatment of stressed hamsters with Tocomin significantly blocked the *in vivo* as well as *in vitro* susceptibility of erythrocytes to lipid peroxidation and reversed the MDA levels close to normal control values, indicating a potent antioxidant property of tocotrienols. Based on our results, it seems plausible that oxygen radicals formed over and above the detoxifying capacity of erythrocytes can cause peroxidative breakdown of phospholipid fatty acids and accumulation of MDA, which may enhance membrane damage. Our study suggests that due to constant availability of antioxidants, tocotrienols, in the body of treated hamsters, the integrity of erythrocytes membrane is significantly improved as shown by substantial protection against *in vivo* and *in vitro* H<sub>2</sub>O<sub>2</sub> induced lipid peroxidation. An increase in erythrocytes MDA concentration was previously reported in LPS treated rats, which were significantly reduced following treatment with vitamin A, C, E or EGB761<sup>14-15</sup>.

Reactive oxygen species and free radicals are part of local host defense mechanisms, because they play a role in killing invading microorganisms and are induced by the same stimuli that induce APR. Due to the presence of persistence inflammation and oxidative stress, lipid peroxidation was increased with a concomitant decline in plasma total antioxidants, which is apparently responsible for cell membrane destruction and cell damage. Oxidized LDL can induce foam cell formation, and oxidative modification of LDL is now recognized as an important process that occurs *in vivo*<sup>8</sup>. In contrast to native LDL, oxidized LDL particles initiate a series of events, including vascular inflammation and macrophage foam cell formation that are central to the atherogenic process<sup>26</sup>. LDL particles with a greater tendency to become oxidized might thus be more likely to participate in proatherogenic events. There is only one published report<sup>12</sup> demonstrating a significant increase in conjugated diene and lipid hydroperoxide levels in LDL fraction from LPS or zymosan treated hamsters.

The data from our study clearly indicates that during acute infection and inflammation, due to exaggerated oxidative stress and depletion of plasma antioxidants, the *ex vivo* and *in vitro* BDC, lipid hydroperoxide and MDA level of LDL was markedly increased in all the three distinct group of stressed hamsters. Tocomin supplementation partially blocked the *in vivo* and *in vitro* LDL oxidizability as seen by a decrease in *ex vivo* and *in vitro* level of lipid peroxidation products and an increase in their lag phase values. Our results also showed that LDL-LPC content, which is known to exert several proatherogenic effect and is a marker for oxidative modification of LDL<sup>8</sup>, was substantially increased (4- to

8-fold) in circulating LDL after LPS, turpentine or zymosan treatment, which indicates that lipoprotein phospholipids are oxidized *in vivo* during APR. A similar increase in LDL associated LPC content in LPS treated hamsters was previously reported by Memon et al.<sup>12</sup>. Feeding of dietary Tocomin significantly blocked the increase in LPC levels of LDL and reduced them by 1.3- to 1.6-fold, indicating a potent hypolipidemic and antioxidant property of Tocomin. Together, these results indicate that LDL isolated from LPS, turpentine or zymosan treated hamsters was more susceptible to *in vitro* Cu<sup>++</sup>-catalyzed oxidation, which suggests that acute phase LDL may alone be more susceptible to further oxidation in the vessel wall.

Several mechanisms could contribute to increased LDL oxidation during APR. Paraoxonase (PON)/arylesterase are HDL-associated enzyme that protects LDL and HDL from oxidative stress<sup>27-28</sup>. This enzyme destroys oxidized phospholipids that are biologically active and thereby prevents the toxic proatherogenic effects induced by oxidized LDL and preserves HDL modification by lipid peroxides<sup>28</sup>. Van Lenten et al.<sup>17</sup> have previously reported that PON activity in HDL decreased in rabbits following injection of croton oil and in humans postoperatively. Our results demonstrate that plasma and HDL arylesterase activity significantly decreases (22 % to 38 %) following LPS, turpentine or zymosan administration. This reduction in arylesterase activity is consistent with earlier reports in LPS treated hamsters<sup>29</sup> or in *H. pylori* infected subjects<sup>18</sup>. However, in those studies protective effect of any drug with antioxidant property was not investigated. In our study, pretreatment with Tocomin significantly prevented the decrease in arylesterase activity and reversed these levels close to normal control values. It has previously been shown that depletion of PON results in the loss of the antioxidant function of HDL, and addition of PON to HDL restores the protective function of HDL<sup>30</sup>.

In addition, Aviram et al.<sup>31</sup> also reported that purified PON is a potent inhibitor of *in vitro* LDL and HDL oxidation. These studies strongly suggest an important role for PON in preventing the oxidation of both LDL and HDL, and hence in the protection of atherosclerosis. Our results indicating a markedly enhanced *in vivo* as well as *in vitro* oxidizability of atherogenic LDL in conjunction with a decreased plasma and HDL arylesterase activity during APR is likely to be a potential mechanism for the increased oxidation of circulating LDL. A decrease in arylesterase activity during acute phase response could therefore be another factor linking the APR with increased atherogenesis.

Our results indicating strong antioxidant impacts of Tocomin in LPS, turpentine or zymosan stressed hamsters are in agreement with earlier findings indicating an inhibition in the formation of conjugated dienes and MDA by tocotrienol rich fraction (TRF) or individual tocotrienols and tocopherols when fed to rats along with an atherogenic diet<sup>32-24</sup>. Support to our results is also obtained from other studies which suggested that in intact membranes, including LDL particles, tocotrienols may have a significantly greater antioxidant effect than tocopherols and they may provide greater protection against CAD<sup>20-21</sup>. Therefore, it appears that tocotrienols as TRF or tocotrienols exert their antioxidant effect on plasma LDL oxidation while being attached to LDL particle. Our combined results strongly suggest that the alleviation of oxidative stress as well as inhibition of LDL oxidation are due to potent free radical scavenging properties of dietary Tocomin (tocotrienols) and, thus, can be used as a dietary supplements in the prevention and treatment of systemic inflammatory process which might induce atherosclerosis.

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