

Effects of Simvastatin and Vitamin E on Diet-induced Hypercholesterolemia in Rats

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Abstract: Hypercholesterolemia is a dominant risk factor for the development and progression of atherosclerosis and its related cardiovascular diseases. The present study aimed to explore the effects of simvastatin combined with vitamin E on diet-induced hypercholesterolemia in rats. In the present study, hypercholesterolemia was induced by feeding rats with cholesterol-rich diet for six weeks. Rats were divided into 5 groups (n = 8): normal control, hypercholesterolemic control, simvastatin (20 mg/kg; p.o.), vitamin E (200 mg/kg; p.o.) and combination of both simvastatin and vitamin E. Drugs were given simultaneously with cholesterol-rich diet for six weeks. Diet-induced hypercholesterolemia resulted in alterations in the lipid profile markers and a state of oxidative stress coupled by compensatory increase in serum level of Nitric Oxide metabolites (NO_x) and decreased aortic endothelial nitric oxide synthase (eNOS) activity parallel to increased Inducible Nitric Oxide Synthase (iNOS) activity, calcium content and aortic wall thickness. Treatment with simvastatin, vitamin E and their combination improved lipid profile and oxidative stress markers. In addition, they attenuated hypercholesterolemia-induced changes in serum NO_x, aortic eNOS and iNOS activities as well as calcium content and aortic wall thickness. The results of combination therapy were better compared to simvastatin monotherapy. Pretreatment of hypercholesterolemic rats with simvastatin and vitamin E attenuated most of the changes induced in rats by cholesterol-rich die to wing to their observed anti-hyperlipidemic and antioxidant properties.

Keywords: Hypercholesterolemia, lipid profile, nitric oxide synthases, oxidative stress, simvastatin, vitamin E

INTRODUCTION

Hyperlipidemia is considered to be a main cause for atherosclerosis and its associated complications such as coronary artery, peripheral vascular and ischemic cerebrovascular diseases (Badimon *et al.*, 2010; Martínez-Martos *et al.*, 2011; Changizi-Ashtiyani *et al.*, 2013). Feeding animals with cholesterol-rich diets has been used to increase serum or tissue cholesterol level or content in order to study the etiology of hypercholesterolemia-related metabolic disorders (Bocan, 1998; Kabiri *et al.*, 2011).

Several studies have demonstrated that oxidative stress plays an important role in the initiation and progression of atherosclerosis through stimulating inflammation and cytokines production (Fernández-Robredo *et al.*, 2008; Hopps *et al.*, 2010; Reuter *et al.*, 2010).

Simvastatin, a 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitor, is commonly used to reduce blood lipids and to treat

cardiovascular diseases including myocardial infarction, stroke and hypertension (Ford *et al.*, 2007; Taylor *et al.*, 2013). It possesses additional pleiotropic effects including antioxidant, anti-inflammatory and immunomodulatory properties (İşeri *et al.*, 2007; Kuzelová *et al.*, 2008; Cumaoglu *et al.*, 2011). It has been suggested that statins have the ability to improve endothelial reactivity through increased bioavailability of nitric oxide (NO) by up-regulation of endothelial nitric oxide synthase (eNOS) or reduction of its oxidative degradation (Laufs *et al.*, 1998; Bonetti *et al.*, 2003) in addition to reduced expression of inducible nitric oxide synthase (iNOS) (Sessa, 2001).

Vitamin E is a potent lipid-soluble antioxidant protecting against lipid peroxidation and LDL oxidation (Maya *et al.*, 2012; Salama *et al.*, 2013). It acts through glutathione peroxidase pathway where it reacts with free radicals produced in the lipid peroxidation resulting in protection of the cell membrane against oxidation (Traber and Atkinson, 2007). It also possesses anti-inflammatory properties, thus exerts

beneficial effects in cardiovascular diseases (Rodrigo *et al.*, 2008). In addition, it has the ability to up-regulate eNOS activity leading to increase in NO production (Ulker *et al.*, 2003).

Accordingly, the present study aimed to investigate the protective effects of simvastatin and vitamin E alone or combined together on diet-induced hypercholesterolemia in rats. To achieve the goals of the study, the effects of the aforementioned agents were evaluated on markers of lipid profile and oxidative stress. As endothelial dysfunction, vascular calcification and aortic wall thickening are among the common features of atherosclerosis (Bonetti *et al.*, 2003; Tang *et al.*, 2006; Attia *et al.*, 2012), the current study aimed to assess the effect of the aforementioned agents on endothelial function, aortic calcification and aortic wall thickness of hypercholesterolemic rats.

MATERIALS AND METHODS

Animals: Adult male Wistar rats (140-180 g) obtained from the animal house of Faculty of Pharmacy, Beni-Suef University (Beni-Suef, Egypt) were used in the present study. The animals were housed eight per cage, kept under suitable environmental conditions (temperature $22\pm 2^\circ\text{C}$; humidity $60\pm 4\%$) with a 12 h light/dark cycle and allowed free access to food and water *ad libitum*. All animal experiments in this study were carried out according to the guidelines of Ethics Committee of Faculty of Pharmacy, Cairo University.

Drugs and chemicals: Simvastatin was provided as a gift from Hikma Company (Egypt), suspended in 1% tween 80 and orally administered in a dose of 20 mg/kg (Jorge *et al.*, 1997) whereas vitamin E (DL- α tocopherol acetate) was purchased from Sigma-Aldrich (USA), dissolved in sesame oil and orally administered in a dose of 200 mg/kg (Aabdallah and Eid, 2004). Pure cholesterol was obtained from Winlab (UK), cholic acid obtained from Biomark (India), lard obtained from MP (France) and methyl thiouracil obtained from Sigma-Aldrich (USA).

Induction of experimental hypercholesterolemia: Hypercholesterolemia was induced by feeding rats with cholesterol-rich diet containing cholesterol (1%), cholic acid (0.2%), lard (4%), egg yolk (7%), methyl thiouracil (0.2%), sodium chloride (1%), wheat bran (6.6%), wheat flour (45%) and corn starch (35%) for six weeks according to the method described by Pengzhan *et al.* (2003).

Experimental design: Rats were randomly divided into 5 groups (n = 8). Group I (normal control) and Group II (hypercholesterolemic control) received saline (i.p.) and sesame oil (p.o.). Groups III-V received simvastatin (20 mg/kg; p.o.), vitamin E (200 mg/kg; p.o.) and their combination, respectively for six weeks. Group I was fed with normal rat chow while groups II-V were fed on cholesterol-rich diet throughout the period of

investigation (6 weeks). At the end of the experimental period, blood samples were collected from 18 h fasted rats in heparinized and non-heparinized tubes. The heparinized blood samples were used for the estimation of blood SOD activity and GSH level while the non-heparinized blood samples were used for serum separation and estimation of levels of Total Cholesterol (TC), Triglycerides (TG), high density lipoproteins cholesterol (HDL-c), Malondialdehyde (MDA) and nitric oxide metabolites.

Immediately after that, animals were sacrificed by decapitation and aortae were isolated. The isolated aortae were washed with Krebs solution, freed from connective tissues and fat, blotted dry and weighed. Part of the aortae was used for the determination of aortic calcium content and the other part was kept in formalin prepared in saline for immunohistochemical determination of aortic eNOS and iNOS activities. In addition, the wall thickness of aortae was measured in all animals.

Estimation of lipid profile markers: Serum TC, TG and HDL-c levels were estimated colorimetrically using commercial reagent kits (Biomed Diagnostic, Egypt) and expressed as mg/dL.

Serum Low Density Lipoproteins cholesterol (LDL-c) level was calculated according to the formula developed by Friedewald *et al.* (1972) as follows:

$$\text{Serum LDL-c} = \text{TC} - (\text{HDL-c} + \text{TG}/5).$$

Atherogenic index (AIX) was calculated according to the formula adopted by Hostmark *et al.* (1991) as follows:

$$\text{Atherogenic index} = (\text{TC} - \text{HDL-c})/\text{HDL-c}.$$

Estimation of nitric oxide metabolites and oxidative stress markers: Serum nitric oxide metabolites were estimated as total nitrate/nitrite (NO_x) according to the method described by Miranda *et al.* (2001) and expressed as $\mu\text{mol/L}$.

Serum lipid peroxides level was estimated by determination of the level of thiobarbituric acid reactivesubstances (TBARS) that were measured as MDA according to the method developed by Satoh (1978) using a commercial reagent kit (Biodiagnostic, Egypt) and expressed as nmol/mL.

Blood SOD activity was determined using the pyrogallol autoxidation method developed by Marklund and Marklund (1974) and expressed as U/mL.

Blood GSH level was determined according to the method described by Beutler *et al.* (1963) using a commercial reagent kit (Biodiagnostic, Egypt) and expressed as mg/dL.

Immunohistochemical estimation of endothelial and inducible nitric oxide synthases: Immunohisto staining was performed as previously described by Martins *et al.* (2011) with slight modifications. Sections

of aorta were cut into 4 μm , then dried in a 65°C oven for 1 h. Slides were placed in a coplin jar filled with 200 mL of triology™ (Cell Marque, USA) working solution for deparaffinization, rehydration and antigen unmasking. The jar was then securely positioned in an autoclave adjusted at 120°C for 15 min after which the pressure was released and slides were allowed to cool for 30 min. Sections were then washed and immersed in tris buffer saline to adjust pH. Quenching endogenous peroxidase activity was performed by immersing slides in 3% hydrogen peroxide for 10 min. Power stain™ 1.0 Poly HRP DAB kit (Genemed Biotechnologies, USA) was used to visualize any antigen-antibody reaction in the tissues. The concentrated primary antibody (rabbit anti-eNOS) (BD Biosciences, UK) was diluted (1:1000) as well the ready to use primary antibody (rabbit anti-iNOS) (Neomarkers, USA) and 2-3 drops were applied, then slides were incubated in the humidity chamber overnight at 4°C. Poly horseradish peroxidase (HRP) enzyme conjugate was then applied to each slide for 20 min. 3, 3'- diaminobenzidine (DAB) chromogen (2-3 drops) was applied on each slide for 2 min followed by counterstaining with Mayer Hematoxylin. Examination of slides was done using image analyzer computer system utilizing ImageJ software (NIH, version v1.45e, USA). Six fields were selected and measured for O.D.

Estimation of aortic wall thickness: Mean wall thickness (μm) of six sections from the aortae of each group was measured. For measuring, a micrometer scale was photographed to allow further conversion of the measurements obtained from the camera in pixels into micrometers, six measurements were recorded which represented the wall thickness of the aortae in each tested group.

Estimation of aortic calcium content: Aortic calcium content was determined according to the method of Essalihi *et al.* (2005). Portions of aortae were dried at 55°C in heating blocks and calcium was extracted with 10% formic acid overnight at 4°C, after which calcium contents were determined colorimetrically through a reaction with o-cresolphthalein using a commercial reagent kit (Spainreact, Spain) and expressed as mg/g wet tissue.

Statistical analysis: Data were expressed as means \pm standard error of mean (S.E.M.). Statistical

analysis was performed using Graph Pad Prism (Graph Pad Software, version 5, Inc., San Diego, USA). Comparison between different groups was done using one way Analysis of Variance (ANOVA) followed by Tukey-Kramer multiple comparisons test. Differences were considered statistically significant when $p < 0.05$.

RESULTS

Effect of simvastatin, vitamin E and their combination on serum lipid profile markers and atherogenic index in cholesterol-rich diet fed-rats:

Feeding rats with cholesterol-rich diet significantly increased serum TC, TG and LDL-c levels as well as atherogenic index while significantly decreased serum HDL-c level (Table 1).

Treatment with simvastatin or vitamin E significantly decreased serum TC level to 81.03 and 73.72%; decreased serum TG level to 70.62 and 45.84%, respectively as compared to hypercholesterolemic control value. Moreover, vitamin E significantly decreased serum TG level to 92.44% as compared to simvastatin group. In addition, combination of simvastatin with vitamin E significantly reduced serum TG level to 68.88% as compared to simvastatin-treated group (Table 1).

Similarly, treatment with simvastatin alone or combined with vitamin E significantly decreased serum LDL-c level to 57.23 and 44.8%, respectively as compared to hypercholesterolemic control value. Likewise, co-administration of simvastatin with vitamin E increased serum HDL-c level to 227.12% as compared to hypercholesterolemic control group and to 242.03% as compared to simvastatin group (Table 1).

In addition, treatment with simvastatin or vitamin E significantly decreased atherogenic index of rats to 35.88 and 73.08%, respectively as compared with hypercholesterolemic control group (Table 1).

Effect of simvastatin, vitamin E and their combination on oxidative stress markers in cholesterol-rich diet fed- rats:

Feeding rats with cholesterol-rich diet significantly increased serum MDA level parallel to reduction in blood GSH level

Table 1: Effect of six weeks daily administration of simvastatin, vitamin E and their combination on serum lipid profile markers (TC, TG, LDL-c and HDL-c) and atherogenic index (AIX) in cholesterol-rich diet fed rats

Parameters/Groups	TC (mg/dL)	TG (mg/dL)	LDL-c (mg/dL)	HDL-c (mg/dL)	AIX
Normal control	51.67 \pm 1.99	50.62 \pm 1.67	17.17 \pm 1.57	30.20 \pm 1.30	0.76 \pm 0.07
Hypercholesterolemic control	107.5 \pm 3.84*	61.25 \pm 1.46*	56.00 \pm 2.79*	22.08 \pm 0.92*	3.01 \pm 0.14*
Simvastatin (20 mg/kg; p.o.)	87.11 \pm 5.65* [⊗]	43.26 \pm 2.57 [⊗]	32.05 \pm 2.07 [⊗]	20.72 \pm 1.55	1.08 \pm 0.19 [⊗]
Vitamin E (200 mg/kg; p.o.)	79.25 \pm 3.58* [⊗]	28.08 \pm 1.79* [⊗]	46.02 \pm 2.87*	28.35 \pm 1.44	2.20 \pm 0.14* ^{⊗b}
Simvastatin +Vitamin E	74.04 \pm 3.45* [⊗]	29.80 \pm 2.70* ^{⊗b}	25.09 \pm 1.88 [⊗]	43.12 \pm 4.47* ^{⊗b}	0.59 \pm 0.16 [⊗]

Values are expressed as means \pm S.E.M. (n = 6-8 rats); Statistics was carried out by ANOVA followed by Tukey-kramer multiple comparisons test; *: Significantly different from normal control value at $p < 0.05$; [⊗]: Significantly different from hypercholesterolemic control value at $p < 0.05$; ^b: Significantly different from simvastatin value at $p < 0.05$

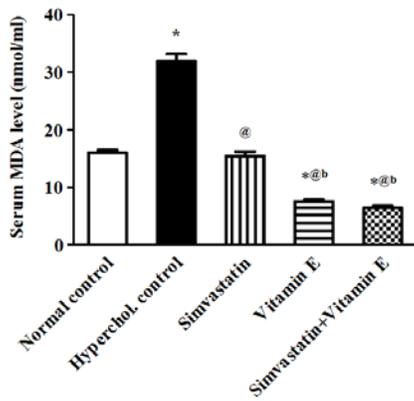


Fig. 1: Effect of six weeks daily administration of simvastatin, vitamin E and their combination on serum malondialdehyde (MDA) level of cholesterol-rich diet fed-rats. Hyperchol. control: hypercholesterolemic control group; Each bar represents the mean±S.E.M. (n = 6-8); Statistics was carried out by ANOVA followed by Tukey-Kramer multiple comparisons test; *: Significantly different from normal control group at p<0.05; @: Significantly different from hypercholesterolemic control group at p<0.05; ^b: Significantly different from simvastatin group at p<0.05

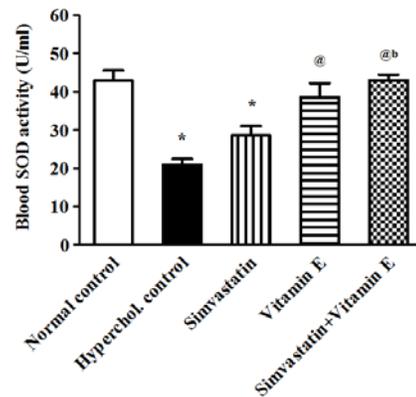


Fig. 3: Effect of six weeks daily administration of simvastatin, vitamin E and their combination on blood superoxide dismutase (SOD) activity of cholesterol-rich diet fed-rats; Hyperchol. control: Hypercholesterolemic control group; Each bar represents the mean±S.E.M. (n = 6-8); Statistics was carried out by ANOVA followed by Tukey-Kramer multiple comparisons test; *: Significantly different from normal control group at p<0.05; @: Significantly different from hypercholesterolemic control group at p<0.05; ^b: Significantly different from simvastatin group at p<0.05

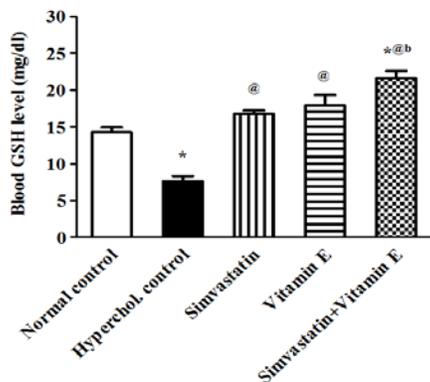


Fig. 2: Effect of six weeks daily administration of simvastatin, vitamin E and their combination on blood reduced glutathione (GSH) level of cholesterol-rich diet fed-rats; Hyperchol. control: hypercholesterolemic control group; Each bar represents the mean±S.E.M. (n = 6-8); Statistics was carried out by ANOVA followed by Tukey-Kramer multiple comparisons test; *: Significantly different from normal control group at p< 0.05; @: Significantly different from hypercholesterolemic control group at p<0.05; ^b: Significantly different from simvastatin group at p<0.05

and SOD activity (Fig. 1 to 3). Treatment with simvastatin or vitamin E significantly decreased serum MDA level to 48.97 and 27.15% (Fig. 1) while increasing blood GSH level by 174.72 and 165.92% (Fig. 2), respectively as compared to hypercholesterolemic control value. Moreover, vitamin

E significantly decreased serum MDA level to 55.45% as compared to simvastatin group. Regarding SOD, vitamin E elevated its activity to 171.18% of hypercholesterolemic control value (Fig. 3).

Moreover, combination of simvastatin with vitamin E significantly reduced serum MDA level by 50.95% (Fig. 1), increased blood GSH level to 137.79% (Fig. 2) and increased blood SOD activity to 140.09% (Fig. 3), respectively as compared to simvastatin group.

Effect of simvastatin, vitamin E and their combination on serum nitric oxide level, aortic calcium content and aortic wall thickness in cholesterol-rich diet fed-rats: Cholesterol-rich diet-fed rats showed significant increase in serum level of NO_x, aortic calcium content and aortic wall thickness.

Simvastatin significantly elevated serum NO_x level to 144.81% of hypercholesterolemic control value (Table 2).

Prophylactic treatment of hypercholesterolemic rats with Simvastatin or vitamin E significantly decreased aortic calcium content to 52.49 and 59.96% coupled by decreased aortic wall thickness to 66.76 and 61.22%, respectively as compared to hypercholesterolemic control value (Table 2, Fig. 4).

Effect of simvastatin, vitamin E and their combination on aortic endothelial and inducible nitric oxide synthase activities in cholesterol-rich diet fed-rats: Feeding rats with cholesterol-rich diet significantly decreased aortic eNOS activity parallel to elevation in iNOS activity (Fig. 5 to 8). Prophylactic

Table 2: Effect of six weeks daily administration of simvastatin, vitamin E and their combination on serum total nitrate/nitrite level (NOx), aortic calcium content and aortic wall thickness in cholesterol-rich diet fed rats

Parameters/Groups	NOx ($\mu\text{mol/L}$)	Aortic calcium (mg/g wet tissue)	Aortic wall thickness (μm)
Normal control	31.36 \pm 2.91	10.42 \pm 0.94	80.79 \pm 1.38
Hypercholesterolemic control	58.60 \pm 4.12*	18.26 \pm 1.24*	124.70 \pm 8.40*
Simvastatin (20 mg/kg; p.o.)	84.86 \pm 5.14* [@]	10.24 \pm 0.85 [@]	83.25 \pm 1.66 [@]
Vitamin E (200 mg/kg; p.o.)	76.17 \pm 4.51*	11.19 \pm 1.00 [@]	76.35 \pm 1.75 [@]
Simvastatin + Vitamin E	66.96 \pm 3.44*	8.37 \pm 0.58 [@]	74.21 \pm 3.92 [@]

Values are expressed as means \pm S.E.M. (n = 6-8 rats); Statistics was carried out by ANOVA followed by Tukey-kramer multiple comparisons test; *: Significantly different from normal control value at p<0.05; [@]: Significantly different from hypercholesterolemic control value at p<0.05

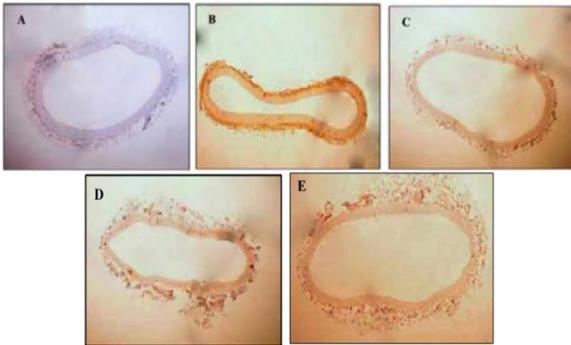


Fig. 4: Microscopic photographs of aortic wall thickness. Thoracic aortae were obtained from: A. Normal control rat, B. Hypercholesterolemic control rat, C. Simvastatin (20 mg/kg)-treated rat, D. Vitamin E (200 mg/kg)-treated rat and E. Simvastatin + vitamin E-treated rat (Magnification: x40)

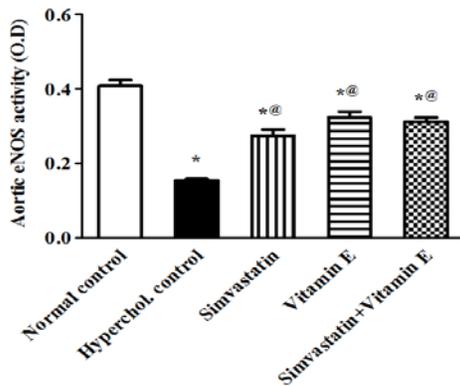


Fig. 5: Effect of six weeks daily administration of simvastatin, vitamin E and their combination on aortic endothelial nitric oxide synthase (eNOS) activity of cholesterol-rich diet fed-rats; Hyperchol. control: Hypercholesterolemic control group; O.D: optical densities; Each bar represents the mean \pm S.E.M. (n = 6); Statistics was carried out by ANOVA followed by Tukey-Kramer multiple comparisons test; *: Significantly different from normal control group at p<0.05; [@]: Significantly different from hypercholesterolemic control group at p<0.05

treatment of hypercholesterolemic rats with simvastatin or vitamin E significantly increased aortic eNOS activity to 180 and 213.33% parallel to decreased aortic iNOS activity to 68.42 and 63.15%, respectively as

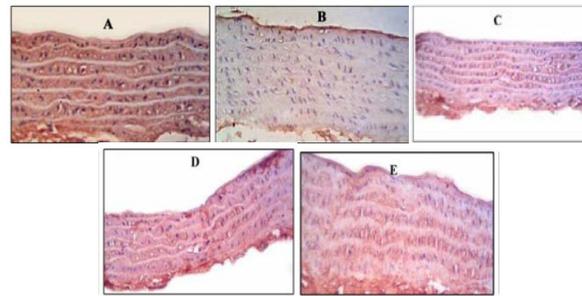


Fig. 6: Immunohistochemistry of endothelial nitric oxide synthase (eNOS) localization in rats' thoracic aorta. There is a significant decrease in eNOS immunoreactivity in hypercholesterolemic control group (B), compared with normal control group (A). In contrast, Vitamin E treated group (D) showed a staining level of eNOS protein comparable to the normal control. In groups (C & E) there is little increase in staining level of eNOS protein compared to group (D). In these microphotographs, eNOS immunoreactivity appears as dark brown staining of the three layers of aorta especially the endothelial monolayer along the interior luminal surface. A. Normal control group, B. Hypercholesterolemic control group, C. Simvastatin (20 mg/kg)-treated group, D. Vitamin E (200 mg/kg)-treated group and E. Simvastatin + Vitamin E-treated group (Mayer Hematoxylin X 200).

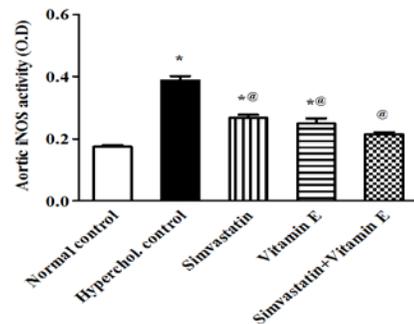


Fig. 7: Effect of six weeks daily administration of simvastatin, vitamin E and their combination on aortic inducible nitric oxide synthase (iNOS) activity of cholesterol-rich diet fed-rats; Hyperchol. control: Hypercholesterolemic control group; O.D: optical densities; Each bar represents the mean \pm S.E.M. (n = 6); Statistics was carried out by ANOVA followed by Tukey-Kramer multiple comparisons test; *: Significantly different from normal control group at p<0.05; [@]: Significantly different from hypercholesterolemic control group at p<0.05

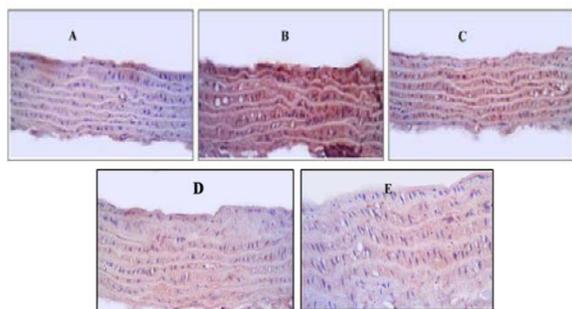


Fig. 8: Immunohistochemistry of inducible nitric oxide synthase (iNOS) localization in rats' thoracic aorta. There is a significant increase in iNOS immunoreactivity in hypercholesterolemic control group (B), compared with normal control group (A). In group (E) a decrease in iNOS expression observed comparable to the normal control and more than groups (C & D). In these microphotographs, (iNOS) immunoreactivity appears as dark brown staining of the three layers of aorta. A. Normal control group, B. Hypercholesterolemic control group, C. Simvastatin (20 mg/kg)-treated group, D. Vitamin E (200 mg/kg)-treated group and E. Simvastatin + Vitamin E-treated group. (Mayer Hematoxylin X 200)

compared to hypercholesterolemic control value (Fig. 5 to 8).

DISCUSSION

Data of the present study revealed marked disturbance in lipid profile of rats fed with cholesterol-rich diet manifested by increased atherogenic index as well as serum TC, TG and LDL-c levels parallel to decrease in serum HDL-c level. Similar results were reported by other investigators (Aziz *et al.*, 2009; Abdel Maksoud *et al.*, 2012; Akdogan *et al.*, 2012; Amanolahi and Rakhshande, 2013).

Prophylactic administration of simvastatin significantly decreased atherogenic index as well as levels of serum TC, TG and LDL-c which is consistent with the work of other researchers (Wu *et al.*, 2009; Kausar *et al.*, 2011). The hypocholesterolemic potency of simvastatin is mainly mediated via inhibition of HMG-CoA reductase activity, the rate-limiting step in cholesterol biosynthesis (Kenis *et al.*, 2005). Moreover, statins have the ability to up-regulate mRNA of LDL-c receptors in rat hepatocytes to increase cholesterol cellular uptake (Kong *et al.*, 2008). The effect of simvastatin on serum TG level could be explained through elevation of lipoprotein lipase activity by increasing lipase mRNA expression (Benhizia *et al.*, 1994) and through suppression of diacyl glycerol acyl transferase, which catalyzes the final step in TG biosynthesis in the rat liver microsomes (Waterman and Zammit, 2002).

Regarding vitamin E-treated group, a significant decrease in atherogenic index as well as serum levels of

TC and TG which finds support in the study of other investigators (Tzanetakou *et al.*, 2012; Salama *et al.*, 2013). The effect of vitamin E could be explained through its radical chain-breaking antioxidant properties (Bjelakovic *et al.*, 2007) which in turn leads to inhibition of free radical-mediated tissue injury. This is supported by improved effects of simvastatin on serum TG and HDL-c levels by its co-administration with vitamin E.

In the current study, feeding rats with cholesterol-rich diet resulted in oxidative stress indicated by a significant increase in serum lipid peroxides level coupled with a significant decrease in blood SOD activity and GSH level. The present results are consistent with the work of other investigators (Luo *et al.*, 2008; Garjani *et al.*, 2009; Abdel-Rahim *et al.*, 2013).

Data of the present work indicated that simvastatin significantly reduced serum MDA level and increased blood GSH level of hypercholesterolemic rats which is in line with the studies of other researchers (Tang *et al.*, 2006; Amin and Abd El-Twab, 2009; Wu *et al.*, 2009) and could be ascribed to antioxidant effect of simvastatin mediated via inhibition of free radical generation from leukocytes and other tissues (Mohamadin *et al.*, 2011), reduction of lipid peroxidation (Tandon *et al.*, 2005) and increasing LDL resistance to oxidation (Deskur-Smielecka *et al.*, 2001).

Regarding vitamin E, a significant decrease in serum MDA level and a significant increase in blood SOD activity and GSH level were observed. Such results were supported by other investigators (Gökkuşu and Mostafazadeh, 2003; Tang *et al.*, 2006) and may be also related to the antioxidant effect of vitamin E (Bjelakovic *et al.*, 2007). Indeed, the combination of vitamin E with simvastatin significantly improved the effects of the latter on serum MDA level, blood SOD activity and blood GSH level.

The current results showed that serum NO level was significantly increased in hypercholesterolemic control rats. Similar results were previously reported in hypercholesterolemic rats (Wang *et al.*, 2011), rabbits (Setorki *et al.*, 2011) and patients (Ferlito *et al.*, 1999) and might be regarded as a defense mechanism to compensate for continuous inactivation of NO by oxygen-derived free radicals in hypercholesterolemia (Kanazawa *et al.*, 1996; Cai and Harrison, 2000). Another possible explanation for the obtained result is increased inducible nitric oxide synthase (iNOS) activity with cholesterol feeding (Minor *et al.*, 1990; Rahman *et al.*, 2001).

Simvastatin significantly increased serum NO level compared to normal and hypercholesterolemic rats which is consistent with the work of Wu *et al.* (2009) and could be explained via the ability of statins to up-regulate eNOS expression and to prevent its down regulation by oxidized LDL (ox LDL) in endothelial cell cultures (Hernández-Perera *et al.*, 1998; Lauf

et al., 1998). In addition, simvastatin decreases the production of superoxide anion (Tang *et al.*, 2006) and increases antioxidant defense mechanisms (Lefer *et al.*, 2001). Hence, simvastatin increases NO bioavailability through both its increased production and decreased oxidative inactivation.

In a similar fashion, vitamin E increased serum NO level which could be explained through the ability of vitamin E to enhance the phosphorylation of eNOS resulting in an amplification of its action and increased level of NO (Heller *et al.*, 2004). In addition, vitamin E increases SOD activity, decreases superoxide anion production, protects LDL from oxidation and scavenges free radicals resulting in decreased oxidative degradation and increased bioavailability of NO (Koh *et al.*, 1999; Tang *et al.*, 2006).

Diet-induced hypercholesterolemia in rats resulted in a significant decrease in aortic eNOS activity coupled with increased aortic iNOS activity which is consistent with the studies of Verbeuren *et al.* (1993) and Li *et al.* (2007). Current findings could be attributed to hypercholesterolemia-induced oxidative stress leading eventually to endothelial dysfunction (Anderson, 1997).

Treatment with simvastatin or vitamin E prevented cholesterol-rich diet-induced changes in aortic eNOS and iNOS activities. It has been reported that simvastatin up-regulates eNOS expression through increasing the stability of eNOS mRNA (Laufs *et al.*, 1998). In addition, the decreased iNOS activity could result from the anti-inflammatory actions of statins leading to reduced expression of pro-inflammatory cytokines and iNOS (Sessa, 2001). Likewise, the modulatory effects of vitamin E could be mediated via its ability to up-regulate eNOS activity (Ulker *et al.*, 2003) and anti-inflammatory properties (Devaraj and Jialal, 2005).

Regarding aortic calcium content, diet-induced hypercholesterolemia resulted in a significant increase in aortic calcium content and wall thickness which is in accordance with the work of other investigators (Wu *et al.*, 2009; Attia *et al.*, 2012). Tang *et al.* (2006) demonstrated that hypercholesterolemia is accompanied by lipid deposition in the vessel resulting in foam cell, plaque formation and vascular calcification. It is also associated with the production of ox LDL which is involved in endothelial injury, vascular calcification and increased aortic thickness (Steinberg and Lewis, 1997; Meisinger *et al.*, 2005).

In the current investigation, simvastatin and vitamin E significantly decreased aortic calcium content of hypercholesterolemic rats. The obtained results were supported by the study of Tang *et al.* (2006) which indicated that simvastatin and vitamin E have the ability to decrease serum ox LDL level, aortic cholesterol ester content and aortic alkaline phosphatase activity. Simvastatin also decreased aortic wall thickness in the present study which is consistent with the studies of other investigators (Lima *et al.*, 2004; Wu *et al.*, 2009)

which revealed a significant correlation between LDL-c lowering effect of simvastatin and atherosclerotic plaque regression as well as luminal area increase leading to decreased aortic wall thickness.

CONCLUSION

In conclusion, the present study revealed that diet-induced hypercholesterolemia resulted in alterations in the lipid profile and a state of oxidative stress coupled by compensatory increase in serum level of total nitrate/nitrite and decreased aortic eNOS activity as well as increased aortic iNOS activity, calcium content and wall thickness. Pretreatment of hypercholesterolemic rats with simvastatin, vitamin E or their combination attenuated most of the changes induced in rats by cholesterol-rich diet. Such findings may be of considerable value in the treatment of hypercholesterolemia and atherosclerosis in clinical practice.

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