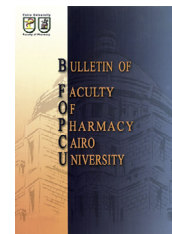




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ORIGINAL ARTICLE

Modulatory role of chelating agents in diet-induced hypercholesterolemia in rats



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KEYWORDS

CaNa₂EDTA;
DMSA;
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Oxidative stress;
Lipid profile;
Nitric oxide synthases

Abstract *Introduction:* Hypercholesterolemia is a major risk factor for the development of atherosclerosis and endothelial dysfunction. Chelating agents may play a modulatory role in atherosclerosis by removal of calcium from atherosclerotic plaques.

Aim: The present study aimed to explore the effects of calcium disodium ethylenediaminetetraacetic acid (CaNa₂EDTA) and meso-2,3-dimercaptosuccinic acid (DMSA) on diet-induced hypercholesterolemia in rats using simvastatin as a reference standard.

Methods: Hypercholesterolemia was induced by feeding rats with cholesterol-rich diet for six weeks. Rats were divided into five groups ($n = 8$): normal control, hypercholesterolemic control, simvastatin (20 mg/kg; p.o.), CaNa₂EDTA (100 mg/kg; i.p.) and DMSA (100 mg/kg; i.p.). Treatments continued daily for the six weeks of diet feeding.

Results: Diet-induced hypercholesterolemia resulted in alterations in the lipid profile markers and a state of oxidative stress coupled by compensatory increase in serum nitric oxide (NO) level and decreased aortic endothelial nitric oxide synthase (eNOS) activity parallel to increased inducible nitric oxide synthase (iNOS) activity, aortic calcium content and aortic wall thickness. Treatment with simvastatin, CaNa₂EDTA and DMSA improved lipid profile and oxidative stress markers. In addition, they attenuated hypercholesterolemia-induced changes in serum NO level, aortic eNOS and iNOS activities, calcium content and aortic wall thickness.

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Conclusion: Pretreatment of hypercholesterolemic rats with simvastatin, CaNa₂EDTA or DMSA attenuated most of the changes induced by feeding rats with cholesterol-rich diet owing to their observed anti-hyperlipidemic and antioxidant properties.

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1. Introduction

Hyperlipidemia is a major cause for atherosclerosis and its associated conditions as coronary artery, ischemic cerebrovascular and peripheral vascular diseases.^{1,2} Feeding animals with cholesterol-rich diet is commonly used as a model for induction of hypercholesterolemia to study the etiology of hypercholesterolemia-related metabolic disorders and the efficiency of anti-hypercholesterolemic agents.^{3,4}

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.^{5,6} It possesses additional pleiotropic effects including antioxidant, anti-inflammatory and immune-modulatory properties.^{7,8}

Calcium disodium ethylenediaminetetraacetic acid (CaNa₂EDTA) and meso-2,3-dimercaptosuccinic acid (DMSA) possess antioxidant properties in addition to their calcium chelating ability.^{9–11} They are widely used for treatment of heavy metal toxicities.^{10–12} Chelating agents can improve atherosclerosis and cardiovascular diseases via their ability to chelate ectopic or metastatic calcium from atherosclerotic plaques.^{13,14} On the other hand, other investigators pointed to the modest role of chelation therapy in atherosclerosis.^{15,16}

The present study aimed to investigate the protective effects of CaNa₂EDTA and DMSA on diet-induced hypercholesterolemia in rats and to compare their effects with that of simvastatin. 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,^{17,18} the current study aimed to assess the effects of the aforementioned agents on endothelial function, aortic calcification and aortic wall thickness of hypercholesterolemic rats.

2. Materials and methods

2.1. 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 ± 2 °C; humidity 60 ± 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.

2.2. Drugs and chemicals

CaNa₂EDTA and DMSA were purchased from Sigma-Aldrich (USA), dissolved in saline and 10% sodium bicarbonate solution, respectively and administered in a dose of 100 mg/kg; i.p.^{12,19} 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.²⁰ Pure cholesterol was obtained from Winlab (UK), cholic acid was obtained from Biomark (India) and methyl thiouracil was purchased from Sigma-Aldrich (USA). Any other chemical used was of the highest analytical grade.

2.3. Induction of hypercholesterolemia

Hypercholesterolemia was induced by feeding rats for six weeks with diet composed of 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%) according to the method described by Pengzhan et al.²¹

Table 1 Effect of six week daily administration of simvastatin, calcium disodium ethylenediaminetetraacetic acid (CaNa₂EDTA) and meso-2,3-dimercaptosuccinic acid (DMSA) on serum lipid profile markers in cholesterol-rich diet fed rats.

Groups	TC (mg/dl)	TG (mg/dl)	LDL-c (mg/dl)	HDL-c (mg/dl)	AIX
Normal control	54.33 ± 1.52	50.62 ± 1.67	17.17 ± 1.57	30.20 ± 1.30	0.76 ± 0.07
Hypercholesterolemic control	113.6 ± 4.5*	61.25 ± 1.46*	56.00 ± 2.79*	22.08 ± 0.92*	3.01 ± 0.14*
Simvastatin (20 mg/kg; p.o.)	87.11 ± 5.65*,@	43.26 ± 2.57@	32.05 ± 2.07@	20.72 ± 1.55	1.08 ± 0.19@
CaNa ₂ EDTA (100 mg/kg; i.p.)	80.33 ± 2.22*,@	39.99 ± 1.68*,@,b	48.58 ± 2.45*	27.81 ± 0.99	2.04 ± 0.17*,@
DMSA (100 mg/kg; i.p.)	70.09 ± 2.51*,@	48.58 ± 1.24@	32.12 ± 3.03*,@	31.29 ± 1.11@,b	1.13 ± 0.11@

TC: total cholesterol; TG: triglycerides; LDL-c: low density lipoprotein cholesterol; HDL-c: high density lipoprotein cholesterol; AIX: atherogenic index.

Values are expressed as means ± 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.

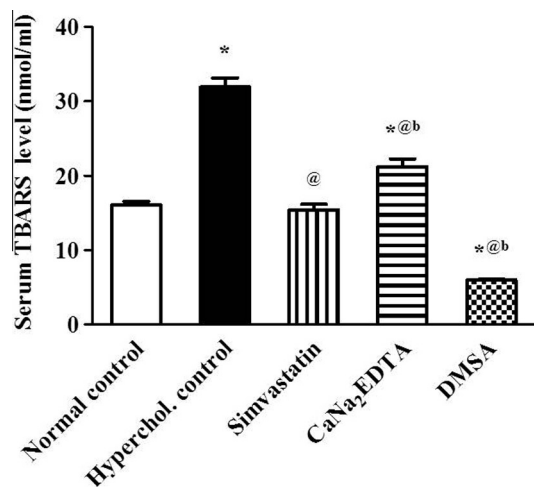


Figure 1 Effect of six week daily administration of simvastatin, CaNa₂EDTA and DMSA on serum thiobarbituric acid reactive substance (TBARS) level of cholesterol-rich diet fed-rats. Each bar represents the mean \pm S.E.M. ($n = 6-8$). Statistics was carried out by ANOVA followed by Tukey-Kramer multiple comparisons test. *Significantly different from the normal control group at $p < 0.05$. @Significantly different from the hypercholesterolemic control group at $p < 0.05$. ^bSignificantly different from the simvastatin group at $p < 0.05$.

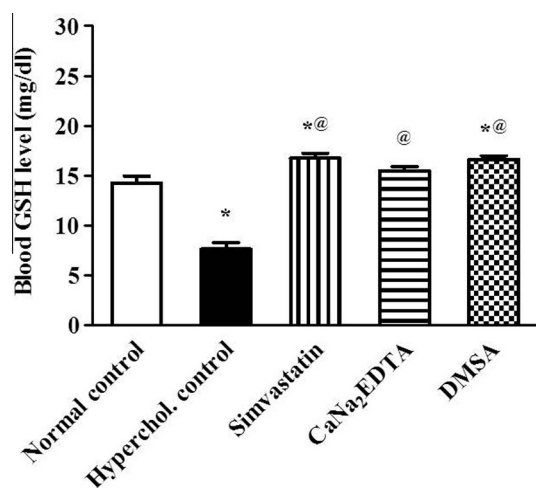


Figure 2 Effect of six week daily administration of simvastatin, CaNa₂EDTA and DMSA on blood reduced glutathione (GSH) level in rats fed with cholesterol rich diet. Each bar represents the mean \pm S.E.M. ($n = 6-8$). Statistics was carried out by ANOVA followed by Tukey-Kramer multiple comparisons test. *Significantly different from the normal control group at $p < 0.05$. @Significantly different from the hypercholesterolemic control group at $p < 0.05$.

2.4. Experimental design

Rats were randomly divided into five groups ($n = 8$). Group I (normal control) and Group II (hypercholesterolemic control) received saline (i.p.). Groups III-V received simvastatin (20 mg/kg; p.o.), CaNa₂EDTA (100 mg/kg; i.p.) and DMSA

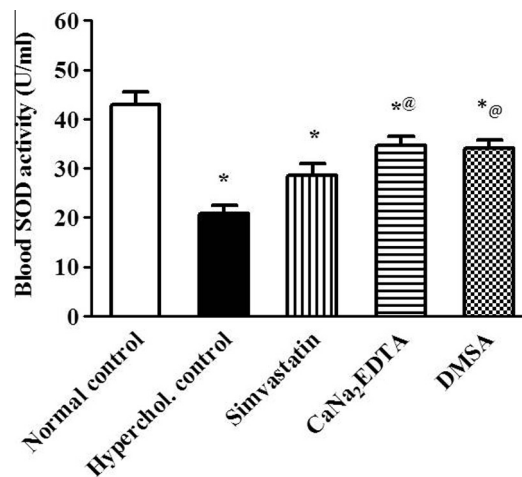


Figure 3 Effect of six week daily administration of simvastatin, CaNa₂EDTA and DMSA on blood superoxide dismutase (SOD) activity level in rats fed with cholesterol rich diet. Each bar represents the mean \pm S.E.M. ($n = 6-8$). Statistics was carried out by ANOVA followed by Tukey-Kramer multiple comparisons test. *Significantly different from the normal control group at $p < 0.05$. @Significantly different from the hypercholesterolemic control group at $p < 0.05$.

(100 mg/kg; i.p.), 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 for estimation of blood superoxide dismutase (SOD) activity and the levels of reduced glutathione (GSH), total cholesterol (TC), triglycerides (TG), high density lipoprotein cholesterol (HDL-c), lipid

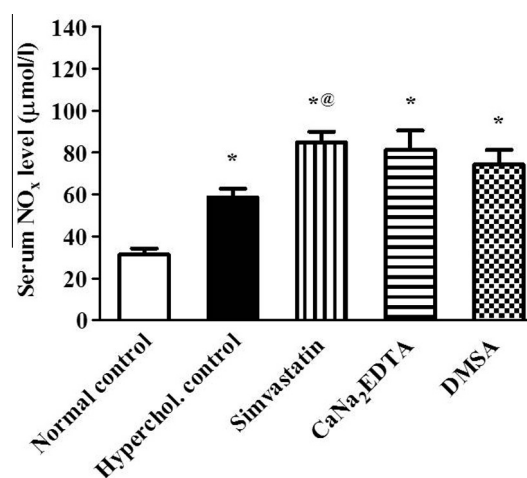


Figure 4 Effect of six week daily administration of simvastatin, CaNa₂EDTA and DMSA on serum total nitrate/nitrite (NO_x) level in rats fed with cholesterol rich diet. Each bar represents the mean \pm S.E.M. ($n = 6-8$). Statistics was carried out by ANOVA followed by Tukey-Kramer multiple comparisons test. *Significantly different from the normal control group at $p < 0.05$. @Significantly different from the hypercholesterolemic control group at $p < 0.05$.

peroxides and nitric oxide (NO). Thereafter, 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 used for immunohistochemical determination of aortic endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS) activities. In addition, the wall thickness of aortae was measured in all animals.

2.5. Biochemical estimations

2.5.1. Estimation of serum lipid profile

Serum TC, TG and HDL-c levels were estimated using commercial reagent

kits (Biomed diagnostic, Egypt) and expressed as mg/dl.

Serum low density lipoprotein cholesterol (LDL-c) level was calculated according to the formula developed by Friedewald et al.²² as follows:

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

Atherogenic index was calculated according to the formula adopted by Hostmark et al.²³ as follows:

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

2.5.2. Estimation of serum nitric oxide

Serum NO level was estimated as total nitrate/nitrite (NO_x) using Griess reagent according to the method described by Miranda et al.²⁴ and expressed as $\mu\text{mol/l}$.

2.5.3. Estimation of oxidative stress markers

Serum lipid peroxide level was estimated by determination of the level of thiobarbituric acid reactive substances (TBARS) that were measured as MDA according to the method described by Mihara and Uchiyama²⁵ and expressed as nmol/ml.

Blood SOD activity was determined using the pyrogallol autoxidation method described by Marklund and Marklund²⁶ and expressed as U/ml.

Blood GSH level was determined according to the method described by Beutler et al.²⁷ and expressed as mg/dl.

2.5.4. Estimation of aortic calcium content

Aortic calcium content was determined according to the method of Essalihi et al.²⁸ Portions of aortae were dried at 55 °C in

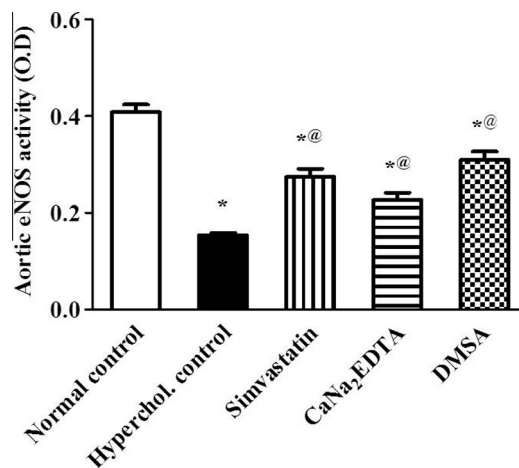


Figure 5 Effect of six week daily administration of simvastatin, CaNa_2EDTA and DMSA on aortic endothelial nitric oxide synthase (eNOS) activity in rats fed with cholesterol rich diet. Each bar represents the mean \pm S.E.M. ($n = 6$). Statistics was carried out by ANOVA followed by Tukey-Kramer multiple comparisons test. *Significantly different from the normal control group at $p < 0.05$. @Significantly different from the hypercholesterolemic control group at $p < 0.05$.

heating blocks and calcium was extracted with 10% formic acid overnight at 4 °C. Calcium contents were then determined colorimetrically through a reaction with o-cresolphthalein using a commercial reagent kit (Spainreact, Spain) and expressed as mg/g wet tissue.

2.6. Immunohistochemical estimation of endothelial and inducible nitric oxide synthases

Tissue staining was performed according to the method described by Martins et al.²⁹ Sections of aortae 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 to adjust pH. Quenching endogenous peroxidase activity was performed by immersing slides in 3% hydrogen peroxide for

Table 2 Effect of six week daily administration of simvastatin, calcium disodium ethylenediaminetetraacetic acid (CaNa_2EDTA) and meso-2,3-dimercaptosuccinic acid (DMSA) on aortic calcium content and aortic wall thickness in cholesterol-rich diet fed rats.

Groups	Aortic calcium (mg/g wet tissue)	Aortic wall thickness (μm)
Normal control	10.42 \pm 0.94	80.79 \pm 1.38
Hypercholesterolemic control	18.26 \pm 1.24*	124.70 \pm 8.40*
Simvastatin (20 mg/kg; p.o.)	10.24 \pm 0.85@	83.25 \pm 1.66@
CaNa_2EDTA (200 mg/kg; p.o.)	10.65 \pm 0.87@	82.73 \pm 3.90@
DMSA (100 mg/kg; i.p.)	6.50 \pm 0.61@	80.48 \pm 1.62@

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 $p < 0.05$.

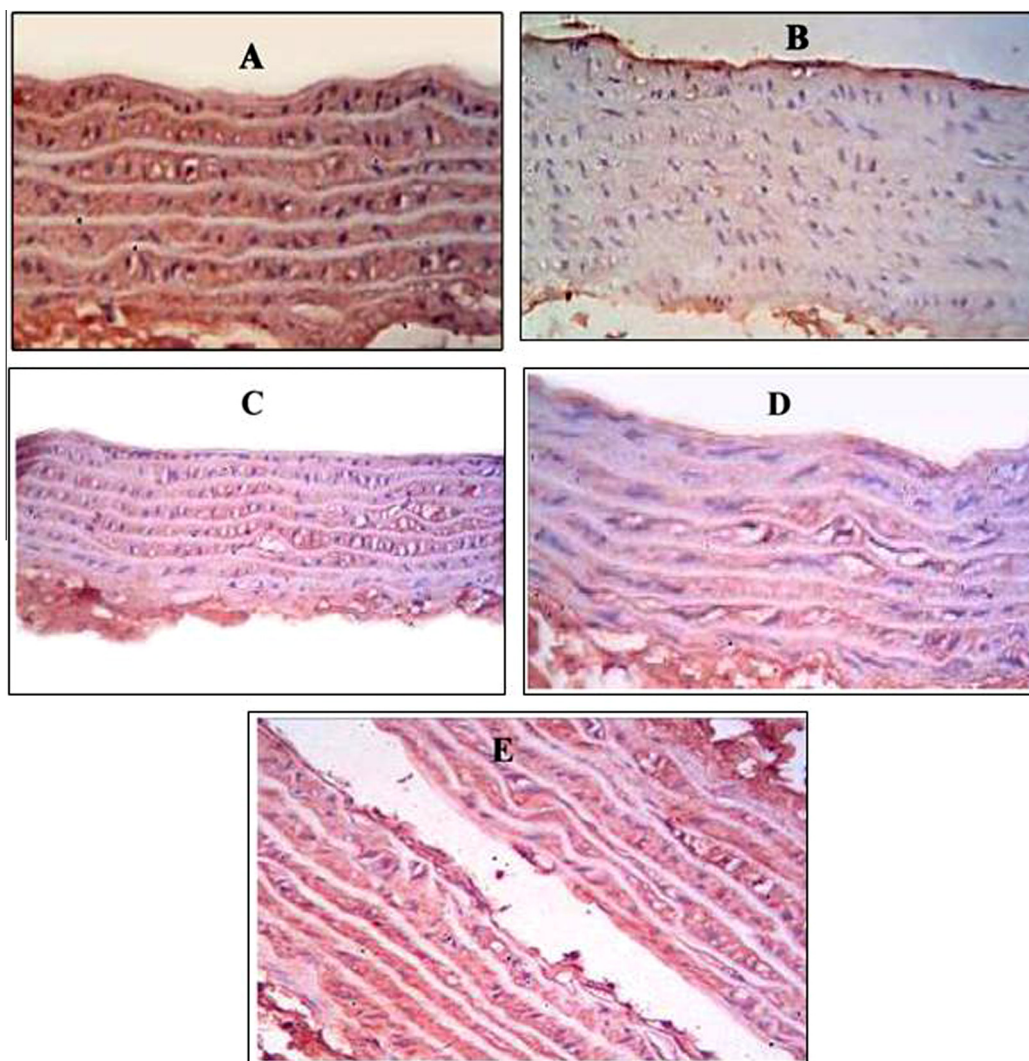


Figure 6 Microscopic photographs of endothelial nitric oxide synthase in rats' thoracic aortae obtained from: (A) Normal control rat, (B) Hypercholesterolemic control rat, (C) Simvastatin (20 mg/kg)-treated rat, (D) CaNa_2EDTA (100 mg/kg)-treated rat and (E) DMSA (100 mg/kg)-treated rat. (Mayer Hematoxylin X 200).

10 minutes. Power stain™ 1.0 Poly HRP DAB kit (Genemed Biotechnologies, USA) was used to visualize any antigen-antibody reaction in the tissues. Rabbit anti-eNOS (BD Biosciences, UK) and anti-iNOS (Neomarkers, USA) were added followed by incubation of slides in a humidity chamber overnight at 4 °C. Poly horseradish peroxidase (HRP) enzyme conjugate was then applied to each slide for 20 min followed by 3,3'-diaminobenzidine chromogen. Counterstaining with Mayer Hematoxylin was done and slides were examined using image analyzer computer system utilizing ImageJ software (NIH, version v1.45e, USA).

2.7. Estimation of aortic wall thickness

Mean wall thickness (μm) of six sections from the aortae of each group was measured using a micrometer scale that was photographed to allow conversion of the measurements obtained from the camera in pixels into micrometers.

2.8. Statistical analysis

Data were expressed as means \pm standard error of mean (SEM). Statistical analysis was performed using GraphPad Prism 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 $p < 0.05$.

3. Results

3.1. Effect of simvastatin, CaNa_2EDTA and DMSA on serum lipid profile markers in rats fed with cholesterol rich diet

Feeding rats with cholesterol-rich diet significantly increased serum levels of TC, TG and LDL-c; meanwhile it decreased serum HDL-c level (Table 1).

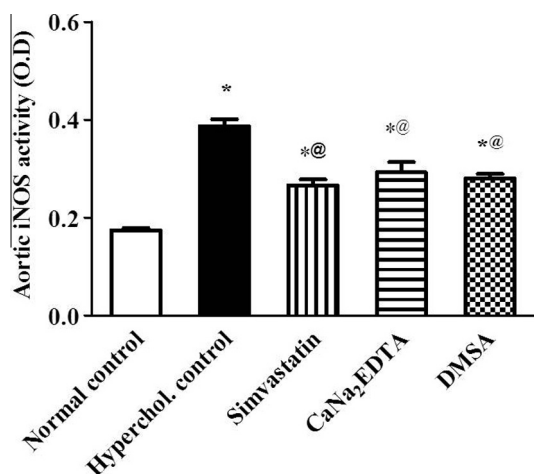


Figure 7 Effect of six week daily administration of simvastatin, CaNa₂EDTA and DMSA on aortic inducible nitric oxide synthase (iNOS) activity in rats fed with cholesterol rich diet. Each bar represents the mean \pm S.E.M. ($n = 6$). Statistics was carried out by ANOVA followed by Tukey–Kramer multiple comparisons test. *Significantly different from the normal control group at $p < 0.05$. @Significantly different from the hypercholesterolemic control group at $p < 0.05$.

Treatment with simvastatin, CaNa₂EDTA or DMSA decreased serum TC level to 76.68%, 70.71% and 61.7%; decreased serum TG level to 70.62%, 65.28% and 79.3%, respectively as compared to the hypercholesterolemic control group. Moreover, simvastatin and DMSA normalized serum TG level (Table 1).

Similarly, treatment with simvastatin or DMSA significantly decreased serum LDL-c level to 57.23% and 57.36%, respectively as compared to hypercholesterolemic control value. Moreover, administration of DMSA significantly increased serum HDL-c level to about 141.71% of hypercholesterolemic control value and to about 151% of simvastatin value. Furthermore, CaNa₂EDTA or DMSA normalized serum HDL-c level (Table 1).

Feeding rats with cholesterol-rich diet significantly increased atherogenic index; meanwhile treatment with simvastatin, CaNa₂EDTA or DMSA decreased atherogenic index of hypercholesterolemic rats to 35.88%, 67.77% and 37.54%, respectively as compared with hypercholesterolemic control value (Table 1).

3.2. Effect of simvastatin, CaNa₂EDTA and DMSA on oxidative stress markers in rats fed with cholesterol rich diet

Feeding rats with cholesterol-rich diet significantly increased serum TBARS level parallel to reduction in blood GSH level and SOD activity (Figs. 1–3). Treatment with simvastatin, CaNa₂EDTA or DMSA decreased serum TBARS level to 48.38%, 66.45% and 18.75% (Fig. 1) while increasing blood GSH level to 319.26%, 302.75% and 317.82% (Fig. 2), respectively as compared to hypercholesterolemic control values. Moreover, CaNa₂EDTA and DMSA significantly decreased serum TBARS level to 137.33% and 38.75%, respectively as compared to the simvastatin group. Regarding SOD, CaNa₂EDTA and DMSA elevated its activity to 165.7% and

163.48% of hypercholesterolemic control value, respectively (Fig. 3).

3.3. Effect of simvastatin, CaNa₂EDTA and DMSA on serum total nitrate/nitrite level in rats fed with cholesterol rich diet

Feeding rats with cholesterol rich diet significantly increased serum NO_x level to 186.86% as compared to the normal control group. Treatment with simvastatin further increased serum NO_x level to 144.81% as compared with hypercholesterolemic control value (Fig. 4).

3.4. Effect of simvastatin, CaNa₂EDTA and DMSA on aortic calcium content and aortic wall thickness in rats fed with cholesterol rich diet

Feeding rats with cholesterol rich diet significantly increased aortic calcium content and aortic wall thickness to 175.24% and 154.35%, respectively as compared with the normal control value. Prophylactic treatment of hypercholesterolemic rats with simvastatin, CaNa₂EDTA or DMSA decreased aortic calcium content to 56.08%, 58.32% and 35.6% parallel to decreased aortic wall thickness to 66.76%, 66.34% and 64.53%, respectively as compared to hypercholesterolemic control values (Table 2).

3.5. Effect of simvastatin, CaNa₂EDTA and DMSA on aortic endothelial and inducible nitric oxide synthase activities in rats fed with cholesterol rich diet

Feeding rats with cholesterol-rich diet significantly decreased aortic eNOS activity parallel to increased iNOS activity (Figs. 5–8). Prophylactic treatment of hypercholesterolemic rats with simvastatin, CaNa₂EDTA or DMSA significantly increased aortic eNOS activity to 180%, 146.66% and 200% parallel to decreased aortic iNOS activity to 68.42%, 76.31% and 73.68%, respectively as compared to hypercholesterolemic control values (Figs. 5–8).

4. 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 levels of TC, TG and LDL-c parallel to a decrease in serum HDL-c level. Similar results were reported by other investigators.^{30,31}

Prophylactic administration of simvastatin, CaNa₂EDTA and DMSA decreased atherogenic index as well as serum levels of TC and TG. Moreover, DMSA decreased serum LDL-c level and increased serum HDL-c levels. Beneficial effects of CaNa₂EDTA in atherosclerosis and other cardiovascular disorders were reported in patients^{14,32} and experimental animals.³³

The mechanism underlying the effect of chelating agents on lipid profile is unclear. However, the obtained results could be attributed to the reported antioxidant effects of CaNa₂EDTA^{9,34,35} and DMSA^{10–12,36} which in turn lead to decreased free radical generation and decreased oxidative damage of the liver, the main organ involved in cholesterol biosynthesis.

Indeed in the current study, oxidative stress was obvious in cholesterol rich diet fed-rats as evidenced by the increase in

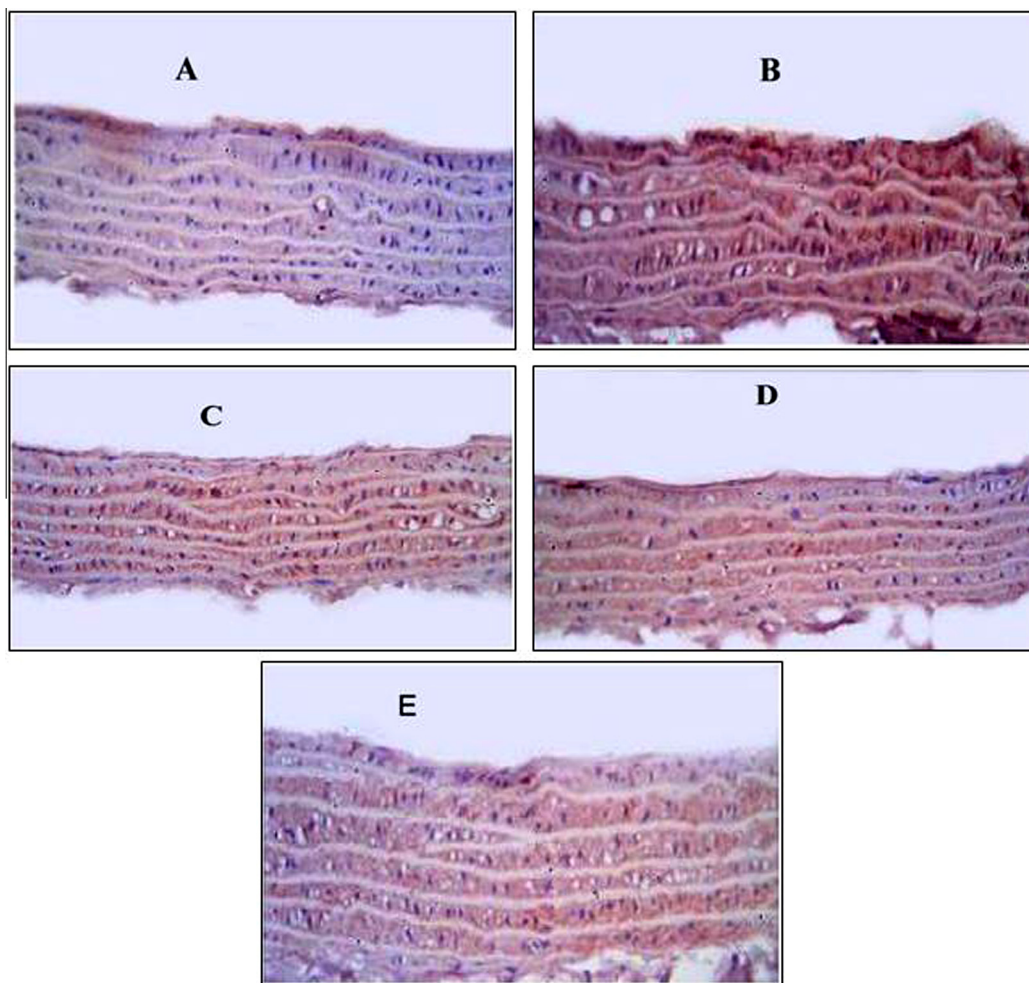


Figure 8 Microscopic photographs of inducible nitric oxide synthase in rats' thoracic aortae obtained from: (A) Normal control rat, (B) Hypercholesterolemic control rat, (C) Simvastatin (20 mg/kg)-treated rat, (D) CaNa_2EDTA (100 mg/kg)-treated rat and (E) DMSA (100 mg/kg)-treated rat. (Mayer Hematoxylin X 200).

serum lipid peroxide level coupled with decreased SOD activity and GSH level. Similar results were previously reported.³⁷

Data of the present work indicated that simvastatin and the chelating agents used significantly decreased cholesterol rich diet-induced oxidative stress. CaNa_2EDTA was reported to inhibit cell-mediated LDL oxidation³⁸ and to chelate transitional metals which are important catalyst for lipid peroxidation, LDL oxidation and free radical formation.⁹ Another explanation for the antioxidant effect of EDTA resides in its ability to chelate calcium.³⁹ Calcium activates phospholipase- A_2 resulting in increased levels of arachidonic acid which in turn result in the production of more free radicals.⁴⁰ Similarly, increased blood GSH level following DMSA administration in lead- and arsenic-intoxicated rats was reported.^{10,36}

The current results showed that serum NO level was significantly increased in hypercholesterolemic control rats. Similar results were previously reported in hypercholesterolemic rats⁴¹ and patients.⁴² Increased serum NO level may be regarded as a defense mechanism to compensate for continuous inactivation of NO by oxygen-derived free radicals in hypercholesterolemia⁴³ or it could be a consequence of increased inducible nitric oxide synthase (iNOS) activity with cholesterol feeding.⁴⁴

Simvastatin significantly increased serum NO level compared to normal and hypercholesterolemic rats which is consistent with the work of Wu et al.⁴⁵ and could be explained via the ability of statins to up-regulate eNOS expression.⁴⁶ In the same context pretreatment of rats with CaNa_2EDTA or DMSA did not affect hypercholesterolemia-induced increase in serum NO level. Foglieni et al.⁴⁷ reported increased expression of eNOS in EDTA-treated rats. Moreover effects of simvastatin, CaNa_2EDTA or DMSA can be explained by their reported antioxidant effects leading to decreased generation of oxygen free radicals, decreased oxidative degradation and increased bioavailability of NO.^{12,18,48}

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 findings of other investigators.⁴⁹ Current findings could be attributed to hypercholesterolemia-induced oxidative stress leading eventually to endothelial dysfunction.⁵⁰

Pretreatment with simvastatin, CaNa_2EDTA or DMSA prevented cholesterol rich diet-induced changes in aortic eNOS and iNOS activities. Laufs et al.⁴⁶ demonstrated that simvastatin up-regulates eNOS expression through increasing the stability of eNOS mRNA. The increased eNOS activity by

CaNa₂EDTA or DMSA could be mediated by their ability to chelate several divalent cations which suppress eNOS activity.⁵¹ DMSA was also reported to decrease cadmium induced-vascular dysfunction and oxidative stress in mice.¹¹

Cholesterol-rich diet feeding resulted in a significant increase in aortic calcium content and wall thickness which is in accordance with the work of other investigators.^{45,52} Tang et al.¹⁸ demonstrated that hypercholesterolemia is accompanied by lipid deposition in the vessel resulting in foam cell, plaque formation and vascular calcification. Hypercholesterolemia is also associated with the production of oxidized LDL (oxLDL) which is involved in endothelial injury, vascular calcification and increased aortic thickness.^{53,54}

In the current investigation, simvastatin significantly decreased aortic calcium content of hypercholesterolemic rats. The obtained results were supported by the study of Tang et al.¹⁸ which indicated that simvastatin has the ability to decrease serum oxLDL 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^{45,55} 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.

Findings of the present study revealed that using chelating agents as CaNa₂EDTA or DMSA significantly decreased aortic calcium content and aortic wall thickness of hypercholesterolemic rats. Similar findings were previously reported.^{13,14} Such findings could be explained through the ability of EDTA or DMSA to chelate calcium and other divalent cations^{39,51} removing the pathologic (ectopic) calcium from both the aortic wall and atherosclerotic plaque.¹³ The observed LDL lowering effect of DMSA may also result in decreased plaque formation, vascular calcification and wall thickness.

5. Conclusions

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 a 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, CaNa₂EDTA or DMSA 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.

6. Conflict of interest

None declared.

Acknowledgement

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