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Original Article

Benificial effect of lipoic acid on nicotine-induced vascular endothelial damage in rabbits

Marwa S. Zaghloul, Manar A. Nader, Hassan El-Kashef, Nariman M. Gamiel

Department of Pharmacology and Toxicology, Faculty of Pharmacy, Mansoura University, Egypt

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Corresponding Author
Manar A. Nader
Pharmacology & Toxicology Department, Faculty of Pharmacy, Mansoura Universty, Mansoura 35516, Egypt.
manarahna@yahoo.com

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Alpha lipoic acid; Endothelial damage; Nicotine; Nitric oxide; Vascular damage

Abstract
This study has been designed to investigate the effect of alpha lipoic acid (ALA) in nicotine-induced vascular endothelial dysfunction (VED) in rabbits. Nicotine (7.5 mg/kg/day, sc, 6 weeks) was administered to produce VED in rabbits. The development of VED was assessed by employing aortic histopathology and estimating serum concentration of nitrite/nitrate (NOx). Further, serum lipid profile was assessed. Moreover, the oxidative stress was assessed by estimating serum thiobarbituric acid reactive substances (TBARS) reduced glutathione (GSH) and superoxide dismutase (SOD) generation. The administration of nicotine produced VED by impairing the integrity of vascular endothelium and subsequently decreasing serum concentration of NOx. Further, nicotine produced oxidative stress, which was assessed in terms of increase in serum TBARS and decreased GSH and SOD levels. However, treatment with ALA (20 mg/kg/day, orally) markedly prevented nicotine-induced VED and oxidative stress by improving the integrity of vascular endothelium, increasing the concentration of serum NOx, decreasing serum TBARS and normalizing SOD and GSH activities. Thus, it may be concluded that ALA reduces the oxidative stress and consequently improves the integrity of vascular endothelium and enhances the generation of nitric oxide to prevent nicotine-induced experimental VED.

INTRODUCTION

Endothelium is a thin monolayer of specialized epithelium comprising of simple squamous cells that covers the inner surface of entire vasculature [1]. It regulates the vascular tone and maintains the free flow of blood in the vessel and possesses antiatherogenic, antiplatelet and antiproliferative properties [2]. Nitric oxide (NO), a potent vasodilatory substance is generated from L-arginine by endothelial nitric oxide synthase (eNOS) in the presence of cofactors such as Ca^{2+}-calmodulin, reduced nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD) and tetrahydrobiopterin (BH_4) in endothelial cells [3]. The partial loss of balance between endothelium-mediated vasodilation and vasoconstriction is described as vascular endothelial dysfunction (VED), which occurs mainly due to reduction in synthesis and release of NO, inactivation of eNOS, excessive generation of reactive oxygen species (ROS), eNOS uncoupling, reduction in endogenous antioxidant defense mechanism and upregulation of asymmetric dimethyl arginine (ADMA), an endogenous inhibitor of eNOS [1, 4]. VED is an insidious condition as it is strongly associated with the pathogenesis of various cardiovascular disorders such as atherosclerosis [5], hypertension [6], heart failure [7] and diabetic nephropathy.

Nicotine exposure via tobacco chewing/cigarette smoking is considered to be a major risk factor involved in the induction and progression of cardiovascular disorders [8, 9]. Accumulating evidence suggests that exposure of nicotine plays a key role in inducing VED [10]. The chronic nicotine exposure by cigarette smoking induces VED by down-regulating the expression of eNOS, increasing the generation of ROS and upregulating ADMA, and thus has been implicated in the pathogenesis of cardiovascular disorders like atherosclerosis and hypertension [11]. Further, exposure of nicotine has been shown to induce

oxidative stress that causes direct endothelial cell damage and disrupts the endogenous antioxidant defense mechanisms by down-regulating catalase (CAT) and superoxide dismutase (SOD) [12]. Concomitantly, different pro-inflammatory mediators such as pro-inflammatory cytokines [13], cellular adhesion molecules including intercellular adhesion molecule-1 (ICAM-1) and vascular cellular adhesion molecule-1 (VCAM-1) [14] are also produced.

Alpha lipoic acid (ALA), also known as 1,2-dithiolane-3-pentanoic acid, or 6,8-thioctic acid has generated considerable clinical interest as a cellular thiol-replenishing and redox-modulating agent [15]. Biologically, ALA functions as a cofactor of oxidative decarboxylation reactions in glucose metabolism to yield energy [16]. It has been used for a long time in the western world to treat complications associated with diabetes [17]. To carry out this function, the disulfide group of the lipoic acid dithiolane ring is reduced to its dithiol form, dihydrolipoic acid (DHLA). Current findings suggest that ALA not only acts as a cofactor in glucose metabolism, but may also acts as an antioxidant *in vitro* and *in vivo* [18]. *In vitro* experiments have shown that both ALA and DHLA are potent scavengers of ROS. ALA quenches singlet oxygen, hydroxyl radical and hypochlorus acid [19] while DHLA scavenges hydroxyl radicals, hypochlorus acid, superoxide anion radicals and peroxyl radicals [20].

In view of these observations, the current study was designed to clarify whether ALA could be able to protect against nicotine-induced VED in rabbits.

MATERIALS AND METHODS

All procedures involving the animals were conducted in accordance with the protocol approved by the committee on Animal Experimentation of Faculty of Pharmacy, Mansoura University. Age matched fifteen male New Zealand White (NZW) rabbits weighing about 1.5-2 kg were employed in the present study. They were fed on standard chow diet and water *ad libitum*. They were acclimatized in the animal house and were exposed to normal light and dark cycle of the day.

Drugs and chemicals

ALA was obtained from Pharmaceutical Industries (El Obour City, Cairo, Egypt). Nitroblue tetrazolium, 1,1,3,3-tetramethoxypropane, Ellman's reagent, and nicotine were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Experimental protocol

Three groups were employed in the present study and each comprised of 5 rabbits: group I (normal group), rabbits were maintained on standard food and water receiving distilled water by subcutaneous (sc) injection; group II (nicotine group), rabbits were administered nicotine (7.5 mg/kg/day, sc) for 6 weeks; group III (ALA group), rabbits were treated with ALA (20 mg/kg/day, orally) and the treatment was started 3 days before the administration of nicotine and it was continued for 6 weeks from the day of administration of nicotine.

Blood parameters measurement

For blood collection from overnight fasted rabbits at the end of 6 weeks, the animals were anesthetized by the administration of ketamine (50 mg/kg) and xylazine (10 mg/kg), intravenously, and blood was collected from the marginal ear vein. Then the animals were euthanized by an overdose of sodium pentobarbital and exsanguinated for aortic assessment.

Collected blood samples were allowed to clot then centrifuged (10,000g), and the sera were collected and stored at −80°C until analyzing the biochemical parameters.

Assessment of lipid profile in serum:

The serum total cholesterol (TC) and high density lipoprotein (HDL) cholesterol were estimated by cholesterol oxidase peroxidase (CHOD-POD) method [21] using the commercially available kit (Biomerieux, Marcyl, Etoile, France). The serum triglycerides (TG) were estimated by glycerophosphate oxidase peroxidase (GPO-POD) method, again using the commercially available kit (Biomerieux). Low density lipoprotein cholesterol (LDL-C) was calculated according to the method described by Friedewald and coworkers [22] using the following equation:

$$\text{LDL-C} = \text{TC} - [\text{HDL-C} - (\text{TG}/5)]$$

Assessment of oxidative stress

Estimation of serum thiobarbituric acid reactive substances (TBARS)

The serum concentration of TBARS was estimated spectrophotometrically to assess oxidative stress. A standard graph using 1,1,3,3-tetramethoxypropane (1-50 μM) was plotted to calculate the concentration of TBARS [23, 24].

Estimation of serum superoxide dismutase activity

The enzymatic activity of SOD was assessed following the Marklund method [25]. SOD activity was determined by computing the difference between auto-oxidation of pyrogallol alone and in presence of the SOD enzyme.

Estimation of serum reduced glutathione (GSH)

Determination of GSH according to the method described by Ellman [26] is based on the reduction of Ellman's reagent [5,5'-dithio-bis (2-nitrobenzoic acid)]

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by sulfhydryl (SH) groups to form 1 mole of 2-nitro mercaptobenzoic acid per mole of SH. The nitro-mercaptobenzoic acid formed has an intense yellow color that can be measured spectrophotometrically.

Estimation of serum nitrite/nitrate (NOx) level

The serum NOx level was determined as an index for NO production. A 0.5% $ZnSO_4$ solution (100 µl) was prepared then added to serum samples (100 µl) and incubated for 5 min at room temperature. The mixture was centrifuged (3,700g) for 20 min at 4°C, and the supernatant was used for measurement of NOx concentration using the Griess method [23, 24].

Histopathological examination

At the end of the experimental protocol, the thoracic aorta was carefully removed and was fixed in 4% paraformaldehyde and embedded in paraffin wax according to standard procedures for the other analyses. Paraffin-embedded slices were serially sectioned at 5 lm. These sections were deparaffinized, rehydrated and then stained with hematoxylin-eosin (H&E).

Statistical analysis

The results were presented as mean ± standard error of mean (SEM). Statistical analysis was carried out using one way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparisons test using Instat-3 (GraphPad Software, version 3.05, San Diego, CA, USA). Statistical significance was considered when $P < 0.05$.

RESULTS

Effect on serum lipid concentration

Compared with the normal group, nicotine administration for 6 weeks produced a significant elevation in serum TC ($P < 0.001$) and TG ($P < 0.01$) levels by 115% and 33%, respectively, with significant decrease in serum HDL-C ($P < 0.001$) by 36.7%. The oral administration of ALA significantly reversed all changes occur in TC ($P < 0.001$), TG ($P < 0.05$) and HDL-C ($P < 0.01$) due to nicotine administration (Table 1).

Effect on serum oxidative stress

An increase in serum TBARS concentration ($P < 0.001$) by 45% was noted in rabbits administered with nicotine. Treatment with ALA significantly reduced serum TBARS concentration ($P < 0.001$) by 26.5% compared to nicotine administered rabbits. Serum SOD ($P < 0.01$) and GSH ($P < 0.001$) activities were found to be significantly decreased in the nicotine-treated group by 15% and 56.5%, respectively, compared to normal group. Treatment with ALA resulted in a reversal of the nicotine-induced reduction of SOD ($P < 0.01$) and GSH ($P < 0.001$) in the serum by 16.5% and 99%, respectively (Table 2).

Effect on serum NOx concentrations

The serum concentration of NOx was noted to be reduced ($P < 0.01$) in nicotine administered rabbits by 38.8% when compared with normal rabbits. However, treatment with ALA significantly attenuated nicotine-induced decrease in serum NOx concentration by 97.3% ($P < 0.001$) (Fig.1).

Histopathological results

Histological examination of the aorta from nicotine treated rabbits showed elevated endothelium with scanty sub-endothelial lipid collection when compared to control. Aorta from ALA treated rabbits showed intact endothelium with no sub-endothelial lipid collection (Fig.2).

Table 1. Effect of ALA on serum TC, TG, LDL-C and HDL-C levels in nicotine administered rabbits (mean ± SEM).

	Normal	Nicotine	ALA
TC (mg/dl)	46.52 ± 1.84	100 ± 3.48^{cc}	53.33 ± 0.76^{nnn}
TG (mg/dl)	101.58 ± 2.37	135.14 ± 9.12^{c}	103.2 ± 4.21^{n}
HDL (mg/dl)	97.04 ± 4.4	61.44 ± 3.57^{cc}	86.86 ± 5.2^{nn}
LDL (mg/dl)	25.69 ± 1.72	65.38 ± 1.41^{cc}	24.73 ± 1.17^{nnn}

ALA: alpha lipoic acid; TC: total cholesterol; TG: triglyceride; LDL-C: low density lipoprotein cholesterol; HDL-C: high density lipoprotein cholesterol. $^{c}P < 0.01$, $^{cc}P < 0.001$ *vs* normal control; $^{n}P < 0.05$, $^{nn}P < 0.01$, $^{nnn}P < 0.001$ *vs* nicotine.

Table 2. Effect of ALA on serum TBARS, SOD and GSH levels in nicotine administered rabbits (mean ± SEM).

	Normal	Nicotine	ALA
TBARS (nmol/ml)	9.59 ± 0.207	13.91 ± 0.544^{cc}	10.23 ± 0.388^{nnn}
SOD (U/ml)	46.38 ± 1.023	39.28 ± 1.405^{c}	45.765 ± 1.2^{nn}
GSH (µmol/ml)	0.223 ± 0.02	0.097 ± 0.007^{cc}	0.193 ± 0.007^{nnn}

ALA: alpha lipoic acid; TBARS: thiobarbituric acid reactive substances; SOD: superoxide dismutase; GSH: glutathione. $^{c}P < 0.01$, $^{cc}P < 0.001$ *vs* normal control; $^{nn}P < 0.01$, $^{nnn}P < 0.001$ *vs* nicotine.

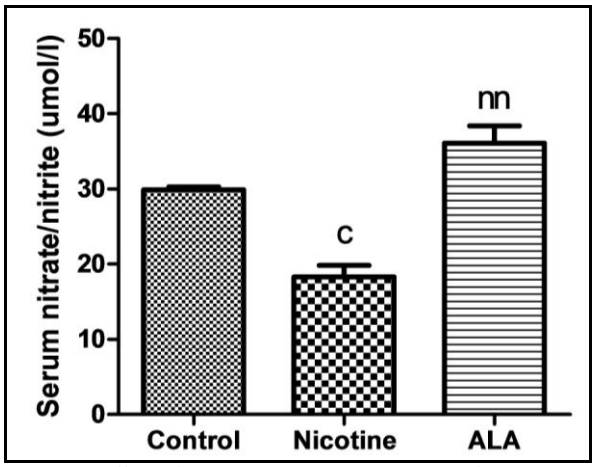

Figure 1. Effect of ALA on serum concentration of nitrate/nitrite (NOx) in nicotine administered rabbits. All values are represented as mean ± SEM. $^{c}P < 0.01$ *vs* normal control, $^{nn}P < 0.001$ *vs* nicotine group.

DISCUSSION

Chronic exposure to nicotine often leads to cardiovascular disorders such as atherosclerosis, hypertension and ischemic heart disease [27]. Studies demonstrated that induction of VED is an early detrimental event associated with nicotine-induced cardiovascular abnormalities [28]. The present study demonstrated the novel therapeutic role of ALA in preventing nicotine-induced VED. Administration of nicotine for 6 weeks in rabbits impaired the integrity of vascular endothelium as proved by histopathological examination, and decreased the serum concentration of NOx. Further, in rabbits administered with nicotine oxidative stress increased as proved by increased TBARS and decreased GSH level and SOD activity. ALA treatment normalized all the previous parameters suggesting that ALA can ameliorate nicotine-induced endothelial damage in rabbits.

Nicotine has been reported to generate ROS through activation of NADPH [29]. Thus, the noted marked induction of VED in nicotine administered rabbits may be due to the development of high degree of oxidative stress and consequent inactivation of NO. Moreover, nicotine was shown to decrease the generation and bioavailability of NO via down-regulation of eNOS and up-regulation of ADMA [29, 30].This is strongly supported by the results of the present study that showed that nicotine administered rabbits exhibited high degree of oxidative stress and marked reduction in serum NOx concentration.

Oxidative stress plays a key role in the development of VED by inhibiting eNOS activation and reducing the generation and bioavailability of NO [4]. Nicotine exposure was shown to generate ROS excessively by activating NADPH oxidase and reducing the antioxidant defense mechanisms via down-regulation of CAT and SOD [31,12]. Thus, it may be suggested that nicotine-mediated development of oxidative stress may be responsible to induce VED. This contention is supported by the results obtained in the present study that administration of nicotine elevated the levels of serum TBARS and decreased the activities of the free radical scavenging enzyme SOD and the level of serum GSH.

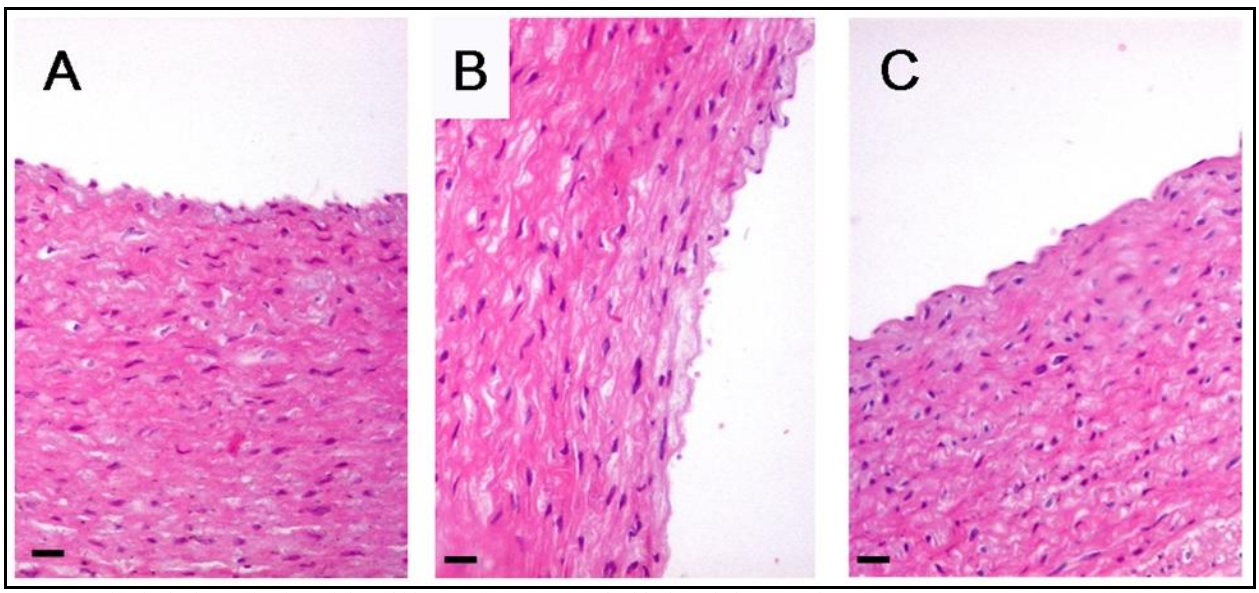

Figure 2. Histological examination of the rabbit aorta (H&E, x400, the black scale bars equal 50 µm): **(A)** Control, intact endothelium with no sub-endothelial lipid collection; **(B)** Nicotine, intact elevated endothelium with scanty sub-endothelial lipid collection; and **(C)** Alpha lipoic acid (ALA), intact endothelium with no sub-endothelial lipid collection.

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Accumulating evidences suggest that elevation in the level of circulating lipids impair the integrity of vascular endothelium and induces VED [32, 33]. It has been shown that nicotine administration alters the lipid profile by increasing the levels of TC and TG and consequently decreasing the levels of HDL-C [34]. Thus, it would be possible in the present study that the alteration in the lipid profile may be additionally involved in the induction of VED in nicotine-administered rabbits. This contention is supported by the results obtained in the present study that administration of nicotine increased the serum levels of TC and TG and consequently decreased the serum levels of HDL. However, treatment with ALA prevented nicotine-induced alteration in lipid profile by reducing the circulating levels of TC and TG, and elevating HDL-C levels. The mechanism on how ALA is able to reduce TC and LDL-C concentration is unknown, but probably via affecting lipoprotein lipase (LPL) activity or cholesterol metabolism by the liver [35]. Chiba and coworkers reported that, LPL activity and HDL-C level is increased in cholesterol fed NZW rabbits after administration of 4-diethoxyphosphoryl-methyl-N-(4-bromo-2-cyanophenyl) benzamide (NO-1886) [36]. ALA is probably capable to initiate LDL-C receptor synthesis in the liver which in turn increase the uptake of cholesterol back to the hepatic system and increase synthesis of apoprotein A component (moeity of HDL-C particles) for reversed cholesterol transport [18].

Another mechanism by which ALA treatment markedly prevented nicotine-induced VED may be through improving the integrity of vascular endothelium and significantly restoring the reduced serum NOx level in rabbits administered nicotine. Thus, endothelium protective properties of ALA may be due to improvement in NO bioavailability. The present finding suggested that ALA was effective in increasing eNOS activity and was also able to improve endothelial function. It has also been shown that ALA activates AMP-activated protein kinase in endothelial cells, an effect that was found to increase eNOS and NO production [37]. Another probable mechanism of action of ALA involves activation of mitogen-activated protein kinase, which in turn will upregulate eNOS and increases NO production [38]. Loss of NO in the microcirculation can result in up-regulation of pro-adhesive cell adhesion molecules [39]. Recent data from experimental and clinical studies indicate that ALA acts as an anti-inflammatory agent that may help preventing cardiovascular disease [40, 41]. ALA exerts anti-inflammatory effects by inhibiting tumor necrosis factor-α (TNF-α)-induced expression of adhesion molecules and monocyte chemoattractant protein-1 (MCP-1), and adherence of monocytes to human aortic endothelial cells [41].

In addition, in the present study, treatment with ALA markedly prevented the nicotine-induced generation of ROS in rabbits by significantly decreasing the levels of serum TBARS and increasing the activities of SOD and GSH which explain the additional antioxidant mechanism involved in the vascular protecting potential of ALA. These findings were parallel to previous reports suggesting that ALA would be a potent metabolic antioxidant source to quench free radicals *in vitro* and *in vivo* [42-45].

On the basis of above discussion, it may be concluded that treatment with ALA markedly prevented the development of experimental VED in nicotine-administered rabbits. The vascular protecting potential of ALA may be attributed to its additional properties such as activation of eNOS and generation of NO and consequent reduction in oxidative stress to improve the integrity and function of vascular endothelium.

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