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Research Article
Influence of Nerolidol on High-cholesterol Diet-Induced Atherosclerosis in Rats via Antioxidant and p38 MAPK Signaling Pathway

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Abstract

Background and Objective: Atherosclerosis and the Cardiovascular Diseases (CVDs) are a severe threat to human health. Consumption of a High Cholesterol Diet (HCD) has been proposed as a significant risk factor for atherosclerosis. Nerolidol can be found in high concentrations in the essential oils of many ornamentals, medicinal and edible or nutritional plants. Nerolidol, one of the sesquiterpenes, is interested in its potential benefits for cancer and cardiovascular disease. The purpose of this study was to investigate the effects of nerolidol on High Cholesterol Diet (HCD)-fed atherosclerosis in Wistar rats. **Materials and Methods:** Twenty-four male Wistar rats were divided into four groups: The first group served as control, the second group was fed with nerolidol (40 mg kg^{-1} b.wt.), the third group was fed with HCD for eight weeks and the fourth group was fed with HCD along with nerolidol (40 mg kg^{-1} b.wt.) for last four weeks. **Results:** Lipid profile, atherogenic index and cardiac markers, antioxidant status and inflammatory levels were determined in HCD-induced atherosclerosis rats. Nerolidol produced a significant anti-atherosclerotic activity in reducing lipid profile, atherogenic index, cardiac markers and improved antioxidant status in HCD-induced treated groups compared to the control. Further, nerolidol significantly inhibited the expression of the p38 MAPK compared to the HCD-induced group. **Conclusion:** In conclusion, the current investigation revealed that nerolidol reduced the HCD induced dyslipidemia in the Wistar rats. The possible mechanism of action may be connected to the antioxidative, down-regulating p-p38 MAPK and anti-inflammatory effect by nerolidol.

Key words: Nerolidol, atherosclerosis, high cholesterol diet, lipid profile, cardiac markers, antioxidants, p38 MAPK

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Atherosclerosis is the principal cause of coronary artery disease (CAD) and is the leading cause of death in both men and women around the world[1]. Atherosclerosis is a chronic progressive disease characterized by the accumulation of lipids (atherosclerotic plaque) and inflammation in the walls of arteries[2]. This process starts with the outflow of Low-Density Lipoprotein (LDL) cholesterol into the subendothelial region, where various factors can change and oxidize[3]. Clinical studies have shown that atherosclerosis and its complications account for almost a third of the deaths worldwide due to the rupture of unstable plaques and related acute thrombotic events[4]. Several genetic, metabolic and environmental factors are involved in the formation and evolution of atherosclerotic plaque[5].

Several risk factors have been identified as being involved in the pathological development of atherosclerosis[6]. Hyperlipidemia is a significant risk factor for the development of atherosclerosis and mounting data indicates a significant positive association between plasma lipid levels and the risk of atherosclerosis[7]. It could be related to an increase in cholesterol and triglyceride levels, as well as an imbalance in lipoproteins of High-Density Lipoprotein (HDL) and LDL and ratios of HDL cholesterol and LDL cholesterol levels in the plasma[8]. Free radical-induced cellular oxidative stress plays an essential part in the pathogenic phase of atherosclerosis. It would result in macromolecular damage like protein oxidation, lipid oxidation, nucleic acid instability and the endothelial dysfunction[9]. Furthermore, the chronic inflammatory response is thought to respond to the entire process of atherosclerosis in the artery walls, including the beginning, growth, progression, complication and plaque rupture[10].

Nerolidol is sesquiterpene alcohol found in various plants and flowers. Nerolidol has been widely used in the food industry as a flavour enhancer in a variety of food products since it was certified as a safe food flavouring ingredient by the United States Food and Drug Administration[11]. Nerolidol can be found in neroli, ginger, citronella, lemongrass, rose and tea tree essential oils[12]. Despite the well-documented anti-inflammatory, antioxidant, antimicrobial and anticancer properties of nerolidol[11,13], no research has yet studied the effects and molecular processes on AS. The current study demonstrated that nerolidol played an important role in HCD-induced AS in rats, linked to the p38 MAPK signalling pathway and antioxidant responses.

MATERIALS AND METHODS

Study area: The present study was carried out in the Department of Cardiology, The Second People's Hospital of Qingyang, Qingyang, Gansu Province, China., from August-November, 2021.

Chemicals: Nerolidol was obtained from Sigma Aldrich (St. Louis, Missouri, USA). All other biochemical kits were obtained from Yehua Biological Technology Company (Shanghai, China).

Experimental rats: A total of 24 male Wistar albino rat's body weights ranging from (150-180 g), were used in this study. All the animals were maintained under standard conditions in the clean air-conditioned room at a temperature (25±2°C) with 50% humidity and a 12:12 hrs dark/light cycle. Animals were providing fed with standard diets and drinking water was assessed to animals and *ad libitum*. All the animals' experimental procedures were approved by the Institutional Animal Ethics Committee.

Experimental design: Rats were divided into four groups (n = 6). At the start of the experiment, control groups were fed a standard pellet diet, the other groups were fed high cholesterol diet and water *ad libitum*. Group I: served as control and were supplemented with standard rat chow and water for 8 weeks. Group II: Nerolidol (40 mg kg^{-1} b.wt.) control rats. Group III: HCD induced rats 5 % cholesterol and 0.5 % cholic acid in powdered rat chow feed for 8 weeks. Groups IV: HCD+Oral administration of nerolidol (40 mg kg^{-1} b.wt.) for the last 4 weeks.

Nerolidol was administered as suspension directly into the stomach using a gastric tube in the morning by mixing with vehicle 0.5% dimethyl sulfoxide.

The total experimental duration was 8 weeks. The animals were anaesthetized by intramuscular doses of ketamine hydrochloride and sacrificed at the end of the experiment period. The blood was collected after sacrificing through the internal jugular vein. The blood sample was collected and isolated the serum and plasma used for the cardiac markers, lipid profile. The heart tissue was immediately removed, washed with PBS and homogenated with appropriate buffer used for biochemical estimation.

Determination of lipids and lipoproteins in plasma and heart tissue: Plasma High-Density Lipoprotein-Cholesterol

(HDL-C), Low-Density Lipoprotein-Cholesterol (LDL-C) and Very-Low-Density Lipoprotein Cholesterol (VLDL-C) in the plasma were measured by kit method. Then, we calculated LDL-C and VLDL-C fractions as per the following formula:

$$\text{VLDL-C} = \frac{\text{TG}}{5}$$

$$\text{LDL-C} = \text{TC} - (\text{HDL-C} + \text{VLDL-C})$$

Apolipoprotein B (APO B) was determined as per the manufacturer's procedure by ELISA kit (Abcam Scientific Company, Shanghai, China).

Tissue lipids were extracted by the method of Radhiga *et al.*[14]. Plasma and heart tissue TC, TG, FFA and PL were estimated by the methods of Li *et al.*[15], Sundaresan *et al.*[16], Diao *et al.*[17] and Chen *et al.*[18], respectively.

Estimation of cardiac markers: The CK-MB is an isoenzyme mainly present in the heart muscle tissue. The activity of CK-MB and CK was assayed according to the methods of Thangaiyan *et al.*[19]. The cTnT and cTnI are the significant regulatory markers that control cardiac actin and myosin interaction. The serum cTn T and I were measured using a commercial kit (ELISA), respectively. The data were quantitatively calculated, as per the kit provided by the manufacturer.

Biochemical estimations: The level of Lipid peroxidative markers such as TBARS was estimated by the methods of Rosmi *et al.*[20] and lipid hydroperoxides including LOOH was determined by the method of Gao *et al.*[21]. Estimation of reduced Glutathione (GSH), Superoxide Dismutase (SOD), Catalase and Glutathione Peroxidase (GPx) were estimated by the following methods[22-25] in heart tissue.

Estimation of the inflammatory markers: We used ELISA kits from Abcam Scientific Company to measure the serum levels of inflammatory cytokines such as TNF-α and IL-6. 50 µL of the sample was added and then 50 µL of antibody cocktail was added to experimental wells. The reaction mixture was incubated at 37°C for 1 hr. The exploratory wells were washed with TMB substrates (100 µL) and set for 10 min. End of the reaction 100 µL stop solution was added to the selected wells and then the colour developed was read OD at 450 nm using an ELISA reader.

Western blot analysis: The heart tissue was homogenized in RIPA buffer containing phenyl methyl sulfonyl fluoride as per the previous report[26]. The protein samples stayed equally loaded into the gel (10% SDS-PAGE) and transferred onto the PVDF membrane. Then nonspecific binding proteins were blocked with 5% BSA for 1 hr. Further, the membrane was incubated using primary antibody p-p38 MAPK overnight at 4°C. After finishing this reaction, the membranes were incubated with HRP linked secondary antibodies for 2 hrs at 37°C. The membranes were washed three times with 10 min intervals and the bands were identified by ECL western blotting substrate reagent

Statistical analysis: All the results were calculated as the Means±Standard Deviation (SD). The data were statistically used to assess by one-way ANOVA followed by Duncan's Multiple Range Test (DMRT) was performed with the statistical analysis (SPSS software package). A value of $p<0.05$ was considered statistically significant.

RESULTS

Effect of nerolidol on HCD-induced lipid profile and lipoproteins in plasma and heart tissue: In the present study, we analysed the effect of nerolidol on HCD-induced rats increased TC, TG, FFA, PL, LDL-C, VLDL-C, apolipoprotein B and decreased HDL-C level in the plasma. Oral administration of nerolidol significantly ($p\leq0.05$) prevented TC, TG, FFA, PL, LDL-C, VLDL-C, apolipoprotein B and enhanced HDL-C levels in HCD-induced rats (Table 1).

HCD-induced rats significantly elevated TC, TG, FFA and PL levels when compared to control rats. Conversely, nerolidol administration significantly ($p\leq0.05$) reduced TC, TG, FFA and PL levels in HCD-induced rats. The nerolidol alone treatment does not alter the lipid profile in heart tissue.

Effect of nerolidol on HCD-induced cardiac marker enzymes in plasma: Table 2 shows the impact of nerolidol treatment on the activities of CK, CK-MB, cTn T and cTn I. HCD-induced rats significantly ($p<0.05$) increased the activity of cardiac marker enzymes. Whereas, treatment with nerolidol significantly decreased the activities of CK, CK-MB, cTn T and cTn I in HCD-induced rats when compared to control rats. The nerolidol alone treatment does not alter cardiac markers activities more than the control rats.

Effects of nerolidol on HCD-induced lipid peroxidative markers and antioxidant status in the heart tissue: HCD rats showed increased lipid peroxidative markers like TBARS and LOOH levels in heart tissue. However, nerolidol administration

Table 1: Effect of nerolidol on the levels of lipids and lipoproteins in plasma and heart tissue of control and HCD-induced rats

Parameters	Control	Nerolidol	HCD	HCD+Nerolidol
Plasma (mg dL^{-1})				
TC	81.98±6.26^{a}	80.13±6.11^{a}	150.37±9.55^{b}	93.23±6.9^{c}
TG	41.51±3.97^{a}	43.38±8.87^{a}	138.19±5.73^{b}	52.07±4.6^{c}
PL	70.31±6.11^{a}	72.32±6.22^{a}	110.19±08.6^{b}	83.59±6.85^{c}
FFA	45.09±3.68^{a}	44.19±3.79^{a}	76.26±5.59^{b}	54.08±4.00^{c}
HDL-C	42.66±3.07^{a}	40.86±3.16^{a}	16.24±1.6^{b}	34.56±2.72^{c}
LDL-C	31.02±2.43^{a}	30.62±2.40^{a}	106.53±8.35^{b}	48.28±3.78^{c}
VLDL-C	8.30±0.65^{a}	8.66±0.67^{a}	27.65±2.16^{b}	10.42±0.81^{c}
APO-B	28.55±1.05^{a}	26.36±1.1^{a}	70.04±5.23^{b}	36.67±2.35^{c}
Heart (mg g^{-1} wet tissue)				
TC	2.55±0.19^{a}	2.49±0.2^{a}	5.5±0.38^{b}	2.85±0.2^{c}
TG	3.28±0.31^{a}	3.00±0.29^{a}	5.7±0.41^{b}	3.71±0.3^{c}
PL	14.36±1.04^{a}	13.95±0.98^{a}	31.41±2.12^{b}	20.43±1.05^{c}
FFA	4.95±0.33^{a}	4.81±0.34^{a}	8.04±0.53^{b}	5.08±0.34^{c}

Values are given as Means±S.D (n = 6), values not sharing a common marking superscript (a, b, c) are different significantly at p-value ≤0.05 (DMRT)

Table 2: Effect of nerolidol on CK, CK-MB, cTn T and cTn I in the serum of control and HCD-induced rats

Parameters	Control	Nerolidol	HCD	HCD+Nerolidol
CK (IU L^{-1})	142.39±12.3^{a}	141.27±14.84^{a}	297.83±20.38^{b}	185.23±15.07^{c}
CK-MB (IU L^{-1})	91.97±7.61^{a}	93.28±7.45^{a}	185.08±18.46^{b}	115.24±9.27^{c}
cTnT (ng mL^{-1})	0.57±0.04^{a}	0.53±0.03^{a}	1.82±0.12^{b}	0.71±0.05^{c}
cTnI (ng mL^{-1})	0.51±0.03^{a}	0.49±0.04^{a}	0.91±0.06^{b}	0.64±0.05^{c}

Values are given as Means±S.D (n = 6), values not sharing a common marking superscript (a, b, c) are different significantly at p-value ≤0.05 (DMRT)

Table 3: Effect of nerolidol on lipid peroxidative markers and antioxidant status in the heart tissue of control and HCD-induced rats

Parameters	Control	Nerolidol	HCD	HCD+Nerolidol
Heart				
TBARS (mmol/100 g wet tissue)	0.52±0.10^{a}	0.50±0.11^{a}	1.24±0.19^{b}	0.75±0.07^{c}
LOOH (mmoL/100 g wet tissue)	51.92±6.25^{a}	49.48±7.1^{a}	98.25±9.52^{b}	64.23±6.17^{c}
SOD (U^{1}/mg protein)	7.34±0.8^{a}	7.49±0.83^{a}	3.28±0.85^{b}	6.9±0.78^{c}
CAT (U^{2}/mg protein)	38.34±6.39^{a}	37.87±7.63^{a}	18.25±4.95^{b}	29.37±4.05^{c}
GPx (U^{3}/mg protein)	6.83±0.46^{a}	6.70±0.59^{a}	3.94±0.68^{b}	6.43±0.73^{c}
GSH (µg/mg protein)	5.43±0.63^{a}	5.26±0.7^{a}	3.04±0.95^{b}	4.85±0.45^{c}

U^{1}: Enzyme required to scavenge the chromogen formed by 50% in 1 min, U^{2}: mmol of hydrogen peroxide decayed per minute, U^{3}: mmol of GSH required per minute and values not sharing a common marking superscript (a, b, c) are different significantly at p-value ≤0.05 (DMRT)

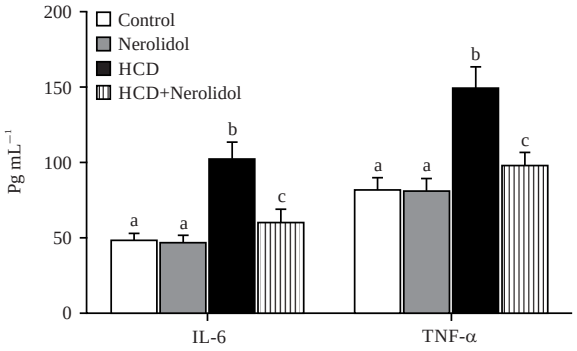

Fig. 1: Effect of nerolidol on inflammatory markers in the serum of control and HCD-induced rats

Values are given as Means±SD (n = 6), values not sharing a common marking superscript (a, b, c) are different significantly at p-value ≤0.05 (DMRT)

significantly (p<0.05) reduced TBARS and LOOH heart tissue compared to the HCD-induced rats. The zingerone alone treatment does not alter these lipid peroxidative markers levels more than the control group (Table 3).

The antioxidants status in the heart tissue of experimental rats (Table 3) was analyzed in experimental rats. Animals were fed with HCD considerably reduced antioxidants such as SOD, CAT, GPx and GSH. Whereas nerolidol administration increased antioxidant status in the HCD-induced rats. The nerolidol treatment alone does not alter the heart tissue antioxidants status compared to the control group.

Effect of nerolidol on Inflammatory marker levels in serum: The impact of nerolidol on HCD-induced Inflammatory marker levels in the serum of control and HCD-induced rats was shown in Fig. 1. HCD-induced the rats show a significant increase in the levels of TNF-α and IL-6. In contrast, nerolidol treatment significantly reduced these inflammatory marker levels (p≤0.05) when compared to HCD-induced rats.

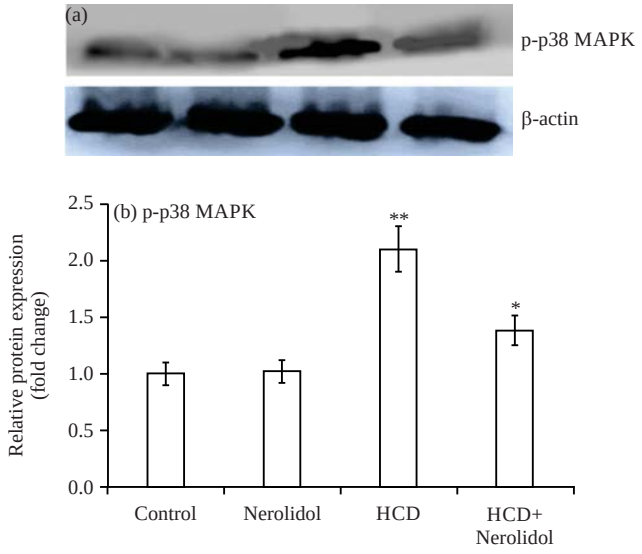

Fig. 2(a-b): Effect of nerolidol on p-p38 MAPK in the heart tissue of control and HCD-induced rats, (a) Effect of nerolidol on p-p38 MAPK in the heart tissue of control and HCD-induced rats by western blot analysis and (b) Graphical representation shows the quantitation of three independent experiments (Means±S.D), with data normalized by defining the respective β-actin.

Effect of nerolidol on p-p38 MAPK protein expression by western blot analysis: The impact of nerolidol on p-p38 MAPK protein expression in the heart tissue of HCD-induced rats was shown in Fig. 2a-b shows quantitative histograms of the Western blot analysis. The up-regulated protein expression of p-p38 MAPK in the HCD-induced group in the heart tissue ($p \leq 0.05$) when compared to control rats. Whereas, nerolidol treatment significantly down-regulated expression in HCD-induced rats. In addition, no differences were observed between the nerolidol treated and the control group.

DISCUSSION

The results of the current study showed that Nerolidol has anti atherosclerosis capabilities against an HCD-induced animal model. This was demonstrated by an improved lipid profile, the restoration of myocyte damage indicators and the suppression of lipid peroxidation. Furthermore, prevention of oxidative stress, inflammation and mediating decrease of the p-p38 MAPK protein signalling processes.

Hypercholesterolemia is strongly linked to atherosclerosis and other coronary artery disorders because excessive levels of plasma cholesterol and triglycerides in hypercholesterolemia can be carried by lipoprotein into the arterial wall, causing endothelial dysfunction and triggering atherogenesis[27]. According to growing data, high LDL-C in plasma has been linked to fat deposition on the artery wall, contributing to atherosclerotic plaques[28]. In contrast, HDL-C may reverse the transport of triglycerides and cholesterol from plasma to the liver, where they are catabolized and removed via bile acids[29]. More importantly, oxidative alteration of LDL would increase LDL deposition in the artery intima and increase monocyte adhesion to endothelium, which is the main factor in the early pathophysiology of atherogenesis[30]. As a result, the hypolipidemic impact was regarded as the primary issue in the management of atherosclerosis. Our findings show that nerolidol supplementation considerably reduces hyperlipidemia, implying that Nerolidol can be helpful for the lipid and lipoprotein profile, slowing the onset and progression of atherosclerosis.

Troponins (T and I), CK and CK-MB are the standard diagnostic indicators of the cardiac cell injury in acute cardiotoxicity[31]. The current findings demonstrate a significant rise in the cardiac markers CK, CK-MB, cTnT and cTnI in the blood of HCD rats, indicating morphological and functional alterations in the heart muscle as well as a disruption in cell membrane integrity[32]. Cardiac indicators enter the bloodstream from cardiomyocytes due to cell membrane rupture and uncontrolled permeability, as seen by enhanced enzyme activity in the HCD group[33]. Oral administration of Nerolidol significantly reduced the CK CK-MB, cTnT and cTnI in HCD-fed rats. The preventive effect of Nerolidol reduces the severity of cardiac anomalies in HCD-induced rats compared to control rats and may be connected to observed changes. Meeran *et al.*[13] observed that Nerolidol affected cardiac indicators, lipid peroxidation markers and antioxidant status in the doxorubicin-induced circulation of experimental rats. Hyperlipidemia would cause an uneven state of cellular redox, resulting in oxidative damage, promoting the pathogenesis of atherosclerosis[34]. Excessive ROS is created and damages the biomacromolecules lipid, protein and DNA in cells, causing harm to the organisms[35]. In the current study, LOOH and TBARS, a representative oxidative stress biomarker of lipid peroxidation, were significantly lower in the nerolidol treatment group compared to the HCD group, which may be attributed to the high ability of Nerolidol to scavenge reactive free radicals discovered in our previous study[36]. Furthermore, compared to the HCD group, nerolidol improved the antioxidant enzymes of SOD, GSH and GSH-Px, indicating that nerolidol could boost the endogenous

antioxidant defense system against oxidative damage in the pathological phase of atherosclerosis. Nerolidol has been demonstrated to exhibit strong antioxidant activities in scavenging free radicals. Previous studies showed that Nerolidol decreased oxidative stress markers, such as malondialdehyde (MDA) and increased the activity of endogenous antioxidants, such as SOD, CAT, GSH and GSH-Px, in various tissues of ISO induced cardiotoxic rats and in diabetic animals[37].

Inflammatory mediators and lipid metabolism abnormalities have also been observed in the aortas of hypercholesterolemic rats. The abnormal inflammatory response may play a role in the onset and progression of AS[38]. Endothelial cell proliferation and macrophage activation, on the other hand, result in the release of proinflammatory cytokines such as TNF- and IL-6. As a result, anti-inflammatory medication may affect atherogenesis[39]. In the current study, our data also indicated that Nerolidol ameliorated high-cholesterol diet-induced AS and attenuated inflammatory mediator releases (TNF-α and IL-6), revealing the vascular protective effects of Nerolidol may be correlated with the diminution of the inflammatory response.

Several studies have linked the p38 MAPK signalling pathway to the inflammatory response[17,40]. TNF-α and IL-6 expression is increased when the p38 MAPK pathway is activated[41]. Our findings revealed that the protein level of p-p38 MAPK was dramatically increased in AS rats and nerolidol downregulated the expression of p-p38 MAPK. These findings suggested that proinflammatory cytokine variations were closely associated with changes in p-p38 MAPK levels. Nerolidol may influence TNF- and IL-6 expression through modulating the p38 MAPK signalling pathways. Because oxidative stress increases the production of p-p38 MAPK, this tendency was linked to the antioxidant benefits of nerolidol.

CONCLUSION

In conclusion, the current study asserts that nerolidol reduced AS via decreasing the inflammatory response and oxidative stress linked to the p38 MAPK pathway. Furthermore, our findings highlighted the usefulness of nerolidol in treating several CVS, including AS.

SIGNIFICANCE STATEMENT

This study will help the researchers know the beneficial effect of nerolidol on treating high cholesterol diet-induced AS that many researchers could not explore. As a result, this can be established as a new therapeutic technique for treating AS. As a result, following a safety evaluation, the medication can be used in pharmaceuticals to treat hyperlipidemia and AS.

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