

# Amlodipine Ameliorates Up-Regulation of ET-1 in Left Ventricle of Hypercholesterolemia Rabbits

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## Abstract

#### **Objective**(s)

In addition to antihypertensive effects, amlodipine may exhibit cardiovascular protective effects in heart tissue. The aim of this study was to evaluate the effects of amlodipine and/or high cholesterol diet on blood, heart tissue concentration and mRNA expression of endothelin-1 (ET-1) in male New Zealand white rabbits.

#### **Materials and Methods**

A total of 40 male New Zealand rabbits were divided into four groups: the normal control group, normal group receiving amlodipine, high-cholesterol diet group and high-cholesterol diet with amlodipine group. After 8 weeks, all the animals anesthetized and blood or tissues samples were collected.

#### Results

After 8 weeks of a high cholesterol diet, the group with such a diet had a significantly higher ratio of left ventricle (LV) weight to body weight than the control group (P=0.0001). After treatment with amlodipine for 8 weeks, ET-1 level was reduced considerably in comparison with the control (P=0.01) and high-cholesterol diet groupes (P=0.01). Amlodipine consumption caused significant reduction (P=0.01) in the level of ET-1 in heart tissues of high-cholesterol diet group but it had no remarkable effect on the reduction of heart tissue ET-1 in amlodipine group compared with the control group.

#### Conclusion

The present study demonstrates that ventricular prepro-ET-1 mRNA quantitatively increases in the highcholesterol diet rabbits which results in development of ventricular hypertrophy. It seems that the treatment with amlodipine retards the progression of LV hypertrophy through attenuation of ET-1 levels independent of lipid changes.

Keywords: Amlodipine, Endothelin-1, Hyperlipidemias, Hypertrophy, Rabbits

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# Introduction

Obesity is a fast growing problem reaching epidemic proportions worldwide (1) and individuals with severe obesity have long been recognized to have a form of cardiomyopathy attributed to chronic volume overload. characterized by left ventricular (LV) dilation, increased LV wall stress, and adaptation LV hypertrophy (2), leading to heart failure (3, 4). Myocardial hypertrophy in response to pathological stimuli has traditionally been seen as an adaptive response of cardiac muscle to the altered conditions of haemodynamic load, during which the increase in wall thickness fulfils the function of regaining normal wall stress (5). Castelli and Anderson, 1986 have reported that LV mass regression reduced cardiovascular complications. Reverse of LV mass has been accepted as a high goal in treatment of cardiovascular disease (6).

The 21- amino acid peptide endothelin-1 (ET-1) is produced by vascular endothelial cells from the 38-amino acid precursor peptide, big ET-1, by the endothelin converting enzyme. ET-1 may contribute to the progression of several cardiovascular disorders such as congestive heart failure, hypertension and ischemic heart disease (7). ET-1 levels have been shown to be increased in hypercholestolemic animals (8) and patients (9). Endothelin in addition to its vasoactive properties, also triggers hypertrophic signaling pathways by activation of extracellular signalregulated kinase in myocardium (10), thereby implying a potential involvement of this peptide in the initiation and progression of In ventricular hypertrophy. vivo. gene knockout of the ET-1 gene has been reported to inhibit ventricular hypertrophy (11).

It has been reported that calcium channel blockers (CCBs) have the potential to prevent the cardiovascular disease and atherosclerosis (12). Amlodipine is considered as one of the 1,4-dihydropyridine calcium antagonists which has a long elimination half-life and has a good indication of the ability to interfere with the cell membrane function which affects on the calcium permeability resulting in calcium inhibition in vascular SMC and reduction of atherosclerotic lesions (13). However, other

studies have been unable to verify this correlation (14), so the issue remains to be clarified. The effect was manifested different in animal experiments (15). Amlodipine may also positively influence on the risk factors that are correlated with atherosclerosis. However, all the mechanisms which are involved are not clear. The main aim of this research was to evaluate the effects of amlodipine and/or high cholesterol diet on the heart tissue concentration, mRNA expression of endothelin and also the progression of ventricular hypertrophy which, so far has not been performed in rabbit model. Thus, the present study probably provides valuable about the pathogenesis information of atherosclerosis. This research may provide also a closer view of amlodipipne effect and may employ strategies to provide evidences to show an anti myocardial hypertrophic role via performing the effects of ET-1 mRNA expression in hypercholesterolemia New Zealand rabbits.

# Materials and Methods Animals

A total of 40 male New Zealand white rabbits (1.4 kg at the beginning) were divided into 4 groups: the normal control group (NC), normal group receiving amlodipine (NA), highcholesterol diet group (HC) and highcholesterol diet with amlodipine group (HE). The control group was fed with normal rabbit chow, whereas the high cholesterol diet groups were under a high cholesterol diet regime (2%). Cholesterol powder (Merck Company) was added to this normal food. NA and HE groups received amlodipine powder (Arya Company, Iran) 5 mg/kg/day. All the animals were housed in an environmentally controlled room. To eliminate the pharmacological effects, 24 hr before the performance of their experiments drugs were withdrawn in each group. All the experimental procedures including rabbit care and handling were performed in agreement with the guidelines provided by Experimental Animal Laboratory and approved by the Animal Care Committee of Tabriz University of Medical Sciences.

## Serum lipid profile

Serum lipid profile including total cholesterol and triglyceride were determined by enzymatic methods using automatic analyzer (Abbott, Alcyon 300, USA).

## The plasma ET-1 level

Because of a local release of ET-1, blood samples gathered from the aortic root and the LV tissue were obtained for measuring of systemic and local ET-1 levels at the end of the study. At completion the of experiment. animals anaesthetized with thiopenton (50 mg/kg/ip). Then left ventricles rapidly isolated after removal of the hearts Blood was also stored in tubes containing EDTA (10 mmol/1 final concentration) treated on ice for determination of the plasma endothelin. After centrifugation (15 min, 4 °C), the plasma (1ml) was stored at -80 °C until analysis. The level of the plasma ET-1 was measured by special kit of ET-1 (Titer Zyme® EIA kit, No: 030806265).

# Cardiac ET-1 level

For the measurement of cardiac ET-1 levels, each left ventricular tissue was weighed individually. The tissues were immediately homogenized with a homogenizer in a buffer containing HCl-20 mmol/l, Acetic acid-1mmol/l which followed by centrifugation of the homogenized preparation (3000 g for 10 min at 4 °C). The light supernatant obtained from homogenization was stored at -80 °C until needed. Following the endothelin measurement time, the supernatant was lyophilized by lyophilizer (Christ Alphal-4) and the level of heart tissue ET-1 was measured by special kit of ET-1.

# RNA extraction and RT-PCR analysis

Total RNA from left ventricular cells was extracted using TRIZOI reagent (*In vitro* gen) according to the manufacturer's instructions. Total RNA (5  $\mu$ g) was treated with 1  $\mu$ g oligo (dT) and AMV reverse transcriptase using first-strand cDNA synthesis kit (Fermentas). The reaction was performed at 42 °C for 60 min. One  $\mu$ l of cDNA was used to perform PCR. The primer sets were as follows: Rab-ET1-F, GCTCCTGCTCCTCGCTGAT and

Rab-ET1-R, AGAGCGAGTGAGAGAGAGTGA,  $\beta$ actin primer-F: 5'- CCC TAA GGC CAA CCG TGA AAA GAT G -3'and  $\beta$  actin primer-R: 5'- GAA CCG CTC ATT GCC GAT AGT GAT G-3'. The cycle profile included denaturation for 60 sec at 94 °C, annealing for 45 sec at 57 °C, and extension for 1 min at 72 °C. The amplified PCR products were electrophoresed on 1% agarose gels, stained with ethidium bromide and visualized by ultraviolet transilluminator. The relative intensities of signals were quantified using scion image software (Scion Corporation USA).

## Statistical analysis

Results were presented as means±SD. Data were analyzed with SPSS. Two-way ANOVA was used to search for possible effects of ET-1 levels, cholesterol levels, and if an F-value was found to be significant, a two-tailed Student's t-test was used to test differences. Correlation between the ratio of LV mass/body weight and ET-1 levels were assessed by Pearson's correlation coefficient. The significant level was assumed at value of P < 0.05.

# **Results**

## The assessment of high cholesterol diets

Our results clearly demonstrated that 8 weeks of high cholesterol diets (2%) significantly increased the level of total serum cholesterol and triglyceride. These observations indicate that atherogenic diet induced hypercholesterolemia in our experimental New Zealand rabbit model (Table 1). After 8 weeks of high cholesterol diet, the hyperlipidemic rabbits had a significantly higher ratio of LV weight to body weight than that of the control group (2.08±0.09 vs. 1.66±0.07 g/kg in controls, P < 0.001). A significant reduction in LV mass occurred after amlodipine treatment by 12% (P < 0.01). There was a significant residual LV hypertrophy after amlodipine treatment, being 4% above that in control (*P*< 0.05).

#### Amlodipine and ET-1 in Hypercholesterolemia Rabbits

Parameters	NC	NA	НС	HA
N	8	8	8	8
Body weight, kg	2.4±0.1	2.4±0.1	2.3±0.1	2.3±0.1
LV weight/body weight, g/kg	$1.66 \pm 0.07$	$1.65 \pm 0.07$	$2.08{\pm}0.09^{*}$	$1.73 \pm 0.07^{**}$
Plasma cholesterol, mg/dl	$49.1\pm0.6$	40.3±0.8	$860.3 \pm 0.6^*$	524.5±5.8**
Plasma triglyceride, mg/dl	95.5±1.7	81.0±0.5	$466.6 \pm 2.5^*$	138.6±1.8**

Table 1. Comparison of the body weight, LV weight/body weight (g/kg) and serum lipid profile changes (mg/dl) among four groups of New Zealand rabbits.

Abbreviations: NC= normal diet control; NA= normal diet with amlodipine; HC= high cholesterol diet control; HA= high cholesterol diet with amlodipine; N= the number of rabbits in each group.

; LV= left venticular

\* Significant difference with NC group (P=0.001); \*\* Significant difference with HC group (P=0.001)

# Circulating, myocardial ET-1 Levels and prepro-ET-1 mRNA in myocardial tissue

The plasma level of ET-1 in atherosclerotic model group was significantly increased as compared with the control group (P < 0.01). After treatment with amlodipine for 8 weeks ET-1 level was reduced significantly in the control (P=0.01) and high-cholesterol diet rabbits P=0.01) (Table 2).

To investigate the possible role of cardiac ET-1 synthesis in the reduction of the plasma ET-1 levels, we determined the ventricular ET-1 levels. Amlodipine consumption caused significant reduction (P=0.01) in the level of endothelin-1 in heart tissue of high-cholesterol diet group but didn't have any significant affect on the reduction of heart tissue endothelin-1 in amlodipine group compared with control (Table 2). The mRNA levels of prepro-ET-1 showed a 1.8±0.2-fold enhancement in the hyperlipidemic rabbits (P= 0.0001; Figure 2). Thus the mRNA levels of prepro-ET-1 were changed in parallel to the tissue peptide levels, indicating that the production of prepro-ET-1 is a critical regulation step for its local activation. Amlodipine administration significantly decreased both prepro-ET-1 mRNA and ET-1 peptides compared with rabbits untreated with amlodipine, implicating that amlodipine has inhibitory effect on ET-1 levels.

#### **Correlation**

The linear regression models showed a significant correlation between tissue ET-1 levels and the ratio of LV mass to body weight (LV mass-to-body weight ratio= 0.004 x tissue ET-1 levels (in pg/mg protein)+1.502, P= 0.000) (Figure 3).

 Table 2. Comparison of plasma and tissue endothelin

 changes among four groups of New Zealand rabbits.

Group	Plasma Endothelin (pg/ml)	Heart Tissue Endothelin (pg/100mgTissue)
 NC	$0.56 \pm 0.01$	$0.31 \pm 0.02$
NA	$0.39 \pm 0.01^{*}$	$0.27\pm0.01$
НС	$0.8 \pm 0.04^{*}$	$0.47 \pm 0.02$ *
HA	$0.6 \pm 0.01$ <sup>#</sup>	$0.37 \pm 0.02$ <sup>#</sup>

Data are expressed as Mean±SEM (n= 8) for each group.

Differences of P < 0.05 were considered significant.

\* NA and HC vs. NC <sup>#</sup> HA vs. HC (Plasma Endothelin) \* NA and HC vs. NC <sup>#</sup> HA vs. HC (Heart Tissue Endothelin)

Abbreviations: NC= normal diet control; NA=normal diet with amlodipine; HC= high cholesterol diet control; HA=high cholesterol diet with amlodipine.



Figure 1. Comparison of left ventricular mass indexed by body weight (BW) among four groups of New Zealand rabbits. \* NA and HC vs. NC  $^{\#}$  HA vs. HC (P < 0.05)

NC= normal diet control; NA=normal diet with amlodipine; HC= high cholesterol diet control; HA= high cholesterol diet with amlodipine.



Figure 2. Comparison of LV prepro-endothelin-1 mRNA levels in among four groups of New Zealand rabbits. \* NA and HC vs. NC<sup>#</sup> HA vs. HC (P< 0.05)

Data are expressed as Mean±SEM (n= 10) for each group. Abbreviations: NC=normal diet control; NA=normal diet with amlodipine; HC= high cholesterol diet control; HA=high cholesterol diet with amlodipine. Each mRNA was corrected for an mRNA level of  $\beta$ - actin. (LV mass-to-body weight ratio= 0.004 x tissue ET-1 levels (in pg/mg protein) + 1.502, *P*= 0.000).



Figure 3. ET-1 concentration of the LV in relation to LV mass/BW.

ETBG: Endotheline gene expression.

LW.BW: Ratio of left ventricular weight to body weight

## Discussion

The results obtained in this research clearly showed that 8 weeks consumption of high cholesterol diets (2%) significantly increased serum total cholesterol and triglyceride. Our observations indicated that atherogenic diet induced hypercholesterolemia in our experimental New Zealand rabbit model (Table 1). The concentration of plasma cholesterol in hyperlipidemic rabbits showed higher effects significantly. Treatment with amlodipine decreased the concentration of plasma cholesterol considerably in comparison hyperlipidemic rabbits. with Amlodepine effect on decreased endotheline gene expression and tissue concentration seems to be related to its effect on the plasma cholesterol concentration. Moreover, GA modifies cholesterol metabolism and regulates the deposition of cholesterol in arterial wall (16). The results of this study show that hyperlipidemic rabbits had significantly higher ratio of LV weight to body weight than that of the control group. A significant reduction in LV mass occurred after amlodipine treatment. There was a considerable residual LV hypertrophy after amlodipine treatment. Figure 1 shows the comparison of left ventricular mass indexed by body weight (BW) among four groups of New Zealand rabbits. The linear regression models demonstrated an important correlation between tissue ET-1 levels and the ratio of LV mass to body weight. Regarding to ET-1 concentration of the LV in relation to LV mass/BW, a remarkable correlation was found between the 2 variables (Figure 3). The present study showed two novel findings utilizing the combined use of molecular and biochemical methods, fascinating new insight into the nature of amlodipine and endothelin. First, hyperlipidemia acting through the increasing levels of ventricular prepro-ET-1 mRNA and tissue ET-1 which results in the progress of ventricular hypertrophy. It in turns may be inhibited by using CBBs. Second, we found that amlodipine administration had the positive effects on the attenuated ventricular hypertrophy at the development stage of LV hypertrophy via attenuation of tissue ET-1 levels. Regarding to hyperlipidemia and LV results showed mass. our that hypercholesterolemia was correlated with increased LV mass. However, attention should be drawn to other potential mechanisms such as local activation of ET-1 which might be associated with the ventricular hypertrophy progression but it depends on the levels of ET-1 in the plasma. As far as we are aware, several studies in hyperlipidemic rabbits, have reported the significant increased levels of ET-1 by vascular endothelium cells (17) but the release of local ET-1 by myocardium has

not been previously addressed. We showed that the enhanced levels of ET-1 in tissues were effective in the progression of ventricular hypertrophy induced in hypercholesterolemia. Our results are in agreement with the previous studies which showed the raised level of ET-1 secreted from coronary vascular cell wall to myocardium. At least 3 classes of signaling pathways in the cardiomyocytes, including mitogen-activated protein kinase, protein kinase C, and the phosphatidylinositol 3kinase/Akt are activated by ET-1. ET receptor blockers suppress the increased synthesis of protein and the activation of these signaling pathways in vitro and in vivo studies. This observation raises the possibility that the  $ET_A$ receptor has been attributed at least in part in ET-1-induced hypertrophic growth (18). Our findings are also in agreement with cardioprotection of amlodipine which is exerted by chronic inhibition of ET-1 levels. However, angiotensin II and free radicals are the other possible mechanisms which are considered to modulate the antihypertrophic effects of amlodipine. Angiotensin II and ET-1, as a complex compose, show positive circuit acting on cardiomyocytes. Studies have shown a correlation between the angiotensin system and ET-1 in vitro so that the angiotensin II may induce ET-1 synthesis in cardiomyocytes (19). Ishiye et al have reported the inhibition of enhancement in ventricular ET-1 content by specific blockade of angiotensin type 1 receptors (20). Amlodipine has been reported which may inhibit the activity of angiotensin II (21), so it is considered as a factor for the down regulation effect on the of ET-1 protein. Moreover. expression blockade of free radicals delays the progress of cardiac hypertrophy (22). Previously it has been shown that cardiac protection of Cablockers may be correlated with the decrease of myocardial oxidative stress (23). Increased production of free radicals activates the mitogen-activated protein kinases which results in induction of cardiac hypertrophy (24). Thus, it seems that amlodipine may have a role to attenuate cardiac hypertrophy by reducing the production of free radicals. Although previous studies in humans have

shown the association of the hyperlipidemia with increased LV mass (25), in this model hypercholesterolemia rabbits developed ventricular hypertrophy. However, this makes it difficult to make a firm conclusion about the direct effects of hyperlipidemia on the cardiac hypertrophy, because there are several factors which are involved in interpreting of LV mass in clinical settings. In the present research to investigate the direct effect of hyperlipidemia on myocyte hypertrophy, we utilized the hyperlipidemic model. We confirmed that amlodipine administration has an effect on the hypertrophic signaling in hyperlipidemia rabbits, via interfering on the activities of ET-1 pathways. The dose of amlodipine (1.2 mg/kg/day) used in this study was considered safe and was similar to the conventional dose of amlodipine utilized in humans (up to 80 mg/kg/day). Therefore, this beneficial effect of amlodipine therapy may have significant application in clinic.

# Conclusion

The present research showed that the level of ventricular prepro-ET-1 mRNA was increased quantitatively bv the highcholesterol diet resulted in increasing of tissue ET-1 levels. This in turn caused in the progress of ventricular hypertrophy. Treatment with amlodipine influences on the development of LV hypertrophy possibly through reduction of ET-1 levels independent of lipid changes. These findings may be considered significant in LV mass-related risk factors of hyperlipidemic patients. Most selected treatments of hyperlipidemia should not only focus on adequate reduction of cholesterol but might also consider concomitant reduction of LV mass, which may help the scientists to fill in the considerable gaps which remains to be clarified in the complex puzzle of the drug interactions with the heart.

# Acknowledgment

This work was supported in part by a grant from Drug Applied Research Center of Tabriz University of Medical Sciences, Tabriz, Iran. We wish to thank Professor Hossein Babaei, for his support and advices.

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