



## Vascular Dysfunction in Short-Term Hypercholesterolemia despite the Absence of Atherosclerotic Lesions

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### ARTICLE INFO

#### Article Type:

Research Article

#### Article History:

Received: 2 May 2011

Accepted: 16 June 2011

ePublished: 20 Aug 2011

#### Keywords:

Hypercholesterolemia

Lovastatin

Atherosclerosis

Vascular Function

### ABSTRACT

**Introduction:** The atherosclerotic effect of hypercholesterolemia on the vascular function is well-known. However, limited studies were done on the effect of hypercholesterolemia without atherosclerotic lesion on the vascular compliance. The aim of this study was to investigate the effects of hyperlipidemia induced by cholesterol rich diet on vessel function in isolated rat aorta in the absence of atherosclerotic lesion. **Methods:** Male wistar rats were randomly divided into 3 groups of 6 animals in each. The rats in normal control group were fed a standard laboratory diet and two other groups were fed a high fat diet for 36 days. A group of high fat fed rats was treated orally with Lovastatin started at day of 16 and continued for last 20 days of the experimental period. At the end of the experiment, inferior vena cava blood was collected to measure the lipid levels and the thoracic aorta was excised and used for isolated vessel preparation and histological study. **Results:** The results of this study indicated that high-cholesterol diet significantly increased total cholesterol and LDL levels in serum ( $p < 0.001$ ). The increase in the serum levels of cholesterol was associated with a profound reduction of endothelium dependent vasodilatation of the thoracic aorta. However, in histopathological study no atherosclerotic lesion was observed. Short-term treatment by Lovastatin (10 mg/kg/day) produced a significant reduction ( $p < 0.05$ ) in the level of total cholesterol and LDL. The endothelium-dependent vasodilatation was improved significantly ( $P < 0.01$ ) by Lovastatin as an anti-hyperlipidemic drug. **Conclusion:** Hypercholesterolemia is associated with endothelial dysfunction in aorta, despite the absence of atherosclerotic lesions.

### Introduction

Atherosclerotic cardiovascular disease is the leading cause of morbidity and mortality worldwide. Dyslipidemia is one of the main risk factors leading to atherosclerosis. Moreover, there is evidence for a role of oxidation in linking lipids and inflammation to development and progression of atherosclerosis. Elevated blood concentration of cholesterol, especially in LDL, constitutes the primary risk factor for atherosclerosis and endothelial dysfunction.<sup>1</sup> Although the impairment of endothelium-dependent relaxation during hypercholesterolemia has been fully elucidated, but the implication of hypercholesterolemia in vascular smooth muscle dysfunction has not been considered.<sup>2-4</sup> A study of the carotid artery in short-term hypercholesterolemic rabbits indicates that hypercholesterolemia with no influence on intimal plaque formation attenuates endothelium-derived relaxation.<sup>5</sup> Rats are resistant to the development of hypercholesterolemia-induced atherosclerosis<sup>6,7</sup> and a study reveals slight endothelial changes in rat aorta only

after they receive a high-fat diet for 12 months.<sup>8,9</sup> In contrast to the cases in many other species, Pisulewski *et al.* believe that hypercholesterolemia in high-fat fed rats dose not develop endothelial dysfunction.<sup>7</sup> Furthermore, not only in rat model but also in other species the effects of hypercholesterolemia on vascular smooth muscle contraction are not fully elucidated.

The purpose of the present study was to determine whether hypercholesterolemia induced by high-fat diet consumption results in endothelium-dependent or -independent unresponsiveness in rat thoracic aorta before the development of atherosclerosis. Further, we speculate that this unresponsiveness not only involve impairment of relaxation but also may comprise impairment of vasoconstriction.

### Materials and methods

#### Animals

Male Wistar rats (110-120 g) were used in this study. The animals were given standard pellet diet and water *ad*

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*libitum*. They were housed in the Animal House of Tabriz University of Medical Sciences at a controlled ambient temperature of  $25\pm 2^\circ\text{C}$  with  $50\pm 10\%$  relative humidity and with a 12-h light/12-h dark cycle.

### **High fat diet**

The high fat diet contained standard Sahand Niroo (Tabriz-Iran) rodent chow powder (62.75%), cholic acid (0.25%), cholesterol (2%), and lard oil (15%), wheat flour (10%), and sucrose (10%).

### **Experimental protocol**

Animals were allocated into three groups of 6 rats each ( $n=6$ ), with food and water freely available. The first group received a standard diet (normal control), while groups 2 and 3 were fed with the high-fat diet for 36 days. Group 2 received 1 ml Carboxyl Methyl Cellulose (0.5%; CMC) in water per day as vehicle (hypercholesterolemic group: HC) and group 3 received suspension of Lovastatin in CMC (10 mg/kg/day; HC+Lovas), using a gavage during the last 20 days treatment period. At the end of experimental period, the rats were fasted for 16 h and then anaesthetized by intraperitoneal (i.p) injection of ketamine plus xylazine. Blood samples were collected from inferior vena cava in centrifuge tubes and centrifuged to obtain plasma and serum. The thoracic aorta was rapidly removed and divided into two sections. A section was placed in Krebs-Henseleit solution for isolated blood vessel preparation and the other was used for histological study.

### **Serum lipids measurements**

Serum concentrations of total cholesterol (TC), HDL, and triglycerides (TG) were determined by enzymatic colorimetric methods using commercially available kits (Randox Laboratories Ltd, UK). The assay was performed according to the manufacturer's instruction. All samples were measured in duplicate. The concentration of LDL was calculated by the following equation:

$$\text{LDL} = \text{TC} - (\text{HDL} + 0.2\text{TG})$$

### **Histological examination**

For histopathological study, biopsies of aorta ( $n=6$ ) of all groups were obtained and fixed in 10% neutral-buffered formaldehyde for 48 h, embedded in paraffin and sectioned at  $5\ \mu\text{m}$ . The sections were stained with haematoxylin and eosin, and examined by light microscopy ( $\times 40$ ).

### **Isolated thoracic aorta preparation**

The thoracic aorta was immediately dissected, transferred to a Petri dish containing Krebs solution,

cleaned of fat and adhering tissues and was cut into ring segments of approximately 3 mm in length; care was taken to avoid any damage to endothelium. The aortic ring was mounted in 10 ml organ bath containing Krebs solution ( $37^\circ\text{C}$ ,  $\text{pH}=7.4$ ). Solution was continuously bubbled with a 95%  $\text{O}_2$ -5%  $\text{CO}_2$  gas mixture. In each ring, two metallic hooks were inserted through lumen of the ring; one was anchored to the organ bath, while the other was vertically attached to a strain gauge force transducers (Lethica Spain), which were connected to an four-channel bridge amplifier (AD Instrument, 4Sp, QUAD Bridge). The isometric force was displayed and recorded on a PowerLab data-acquisition system with a computerized analysis program (Chart 5.4.2, AD Instruments). Aortic rings were allowed to equilibrate for 60 min at a resting tension of 2 g, with the bath medium changed every 15 min. After the equilibration period, aortic rings were contracted with phenylephrine ( $10\ \mu\text{M}$ ) and exposed to carbachol (Carb; 0.3 to  $11\ \mu\text{M}$ ) or to sodium nitroprusside (NSP; 5 to  $320\ \text{nM}$ ) to test the endothelium-dependent and independent relaxation. The responses are expressed as the percent relaxation of phenylephrine-induced tone.

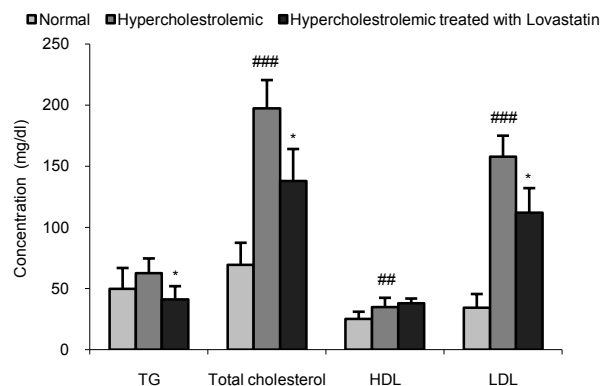
### **Statistical analysis**

Data were presented as mean $\pm$ SEM. Comparisons between groups were made with ordinary ANOVA. If ANOVA analysis indicated significant differences, a Student-Newman-Keuls post test was performed to compare mean values between treatment groups and control. Differences between groups were considered significant at  $p<0.05$ .

## **Results**

### **Serum lipid profile**

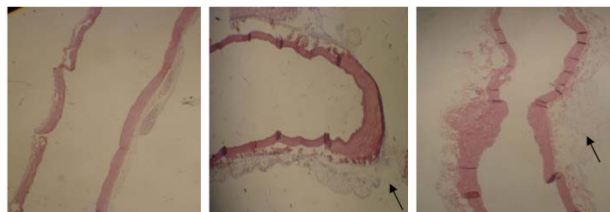
Compared with the normal rats, food intake and body weight gain were high in hypercholesterolemic animals throughout the experiment period (data not shown). Compared with the normal control rats, the high-fat diet for 36 days produced a significant increase in serum total cholesterol, LDL ( $p<0.001$ ), and triglycerides by 185%, 364%, and 26%, respectively (Figure 1). There was also a significant ( $p<0.05$ ) increase in serum HDL by 40%. The oral administration of Lovastatin (10 mg/kg/day) for 20 days to hypercholesterolemic (HC) rats caused a significant ( $p<0.05$ ) decline in serum total cholesterol and LDL. These declines were accompanied by a significant ( $p<0.05$ ) reduction of serum triglycerides in Lovastatin treated group. There was no significant change in the serum level of HDL in Lovastatin (as a positive control group) group.



**Figure 1.** Effects of high fat diet on serum lipid profiles as triglycerides (TG), total cholesterol, HDL, and LDL. Data are expressed as mean±SEM. Number of rats per group n=6. ###p<0.001 and ####p<0.0001 compared with normal control group. \*p<0.05 compared with hypercholesterolemic rats using ordinary ANOVA test.

### Effect of high fat diet on lipid accumulation in aorta

At the end of the experiment, a section of thoracic aorta was examined by histological examination for lipid accumulation. A microscopic study of the tissue slices from the aorta (Figure 2) did not reveal intimal plaque formation or atherosclerotic changes in all studied groups. However, there was a moderate lipid accumulation outside the vessels obtained from the high fat fed rats.

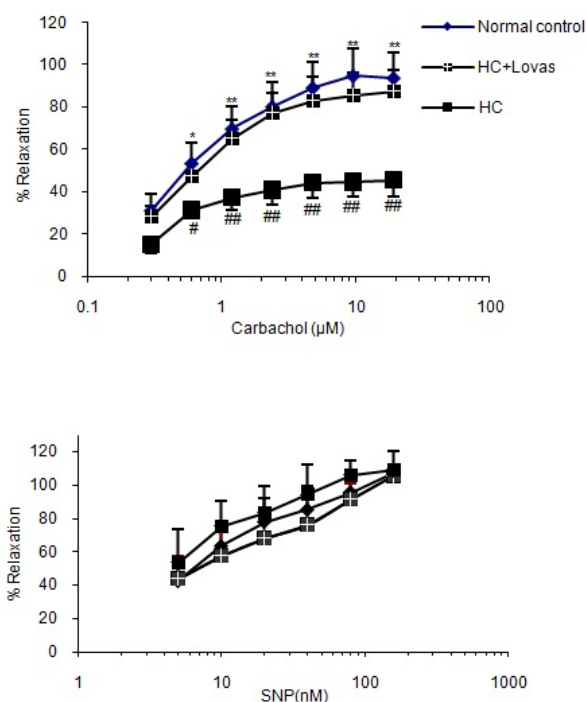


**Figure 2.** Microphotography of thoracic aorta slice (Haematoxylin and Eosin stain) from control normocholesterolemic rat (left), hypercholesterolemic rats (middle), and lovastatin treated HC rats (right). Aorta from control normocholesterolemic rats demonstrates lack of lipid deposition outside the vessel. Vessels from all groups demonstrate lack of atherosclerotic plaque in the intimal of the vessels. Arrows indicates a slight lipid accumulation outside the wall. Original magnification, 40×.

### Effect of hypercholesterolemia on the relaxation induced by carbachol or SNP in aorta

The arteries from all groups were contracted by phenylephrine (10 μM) and then exposed to different concentrations of either carbachol or sodium nitroprusside (SNP). Generally, the vessels obtained from hyperlipidemic rats demonstrated a weak contraction in response to phenylephrine. Compared

with the control normal rats the endothelium-dependent relaxation, induced by carbachol, was strongly ( $p<0.001$ ) decreased in the hypercholesterolemic group (Figure 3 upper). The maximum relaxation induced by 9.6 μM of carbachol was  $95\pm12\%$  in the control group while, the relaxation induced by 19.2 μM of carbachol was only  $34\pm7\%$  in arteries isolated from the hypercholesterolemic rats. Lovastatin (10 mg/kg/day), a 3-hydroxy-3-methylglutaryl CoA reductase inhibitor (statin), significantly increased vasodilatation responses to carbachol in the hypercholesterolemic rats. The carbachol induced relaxation in lovastatin treated rats (maximum  $88\pm10\%$ ) was similar to that of the normal control group (Figure 3 upper). Contrary to the case of endothelium-dependent relaxation, hypercholesterolemia did not affect endothelium-independent vascular relaxation induced by SNP (Figure 3 lower). In addition, lovastatin treated groups presented relaxations similar to that of the normal control group.



**Figure 3.** Relaxation of precontracted isolated thoracic aorta to carbachol (upper trace) and sodium nitroprusside (SNP; lower trace). Isolated arteries were constricted by 10 μM Phenylephrine throughout the experiment. Carbachol or SNP were added cumulatively to construct a vasodilatory concentration-response curve. Data are expressed as mean±SEM. Number of rats per group n=6. \*p<0.01 and ##p<0.001 compared with normal control group. \*p<0.05 and \*\*p<0.01 compared with hypercholesterolemic rats using ordinary ANOVA test. HC = hypercholesterolemic rats; Lovas = Lovastatin.

## Discussion

Hyperlipidemia is one of the major risk factors of atherosclerosis and endothelial dysfunction.<sup>1</sup> In this study adding cholesterol (2%) and lard oil (15%) to the diet of rats for 36 days led to hypercholesterolemia as indicated by significant increases in serum total cholesterol (185%;  $p<0.001$ ), LDL-C (364%;  $p<0.001$ ), and a non significant increase in the level of triglycerides (26%). These increases was along with a light but significant raise in serum HDL-C ( $p<0.05$ ). In this study, we hypothesized that hypercholesterolemia even without atherosclerotic lesions can cause vascular dysfunction. Vascular studies in humans and in animal models of hypercholesterolemia/atherosclerosis indicate that endothelial function is impaired during hypercholesterolemia.<sup>2-4</sup> Although the mechanisms underlying the impairment of endothelium-dependent relaxation have not been fully elucidated, a number of evidence demonstrate that nitric oxide (NO) inactivation by oxygen-derived free radicals may play a critical role.<sup>9,10</sup>

The result of present study shows that responses to carbachol in thoracic aorta were reduced in high-fat fed rats, compared with responses in control animals. While, sodium nitroprusside-induced relaxation of the artery did not differ between the control and all treated or untreated hypercholesterolemic rats. Histological examination of tissues from hypercholesterolemic rats did not show morphological changes in the aorta. These findings suggest that increased plasma level of cholesterol, even without morphological and atherogenic changes are associated with endothelial dysfunction in aorta.

A finding of the present study is that impaired endothelium-dependent relaxation of thoracic aorta in hypercholesterolemic rats was improved by treatment with lovastatin. The effects of lovastatin on improvement of carbachol-induced relaxation in hypercholesterolemic rats, however, may be mediated not only by their lipid-lowering properties but also by an effect on the activity of endothelial nitric oxide synthase (eNOS).<sup>11</sup>

Vasodilatation produced by acetylcholine or carbachol has been shown to be dependent on the presence of vascular endothelial cells.<sup>12,13</sup> An endothelium-derived vasodilator factor, EDRF, has been identified. EDRF is considered to be nitric oxide (NO), which is produced by NO synthase (NOS) from L-arginine and relaxes smooth muscle cells by increasing their intracellular cyclic GMP level.<sup>14</sup> The most commonly abnormalities in the regulation of the lumen of vessels relate to endothelial cell dysfunction. In this context, endothelial cell dysfunction has been defined by blunting of the vasodilatory response to carbachol which is known to produce NO-dependent vasodilatation. Hyperlipidemia and increased levels of oxidized LDL are important pathogenic mechanisms of endothelial dysfunction.

Since, it has been shown that treatment with hypolipidemic agents improves endothelial function in hyperlipidemic patients<sup>15</sup>; it becomes very tempting to find out whether hyperlipidemia itself alone in the absence of any vessels lumen lesion may results in endothelium dependent dysfunction of arteries. The present study found that lipid concentration in blood is a key determinant of the endothelial function independent of atherogenesis and of intimal plaque formation.

## Acknowledgments

The study was supported by a grant from the Research Vice-Chancellorship of Tabriz University of Medical Sciences, Tabriz, Iran.

*Ethical issues:* This study was performed in accordance with the Guide for the Care and Use of Laboratory Animals of Tabriz University of Medical Sciences, Tabriz, Iran (National Institutes of Health Publication No 85-23, revised 1985).

*Conflict of interests:* No conflict of interest to be declared.

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