Endothelial Dysfunction in Experimental Atherosclerosis in the Rabbit with Extraction of Instantaneous Changes in the Arterial Wall

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Received 27 December 2011; Accepted 15 April 2012

Abstract

Background: In this study, we used a new computerized analytical method for the measurement of the endothelial function in sequential ultrasound images and compared it with histological studies, using the abdominal aorta in normal and atherosclerotic rabbits.

Methods: Six rabbits received a standard rabbit chow as the normal group and the other 6 rabbits were fed a high cholesterol diet for four weeks as the atherosclerotic group. B-mode images of the abdominal aorta with 46 frames per second were saved over three cardiac cycles at baseline and during acetylcholine or nitroglycerin drug infusion in the normal and atherosclerotic rabbits. In order to evaluate endothelial-dependent relaxation, acetylcholine-mediated dilation (AMD) was measured during the infusion of acetylcholine at a rate of 0.5 µg/kg/min and endothelial-independent relaxation was evaluated by measuring nitroglycerine-mediated dilation (NMD) during the infusion of nitroglycerin at a rate of 5 µg/kg/min. In addition, the ultrasonic evaluation was confirmed by histopathological evaluation of the abdominal aorta.

Results: Significant differences in AMD were detected between the normal and the four-week cholesterol-fed rabbits (p value < 0.05), whereas there were no significant differences in NMD between the two groups (p value > 0.05). No microscopic intimal lesions were seen in the normal rabbits, but intimal thickening was observed in the histological studies in the four-week cholesterol-fed rabbits. Additionally, the total cholesterol, triglycerides, low-density lipoprotein cholesterol, and high-density lipoprotein cholesterol levels were remarkably increased in the sera of the four-week cholesterol-fed rabbits (p value < 0.05).

Conclusion: A new automatic method can help accurately evaluate the endothelial function in normal and hypercholesterolemic rabbits.

J Teh Univ Heart Ctr 2012;7(3):128-135

This paper should be cited as: Rahmani-Cherati T, Mokhtari-Dizaji M, Vajhi A, Rostami A, Mehrad H, Mohsenifar A. Endothelial Dysfunction in Experimental Atherosclerosis in the Rabbit with Extraction of Instantaneous Changes in the Arterial Wall. J Teh Univ Heart Ctr 2012;7(3):128-135.

Keywords: Atherosclerosis • Ultrasonography • Rabbits • Vasodilation

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Introduction

The most important role of vascular endothelium is the control of vascular tone by producing contracting and relaxing agents. Nitric oxide is one of these factors which are released after the stimulation of endothelial cells by shear stress and some hormones like acetylcholine and serotonin. Risk factors such as oxidative stress and hypercholesterolemia alter the physiological processes of the endothelium system and thereby damage the endothelium. Endothelial dysfunction is associated with the diminished activity of endothelium-dependent hyperpolarizing factor and the decreased production of nitric oxide. Therefore, it leads to clinical consequences that include an increased inflammation, vasoconstriction, and development of atherosclerotic lesions. Some in vivo studies have shown endothelial dysfunction in the arteries without angiographically detectable lesions in hypercholesterolemic patients. Since impaired endothelium-dependent relaxation in the arteries is the most sensitive marker for the early diagnosis of atherosclerosis, a non-invasive, reproducible, and accurate method is required for the measurement of the endothelial function.

Non-invasive evaluation of the endothelial function is an accurate method, which involves measuring acetylcholine-mediated dilation (AMD) of the vascular lumen. In experimental conditions, the evaluation of nitric oxide production is the most useful method for the estimation of the endothelial function. Non-invasive testing involves calculating the changes induced by acetylcholine administration in the diameter of vessels with an ultrasound device.

Most researchers have used manual tracing for the measurement of the arterial diameter in ultrasound images. The manual tracing method is not only based on subjective operator assessment but also time-consuming. Several studies have suggested automated algorithms for the measurement of the arterial diameter. Use of such methods increases the reproducibility of the results and also reduces the duration of image processing.

In this study, endothelium-dependent dilation produced by acetylcholine as well as nitroglycerine-induced endothelium-independent dilation was evaluated on the abdominal aorta of normal and atherosclerotic rabbits, using a B-mode ultrasound device. In our previous study, we presented a new automatic analyzing method for the detection of instantaneous changes in the arterial wall of rabbits’ abdominal aorta in sequential images of B-mode ultrasound. We showed that this automatic method is accurate and highly reproducible and also the processing time can be efficiently reduced. Dynamic programming algorithm has the highest accuracy and maximum gradient algorithm has the lowest computation complexity for ultrasound image processing. Therefore, we combined the two algorithms for the automated measurement of instantaneous changes in the arterial diameter. In the present study, we utilized a new computerized analysis method for the measurement of AMD and nitroglycerine-mediated dilation (NMD) in sequential ultrasound images on the abdominal aorta during acetylcholine or nitroglycerine infusion in normal and atherosclerotic rabbits and compared the results obtained from the ultrasonic studies with those of histological evaluations.

Methods

A total of 12 healthy male New Zealand white rabbits, weighing 2.5-3 kg, were purchased from Pasteur Institute of Iran (Tehran, Iran). The rabbits were involved in this study after two weeks of adaptation in housing facilities. All the animals were handled in accordance with the guidelines of Universities Federation for Animal Welfare (UFAW). The animal experiments and protocols were evaluated and approved by the Animal and Ethics Review Committee of the Tarbiat Modares University (Tehran, Iran).

The animals were individually housed in metal cages in air-conditioned room (22 ± 1 °C). The rabbits were randomly divided into a control group (A), in which the rabbits consumed a standard chow diet (n = 6), and a damaged group (B), in which the rabbits were fed with standard rabbit chow supplemented with 2% cholesterol for four weeks (n = 6).

Ultrasound examination of the endothelial function of the rabbits’ aorta was performed once at the commencement of the study and once at four weeks after the start of high cholesterol diet for the hypercholesterolemic and the normal rabbits. In order to reduce the movement and gas artifact of the rabbits’ intestines, all the animals were kept off feeds for twelve hours. The rabbits were anesthetized with 50 mg/kg ketamine and 5 mg/kg xylasine (Alfasan Co., The Netherlands) by intramuscular injection. Heart rate and blood pressure were recorded via a monometer on the animal forearm, using a non-invasive blood pressure measurement device (pet MAP graphic, RAMSEY Med. Inc., FL, USA, ± 1 mmHg).

The rabbits’ abdomen was shaved and the animals were put in dorsal decubitus position. The marginal ear vein was cannulated for drug infusions. All the animals underwent ultrasonography (GE Med Systems, Voluson 730 Pro, Kretztechnik GmbH and Co. OHG, Austria) with a nine-MHz linear array transducer (6-12 MHz). The ultrasound probe was placed at the abdominal aorta, 1.5 cm below the renal artery (Figure 1A). A longitudinal axis view of the abdominal aortic artery was obtained from the healthy and atherosclerotic groups (Figure 1B). The transducer was placed with the least possible pressure and allowed the expansion of the abdominal aortic artery in all directions. Ultrasonic examination of the abdominal aortic artery was...
performed after at least thirty minutes of rest in the supine position and when the heart rate and blood pressure had reached a steady state. A data acquisition system was used for monitoring and grabbing the changes in the arterial wall at a rate of forty-six frames per second. Sequential images of B-mode ultrasound were acquired at baseline and drug infusions (acetylcholine and nitroglycerine). The animals received acetylcholine drug at a rate of 0.5 µg/kg/min for 15 seconds. Blood pressure was returned to baseline for at least five minutes after the end of drug infusion. Then, nitroglycerine infusion was started at a rate of 5 µg/kg/min for 15 seconds. During the acetylcholine and nitroglycerine infusions, ultrasound images were recorded. In off-line analysis, instantaneous changes in the arterial diameter at baseline and during acetylcholine or nitroglycerine infusion were obtained, and the endothelial function in the normal and atherosclerotic rabbits was estimated.

The image size was 512 × 440 pixels. All the images were exported to MATLAB for image processing in MATLAB software version 7.0.1 (Mat Software Co., Mathworks, USA). Pixel size in digitized B-mode ultrasound image was 0.06 × 0.06 mm. For off-line analysis, a computerized analyzing method, which was presented in our previous study, was employed for detecting the instantaneous changes in the far and near walls in conjunction with the lumen diameter of the artery in sequential B-mode ultrasound images. Briefly, this method is based on the maximum gradient algorithm, and some characteristics of the dynamic programming algorithm were added for our applications. In this method, cost function and reference points were based on the maximum gradient and dynamic programming algorithm, respectively. Application of the algorithm to all of the images generated a sequence of artery diameters throughout three cardiac cycles.

In order to evaluate endothelial function, acetylcholine was used as an endothelial-dependent vasodilator. AMD was calculated as the ratio of the abdominal aorta diameter after acetylcholine infusion to the baseline diameter (expressed as percent change). Also, NMD was calculated in the same manner. AMD and NMD were calculated as follows:

1) \[ AMD\% = \frac{D_{\text{Max-ACh}} - D_{\text{Mean-Base}}}{D_{\text{Mean-Base}}} \times 100 \]

2) \[ NMD\% = \frac{D_{\text{Max-NG}} - D_{\text{Mean-Base}}}{D_{\text{Mean-Base}}} \times 100 \]

Where \( D_{\text{Max-ACh}} \) is the maximum systolic diameter after acetylcholine infusion, \( D_{\text{Max-NG}} \) is the maximum systolic diameter after nitroglycerin infusion, and \( D_{\text{Mean-Base}} \) is the mean diameter of the rabbits’ abdominal aorta over three cardiac cycles at baseline.

Blood samples were taken via cardiac puncture after six weeks, and total cholesterol, triglycerides, low-density lipoprotein cholesterol (LDL), and high-density lipoprotein cholesterol (HDL) levels were measured with a commercially available spectrophotometric assay kits to compare between the two groups.

In order to confirm the ultrasonic results, the histopathological method was used for detecting atherosclerotic lesions. Consequently, after the end of the experiment procedures, all the animals were sacrificed with a lethal dose of intravenous pentobarbital sodium for histopathological analysis. The abdominal aortic artery was quickly removed from the sacrificed rabbits and washed with phosphate-buffered saline. The segments (approximately 0.5 cm in length) of the abdominal aorta were then excised and cut into three equal-sized pieces. Some specimens were opened longitudinally and mounted flat with the intimal side facing up on the slide. The arteries were fixed in 10% buffered formalin and embedded in paraffin. To characterize
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the general architecture of the lesions, four-μm thick cross-sections were cut and mounted on glass slides (twelve sections in a lamella), and thereafter aortic rings were stained with hematoxylin and eosin for light microscopy (Olympus, BX51, Tokyo, Japan, magnification × 200). Digital image acquisition using digital image processing software (Image Tools Microsoft, San Antonio, Texas) was subsequently employed. The intima-media thicknesses of the abdominal aorta in the normal and atherosclerotic rabbits were measured.

All the values are presented as mean ± SD. Comparisons of the data obtained from the normal and atherosclerotic groups were performed using the independent Sample t-test and 95% confidence interval (CI). A p value < 0.05 was considered statistically significant. All the statistical analyses were performed using the SPSS software package (SPSS V. 11.5, Inc. Chicago, IL, USA). Reproducibility of each experiment was reported as coefficient of the variance percent (CV).

Results

The automated algorithm was run for measuring the instantaneous changes in the far and near walls and also the lumen diameters of the abdominal aorta in the normal and atherosclerotic rabbits at baseline and during acetylcholine or nitroglycerine infusion. Examples of the instantaneous changes in the arterial diameter at baseline and during acetylcholine or nitroglycerine infusion in the normal and atherosclerotic rabbits are shown in Figures 2 (A-F).

The demographic measurements of the rabbits are depicted in Table 1 as mean ± SD. The rabbits had a normal cardiac function and were comparable in terms of weight, heart rate, systolic and diastolic blood pressures, mean blood pressure, and pulse pressure.

There were no differences in systolic and diastolic blood pressures, mean blood pressure, pulse pressure, heart rate, and weight between the normal and the cholesterol-fed rabbits (p value > 0.05).

Total cholesterol, triglycerides, LDL, and HDL levels were remarkably increased in the serum of the four-week cholesterol-fed rabbits. Significant differences in total cholesterol, triglycerides, LDL, and HDL levels were seen between the normal and the four-week cholesterol-fed rabbits (Table 2).

There were no visible intimal lesions in the aortas of the control group in the microscopic studies. Internal and external elastic laminae could be identified; and as expected, no smooth muscle cells were observed in the intima.
The intima-media thickness was 0.16 ± 0.00 mm (Figure 3A). Intimal thickening with fatty deposition was observed in all the hematoxylin-eosin stained specimens of the aortas of the four-week cholesterol-fed rabbits (Figure 3B). After four weeks, elastic laminae could not be identified and macrophage and lipid accumulation within the disorganized elastic laminae caused an increase in intimal thickness. The intima-media thickness was 0.21 ± 0.02 mm, which became comparable with the control values. In the atherosclerotic group, the intima-media thickness was significantly higher than that of the normal artery (p value < 0.05). In the normal arteries, the media consisted of multiple layers of circularly oriented smooth muscle cells.

Table 1. Demographic measurements in the normal group and the four-week cholesterol-fed group (atherosclerotic group)*

| Variables                        | Normal group | Atherosclerotic group | P value |
|----------------------------------|--------------|-----------------------|---------|
| Systolic blood pressure (mm Hg)  | 123.1±8.3    | 129.1±7.0             | 0.123   |
| Diastolic blood pressure (mm Hg) | 80.2±7.4     | 77.2±6.1              | 0.562   |
| Mean blood pressure (mm Hg)      | 93.1±7.0     | 95.3±7.0              | 0.687   |
| Pulse pressure (mm Hg)           | 43.0±5.1     | 49.2±5.1              | 0.060   |
| Heart rate (min⁻¹)               | 141.0±29.0   | 134.0±43.0            | 0.725   |
| Weight (g)                       | 2610.0±204.0 | 2754.3±239.2          | 0.755   |

*Data are presented as mean±SD

No differences in the mean abdominal aorta diameter were observed at rest status and during acetylcholine (Ach) or nitroglycerine (NG) infusion for the normal artery. Significant vasodilation effects were detected after acetylcholine administration in the control group and the four-week cholesterol-fed group (p value < 0.05). AMD was 18.49 ± 1.62 and 10.20 ± 1.77 percent in the normal group and the four-week cholesterol-fed group, respectively (Figure 4). Similar vasodilation effects were observed by the infusion of nitroglycerine in the two groups. NMD was 15.54 ± 2.35 percent and 14.64 ± 1.54 percent in the control group and the four-week cholesterol-fed group, respectively (Figure 4). There was no significant vasodilation effect after the infusion of nitroglycerine in the control group and the four-week cholesterol-fed group (p value > 0.05).

Table 2. Serum parameters changes in the normal group and the four-week cholesterol-fed group*.

| Concentration (mg/dl) | Normal group | Atherosclerotic group | P value |
|-----------------------|--------------|-----------------------|---------|
| TCH                   | 54.8±5.9     | 1544.3±82.5           | < 0.001 |
| TG                    | 33.3±3.8     | 270.8±35.2            | < 0.001 |
| HDL                   | 25.0±3.3     | 40.7±5.3              | 0.006   |
| LDL                   | 30.5±3.1     | 814.7±104.6           | < 0.001 |

*Data are presented as mean±SD

TCH, Total cholesterol; TG, Triglyceride; HDL, High-density lipoprotein cholesterol; LDL, Low-density lipoprotein cholesterol

Instantaneous changes in the abdominal aorta diameter at baseline and during acetylcholine or nitroglycerine infusion were obtained, using sequential B-mode images. Peak systolic, end diastolic, and mean diameters of the abdominal aortic artery at baseline and during acetylcholine or nitroglycerine infusion are presented in Table 3. No significant differences were shown for these parameters between the normal and the four-week cholesterol-fed rabbits.
Figure 3. Hematoxylin-eosin staining of transversal sections of the rabbit abdominal aorta in: A) the normal group and B) in the four-week cholesterol-fed rabbit group. The arrow shows thickening with fatty deposition.

Table 3. Peak systolic and end diastolic diameters (mm) in the abdominal aorta for the normal group and the four-week cholesterol-fed rabbits (atherosclerotic group) at rest status and during acetylcholine (Ach) or nitroglycerine (NG) infusion, extracted by the automated method

| Diameter (mm) | Status   | Normal group (mm) | Atherosclerotic group (mm) | P value |
|--------------|----------|-------------------|---------------------------|---------|
| Peak systolic| Rest     | 3.1±0.2           | 3.0±0.1                   | 0.815   |
|              | Ach      | 3.3±0.2           | 3.1±0.1                   | 0.088   |
|              | NG       | 3.2±0.2           | 3.2±0.2                   | 0.884   |
| End diastolic| Rest     | 2.6±0.1           | 2.6±0.1                   | 0.900   |
|              | Ach      | 2.8±0.2           | 2.7±0.1                   | 0.233   |
|              | NG       | 2.8±0.2           | 2.8±0.1                   | 0.990   |
| Mean         | Rest     | 2.8±0.2           | 2.8±0.1                   | 0.962   |
|              | Ach      | 3.0±0.2           | 2.8±0.1                   | 0.077   |
|              | NG       | 3.0±0.2           | 3.0±0.2                   | 0.882   |

*Data are presented as mean±SD

Discussion

Vascular endothelium-dependent vasodilation is impaired by hypercholesterolemia both in human and animal models. Experimental and epidemiological data have demonstrated that a high cholesterol diet is highly related to the development of hypercholesterolemia. Loss of endothelium-derived relaxing factor is the earliest alteration to endothelium after the start of a high cholesterol diet. Hypercholesterolemia impairs vascular endothelial function by reducing nitric oxide. Endothelial dysfunction, which is measured by AMD or flow-mediated vasodilation, is the most important early event in atherosclerosis. Several studies have demonstrated that the endothelial dysfunction of peripheral arteries is a useful predictor for coronary artery disease and other cardiovascular risk factors. Therefore, a non-invasive, safe, accurate, and reproducible method is required for evaluating the endothelial function.

It seems feasible that AMD could be drawn upon as an index of the endothelial function under physiologically-induced shear stress. Whether or not this method can detect changes in the endothelial function, however, has not been examined. Non-invasive measurement of flow-mediated vasodilation using B-mode ultrasound has been applied in human cardiovascular research. Moreover, methodological research must be focused on establishing extraction protocols of images to measure the endothelial function. Thus, in this study, AMD and NMD of the abdominal aorta in normal and atherosclerotic rabbits were estimated and compared using sequential ultrasound images as the automated method. The instantaneous changes in the lumen diameter under chemical stimulus could be useful for evaluating the endothelial function.

In animal studies, the classical method of ex vivo organ bath has been widely used for evaluating endothelial function. Be that as it may, the results obtained from ex vivo...
studies could not be directly applied to in vivo studies. Some stimulations such as shear stress have a useful effect on the release of endothelium-dependent vasodilation in arteries in vivo. In addition, it has been noted that in ex vivo studies, arteries are preconstricted before the evaluation of relaxation caused by several vasodilators. Invasive assessment of the arterial endothelial function by intravascular ultrasound is widely used. Intravascular ultrasound has been employed in human as well as in animal models and yields an accurate assessment of the arterial function and morphology; it has, nonetheless, been restricted to invasive applications.

We used non-invasive B-mode ultrasound for evaluating the endothelial function in normal and atherosclerotic rabbits. The automatic analyzing method was drawn upon for off-line analysis of sequential B-mode ultrasound images. Our previous study showed that this method can automatically detect instantaneous changes in the arterial wall without any manual correction. Our method, however, has some limitations due to the difficulty in obtaining artifact-free, high-quality ultrasound images of both far and near vessel walls. Moreover, movement of the probe and respiratory movement during image acquisition may cause artifacts, which should be excluded from analysis.

In this study, significant endothelial dysfunction was seen in the rabbits given a high cholesterol diet for four weeks. Jayakody et al. showed that the AMD of aortic rings in rabbits fed a 2% cholesterol-rich diet was significantly impaired as early as four weeks after the start of a high cholesterol diet. Also Furchgott et al. demonstrated that the AMD of the isolated aortic rings in rabbits depended on the endothelial function. The authors also reported that, without endothelium, they only detected vasoconstricting effect.

Many studies have reported that AMD is impaired in atherosclerotic arteries with histological lesions in cholesterol-fed animal model or coronary arteries in human in vitro. Our study showed intima thickening in aortas in the four-week cholesterol-fed rabbits, whereas no microscopic visible intimal lesions were detected in the normal rabbits. Some animal studies have suggested that high plasma cholesterol alone might decrease endothelium-dependent vasodilation. In contrast, another research group demonstrated that hypercholesterolemia alone did not impair the endothelial function in the aorta of cholesterol-fed rabbits. The group showed that endothelial dysfunction was detected only in arteries with intimal lesions. Our data showed endothelial dysfunction in the arteries with histological changes in the intima in the cholesterol-fed rabbits.

**Conclusion**

In this study, we used a computerized analyzing method for evaluating endothelium-dependent and independent relaxation in sequential ultrasound images of hypercholesterolemic rabbits. Our method offers the option of evaluating the endothelial function repeatedly and accurately and enables us to investigate functional and structural alterations during the progression of atherosclerosis. We can apply these results for human arteries and thus evaluate arterial walls during the progression of atherosclerosis, especially in initial symptoms.

**Acknowledgment**

This study was approved and funded by Tarbiat Modares University.

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