Original Article

Developing a Rabbit Model of Neointimal Stenosis and Atherosclerotic Fibrous Plaque Rupture

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Abstract

Background: A precise understanding of the mechanism of human neointimal stenoses and atherosclerotic fibrous plaques, which give rise to thromboses in vital arteries, requires a suitable animal model that would mimic the same characteristics well. We developed a rabbit model of neointimal stenosis and fibrotic plaque rupture in the carotid artery to visualize the lesion progress and to characterize the lesion types according to the American Heart Association classification.

Methods: Twenty-eight healthy male New Zealand white rabbits were randomly divided into two groups: The rabbits in group A (n = 14) consumed a standard chow diet, and those in group B (n = 14) were injured via perivascular cold injury using liquid nitrogen at the right common carotid artery before being fed a high cholesterol diet (1.5%) for eight weeks. Plasma lipid evaluation was performed before the sacrificing of the rabbits. At the end of every week, at least 1 rabbit from group B was sacrificed for an analysis of lesion histopathology and calculation of the area ratios of the intima to media.

Results: The plasma lipid level in group B was significantly higher than that in group A (p value < 0.05). The histopathological results revealed atherosclerosis characteristics such as endothelial layer destruction, fatty streaks and lipid-containing macrophages (foam cells) formation in the intima and media layers, extracellular lipid collections, smooth muscle cells proliferation and migration, neointima formation, intima thickening and deformation, fibrotic plaque formation, and finally plaque rupture. Statistical analysis revealed a significant increase in the intima-to-media ratio at the end of the eighth week (6.41 ± 0.27 , p value < 0.05).

Conclusion: We successfully developed a rabbit model of neointimal stenosis and atherosclerotic fibrous connective tissue plaque rupture, which is not only quickly and easily reproducible and inexpensive but also without mortality. The merits of our model render the evaluation of neointimal stenoses and fibrotic plaques and their treatment strategies more feasible in humans.

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Keywords: Atherosclerosis • Carotid arteries • Plaque, atherosclerotic • Rabbits • Animals

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Introduction

Atherosclerosis, the leading cause of mortality and morbidity in modern societies, is a disease of the vessel wall that occurs in coronary arteries, carotid arteries, aortas, and other peripheral arteries.¹ Atherosclerosis is a progressive disease process of large arteries and involves a gradual accumulation of lipids, inflammatory cells, neointima formation, and fibrous elements in plaques in vessel walls. The pathogenesis appears to be far more complex than a single causative element, but is instead a multifactorial process that involves environmental, dietary, genetic, hemodynamic, and metabolic factors in its development.²

Atherosclerosis has been classified by the American Heart Association (AHA). In brief, Type 0: No intimal thickening, Type I: Initial lesion; not grossly apparent plaque, isolated macrophages contain oxidized lipid droplets (foam cells), Type II: Fatty streak; lesion grossly apparent with Sudan III staining, foam cells and smooth muscle cells contain lipid droplets, Type III: Preatheroma; raised fatty streak in gross morphology, multiple but small extracellular lipidic cores, foam cells contain lipid droplets, increasing number of smooth muscle cells, Type IV: Atheroma; single and massive extracellular lipid pool (lipid core), grossly visible, well delimited, covered by a proteoglycan-rich layer infiltrated with foam cells and smooth muscle cells with and without lipid droplet inclusion, Type Va: Fibroatheroma; Type IV with a cap rich in fibrosis (collagen), possible small calcifications, Type Vb: Calcified plaque; lesion with a lipid core or fibrotic tissue, with large calcifications, Type Vc: Fibrotic plaque; fibrous connective tissue, no lipid core, Type VIb: Wide hemorrhage, and Type VIc: Thrombosis.³

Atherosclerotic lesions are advanced when the accumulation of lipid, cells, and matrix is associated with the disorganization, repair, and thickening of the intima and the deformity of the arterial wall. Type IV lesions, also called atheromas, are advanced lesions in which extracellular lipid occupies a well-defined and extensive area in the intima, called the lipid core, which develops through the coalescence of smaller isolated pools of extracellular lipid.⁴ Evaluation of the atherosclerotic fibrous plaque is difficult directly in humans. In addition, direct human research is limited by the inability to control experiments and by the slowness of lesion development. An appropriate animal model could, therefore, prove critical to the research and development of new diagnostic and therapeutic modalities.⁵

Almost one hundred years ago, in 1908, Ignatowski discovered that diets of milk, meat, and eggs resulted in atherosclerotic lesions in the rabbit. In 1913, Anitschkow and Chalatow demonstrated that the atherogenic component of the diet was cholesterol. Their pioneering experiment, in which dietary cholesterol was used to induce hyperlipidemia that resulted in atherosclerotic lesions, established the rabbit as an experimental model of atherosclerosis that is still used today.⁶

Several characteristics of the rabbit make it an excellent model for the assessment of the effects of human atherosclerosis susceptibility: a) Rabbit apolipoprotein B (apoB), which contains lipoproteins, is similar to that of humans in its chemical composition and apoprotein content; b) rabbit liver does not edit apoB mRNA and, thus, produces apoB-100, which contains very low-density lipoproteins as does the human liver; c) cholesteryl ester transfer protein, which plays a central role in the atherosclerotic process, is abundant in both human and rabbit plasma; and d) rabbits are very susceptible to diet-induced atherosclerosis.⁷ In rabbits, lesion morphology is altered by the percentage of cholesterol added to the diet and the duration of the diet. Diets that are short in duration and with a percentage of cholesterol of more than 1% cause hypercholesterolemia, and atherosclerotic lesions rich in foam cells originate from macrophages.^{8,9}

Several models of arterial injury have been used to induce short-time atherosclerosis formation in rabbits in combination with high cholesterol diets. These can be classified in models that use an intravascular approach such as balloon catheters,¹⁰⁻¹⁴ wires, photochemicals, hypotonic solutions, and air¹⁵ or models that employ a perivascular approach such as electric currents,^{16, 17} endotoxin, hot temperature or a collar,¹⁸ cold gas injury,¹⁹⁻²⁰ and ligation.²¹

Neointimal stenoses and atherosclerotic fibrotic plaques and concomitant ruptures via perivascular liquid nitrogen (-196 °C) injury have not been established in an animal model yet. In this study, we aimed to characterize the different types of lesions and their respective formation duration histopathology in accordance with the AHA classifications.

Methods

A total of 28 healthy male New Zealand white rabbits (2.0-3.0 kg body weight) were recruited in this study. The animals were treated in keeping with the Guide to the Care and Use of Experimental Animals published by the American Council and also Animal Care and Protection Committee of Tarbiat Modares University. The animals were housed individually in cages and randomly assigned to two groups: Group A (n = 14), in which normal rabbits consumed a standard chow diet, and group B (n = 14), in which the rabbits were injured at the right common carotid artery via perivascular cold injury using liquid nitrogen (-196 °C) before being fed an atherogenic diet (chow supplemented with 1.5% cholesterol) for eight weeks. For cold injury, the rabbits were anesthetized with an intramuscular injection of xylazine (5 mg/kg) and ketamine (35 mg/kg) and were subsequently placed in dorsal recumbency on a surgery table so as for their necks to be shaved. A midline skin incision was thereafter made, and the right common carotid artery was surgically exposed and dissected from the surrounding tissues. The common carotid artery was injured perivascularly 1cm below the bifurcation by dripping 0.5 ml liquid nitrogen. For this purpose, a one-cm segment was isolated with two artery clamps, and a one-ml syringe needle was immediately inserted into the segment. Blood was rinsed from the segment with phosphate-buffered saline, and the segment was evacuated completely in order that the needle could be withdrawn from the artery (Figure 1).





Figure 1. Surgical exposure of the common carotid artery (A), the rinsing of the blood from the segment with phosphate-buffered saline (B), edema progression a few minutes after perivascular liquid nitrogen cold injury (C), closure of the surgical incision (D)

Liquid nitrogen (about 0.5 ml) was dripped as quickly as possible on the artery through an especial probe, and it acted on the basis of physical pressure and thermal laws. Immediately after liquid nitrogen injury, a bright zone was seen. A few minutes later, edema progressed and initiated an inflammation response in the artery. The segment was then rinsed with phosphate-buffered saline, and the artery clamps were loosened from the artery. Circulation was established, with the injury area looking dark at this point. In the next step, antibiotic powder containing sulfathiazole and neomycin was applied to the surgery position before the closure of the surgical incision. Postoperatively, the animals were allowed to recover and conventional antibiotics were required to prevent infection. The injured rabbits consumed a high fat diet (1.5% cholesterol) for eight weeks. Plasma samples were drawn from the heart of group B rabbits every week and were collected to measure total cholesterol (TC), triglyceride (TG), low-density lipoprotein (LDL) cholesterol, and high-density lipoprotein (HDL) cholesterol for a comparison with group A. All the rabbits survived until the time of sacrificing, before which they were all weighed to measure weight changes in the two groups every week. The animals were sacrificed using an overdose of intravenous pentobarbital sodium for histopathology. In group B, 1 rabbit was sacrificed at the end of every week for seven weeks and then 7 were sacrificed at the end of the eighth week. The right common carotid arteries (1 cm in length) were quickly removed from the sacrificed rabbits and washed with phosphate-buffered saline. The specimens were afterward cut into pieces; some specimens were opened longitudinally and mounted flat with the endothelial side up on the slides. The pieces were fixed in 10% buffered formalin and embedded in paraffin. The general architecture

B

of the lesions was characterized by cutting serial 4- μ m thick cross sections and mounting them on glass slides before staining with hematoxylin and eosin for light microscopy. (Olympus, BX51, Japan, Tokyo, magnification × 200). In the histopathological samples, the area ratios of the intima to media were measured with Image Tools software (Microsoft, San Antonio, Texas). Histopathologically, the intima-tomedia ratio results in the injured group (B) were compared with those obtained from the uninjured rabbits (A).

All the values are presented as mean \pm SD, and a p value < 0.05 is considered statistically significant. All of the statistical analyses were performed using the SPSS software package (SPSS V. 13.5, Inc. Chicago, IL, USA).

Results

The weight of the animals was monitored on a weekly basis to ensure that no complications developed at the surgical site. The body weight of the rabbits of group A at baseline was 2268 ± 106 g, which increased to 2504 ± 147 g at the end of the eighth week. In group A, an increase in the mean weight was seen each week during the eight-week period, whereas group B had a drop in the first week (from 2221 ± 108 g to 2094 ± 860 g), followed by weight gain during the next seven weeks (from 2094 ± 860 g to 2860 ± 268 g) (Figure 2).



Figure 2. The mean and standard error of weight changes for group A (without cholesterol) and group B (surgery and cholesterol feeding) during eight weeks of regimen

After the eighth week, the results of the biochemical analysis of the atherogenic diet group (B), in comparison with those of the normal diet group (A), showed a highly significant increase in TC, LDL, and TG (from 74 ± 22 to 1018 ± 221 , from 34 ± 21 to 936 ± 180 , and from 58 ± 19 to 148 ± 27 mg/dl, respectively; p value < 0.05) and modified

changes in HDL cholesterol (from 21 ± 9 to 32 ± 7 mg/dl) (Figure 3).



Figure 3. Serum parameters changes in group A and group B after eight weeks LDL, Low-density lipoprotein cholesterol; HDL, High-density lipoprotein cholesterol; TG, Triglyceride; TC, Total cholesterol

In the uninjured arteries (group A), the adventitia, multiple layers of the circularly-oriented smooth muscle cells (media), intima, and endothelial cells were clearly visible. The internal and external elastic laminae could be identified; and as expected, no smooth muscle cells were observed in the intima. The intima-to-media ratio was 0.096 ± 0.00 (Figure 4A).

The damage to the endothelium, media, and adventitia with liquid nitrogen and cholesterol-rich diet regimen gave rise to the disorganization of the cells across the adventitia and endothelium after one week. The elastic laminae could not be identified, and the media was of dishomogeneous thickness, characterized by the penetration of lipoproteins into the smooth muscle cells. The predominant cell type in the media and intima was the lipid-containing smooth muscle cells (fatty streaks) and lipid containing macrophages (foam cells). What was observed, therefore, was advanced atherosclerotic lesion, accompanied by type I and II, based on the AHA classifications within one week after surgery.^{3, 4} The intima-to-media ratio was 0.19 ± 0.01 .

One week after liquid nitrogen damage, by comparison with the uninjured carotids, a substantial reduction in medial thickness and increase in the intima was observed, which was mostly due to the diffuse macrophages' penetration into the intima (Figure 4B).

After the second week, a rearrangement of the smooth muscle cells in the arterial lesion was observed. In fact, although the macrophages still populated the medial layer, the medial lipid accumulation had an increase by comparison with earlier time points, which was followed by a drop (0.15 \pm 0.01) in the intima-to-media ratio (Figure 4C).





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Figure 4. Hematoxylin-eosin staining of transversal (A, B, C, D, E) and longitudinal (F, G, H) sections of rabbit common carotid arteries in uninjured group (A) and in injured group (B, C, D, E, F, G, H) from week one to week eight

After three weeks, the macrophage accumulation within the disorganized elastic laminae caused an increase in intimal thickness and the intima-to-media ratio (0.35 ± 0.02) , which became comparable with the control values (Figure 4D). In some neointimal areas, a massive lipid was visible, apparently constituted by both intracellular and extracellular lipids. The histopathological analysis of the sections showed that diffuse and cushion neointima stenoses were formed by a mixed population of smooth muscle cells and macrophages in some areas. The intima-to-media ratio was 0.48 ± 0.03 (Figure 4E).

By the fifth week, the macrophages of the media had been mostly replaced by smooth muscle cells and collagen, which led to fibrotic plaque stenosis and a rise in the intima-tomedia ratio (0.70 ± 0.04) (Figure 4F).

Another interesting feature of the lesion progression over time was a remodeling of the lesion shape. The neointimal formation in the fourth week distributed in a diffuse fashion up to seven weeks into an initial fibrotic plaque (V_c) (Figure 4g). Finally, at the end of the eight-week period, plaque rupture and concomitant thrombosis was seen with an intima-to-media ratio of 6.40 ± 0.27 (Figure 4H).

At each analyzed time interval, the intima-to-media ratio was higher in the injured carotids than in the uninjured ones, with the exception of week one. Specifically, the thickness within the internal elastic laminae and fibrous cap (neointima area) increased progressively between weeks five and eight after injury (Figure 5). In the uninjured arteries, the media consisted of multiple layers of circularly-oriented smooth muscle cells, shown below as week zero.



Figure 5. The intima-to-media ratios in injured carotid group during an eight-week period following injury and a high fat diet (1.5% cholesterol). In week zero, carotid arteries were healthy

Discussion

In our rabbits, injury to carotid arteries with liquid nitrogen (-196 °C), followed by a cholesterol rich diet, induced neointimal stenosis and atherosclerotic fibrous plaque rupture; this can be characterized by lipid-containing macrophages (foam cells) and lipid-containing smooth muscle cells (fatty streaks) in the media and intima, intima thickening by fibrous tissue proliferation and smooth muscle cells migration, media attenuation and arterial wall remodeling, neointima formation, small lipid vacuoles and extracellular lipid droplets in media and neointima, and soft and fibrous plaque formation. Also, the plasma lipid level in group B (injured arteries) was significantly higher than that in group A (control group), and the intima-to-media ratio increased from 0.09 ± 0.00 at week zero (group B) to 6.40 ± 0.27 at week eight. All of these features indicate that the neointimal stenoses and plaque ruptures induced in this present study are similar to those observed in patients.^{3, 4}

The mechanisms of atherosclerosis include endothelial dysfunction, lipid accumulation, and enhanced inflammatory involvement. Convincing evidence has emerged over recent years to support the theory that endothelial injury is the initiation of the arteriosclerosis process. Under normal conditions, the endothelium regulates vascular homeostasis through a variety of factors, including nitric oxide (NO), prostacyclin, and endothelin, which act locally in the vascular wall and lumen. Endothelial dysfunction contributes to enhanced vasoconstrictor responses, adhesion of platelets and monocytes, foam-cells formation, and subsequently the proliferation and migration of vascular smooth muscle cells, which makes fatty streaks as well as fibrous and soft plaque formation.

A large body of evidence suggests that high-plasma cholesterol concentrations, especially of LDL cholesterol, result in atherosclerotic lesion formation. Oxidized LDL particles are a potent atherogenic agent. Inflammation is thought to be the link between hyperlipidemia and atherosclerosis.²¹⁻²³ Evaluation of atherosclerosis by its nature is difficult directly in humans. As a result, animal models offer an alternative, wherein the physiopathology aspects of atherosclerosis could be simulated, variables could be controlled, and statistical data could be obtained in a short period of time.⁵ In our model, cold injury induced a modified injury, destroying some cells across the vessel wall, including the smooth-muscle cells in the media, endothelial cells in the intima, and fibroblasts in the adventitia. Furthermore, cold liquid nitrogen damages the vasa vasorums in the adventitial layers, preventing the supply of nutrients and oxygen to the adventitia and media.24,25 This injury causes transient plateletand fibrin-rich thrombosis, and matrix debris by infiltrating the leukocytes in the intima, media, and adventitia during the first week after injury. Because of the complete cell necrosis across the injury, the healing of the vascular wounds initiates from the adjacent uninjured borders. Intimal thickening or neointima formation in response to vascular injury results from the excessive accumulation of smooth muscle cells and deposition of extracellular matrix in the intimal layer of the vessel wall. Diffuse intimal thickening commonly occurs in the arteries of humans and increases progressively throughout life. Eccentric intimal thickenings (intimal cushions), associated with branches and orifices, have been observed in human arteries from the first week of life.²⁶⁻³⁰ Both the diffuse intimal thickenings and the intimal cushions are considered to be susceptible sites for atherosclerosis. The growth of the intima of large blood vessels is a key early event in the development of atherosclerosis and regarded as "the soil" essential for lesion development and growth. Excessive neointima growth is also a major cause

of restenosis following percutaneous transluminal coronary angioplasty, significantly narrowing the vessel lumen at the injury site.³⁰

Cholesterol injury leads to atherosclerotic lesion formation in neointima areas because of primary endothelium injury.²⁹ Healthy endothelium releases nitric oxide, a gas that keeps the arterial wall healthy but that is rapidly inactivated by blood. According to the response-to-injury hypothesis of atherosclerosis, in response to cold injury and following exposure to atherogenic stimuli, endothelial cells express adhesion molecules and elaborate growth factors, which lead to the recruitment of leukocytes in an inflammatory response to injury. Leukocytes adhere and migrate into the vessel wall, localize subendothelially, and develop into lipid-laden macrophages (foam cells). Foam cells, in turn, release growth factors and cytokines, which promote the recruitment of smooth muscle cells and stimulate neointimal proliferation, continue to accumulate lipid, and support endothelial cell dysfunction. Collectively, these events promote the development of a lipid-rich atheromatous lesion. Subsequent denudation of the endothelium exposes circulating platelets and coagulants to the underlying matrix, thereby initiating thrombosis and triggering a cascade of events giving rise to a fibro-proliferative lesion and luminal narrowing.^{3, 21-23}

As smooth muscle cells secrete extracellular matrix proteins, fatty streaks may progress to fibrous plaques, contributing to the narrowing of the lumen. Advanced lesions occur with the rupture of the plaque, which allows blood components to come into contact with plaque lipids and tissue factor, resulting in the formation of a thrombus. If a thrombus is occlusive, it will lead to myocardial infarction and ischemic stroke.⁴

The tissue characteristics in our group B rabbits after four weeks were very similar to neointimal hyperplasia formation in restenosis in stent implantation. The use of stent implantation is one of the current procedures for enlarging neointimal and fibrous plaque lumen. Instent restenosis, however, remains a pivotal limitation after coronary and peripheral stenting. Both neointimal hyperplasia and matrix production, which are initiated by activated smooth muscle cells in response to vessel wall injury, have been shown to play an important role in restenosis. Restenosis is considered a local biological response to catheter-induced injury in routine stenting. Neointima is basically an accumulation of smooth muscle cells within a proteoglycan matrix that narrows the previously enlarged lumen. Coronary stents provide mechanical scaffolding that virtually eliminates recoil and remodeling. Nonetheless, stents do not reduce neointimal growth. Recently, sonotherapy has been employed to decrease smooth muscle cell proliferation and neointimal formation.²⁶⁻²⁸ Also diffuse intimal thickening commonly occurs in the arteries of humans and increases progressively throughout life. Both diffuse intimal thickenings and intimal cushions were seen in our surgery group after four weeks.

Additionally, at the seventh and eighth postoperative weeks, fibrous plaque formation and concomitant rupture could be observed, which created thromboses in the rabbits in the same manner as the process in humans.⁴

Conclusion

We successfully developed a rabbit model of atherosclerosis formation caused by perivascular cold-induced artery injury in high-cholesterol fed rabbits. We observed lipid-containing macrophages (foam cells), extracellular lipid collections, neointima formation, and fibrous plaque in the surgery group in the pathology evaluation. The serum lipid level in the injured rabbits was significantly higher than that in the control group. All of these features indicate that the atherosclerosis induced in the present study is similar to that observed in patients with coronary heart disease.⁴

Balloon and electrically-induced endothelial injury rabbit models are very labor intensive and expensive, and animals frequently require over eight weeks to develop significant lesions. Also in these models, endothelial injury is difficult and only the initial stages of atherosclerosis or soft plaques can be produced.^{16, 17} By comparison, not only is our model inexpensive and quickly and simply reproducible, but also it enables us to observe the initial, middle, and last stages of atherosclerosis (fibrous plaque and its rupture). Furthermore, neointima formation, which is responsible for stenosis before and after stenting, formed within four weeks in our model.

In light of the results of the present study, it can be concluded that our simple technique on New Zealand rabbits can provide a model of induced neointima stenosis and fibrous plaque rupture with a high degree of morphological similarity between the animal model and the human. The strong points of our model can facilitate the evaluation of neointimal stenoses and fibrotic plaques in humans and the development of treatment modalities such as sonotherapy, balloon angioplasty, and stenting in the prevention of neointimal thickening, atherosclerotic stenosis, plaque rupture, and concomitant thrombosis.

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