Establishment of Rabbit Abdominal Aortic Atherosclerosis Model by Pancreatic Elastase Infiltration Associated with High Fat Diet

Song-Nian Liang, Ke Xu and Hong-Shan Zhong

Background: The aim of this study was to evaluate the feasibility of a high fat diet (HFD) associated with pancreatic elastase (PE) infiltration, in establishing the rabbit aortic atherosclerosis model.

Methods: The HFD+PE method and the HFD+saccule injury (SI) method were simultaneously used to prepare the rabbit atherosclerosis model; the control group was established with the normal diet. Biochemical indicators, radiological imaging, pathomorphology and immunohistochemistry were used to evaluate the HFD+PE modeling results.

Results: There were significant changes in the blood lipid contents, as well as the pathomorphological and immunohistochemical results between the two experimental groups and the control group (p < 0.05). However, there was no difference between the two experimental groups. The rabbit aortic atherosclerosis model prepared by the HFD+PE method had no significant difference in the local vascular pathomorphological and immunohistochemical results with the traditional HFD+SI method.

Conclusions: The use of HFD with PE infiltration is feasible in establishing the rabbit aortic atherosclerosis model.

Key Words: Animal model • Atherosclerosis • Rabbit

INTRODUCTION

Cardiovascular disease has become the number one eradicator of human life and substantial health challenge around the world. Studies have also found that atherosclerosis (AS) is the main pathological basis of cardiovascular and cerebrovascular diseases, such as stroke and myocardial infarction. Therefore, the early diagnosis of AS would be conducive to the early diagnosis and treatment of cardiovascular and cerebrovascular diseases, which could be of great significance in an effort to help improve the lives of people. The characteristic manifestation of AS is the formation of atherosclerotic plaque, including core necrosis, calcification and appearance of inflammatory smooth muscle cells and foam cells. The characteristics of atherosclerotic plaques indicate that AS is a type of complex disease, including multiple factors such as vessels, metabolism and immune system.

Atherosclerotic plaque research is almost daily expanding and the animal model plays a very important role in this onward push forward. The establishment of an animal model depends on the focal accumulation of multiple cells, intracellular lipid, fibrous tissues and complex proteoglycans inside the arterial intima. The high fat diet (HFD) and mechanical intimal injury are the two most classic methods, in the former, modeling would take a long time, while the latter might be sub-
ject to the limitations of experimental equipment. Studies on a rabbit model of abdominal aortic aneurysm induced by papain infiltration with normal diet have resulted in a prospective outcome. They focused on the rules of changes in pathomorphology and immunohistochemistry of aneurysm, which were somewhat similar to that of AS and also accompanied by intimal injury. It was conceivable that based on the impaired intima, hyperlipidemia might increase the deposition of lipid, and promote intimal hyperplasia and plaque formation during the repairing process.

The aim of this experimental study was to evaluate the feasibility of a new method to establish rabbit aortic atherosclerosis model with HFD and pancreatic elastase (PE) infiltration.

**MATERIALS AND METHODS**

**Animals and grouping**

We used 20 healthy male New Zealand white rabbits purchased from the Laboratory Animal Center of China Medical University, each weighing 2 ± 0.2 kg and between 3-4 months old. Each rabbit was fed separately at the Key Laboratory of Imaging Diagnosis and Interventional Treatment of Liaoning Province, at a temperature of 22 ± 2 °C and a relative humidity of 40-60%. The 20 rabbits were randomly divided into three groups, the normal diet group (n = 4), the saccule injury (SI) group (n = 8), and the PE group (n = 8). The control group was given the normal diet, while the other two groups were given the HFD (1% cholesterol, 5% egg yolk powder, 5% lard and 89% normal diet). The intake of each rabbit was 50 g/(kg · d), all rabbits had free access to water and were weighed every 4 weeks. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the First Affiliated Hospital of China Medical University.

**Modeling methods**

For the HFD+PE group, after 4 weeks of ingesting an HFD diet, the rabbits were anesthetized with 3% pentobarbital (Sigma-Aldrich P3761, US, 30 mg/kg body weight, im) and xylazine hydrochloride (Jilinhuamu, Co., Ltd, Jilin, China, 0.5 ml/kg body weight, im). The rabbits were then injected with the visualization contrast agent (Iopromide, Bayer Xianling Pharmaceutical Co., Ltd, Guangdong, China) 10 ml via the ear vein to evaluate the aortic conditions. Thereafter, the abdomen was accessed by a midline laparotomy to expose and free the abdominal aorta, with a customary length of 2 cm. A curved plastic sheet was inserted into and beside the dorsal artery to isolate the surrounding tissues. Then, the sterile gauze was used to wrap the arterial surroundings, and 80 μl PE (Shanghaikayon Biological Technology Co., Ltd, Shanghai, China, 1 u/μl) was instilled into the gauze for 15-min infiltration. After the local aortic walls exhibited extension and hyperemia, we removed the plastic sheet and gauze, and then twice rinsed the local aorta with saline to remove the residual protease and thereafter closed the abdomen.

For the HFD+SI group, after 4 weeks of administration of HFD, the rabbits were subject to anesthesia and angiography with the same method as noted above. Then, we prepared the skin of the right inguinal area, incised and exposed the right femoral artery, and then punctured the right femoral artery with direct visual confirmation. Under the guidance of a microguiding wire, the 4F Fogarty balloon catheter (Baxter, Deerfield, Illinois, USA) was directed to the diaphragmatic level and inflated to 4 mm diameter by injected contrast agent. This uniformly dragged the balloon down to bifurcation of the aortal distal iliac artery. We repeated the above action three times, and then exited the balloon and ligated the right femoral artery. Ultimately, we were able to compare the visualization again.

**Biochemical indicators**

After the groupings were established, biochemical tests were performed on each group member at the 0, 4th, 8th and 12th week. From the 3 groups, 3 ml ear vein blood was taken each time, and centrifuged at 2000 g for 20 min to extract the serum, after which an automatic biochemical analyzer was used to determine the levels of total cholesterol (TC), triglyceride (TG), low density lipoprotein cholesterol (LDL-C) and high density lipoprotein cholesterol (HDL-C). Thereafter, we used the chemical enzyme method, relative to the occurrence and development of AS.
Radiographic evaluation

After grouping, angiography was performed on the 3 groups at the 4th, 8th and 12th week. Utilizing the aforementioned method of anesthesia, animals were fixed on the DSA equipment (ARCADIS Varic, Siemens, Muenchen, Germany) and injected with 5 ml of contrast agent via the ear vein. After allowing several seconds for circulation, the abdominal aorta was visualized under x-ray. For further evaluation of the lumen morphology and hemodynamics, on the 4th week, the SI group and the PE group underwent angiography before treatment for further comparison.

Histopathological and morphological analysis

Subsequent to 12 weeks of feeding, ear vein blood specimen samples were obtained from each animal, after which visualization of abdominal aorta after anesthesia was performed. The abdominal aortic lesion was freed and excised, and then fixed in 10% formalin solution for more than 48 hours. Then the segments were subjected to paraffin embedment, with a slice thickness of 6 μm used for HE and elastic Van Gieson (EVG) staining (Baso Diagnostics Inc., Zhuhai, China). All the slices were examined by way of an Olympus BX 40 Light Microscope (Olympus Corporation, Tokyo, Japan). The elastin was then analyzed semi-quantitatively with ImageJ 1.41 software as previously reported. The elastin ingredients were calculated by distinguishing the elastin area of the aortal wall in the EVG staining slice.

Immunohistochemistry

The ultrasensitive streptomyces antibiotic protein-peroxidase immunohistochemical method was applied to identify the macrophages with monoclonal mouse anti-rabbit macrophage clone RAM11 (Dako, Denmark) and matrix metalloproteinase (MMP)-2, 9 with mouse monoclonal antibodies (Abcam, Hong Kong, China). The tissue sections were incubated with 0.1% hydrogen peroxide for 30 min to remove the peroxidase activity. This was followed by incubation with 10% goat serum-diluted RAM11 (1:100), MMP-2 (1:150) and MMP-9 (1:300) antibodies overnight at 4 °C. The slices, which were incubated with the primary antibody-free goat serum, were used as the negative control. They were then stained by 3,3'-diaminobenzidine (DAB) and hematoxylin (ZSGB-Bio Co., Ltd, Beijing, China). All slices were viewed and tabulations made by use of a 200x microscope, and then graded according to expression levels into grade I (< 10%), grade II (10-50%) and grade III (> 50%).

Statistical analysis

The data were expressed as (x ± s), and the bilateral t-test and ANOVA were performed to analyze the intergroup difference (Prism 5.0, GraphPad Software), with p < 0.05 considered as the level of statistically significant difference.

RESULTS

Body weight and lipid levels

The body weights of the 3 groups did not exhibit a significant difference at the 0, 4th, 8th and 12th week (Table 1). However, the 8th week measurement revealed that the body weights of the PE group decreased slightly, which might be related to poor eating appetite after laparotomy.

Compared with the normal group, the serum TC, TG, LDL-C and HDL-C contents in the PE group and the SI group were significantly higher on the 4th, 8th and 12th week (p < 0.05) (Table 2), among which the 12th week increase was particularly significant.

Angiographic results

The first angiograms of all 20 rabbits on the 4th week exhibited smooth aortic walls and fluent blood flow, with a diameter of 1.8-2.5 mm. The SI group exhibited no significant change of abdominal aortic lumen

| Table 1. Body weight changes of each group (kg)* |
|-----------------|---------------|-----------------|-----------------|-----------------|-----------------|
| Group          | Number | 0 (baseline) | 4th week | 8th week | 12th week |
| Control        | 4      | 2.11 ± 0.20  | 2.25 ± 0.18 | 2.40 ± 0.17 | 2.78 ± 0.32 |
| SI             | 8      | 2.12 ± 0.14  | 2.26 ± 0.18 | 2.30 ± 0.16 | 2.67 ± 0.16 |
| PE             | 8      | 2.10 ± 0.19  | 2.24 ± 0.18 | 2.20 ± 0.16 | 2.70 ± 0.19 |

* There was no statistical difference in the body weight of each group. PE, pancreatic elastase; SI, saccule injury.
upon angiographic imaging at the 8th week, and the blood flow was fluent; by the 12th week angiography, the rabbit abdominal aortic lumen showed irregular stenosis associated with various degrees of stenosis-posterior stretching. Regarding the PE group, angiography at the 8th week showed that the abdominal aorta exhibited focal cystic expansion, and the expansion scope was consistent with the range of PE infiltration, with an average diameter of 3.8 ± 0.3 mm, while the normal lumen diameter at the proximal end of expansion was 2.4 ± 0.3 mm. Angiography at the 12th week exhibited local lumen of the PE group more narrowed than before, with an average of 2.8 ± 0.4 mm (p < 0.01), where the normal lumen diameter at the proximal end of expansion was 2.3 ± 0.4 mm (Figure 1). There was no significant change in the control group when compared with the first angiography, the abdominal aortic wall was normal, and the blood flow was fluent.

Histological and morphological evaluation

Utilizing light microscopy, the aorta in the control group exhibited the clear 3-layer structure, with smooth and complete intima and the smooth muscle cells arranged neatly. The aorta in the SI group significantly thickened, with different-size and irregular foam cells that could be seen accumulated, and a lot of lipids that could be seen deposited among the elastic plate and the intercellular spaces, and the smooth muscle cells arranged irregularly. In the PE group, the aortic intima thickened, accompanied by lipid deposition, visible plaque formation, internal elastic membrane damage and irregular arrangement of smooth muscle cells. The semi-quantitative analysis of elastic protein components of each group exhibited that the control group was 28.23% ± 5.7%, the SI group was 18.29% ± 6.9% (compared with the control group, p < 0.05), and the PE group was 17.61% ± 7.2% (compared with the control group, p < 0.05). There was no significant difference between the SI group and the PE group. The hematoxylin and eosin and EVG staining of aortic slice of each group were shown in Figure 2.

Immunohistochemical examination

The immunostaining of MMP-9, MMP-2 and RAM11 in the SI group and the PE group, compared with the control group, showed higher protein expression levels (p < 0.01), with the MMP-9 and MMP-2 secreted by the irregularly-arranged smooth muscle cells and macro-

Table 2. Blood lipid changes of each group (mmol/L)

<table>
<thead>
<tr>
<th>Item</th>
<th>Group</th>
<th>Number</th>
<th>0 (baseline)</th>
<th>4th week</th>
<th>8th week</th>
<th>12th week</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC</td>
<td>Control</td>
<td>4</td>
<td>1.14 ± 0.17</td>
<td>1.33 ± 0.45</td>
<td>1.51 ± 0.13</td>
<td>1.50 ± 0.34</td>
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<td></td>
<td>SI</td>
<td>8</td>
<td>1.10 ± 0.45</td>
<td>9.38 ± 4.69⁹</td>
<td>11.99 ± 3.39⁹</td>
<td>21.04 ± 5.23⁸</td>
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<tr>
<td></td>
<td>PE</td>
<td>8</td>
<td>1.13 ± 0.25</td>
<td>8.58 ± 3.57⁹</td>
<td>9.62 ± 4.39⁹</td>
<td>19.38 ± 4.79⁸</td>
</tr>
<tr>
<td>TG</td>
<td>Control</td>
<td>4</td>
<td>1.04 ± 0.36</td>
<td>0.89 ± 0.07</td>
<td>0.86 ± 0.34</td>
<td>0.87 ± 0.20</td>
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<tr>
<td></td>
<td>SI</td>
<td>8</td>
<td>1.09 ± 0.44</td>
<td>1.26 ± 0.47*</td>
<td>1.05 ± 0.44*</td>
<td>1.44 ± 0.51*</td>
</tr>
<tr>
<td></td>
<td>PE</td>
<td>8</td>
<td>1.07 ± 0.34</td>
<td>1.29 ± 0.46*</td>
<td>1.09 ± 0.44*</td>
<td>1.39 ± 0.34*</td>
</tr>
<tr>
<td>LDLC</td>
<td>Control</td>
<td>4</td>
<td>0.54 ± 0.15</td>
<td>0.39 ± 0.13</td>
<td>0.48 ± 0.16</td>
<td>0.89 ± 0.21</td>
</tr>
<tr>
<td></td>
<td>SI</td>
<td>8</td>
<td>0.58 ± 0.12</td>
<td>3.10 ± 1.74⁹</td>
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<td>13.73 ± 4.09⁸</td>
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<tr>
<td></td>
<td>PE</td>
<td>8</td>
<td>0.56 ± 0.14</td>
<td>3.57 ± 1.88⁹</td>
<td>3.40 ± 2.74⁹</td>
<td>11.10 ± 5.24⁸</td>
</tr>
<tr>
<td>HDLC</td>
<td>Control</td>
<td>4</td>
<td>0.69 ± 0.18</td>
<td>0.63 ± 0.07</td>
<td>0.53 ± 0.08</td>
<td>0.37 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>SI</td>
<td>8</td>
<td>0.66 ± 0.19</td>
<td>1.44 ± 0.69⁹</td>
<td>1.89 ± 0.48⁹</td>
<td>3.86 ± 0.92⁸</td>
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<tr>
<td></td>
<td>PE</td>
<td>8</td>
<td>0.68 ± 0.22</td>
<td>1.53 ± 0.44⁹</td>
<td>1.68 ± 0.78⁹</td>
<td>3.66 ± 0.72⁸</td>
</tr>
</tbody>
</table>

* p < 0.05; ⁹ p < 0.01, compared with the control group.

HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; PE, pancreatic elastase; SI, saccule injury; TC, total cholesterol; TG, triglyceride.

Figure 1. Angiographic changes. (A) 8th week angiography of the SI group, which showed the abdominal aortic lumen was irregular, with multiple stenotic changes; (B) 4th week angiography after PE treatment, the focal lumen still enlarged; (C) 8th week angiography after PE treatment, the focal lumen narrowed than before, while the wall was stiff.
phages. The control group itself had no MMP-2, MMP-9 and RAM11 expression, and the two experimental groups exhibited no significant difference (p > 0.05) (Figure 3).

**DISCUSSION**

AS is closely related to cardiovascular diseases, and its pathogenesis is extremely complex. Currently, most scholars support the “endothelial injury theory”, and the formation of atherosclerotic lesion is the inflammatory fibroproliferative results that occur when the artery responses to the intimal injury.\(^{16,17}\) Therefore, the HFD and SI methods are currently the most commonly used methods of preparing an AS model.\(^{18,19}\) Long-term HFD might cause the increase of in vivo lipids and its deposition in the artery; the balloon with the appropriate diameter could cause damage to the arterial intima, thus accelerating the accumulation of lipids in the arterial walls, and promoting the formation of atherosclerotic plaque.\(^{20}\) This method is well-accepted as a preparation for use of the rabbit AS model, the stages of which are relatively similar to those of human AS formation.\(^{21-23}\) In this experiment, the pathological and immunohistochemical testing results of the rabbit abdominal aorta in the SI group had further confirmed the validity of this method in preparing the AS model. But during the process, the balloon needed to be inserted through the femoral artery, while the rabbit femoral artery was fine, so the operations such as piercing would be more difficult, as well as the risk of bleeding also existed. Meanwhile, the balloon extension degree and the pulling speed would also have an impact towards the modeling results.\(^{23}\) In addition, the operation needed X-ray monitoring, which required a higher standard of attention towards the laboratory equipment and laboratory. So, we wanted to establish an AS model with a new method, which was different from the conventional process.

It has been reported that incubating normal dietary rabbit abdominal aortic periphery with protease for 20 min could induce the formation of local aneurysm, which was also accompanied by the intimal injury—the inflammatory cells (primarily the macrophages) infiltrated, the smooth muscle cells significantly reduced and elastic fibrosis.\(^{12}\) Three weeks later, the intimal hyperplasia became obvious, the infiltrated macrophages and smooth muscle cells increased gradually, and thus shrank the aneurysm, suggesting that the aneurysm had the self-repairing ability.\(^{12-14}\)

Based on the above reports, we used PE infiltration with HFD to make the rabbit aortic AS model. The experimental animals were given HFD 4 weeks before the modeling until the experiment ended, aiming to prompt the abnormal lipid metabolism in vivo, and the 4\(^{th}\)-week serological status confirmed the hyperlipidemia status in the experimental rabbits. After the modeling and the repairing process, the intima exhibited hyperplasia, which increased the deposition of lipid, the secretion of MMP-2, 9 and the infiltration of smooth muscle cells and inflammatory cells. It was conducive to the formation of AS.

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**Figure 2.** Morphological changes. The SI group and the PE group showed obvious intimal hyperplasia and lipid infiltration, the arrows pointed at the residual internal elastic membrane, and the tissue above it was the hyperplastic intima. The elastic protein substances were significantly reduced. NS, p = 0.086, *, p < 0.01.
At the beginning of this experiment, the testing and angiography of each group exhibited no significant difference. After 8 weeks of pancreatic elastase treatment of the rabbit abdominal aorta, the biopsy showed the aortic intima thickened, the plaque formed, different-size irregular foam cells aggregated, the smooth muscle cells increased and were arranged irregularly. The immunohistochemical examinations suggested that the contents of MMP-2, 9 and macrophage significantly increased, which were all consistent with the atherosclerotic changes and exhibited no significant difference with the SI modeling method. This confirmed the effectiveness of intimal injury caused by the elastase-infiltrating-arterial-periphery, while the control group exhibited no significant change with the early stage of trial.

As mentioned above, in this study, based on the HFD, the PE infiltration method could injure the intima efficiency and provide the same pathomorphological and immunohistochemical appearance with atherosclerotic intima.

In the SI group, the saccule contacted the arterial intima intimately and the mechanical endothelial injury was direct. While in the PE group, periarterial pancreatic elastase infiltrated through media into the intima, although the intimal injury was complicated with the destruction of media. The degeneration of smooth muscle cell and reduction of extracellular matrix in media resulted in the attenuation of media and the dilation of lumen. So, angiography and macroscopic observation revealed that the focal lumen were slightly enlarged instead of typical stenosis. Another manifestation was that the dilation reduced significantly 4 weeks later, suggesting the recovery of impaired media was later than that of intima. Other studies had also reported that the aneurysm produced by elastase had self-repairing ability. Origuchi et al. reported that aneurysms in-

Figure 3. Expressions of MMP-2, MMP-9 and RAM11. There were lots of brown spots in hyperplastic intima of PE group and SI group ($p > 0.05$), especially in the cross section detected for MMP-2 and RAM11. It was means the expressions of MMP-2, MMP-9 and RAM11. There was no difference between the two experimental groups. However, the expressions mentioned above were nearly not found in the control group.
duced by adventitial elastolysis did not progress, and after 42 days showed gradual shrinkage. By day 90, aortic diameters had nearly normalized, aortic walls also returned to their pre-elastase application thickness and some mature medial elastic lamellae showed regeneration, medial smooth muscle cells reverted to the contractile type.

Therefore, further narrowing following the recovery of media and hyperplasia of intima was possible, which still needed long-term observational study. In addition, the dosage and the infiltration time of elastase could be appropriately reduced in order to effectively damage the focal intima, while reducing the aneurysmal dilatation degrees and shorten the recovery time.

Compared with the traditional SI method, we used the open-abdomen method to perform the rabbit aortic intimal injury, which ensured the integrity of animal femoral artery, and thus was conducive to further research. It could be assumed that if the contact area of the free artery and elastase was increased, an AS model with larger range could be obtained. In short, the HFD+PE method could be used to prepare the rabbit abdominal aortic AS model, and the method was simple and controllable, without the assistance of an X-ray or other imaging devices, especially suitable for such cutting-edge research areas as evaluating the tectorial membrane-stent-mediated gene therapy towards the aortic aneurysms, and preventing the drug-eluting stent-caused restenosis and other potential problems.

CONCLUSION

The use of HFD with PE infiltration is feasible in establishing the rabbit aortic atherosclerosis model.

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