Performance of a Modified Rabbit Model of Abdominal Aortic Aneurysm Induced by Topical Application of Porcine Elastase: 5-Month Follow-up Study

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WHAT THIS PAPER ADDS?
This study describes a modified method for creating an abdominal aortic aneurysm (AAA) model, with a simplified surgical procedure and shortened elastase induction time. This simpler aneurysm model is valuable to investigate the pathogenesis of aortic aneurysm, and is likely to become a popular tool for testing medical intervention for AAAs.

Objectives: To modify the method for creating an abdominal aortic aneurysm in rabbits, and to study its performance.
Materials and methods: A total of 24 New Zealand white rabbits were induced topically with 10 μl of porcine elastase (0, 0.1, 5 and 10 units μl⁻¹) to define the optimal concentration (groups A–D). Twelve aneurysms were induced with 10 units μl⁻¹ of 10 μl elastase to serve as a follow-up group (group E) to serve as a follow-up. A 1.5-cm aortic segment was isolated and induced with elastase solution for 30 min.

Results: All animals in groups D and E developed AAA by day 5. Aneurysms in Group E were stable over 100 days. Partial destruction to disappearance of elastic lamellae and smooth muscle cells (SMCs) was seen in elastase-treated animals by day 5. Regenerated elastin and proliferated SMCs were present in group E. Matrix metalloproteinases 2 and 9 and RAM11 showed strong expression in group D, but expression decreased in group E after day 15.

Conclusions: The rabbit AAA model induced via topical application of porcine elastase at 10 units μl⁻¹ for 30 min appears easy and simple, with shorter induction and more rapid aortic dilation. The model is stable over 100 days and is useful to study the formation and progress of AAAs.
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Abdominal aortic aneurysm (AAA) is defined as focal aortic dilation with a diameter of 1.5 times greater than normal,¹ which is characterised by smooth muscle cell (SMC) depletion,²,³ chronic medial and adventitial inflammation, elastin degeneration and medial attenuation.⁴ The pathogenesis of AAA has not been completely elucidated, although studies indicate that matrix metalloproteinases (MMPs), particularly MMP2 and MMP9, may play a vital role in AAA formation.⁵–⁹
Several experimental aneurysm models have been developed to study the pathogenesis of AAA and to mimic clinical scenarios for translational research,⁷,¹⁰–¹⁵ including an elastase-induced AAA model reported by Anidjar et al.,¹⁶ one of the most popular. Since this model is histologically and haemodynamically similar to natural human aneurysm, elastase perfusion is widely used to induce aneurysm formation in mice and rabbits.¹²,¹⁵–²⁰ However, such AAA models require insertion of a cannula into the common iliac artery, making the surgical procedure complex and difficult. In addition, leakage of the elastase solution during perfusion may cause surgical failure and death.
Topical elastase induction has been used to induce aneurysms of the carotid artery of rabbits.¹⁰,²¹–²³ In addition, AAA models have also been successfully created by adventitial elastolysis, in which the aortic segment is bathed in elastase solution (20 units ml⁻¹) for 70 min.²⁴ Miyake et al. and Origuchi et al. required 2–3 h to create an effective AAA model.²⁵,²⁶ However, lengthy surgical procedures may increase the chance of infection and result in animal death and procedure failure. On the other hand,
shorter elastase exposure times can be used to create AAA in rats. In this study, we hypothesised that a rabbit AAA model might be further optimised by an intense topical application of a higher concentration of elastase and a shorter period of induction. In addition, a 5-month follow-up was performed in order to study the durability and value of our modified AAA model.

MATERIALS AND METHODS

Production of AAA model

The animal study was approved by our institutional committee for the care and use of laboratory animals. A total of 24 male New Zealand white rabbits weighing 2.40 ± 0.38 kg were used in the study, and divided into four groups corresponding to different concentrations of porcine elastase solution (≥30 units mg⁻¹, PI 9.5, pH 8.1—8.9, Shanghai Kayon Biological Technology Co., Ltd., Shanghai, China). Groups B–D received 0.1, 5 and 10 units µl⁻¹ of elastase solution, respectively, while physiological saline without elastase was used for the control (group A). Rabbits were anesthetised with intravenous injection of 30 mg kg⁻¹ sodium pentobarbital. The induced segments were harvested and placed under the isolated aortic segment to support the artery without complete blockage of the circulation. A piece of sterile cotton gauze (3 × 4 mm) was used to wrap the middle part of the exposed aorta. Ten microlitres of elastase solution at four different concentrations (0, 0.1, 5 and 10 units µl⁻¹) were applied to the sterile gauze coating the artery and left in place for 30 min to determine the optimal concentration. The gauze and forceps were then removed, and the arterial segments were washed twice with physiological saline to clear any remaining elastase (Fig. 1). The outer diameter of the incubated segment was measured by a micrometre prior to and at 10, 15, 20, 30 min and 5 days after incubation. Using the same procedure, AAAs were induced in an additional 12 rabbits with 10 µl elastase solution at the optimal concentration of 10 units µl⁻¹ determined previously, to serve as the follow-up group. All animals were fed a chow enriched in 1% cholesterol after the procedure.

Intravenous digital subtraction angiography (IVDSA)

Diameter measurement was performed with IVDSA, an alternative and feasible method for evaluating elastase-induced aneurysms in rabbits. All studied animals in groups A–D underwent imaging before and immediately after surgery as well as on days 3 and 5 after surgery. In group E, serial IVDSA was performed before surgery and on days 5, 15, 40, 100 and 150 after the procedure. After placing a 24-gauge angiocatheter in the ear vein, 6 ml of iodinated contrast material was infused within 3 s by manual injection. As previously reported, the diameter of the aortic segment was measured in reference to an external sizing device (1 cm in length) placed under the abdomen during IVDSA. AAA was defined as an aortic lumen diameter that was dilated 50% or more than the diameter before induction with elastase solution.

Histological analysis

All animals in groups A–D on day 5 and every fourth rabbit in group E on days 15, 40 and 150 were sacrificed just after imaging by intravenous injection of an overdose of sodium pentobarbital. The induced segments were harvested and fixed in 10% buffered formaldehyde. Transverse sections (5 µm thick) of aortic segments from each animal were stained with haematoxylin and eosin (HE), elastic van-Gieson (EVG) and Picrosirius red (PSR) dyes. Elastin and collagen contents, expressed as a percentage, were semi-quantified with the use of ImageJ 1.41 software as previously reported. The elastin/collagen ratio was calculated by dividing elastin content in the EVG-stained sections by the collagen content in the PSR-stained sections with the same nonoverlapping high-power fields. The intima/media ratio was calculated by the similar method in the HE-stained sections.

Immunohistochemical analysis

The UltraSensitive streptavidin-peroxidase method (KIT-9701, Maixin, Bio, China) was used for detection of MMP2, MMP9 (mouse monoclonal antibody, Abcam, HongKong, China) and macrophages (monoclonal mouse anti-rabbit macrophage, clone RAM11, Dako, Denmark). Fixed tissues were embedded in paraffin and cut into 5-µm-thick sections. Slides were incubated with 1% hydrogen peroxide for 10 min at room temperature to block endogenous peroxidase activity. The aortic sections were blocked with 10% goat serum, and then incubated at 4 °C overnight with primary antibodies specific to MMP2 (1:150 dilution), MMP9 (1:300 dilution) and RAM11 (1:100 dilution). Sections incubated with goat serum lacking a primary antibody served as a negative control. The aortic sections

Figure 1. Photograph during surgical procedure. A segment of abdominal aorta proximal to the bifurcation was isolated and supported by a pair of curved-tip forceps.
were visualised by incubating with diaminobenzidine tetrahydrochloride and counterstaining with haematoxylin. Every four sections in each rabbit were studied and scored at 200× magnification. Any sections that exhibited strong expression (>50%) were scored as 3, moderate expression (10%–50%) as 2 and weak expression (<10%) as 1.

For immunofluorescence staining of smooth muscle cells (SMCs), the aortic sections were incubated at 4 °C overnight with mouse monoclonal anti-alpha smooth muscle actin (1:150 dilution, clone 1A4, Sigma Aldrich Trading Co. Ltd., Shanghai, China). After three washes with PBS, sections were then incubated with FITC-conjugated goat anti-mouse IgG for 1 h at room temperature. SMC content was calculated by dividing the alpha-actin positive staining by the aortic wall area using ImageJ 1.41 software.

Statistical analysis
Results are expressed as means ± SD. The data were analysed using one-way analysis of variance (ANOVA) or repeated-measures ANOVA followed by Bonferroni post-hoc tests (Prism 5.0, GraphPad Software, Inc., SanDiego, CA). Differences were considered statistically significant at p < 0.05.

Figure 2. Diameter changes of induced segments measured by intravenous digital subtraction angiography. (a) Anteroposterior aortography by intravenous digital subtraction angiography (IVDSA) on day 5. Diameters of induced segments shown in groups A to D are 2.0 mm, 2.7 mm, 3.9 mm and 6.6 mm. Arrows show renal arteries. (b) Profile plots of inner diameters for different groups by day 5 (n = 6/group), and inner diameter change within 5 months in group E (n = 12). *p < 0.0001, compared to group A, †p < 0.05, compared to group C. (c) Profile plots of outer diameters for different groups by day 5. *p < 0.05, compared to group A and group B. Baseline: time-point before operation or arteriospasm during isolation of aortic segment.
RESULTS

Influence of elastase solution on diameter enlargement

All rabbits survived the procedure, and no rabbits died during imaging. All animals in group D developed AAA, while only one aneurysm occurred in group C and none occurred in groups A or B (Fig. 2a). Repeated-measure ANOVA revealed significant effects of group and induction time, as well as significant group × time interaction in inner diameter ($F = 29.53, p < 0.001$). Inner diameter in group D

![Image](image_url)

Figure 3. Histomorphometric changes in groups A to D on day 5 and group E during a 150-day follow-up. HE, Haematoxylin and eosin stain; EVG, elastic Van-Gieson stain; PSR, Picrosirius red. Group E-15, group E on day 15; group E-40, group E on day 40; group E-150, group E on day 150. NS, not significant, *$p < 0.05$, group**$p < 0.01$, ***$p < 0.0001$. Bar = 100 µm, original magnification ×40.
increased significantly when compared to groups A and C from day 0 to day 5 (\(p < 0.0001\) and \(p < 0.05\), respectively). Five days after surgery, rabbits in group E showed dilation of the aortic diameter of 1.66 ± 0.09 times that recorded before induction, which remained stable over 100 days (1.72 ± 0.16, 1.68 ± 0.01 and 1.56 ± 0.08 on days 15, 40 and 100, respectively). After day 100, diameters decreased to 1.36 ± 0.28 times the initial diameter by day 150 (Fig. 2b). For outer diameter, repeated measures ANOVA showed significant main effects of group and time, as well as group \(\times\) time interaction (\(F = 7.69, p < 0.001\)). Outer diameter in group D increased significantly compared with groups A and group B from 15 min to 5 days (\(p < 0.01\) and \(p < 0.05\), respectively, Fig. 2c). There were no significant differences between groups C and D, or between groups B and C.

### Aortic wall thickness changes

The intima–media thickness (IMT) tended to increase in group C, compared to segments from group A (\(t = 4.58, p < 0.0001\)), while there was a significant decrease in IMT between group A and group D (\(t = 3.95, p < 0.01\)). In group E, there was an obvious tendency towards larger IMT on day 150 when compared to groups A or D on day 5 (\(t = 11.03, p < 0.0001\) and \(t = 14.99, p < 0.0001\), respectively). In Group E, intimal hyperplasia was observed starting at day 15 after surgery, and the degree of intimal hyperplasia, which was assessed by the intima/media ratio tended to increase by day 40 (0.61 ± 0.27, \(t = 3.38, p < 0.01\) and day 150 (1.67 ± 0.81, \(t = 10.92, p < 0.0001\)) compared to group D on day 5 (0.12 ± 0.11, Fig. 3).

### Elastin degeneration and regeneration

Histological examination of the control segments revealed that the aortic wall was regular, SMCs were normal and medial lamellae as well as the elastic interna and externa were intact. The elastic externa and lamellae in the media were fragmented in groups B and C. In group D, the aortic wall became disorganised with a large amount of red blood cells and inflammatory cells present, and the elastin fibres had almost entirely disappeared, except for some remnant elastic interna. Fragmented elastic fibres were seen in the

![Figure 4](image-url). Immunohistochemical findings and results of semiquantitative analysis. L, lumen, group E-15, group E on day 15; group E-40, group E on day 40; group E-150, group E on day 150. **\(p < 0.01\), ***\(p < 0.0001\). Bar = 20 μm, original magnification ×200.
media and the disorganised newborn elastin and migrated SMCs were seen in the hyperplastic intima in group E (Fig. 3). Elastin content decreased significantly from groups A to D and increased significantly during the follow-up in group E ($p < 0.0001$). Collagen content did not decrease significantly after elastase induction, but increased significantly in group E by day 40 ($t = 4.15$, $p < 0.01$). The elastin/collagen ratio in groups B to D decreased significantly compared to group A ($1.03 \pm 0.50$, $p < 0.0001$), but increased significantly in group E on day 40 ($t = 3/54$, $p < 0.05$). As shown in Figure 3.

**MMP2 and MMP9 expression and inflammatory infiltration**

MMP2 expression was increased in all elastase-induced segments. This moderate expression was shown during follow-up in group E, but only the expression in group D was significant compared to group A on day 5 ($t = 4.31$, $p < 0.01$). Five days after surgery, expression of MMP9 was present in all elastase-treated groups, while no expression was seen in the control group (Fig. 4). Very weak expression of MMP9 was seen in adventitial and medial areas in groups B and C. Sections from group D rabbits exhibited strong MMP9 staining throughout the aneurysm wall, particularly within inflammatory cells and certain myofibroblasts. Inflammatory cells, mainly macrophages, were observed to infiltrate the adventitial wall in groups A–C, and RAM11-positive cells were shown throughout the induced wall in group D. Group E showed weak expression of both MMP9 and RAM11 in adventitia during the 150-day follow-up, with positive expression of RAM11 shown in hyperplastic intima on day 150.

**Loss and regeneration of SMCs**

Immunofluorescent assay of SMCs revealed an obvious tendency towards larger loss of SMCs with increased elastase concentration on day 5, and SMCs in all elastase-induced sections decreased significantly compared to group A ($p < 0.01$). Regeneration of SMCs was seen in group E 15 days after surgery, and SMCs increased significantly after day 15 during follow-up ($p < 0.0001$, vs. day 5) and almost recovered to normal status 5 months later (Fig. 5).

**DISCUSSION**

We have successfully developed an AAA model in rabbits through periarterial application of 10 µl porcine elastase

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**Figure 5.** Smooth muscle cells (SMCs) attenuation and proliferation by immunofluorescence. (a) Influence of elastase on SMCs in groups A–D on day 5. (b) Changes of SMCs in group E during follow-up. (c) Semiquantitative analysis of SMCs. group E-15, group E on day 15; group E-40, group E on day 40; group E-150, group E on day 150. Group**$p < 0.01$, ***$p < 0.0001$. Bar = 20 µm, original magnification $\times 200$. 

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solution (10 units ml$^{-1}$) using a shortened 30-min induction time. We found that the aortic diameter increased with increasing elastase concentration. Lower concentrations of elastase were unable to reliably induce AAA formation, other than medial hyperplasia. Increase of the intima/media ratio and elastin content, and regeneration of SMCs during follow-up, indicated that SMCs and elastin degradation were essential for aneurysm formation, SMC proliferation and elastin regeneration accounted for the self-healing tendency seen in an elastase-induced aneurysm model. In fact, SMCs must play a critical role in aneurysm model formation and progression, since elastin was synthesised by vascular SMCs. Rabbits in group D exhibited strong MMP2, MMP9 and RAM11 staining throughout the aneurysmal wall, while both MMP9 and RAM11 showed weak expression in adventitia after 15 days during follow-up. Interestingly, MMP2 showed a moderate expression even by day 150, and positive expression of RAM11 was observed on day 150, which might have been caused by the infiltration of macrophages into the hyperplastic intima.

Models of AAA have been induced in rodents and rabbits by using porcine elastase perfusion. In this model, a cannula is inserted via the common iliac artery, and 100 units ml$^{-1}$ elastase solution is perfused for 5–10 min at a peak pressure between 300 and 400 mmHg. The abdominal aorta is clamped to block blood circulation during perfusion, and the arteriotomy requires suturing. This model is more complex and less accessible than a model created by adventitial elastolysis,\cite{1} in which a 3-cm segment of the infrarenal aorta is bathed in 20 units ml$^{-1}$ elastase solution for 70 min and results in more than 90% of rabbits developing aneurysms.\cite{2} However, accurate dosage could not be determined during induction in this experiment. By contrast, in our study, 10 l of elastase solution was efficient enough to induce AAA formation without haemorrhage and aneurysm rupture, and our induction time was considerably shorter than previous reports.

Origuchi et al.\cite{3} reported that the aneurysm induced by adventitial elastolysis exhibits spontaneous healing. Additionally, they found that aneurysm gradually shrinks by 42 days, becoming almost normal by day 90. However, in our study, the aneurysm remained stable over 100 days, although a tendency for the aneurysm to shrink after 5 months was noted. The aneurysmal inner diameter gradually decreased and the elastin/collagen ratio recovered to normal status after 5 months, although IMT increased significantly due to intimal hyperplasia. The induced aorta could not completely return to normal conditions, considering that elastin was almost nonexistent in the media and newborn elastin in hyperplastic intima was disorganised with a lack of normal structure of the elastic lamina. In addition, the aortic wall was notably thickened, mainly due to an increase in IMT.

In conclusion, we present an easy, efficient and reproducible way to induce rapid dilation of rabbit aortic arteries to create a model of AAA. This aneurysm model is stable over 100 days, and is useful for studies of the formation and progress of aneurysm in rabbits.

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CONFLICT OF INTEREST

None declared.

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