Perfusion pressure of elastase impacts the formation ratio and diameters of abdominal aortic aneurysms in rats

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Abstract. Intra-arterial perfusion with elastase is a common method used to create abdominal aortic aneurysms (AAA) models. The present study aimed to explore the impact of porcine pancreatic elastase (PPE) perfusion pressure on the morphology of abdominal aortic aneurysms. A total of 40 male Sprague Dawley rats were randomized into four groups. The elastase was perfused at pressures in the aortic lumen of 300, 100 and 0 mmHg in three groups, respectively. Rats perfused with saline at 300 mmHg were used as controls. The maximum diameters of the AAA were monitored with ultrasound at 7, 14 and 28 days after the operation. Elastin degradation and inflammatory cell counts were determined using histochemical staining. All rats were successfully perfused at the scheduled pressure. After 7 days, the AAA formation ratio of PPE-300, PPE-100 and PPE-0 was 100, 50 and 0%, respectively. After 14 days, the AAA formation ratio in PPE-100 and PPE-0 reached 90 and 20%, respectively. After 28 days, the diameters of the isolated aorta in PPE-300, PPE-100, PPE-0 and NaCl-300 were (mean ± standard deviation) 7.34±1.81, 4.02±0.40, 2.92±0.32 and 2.49±0.07 mm, respectively, and the difference between groups was statistically significant ($P<0.05$). The formation ratio in PPE-300, PPE-100, PPE-0 and NaCl-300 was 100, 100, 20 and 0%, respectively. Elastase perfusion pressure could impact the AAA formation ratio at an early stage and the maximum diameter of the aneurysm without increasing animal mortality. Elastase perfusion with high pressure could accelerate aneurysm formation and represents a potential method for building large-size abdominal aortic aneurysms. However, the underlying mechanisms need further investigation.

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Introduction

An abdominal aortic aneurysm (AAA) is a life-threatening disease (1,2). The primary danger is the risk of rupture and death from hemorrhage. The AAA may be found in up to 8% of men aged >65 years and it is the 13th leading cause of death in the USA (3). AAA is diagnosed if the diameter of the abdominal aorta is >1.5 times (or ≥3 cm) the normal value (4). The single most important predictor of rupture is the diameter of AAA, with the risk of rupture increasing for larger aneurysms (5). The goal of medical management is to repair the AAA before rupture. Currently, surgical treatment is the only way to prevent the rupture of AAA and there are no reliable pharmacological agents that limit AAA expansion (6). This is partially associated with a lack of understanding of the underlying AAA expansion mechanisms (7).

Animal models have been used to investigate the mechanisms involved in AAA development and determine measures for early prevention and treatment (8). Common AAA animal models comprise the transient intra-arterial perfusion with porcine pancreatic elastase (PPE) and the periaortic application of calcium chloride ($CaCl_2$) (9-11). Due to the similar pathological characteristics of human AAA, intra-arterial perfusion with elastase has been frequently used for AAA modelling (12,13). However, several factors such as elastase concentration and the perfusion time of elastase can also impact the AAA formation ratio and animal mortality (14,15).

Intra-arterial perfusion with PPE was able to induce the destruction of the medial elastic fibres, eventually causing an AAA (16,17). To the best of the authors' knowledge, the effects of elastase perfusion pressure on the aneurysmal morphology and mortality in experimental animals remain to be elucidated (18). The present study aimed to determine if the perfusion pressure impacts the AAA formation ratio and maximum diameter, by exploring three different levels of perfusion pressure of elastase. The impact of perfusion pressure on animal mortality was also investigated in the present study. The current findings could facilitate the understanding of more characteristics of the elastase-induced AAA models,

helping researchers to choose appropriate animal models to explore the pathogenesis and treatment of AAA.

Materials and methods

Animals. A total of 40 male 12-15 week old Sprague Dawley rats (weight, 550-600 g) were purchased from Vital River Laboratory Animal Technology Co., Ltd. and housed in a standard laboratory environment with ambient temperature of 22-25˚C, humidity of 55-65% and a 12-h light/dark cycle. Animals had free access to standard solid claviform food and autoclaved tap water. The procedures were performed following the National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH publications no. 85-23, revised in 1996) and the study was approved by the Animal Care and Use Committee of Fuwai Hospital (Beijing, China; no. 0099-1-8-HX).

Abdominal aortic aneurysm models. The elastase-induced AAA models were prepared using PPE (type I; 8.5 ml; 11.8 mg protein/ml; 4 U/mg protein; Sigma-Aldrich; Merck KGaA) according to an improved method (19). Briefly, rats were anaesthetized with 2% pentobarbital sodium (60 mg/kg; intraperitoneally). A laparotomy was performed via a midline abdominal incision under sterile conditions. The infrarenal abdominal aorta was separated from the left renal vein to the bifurcation of the aorta. The primal and distal parts of the isolated aorta were temporarily ligated using a 4-0 suture. In addition, all the collateral arteries from the isolated segment (including the lumbar artery) were ligated with a 6-0 suture. The isolated segment was punctured using 22-GA venous indwelling needle (BD Angiocath; BD Biosciences) and the leakproofness inside the isolated aorta was confirmed by infusing saline solution (0.9% NaCl). Only the rats with good leakproofness in the isolated aorta were included in PPE or NaCl perfusion.

Perfusion pressure of porcine pancreatic elastase. To observe the impact of perfusion pressure on AAA morphology, the animals were randomized into four groups (10 rats per group). The first group was named PPE-300, in which the rats were perfused with PPE at 300 mmHg pressure. The second group was named PPE-100 in which the rats were perfused with PPE at 100 mmHg pressure. The third group was named PPE-0, in which the rats were perfused with PPE without pressure. The rats in the fourth group, which was named NaCl-300, were used as controls and perfused with 0.9% NaCl at a pressure of 300 mmHg.

The perfusion pressure of PPE or NaCl was controlled using a pressure pump. Importantly, The PPE concentration and perfusion time were identical among the groups; the concentration of PPE was 47 U/ml and the perfusion time was 30 min. After perfusion, the abdomen of the rat and the isolated aorta were washed three times with 0.9% NaCl. The 22-GA venous indwelling needles were subsequently removed and the puncture sites were sutured with 7-0 Prolene™ (W8702; Johnson & Johnson). When the blood flow was restored, the vessel diameters were measured using a Vernier caliper (0-150 mm; Standard Gage Co., Inc.). Finally, the incision was closed and the rat was returned to their cages.

Ultrasonography. The measurement of the aortic diameters was performed using an ultrasound imaging instrument according to previously described methods (20). To dynamically monitor the diameters of the aneurysm, ultrasound measurements of the aorta were performed at 7, 14 and 28 days postoperatively. In brief, the animals were anaesthetized by isoflurane inhalation. Anesthesia was induced with 3.5% isoflurane and was maintained using 2.0% isoflurane. Then the animals were shaved and placed in dorsal recumbency. Aortic imaging was performed using a Vevo® 2100 imaging system (VisualSonics, Inc.). The transducer MS-250 (13-24 MHz; VisualSonics, Inc.) was then oriented to provide axial images from the left renal vein to the aortic bifurcation. The maximum diameter planes were obtained for the subsequent analysis. The images were analyzed using Vevo LAB version 1.7.1 software (VisualSonics, Inc.) using the following formula: Dilation ratio (%)=(aneurysmal diameter/normal aortic diameter) x100%. Normal aortic diameters were defined as mean values corresponding to the isolated segment. AAA was defined as a dilation ratio >150%.

Histology. At 28 days, the animals were euthanized by an overdose of pentobarbital sodium (200 mg/kg, intraperitoneally). If the rat's pain response disappeared and the heartbeat and respiration stopped, the rat was judged to be dead. The abdominal aorta tissue of rats was harvested immediately after the administration of the anesthetic. The tissue samples were fixed in 4% methanol solution for at least 48 h at room temperature and processed using standard procedures in graded alcohols and xylene, and embedded in paraffin. Paraffin-embedded slices were serially sectioned at 4 μm intervals. The slices were stained using H&E staining and images were captured with a microscopic system (IX71; Olympus Corporation). The maximum diameters of AAA were measured based on H&E staining results and considered as the diameter of the aneurysm.

Elastin degradation is an important pathological characteristic of AAA. To observe the elastin degradation, elastic van Gieson (EVG) staining was performed at room temperature and the process was observed in real time under microscope (DM750; Leica Microsystems GmbH) until the staining was successful, as previously described (19). In addition, the cell types in the aneurysms were identified using immunohistochemistry (IHC). In brief, the slices were incubated overnight at 4˚C with anti-α-smooth muscle actin (α-SMA; 1:1,000; cat. no. ab5694; Abcam), anti-CD8 (1:500; cat. no. ab217344; Abcam) and anti-CD68 (1:1,000; cat. no. ab213363; Abcam) primary antibodies to identify vascular smooth muscle cells, lymphocytes and macrophages, respectively. Signal amplification was performed the following day using goat anti-rabbit IgG secondary antibody (Beijing Zhongshan Jinqiao Biotechnology Co., Ltd.; OriGene Technologies, Inc.). Tissue images were captured using a fluorescent inverted microscope (IX71; Olympus Corporation). The elastin content in the abdominal aorta was analyzed using ImageJ version 1.45 software (National Institutes of Health; https://imagej.nih.gov/ij/).

Statistical analysis. Statistical analysis was performed using GraphPad Prism version 8.0 (GraphPad Software, Inc.). The results are expressed as the mean ± standard deviation. Data

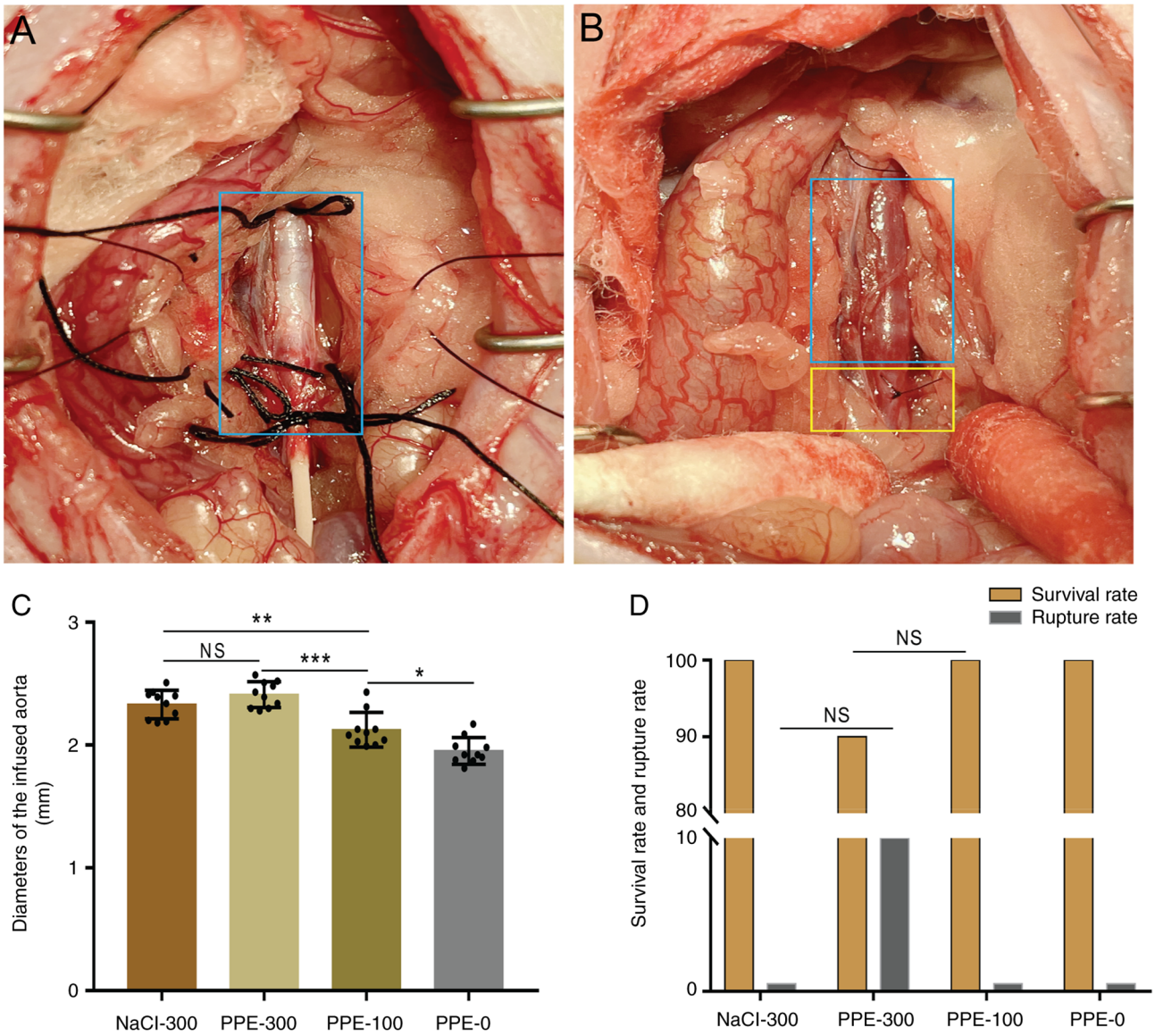

Figure 1. Outcomes of the operation and the survival rate of rats. (A) Representative image of an isolated aorta during elastase perfusion. The blue box area indicates the infused aorta. (B) Representative image of an infused aorta immediately after perfusion. Blue and yellow box areas represent the infused and normal aorta, respectively. (C) Diameters of the infused aorta in four groups were measured using Vernier calipers soon after the perfusion (n=10 independent experiments per group). (D) The AAA rupture rates and survival rates of rats 28 days after the operation (n=9 in PPE-300; n=10 in the other three groups). Differences among groups were analyzed using a one-way analysis of variance followed by Tukey's HSD for multiple comparisons. *P<0.05, **P<0.01 and ***P<0.001. NS, not significant; AAA, abdominal aortic aneurysm; PPE, porcine pancreatic elastase; PPE-300, PPE perfusion at 300 mmHg; PPE-100, PPE perfusion at 100 mmHg; PPE-0, PPE perfusion without pressure; NaCl-300, saline solution perfusion at 300 mmHg.

were analyzed using a one-way analysis of variance followed by Tukey's highly significant differences test for multiple comparisons. P<0.05 was considered to indicate a statistically significant difference.

Results

Operation outcomes and survival rates of animals. The PPE or NaCl was successfully infused in the isolated aorta of all the experimental rats (100%) under scheduled pressure. No aortic rupture occurred during perfusion (Fig. 1A). At the end of perfusion, the mean diameter of the infused aorta in PPE-300 (2.41±0.01 mm) was significantly larger than that in PPE-100 (2.12±0.21 mm) and PPE-0 (1.95±0.01 mm). The mean diameter of the infused aorta in PPE-100 was larger than that in PPE-0, while no difference was found between PPE-300 and NaCl-300 (2.33±0.01 mm; Fig. 1B and C). Similarly, the mean diameter of the infused aorta in NaCl-300 was larger than that in PPE-100 and PPE-0.

During the 28-day follow-up, only one rat (10%) in the PPE-300 group died of AAA rupture. The survival rate in NaCl-300, PPE-300, PPE-100 and PPE-0 was 100, 90, 100 and 100%, respectively. The rate of AAA rupture in PPE-300 was 10%, while no AAA rupture occurred in the other three groups (Fig. 1D).

Formation ratio of AAA with time. Aortic ultrasound was performed 7, 14 and 28 days after the operation to measure the aneurysm diameters and calculate the formation ratio of the AAA. After 7 days, the aortic ultrasound indicated that

the diameters of the AAA increased with the increasing of perfusion pressure (Fig. 2A). The AAA formation ratio in the PPE-300, PPE-100 and PPE-0 was 100, 50 and 0%, respectively (Fig. 2B). After 14 days, the formation ratio of AAA in PPE-100 and PPE-0 increased to 90 and 20%, respectively, with a significant difference between these values. After 28 days, the diameter of isolated aorta in PPE-300, PPE-100, PPE-0 and NaCl-300 was 7.34±1.81, 4.02±0.40, 2.92±0.32 and 2.49±0.07 mm, respectively. The AAA formation ratio in PPE-300, PPE-100 and PPE-0 was 100, 100 and 20%, respectively (Fig. 2B and C). The formation ratio of AAA in PPE-0 was significantly lower than that in PPE-300 and PPE-100 ($P<0.001$). No AAA was found in the NaCl-300 group during the 28-day follow-up.

Dilation ratio of AAA based on H&E staining. Aortic tissues were collected for histopathological staining 28 days after the operation. The H&E staining results showed that the normal diameter of the aorta was 1.56±0.01 mm. According to the >150% normal diameter criterion, the diagnostic diameter of the AAA was >2.34 mm (Fig. 3A). The maximum diameter of the AAAs was measured and the dilation ratio was calculated based on the H&E results. The maximum diameter in PPE-300, PPE-100 and PPE-0 and NaCl-300 was 5.21±2.14, 3.34±0.35, 2.19±0.29 and 1.68±0.03 mm, respectively (Fig. 3B). The maximum dilation ratio of aneurysm in NaCl-300, PPE-300, PPE-100 and PPE-0 was 106.25±0.43, 333.91±88.07, 214.29± 14.26 and 140.20±7.81%, respectively (Fig. 3C).

Pathological characteristics of AAA. Elastin degradation is a pathological feature of AAA (21). Aneurysm tissues were obtained 28 days after surgery and EVG staining was performed to observe the impact of elastase perfusion pressure on the elastin degradation. The results showed that the elastin levels in PPE-300, PPE-100, PPE-0 and NaCl-300 were 3.45±0.25, 14.54±6.31, 33.72±3.86 and 35.54±1.82%, respectively. In addition, nearly no elastin was found in the aneurysm vessels of PPE-300 (Fig. 4A). Inflammatory cell infiltration is an important feature of aneurysms (22). In the present study, IHC analysis suggested that some macrophages were found in aneurysms from the PPE-300 group and they were mainly located in the vascular adventitia areas (Fig. 4B). However, macrophages were not observed in the other three groups (data not shown). Unexpectedly, some intimal hyperplasia was found in the aneurysms in the PPE-300 group. IHC staining showed that the cells in the intimal hyperplasia areas mainly consisted of vascular smooth muscle cells (Fig. 4C).

Discussion

The present study explored the impact of elastase perfusion pressure on the aneurysm morphology. Based on the present results, two conclusions can be made. First, the elastase perfusion pressure could impact the formation ratio of AAA at an early stage. Second, the perfusion of elastase with high pressure could increase the maximum diameters of AAA in rats. Moreover, increasing the elastase perfusion pressure up to 300 mmHg did not increase the mortality of the animals. In addition, the current findings indicated that perfusion of elastase with high pressure represents a method to create a considerably large AAA. The present study contributes to refining elastase-induced AAA models and can also help researchers to select appropriate models for their studies.

Perfusion time and elastase concentration are important factors affecting elastase-induced aneurysm models (15,16). In the present study, identical perfusion times and elastase concentrations were applied to different groups, which contributed to the observation of the impact of elastase perfusion pressure on the aneurysmal morphology. The perfusion time of elastase reported in previous studies varied from 7-120 min (23). Of course, the perfusion time of elastase is always different in different animal species and the commonly used perfusion time of elastase in rats is 30 min (24). Given that the mortality increases with the increase of perfusion time, the perfusion time used in the present study was set at 30 min. Elastase concentration used in previous studies varied from 5-200 U/ml (25). In the present study, a commercial PPE solution without any dilution was used.

The elastase perfusion pressure is also influenced by the leakproofness of the isolated aorta (26). In the present study, the lateral branches of the isolated aorta, including the lumbar arteries, were completely ligated before perfusion to obtain good leakproofness and maintain perfusion pressure. Given that the normal blood pressure of rats was about 100 mmHg, the commonly used elastase perfusion pressure in previous studies was always 100 mmHg (27,28). The current study compared three different perfusion pressures of elastase including the commonly used values (100 mmHg). The present results suggested that elastase perfusion pressure can significantly affect aneurysm morphology. First, the formation ratio of AAA was proportional to the elastase perfusion pressure at 7 days postoperatively. All rats (100%) in the high-pressure group (PPE-300) reached the criterion of aneurysm 7 days post-operation (>150% normal vessel diameter), while the formation ratio of the aneurysm in medium-(PPE-100) and low-pressure (PPE-0) group were 50 and 0%, respectively. In addition, the formation ratio in medium and low perfusion pressure showed an increasing trend, reaching 90 and 20% at 14 days, respectively. Moreover, their formation ratios were up to 100 and 20%, respectively, 28 days after the operation. The perfusion of elastase with high pressure could accelerate AAA formation. Second, the elastase perfusion pressure could also impact the maximum diameters of the aneurysm. The present findings suggested that the maximum aneurysm diameters in the high-pressure group were significantly larger than those in the medium- and low-pressure groups. Similarly, the maximum diameters of aneurysms in the medium-pressure were larger than those in the low-pressure group. These results suggest that the perfusion of elastase with high pressure is a potential method to create a considerably large-size AAA. To the best of the authors' knowledge, large-size AAA models have not been reported in the literature and these need to be explored in the future.

In addition to aneurysm morphology, elastin degradation and infiltration of inflammatory cells are important characteristics of AAA (29). The present results showed that the degree of elastin degradation was proportional to the elastase perfusion pressure. As expected, almost no elastic fibers were found in aneurysms from the high-pressure group, which can be explained by the excessive dilation of the aorta under high pressure and more elastase permeation into vessels. Elastic

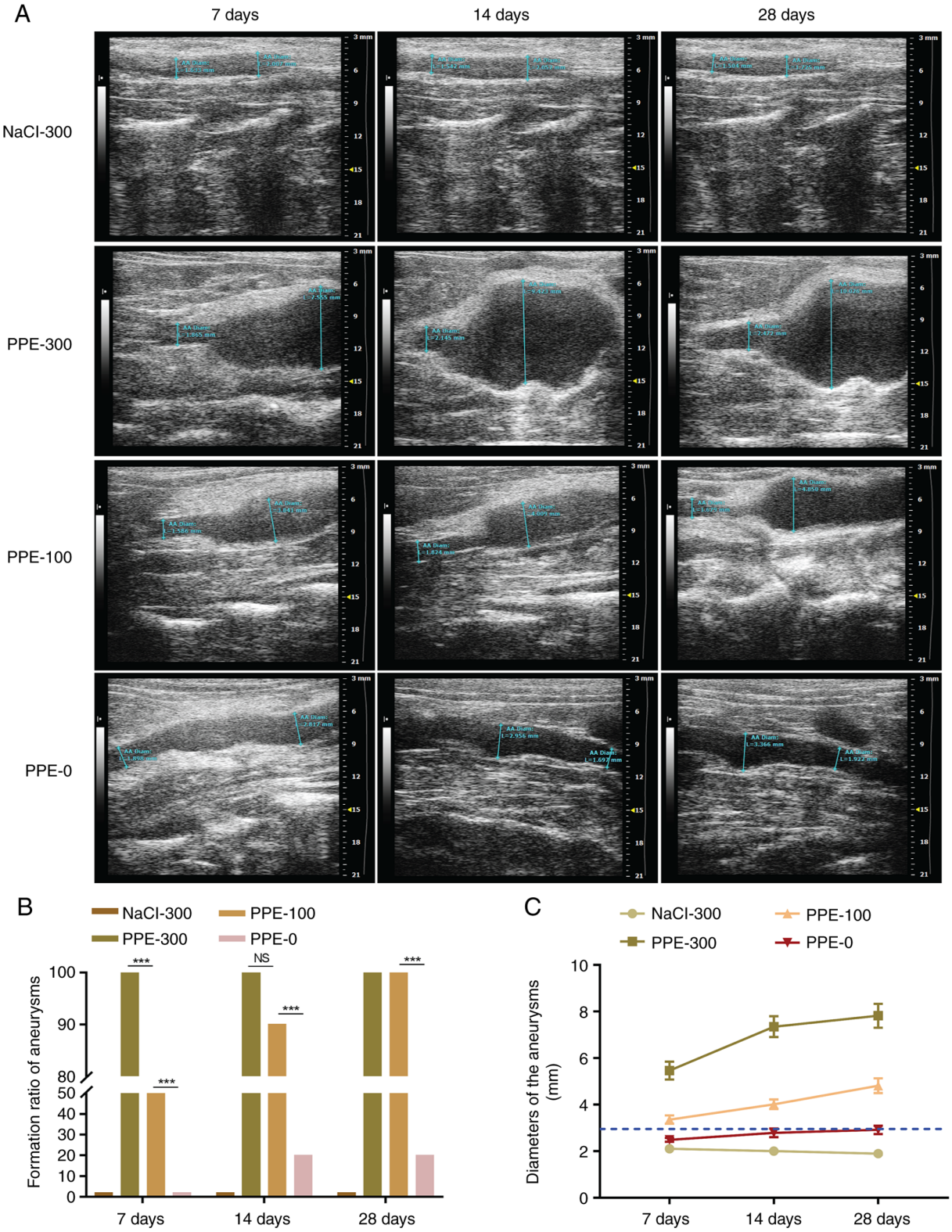

Figure 2. Formation ratio of AAA at different time points. (A) Ultrasound representative image of AAA morphology. Green lines represent the diameter of normal vessels and aneurysms. (B) Formation ratio of AAA at different times. (C) Diameters of AAA at different time points. Data are shown as the mean ± standard deviation. Differences among groups were analyzed using a one-way analysis of variance followed by Tukey's HSD for multiple comparisons. ***P<0.001 (n=9 in PPE-300 and 10 in the other three groups). NS, not significant; AAA, abdominal aortic aneurysm; Diam, diameter; PPE, porcine pancreatic elastase; PPE-300, PPE perfusion at 300 mmHg; PPE-100, PPE perfusion at 100 mmHg; PPE-0, PPE perfusion without pressure; NaCl-300, saline solution perfusion at 300 mmHg.

fibers showed significant degradation in the medium-pressure group (100 mmHg). At 100 mmHg, the degree of elastin degradation in the present study was more evident than that reported in the previous studies (30,31). This divergence may be explained by the leakproofness of the isolated aorta and the difference in elastase concentrations.

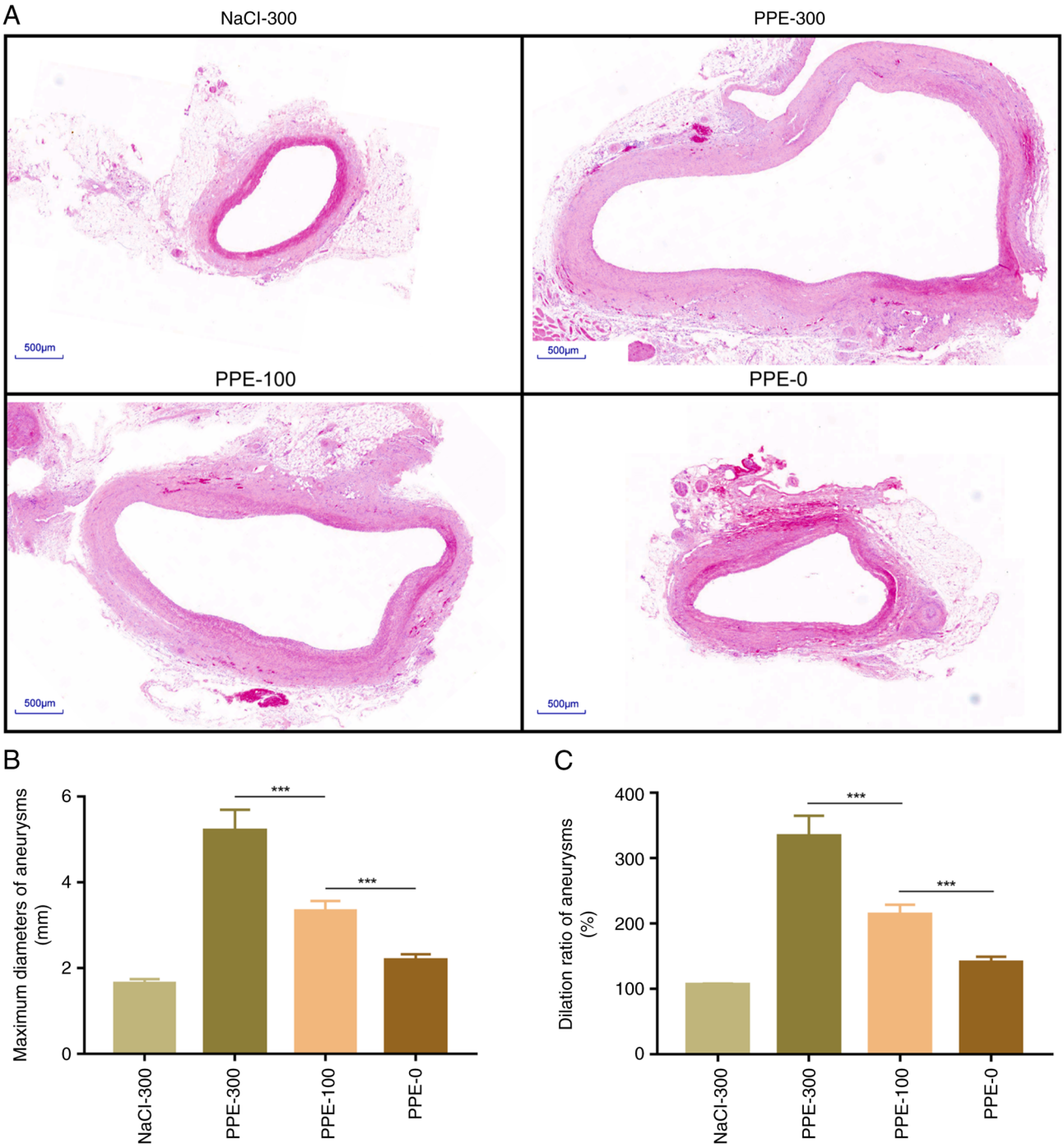

Figure 3. Dilation ratio of AAA 28 days after the operation. (A) Representative H&E staining image of AAA in the four groups. Scale bar, 500 μm. (B) Maximum diameters of aneurysms in the four groups. (C) Dilation ratio of aneurysms 28 days after the operation. The aortic tissue was collected 28 days after the operation and the dilation ratio was calculated based on H&E results. Data are presented as the mean ± standard deviation. Differences among groups were analyzed using a one-way analysis of variance followed by Tukey's HSD for multiple comparisons. ***P<0.001 (n=9 in PPE-300 and 10 in the other three groups). AAA, abdominal aortic aneurysm; PPE, porcine pancreatic elastase; PPE-300, PPE perfusion at 300 mmHg; PPE-100, PPE perfusion at 100 mmHg; PPE-0, PPE perfusion without pressure; NaCl-300, saline solution perfusion at 300 mmHg; H&E, hematoxylin and eosin.

As for the inflammatory cells, some macrophages were found in the vessels of the high-pressure group, which were mainly localized in the adventitia areas, while there were almost no inflammatory cells in the other groups. This phenomenon may be associated with the advanced stage of AAA because there is always a significant inflammatory reaction at the early stage of AAA progression (32). Interestingly, some intimal hyperplasia was found in the aneurysms in the high-pressure group. IHC staining showed that the cells in the intimal hyperplasia area were mainly vascular smooth muscle cells. The present study hypothesized that the reason for the intimal hyperplasia in these vessels may be associated with the loss of elastin and the abnormal hemodynamic changes. A previous study reported that aortic wall smooth muscle cell proliferation may limit the progression of AAA (33). Therefore, the present study hypothesized that the intimal hyperplasia in

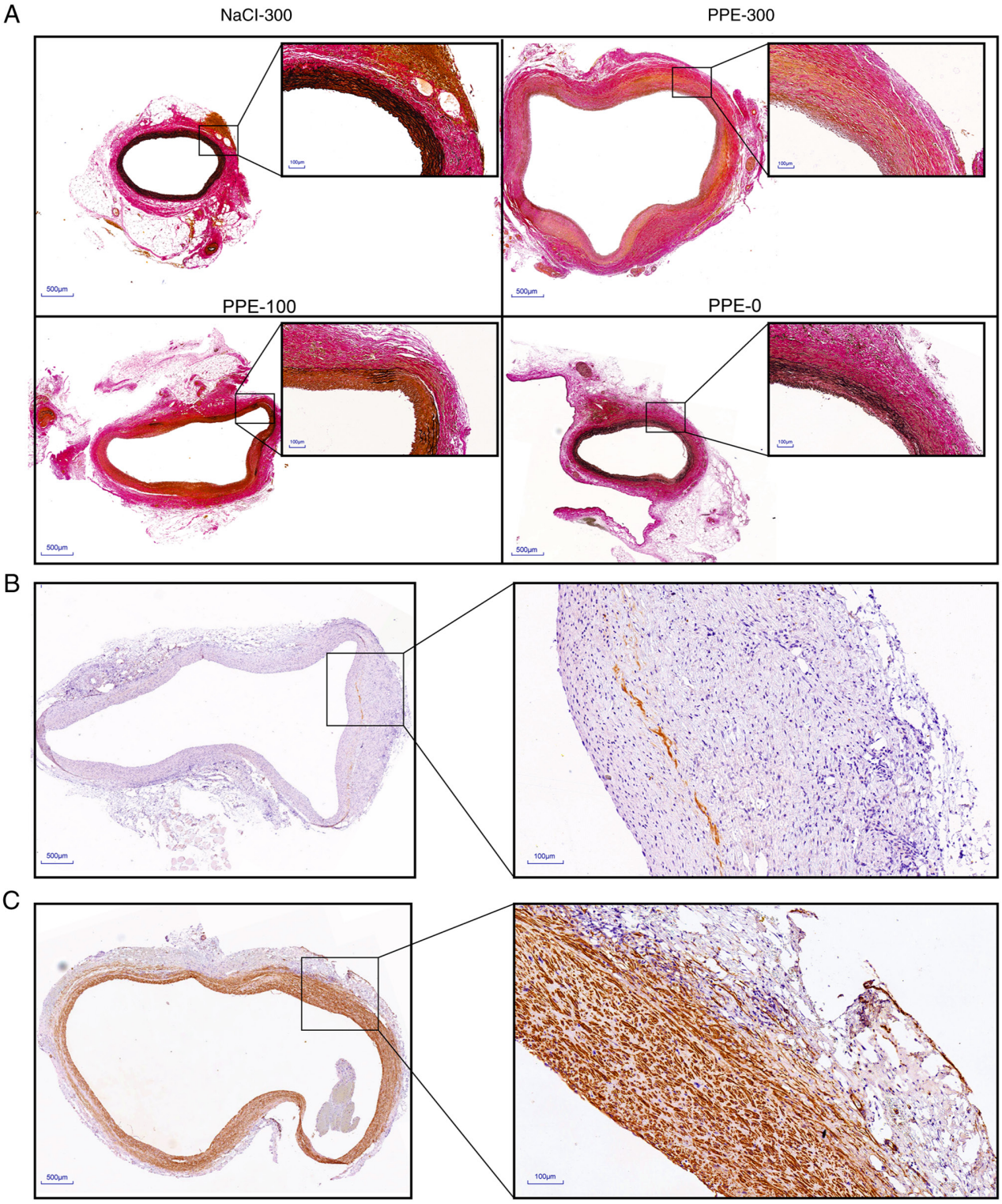

Figure 4. Pathological characteristics of abdominal aortic aneurysm 28 days after the operation. (A) Representative image of PPE concentrations in groups shown with Elastic Van Gieson staining. (B) IHC staining showed that some macrophages were found in aneurysms. This image is from the PPE-300 group. (C) The IHC results indicated that the cell types in intimal hyperplasia were mainly vascular smooth muscle cells. This image is from the PPE-300 group. Main image scale bar, 500 μm (insert scale bar, 100 μm). PPE, porcine pancreatic elastase; PPE-300, PPE perfusion at 300 mmHg; PPE-100, PPE perfusion at 100 mmHg; PPE-0, PPE perfusion without pressure; NaCl-300, saline solution perfusion at 300 mmHg; IHC, immunohistochemistry.

the aneurysms from the high-pressure group may have been a protective reaction under the abnormal hemodynamic changes.

In the present study, only one rat in the high-pressure group underwent an aneurysm rupture and died 5 days after the operation, which was confirmed by exploratory laparotomy. No deaths occurred in the other three groups. The mortality rate in the present study was lower than that reported in the literature (34). Mortality can be associated with several factors including the operation method, perfusion time and elastase concentration (14). The present study hypothesized that, given the lateral

vessels of the isolated aorta were completely ligated, there was limited elastase flowing into the blood cycle, which contributed to a decrease in animal mortality. In addition, the abdomen of the rats was washed three times with saline solution after perfusion. Similarly, the elastase solution inside the isolated aorta was also removed and washed three times with saline solution. The operation method was a factor that impacted animal mortality. A method similar to that described by Hu *et al* (19) was used in the current study, which can also partly explain the lower mortality.

The present study has some limitations. First, the follow-up was 28 days. During follow-up, the elastase perfusion pressure was found to impact the formation ratio at an early stage and the maximum diameters of the AAAs. However, the effects of elastase perfusion pressure on the morphology of aneurysms need to be further explored in long-term follow-up studies. Second, an inflammatory reaction in the early stages of AAA formation cannot be confirmed. To continuously monitor the changes in aneurysm diameters using non-invasive ultrasound, no aneurysm samples were obtained during the early stages of AAA formation, which prevented observing the possible infiltration of inflammatory cells in the vessels. Third, the current findings were obtained in rats, if other animals could present similar results remain to be elucidated. Fourth, the study sample size is limited and the current findings still need to be further confirmed by studies with larger samples.

The diameter of the aneurysm is proportional to the pressure of the perfusion, which more insights into the characteristics of elastase-induced AAA models. The current findings indicated that for some intervention studies a constant perfusion pressure should be maintained to avoid bias in the study results due to perfusion pressure.

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Availability of data and materials

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XL performed the experiments. CQ analyzed the data. JF prepared the manuscript. LT analyzed and interpreted the results. YZ revised the manuscript and CS designed the study. XL and CQ confirm the authenticity of all the raw data. All authors were involved in discussions and commented on the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Animal Care and Use Committee of Fuwai Hospital (Beijing, China; no. 0099-1-8-HX).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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