

Benazepril Inhibits the Formation of Abdominal Aortic Aneurysms in RabbitsYang Fu¹, Jianhua Huang², Huihuan Tang², Xiaocheng Li³, Qi Zhang²¹Department of Vascular Surgery, the First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan 450000, China²Department of Vascular Surgery, Xiangya Hospital of Central South University, Changsha, Hunan 410078, China³Department of General Surgery, the First Affiliated Hospital, Liuzhou, Guangxi 545006, Chinagentlem0423@hotmail.com

Abstract Background: The purpose of this study was to observe the effect of benazepril on the formation of abdominal aortic aneurysm (AAA) in rabbits. **Methods:** Male New Zealand white rabbits were randomly divided into six groups according to the perfusion solution (saline, elastase, and elastase combined with benazepril intervention) and postoperative observation time (two days and seven days). Morphological changes of the abdominal aorta after perfusion and blood pressure changes were observed. The expression of matrix metalloproteinase-9 (MMP-9) and nuclear factor kappa B (NF- κ B) were measured. **Results:** Among the three groups at postoperative day two, there was no significant difference in the mean dilation rate of the abdominal aorta ($P=0.055$). At postoperative day seven, the mean dilation rates were 7.50% (saline perfusion), 120.62% (elastase perfusion), and 39.20% (benazepril intervention). Blood pressure is not significantly correlated with the mean dilation rates of the abdominal aorta ($r=-0.137$). Benazepril partially reduce degradation of elastic fibers and inhibit inflammatory cell infiltration ($P<0.01$). In the benazepril intervention groups, the expression of MMP-9 were decreased in each time group compared with that in the elastase groups ($P<0.01$), and the intranuclear expression of NF- κ B p65 was also decreased compared with that in the elastase groups ($P<0.01$). **Conclusion:** Benazepril can significantly inhibit AAA formation in rabbit models; the mechanism may be related to inhibition of inflammatory infiltration, multilevel down regulation of degradation of extracellular matrix, and protection of elastic fibers.

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Key words: AAA, Inflammation, MMP-9, NF- κ B

1.**Introduction**

Abdominal aortic aneurysm (AAA) is a common life-threatening arterial degenerative disease; currently, the main treatment of AAA is elective surgery based on imaging monitoring, and typically, an AAA diameter greater than 5.5 cm is considered to be an indication for surgery. A large-scale randomized controlled trial found that early elective surgery for small AAAs (diameter 4.0-5.5 cm) could not improve the survival time of the patients^{1,2}. Therefore, drugs that can inhibit AAA formation and growth would greatly improve the current treatment of AAA.

Studies have found that chronic transmural inflammation and destructive remodeling of structural proteins in the medial aortic layer are the main pathophysiological changes of AAA³⁻⁵. Activation of matrix metalloproteinases (MMPs) is the main cause of structural protein degradation in the process of AAA formation. Large amounts of data have shown that macrophage-derived gelatinase MMP-9 is the main enzyme for elastase degradation and plays a critical role in the formation of AAA^{6,7}. As a junction of the signal transduction pathway, nuclear factor kappa B (NF- κ B) is closely related to the development of

inflammation. In its non-activated state, NF- κ B (p50/p65 dimer) is bound by its inhibitory protein I κ B in the form of a trimer and is sequestered in the cytoplasm. With stimulation of external signals, I κ B is phosphorylated by specific kinases and subsequently degraded, which results in the release of a p50-p65 dimer. The free p50-p65 dimer then translocates to the nucleus, binds to κ B sites in the promoter region of its target genes, and regulates the expression of its target genes. These target genes include many inflammatory mediators, including interleukin-1 (IL-1) and interleukin-6 (IL-6), and some matrix metalloproteinases, including MMP-1,3, and 9⁸⁻¹³. Pyrrolidine dithiocarbamate (PDTC, a specific inhibitor of NF- κ B) inhibits the activity of MMP-9 and can effectively inhibit the formation of AAA in experimental rabbits¹⁴.

Angiotensin-converting enzyme inhibitors (ACE-I) not only have an anti-hypertensive effect but can also directly inhibit cardiovascular remodeling^{15,16}. A retrospective analysis of 15,326 patients with AAA in Canada showed that the incidence of rupture was significantly lower in patients treated with ACE-I, while other anti-hypertensive drugs did not have this

effect¹⁷. In order to explore the effect and mechanism of ACE-I on the formation of AAA, we established an elastase perfusion AAA rabbit model.

2. Material and Methods

2.1 Surgical procedures After obtaining approval from the Laboratory Animal Science Committee of Central South University, we selected 42 healthy male New Zealand white rabbits (1800-2200 g) and perfused the infrarenal abdominal aorta with elastase¹⁸. After entering the rabbit abdominal cavity and exposing the abdominal aorta and inferior vena cava, a segment of the abdominal aorta (approximately 1 cm long) without branches in the anterior and lateral wall was selected (if the branches could not be avoided, they were ligated with #2-0 silk sutures); lumbar arteries in the posterior wall of this segment did not need to be fully mobilized. Then, the proximal end of this segment of the abdominal aorta was blocked with a noninvasive bulldog clamp, the common iliac artery on one side was punctured with a BD intima-II closed intravenous catheter (0.7*19 mm), and the needle after the catheter entered this segment of the abdominal aorta. Next, the catheter was advanced until it was 2 cm away from the bulldog clamp, the abdominal aorta was ligated at the site close to its branches by tying a slipknot with #0 silk suture to fix the catheter, and the lumbar artery was blocked behind the abdominal aorta occluded with a bulldog clamp (Figure 1). The catheter was connected with a microperfusion pump with the perfusion pressure maintained at 100 mmHg. After 30 minutes of perfusion, the catheter was withdrawn, and the punctured site on the iliac artery was sutured with #6-0 silk suture to restore blood flow in the abdominal aorta. Then the abdominal cavity was closed layer by layer with #0 silk suture. Animals were kept in separate cages after surgery.

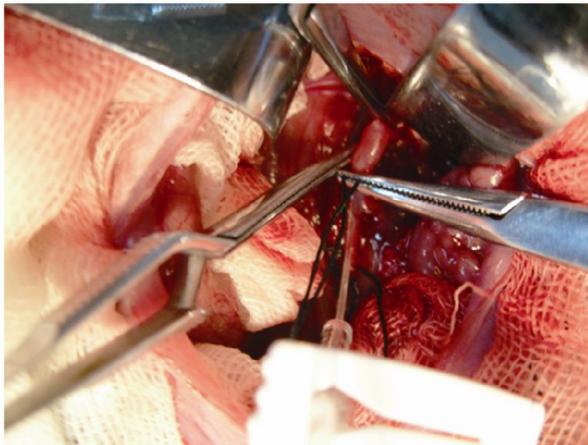


Figure 1. A blocked segment of the infrarenal abdominal aorta for catheterized perfusion.

During surgery, we measured the diameter of the abdominal aorta before perfusion (AD pre) and after perfusion (AD post, 5 minutes after blood flow resumption) using an operating stereomicroscope (Leica, Deerfield, Ill) and a calibrated ocular grid. The second surgery was performed in rabbits two days or seven days later, and the final abdominal aortic diameters (AD final) were measured. After the second surgery, the perfused segment of abdominal aorta was harvested and divided into two segments; one segment was placed into liquid nitrogen and then immediately transferred into a -70°C freezer for preservation, and the other segment was placed into 10% neutral formalin and fixed overnight for routine paraffin-embedding.

2.2 Animal grouping and treatment Rabbits were numbered and randomly divided into the following groups: saline perfusion control group (group A, 10 rabbits), elastase perfusion control group (group B, 16 rabbits), and benazepril experimental group (group C, 16 rabbits). After surgery, rabbits in each group were randomly and equally divided into two groups, the two-day group and the seven-day group. The perfusion solution in group A was saline and that of groups B and C was type I porcine pancreatic elastase solution (E1250, Sigma, US). Rabbits in group C were administered 3 mg/kg body weight/day benazepril intragastrically from one day before perfusion until one day before harvesting the samples.

2.3 Measurement of blood pressure OMP-7201 Life Scope monitor was used to measure the systolic pressure and diastolic pressure of three rabbits per group when punctured with a catheter before the first surgical perfusion, and then the mean arterial pressure was calculated. Mean value of three times of measurement in five minutes was calculated as blood pressure before perfusion (BP pre). The same method was employed in the second surgery to determine the mean arterial pressure as the final blood pressure (BP final).

2.4 Histological staining Aortic tissue cross sections (5 μ m) were examined with hematoxylin-eosin (HE) and verhoeff-van gieson (VVG) staining. Inflammation was detected by HE staining. Sections from three animals in each group were scored using a one to five-point scale for the extent of inflammation by three observers blinded to the experimental design. Elastic fiber was stained to black by VVG staining and quantitatively evaluated for changes in the aortic wall. The percentage of elastin content in the entire aortic wall was calculated by a morphometry system (MacSCOPE, Version 2.2, Mitani Corporation, Japan).

2.5 Western blot Tissue MMP-9 total protein and NF- κ B nucleoprotein were extracted using protein extraction kits (Pierce Chemical, Lot No:78503 for MMP-9, Lot No:78833 for NF- κ B) and quantified by

Coomassie brilliant blue staining. The nucleoprotein was extracted as follows: frozen issue was dissolved in PBS and homogenized, after 5 minutes standing on ice, the mixture was centrifuged for 2 minutes at 500 rpm under 4°C and the supernatant was discarded; then Cytoplasmic Extraction Reagent and protease inhibitors were added and the solution underwent 15 seconds high-speed vortex and 10 minutes standing on ice; after that, 5 seconds high-speed vortex and 5 minutes centrifugation at 16000 rpm under 4°C were performed and the supernatant was discarded. Nuclear Extraction Reagent and protease inhibitor were added in precipitation and 15 seconds high-speed vortex was done. The mixture was placed on ice for 40 minutes and 15 seconds high-speed vortex was done every 10 minutes. After 5 minutes centrifugation at 16000 rpm under 4°C, the supernatant was collected, which was the nuclear protein. Samples were subjected to SDS-polyacrylamide gel electrophoresis for 1 hour, and then transferred to polyvinylidene difluoride (PVDF) for 70 minutes. Blots were blocked overnight with 1% Casein, sectioned, and incubated with appropriate primary antibodies for 2 hours. Primary antibodies included rabbit anti-NF- κ B p65 (polyclonal antibody, 1:500, BD, NJ) and rabbit anti-MMP-9 (polyclonal antibody, 1:500, Chemicon, US). GAPDH (1:1000, Sigma-Aldrich, US) and PCNA (1:500, Santa Cruz, US) were used as internal controls for MMP-9 and NF- κ B p65, respectively. The blots were then incubated for 1 hour in the appropriate secondary antibodies. Proteins were visualized using an enhanced chemiluminescence system (ECL, Pierce Chemical). Bands were quantified by densitometry.

2.6 Statistical analysis The data was analyzed using a SPSS 17.0 software package. Data in each group was shown as mean \pm standard deviation (mean \pm SD); the differences of means between two groups were compared using independent sample *t* tests and were compared using single factor analysis of variance (ANOVA) among multiple groups. The related samples were analyzed by paired *t* test. Person test was used to analyze correlation. $P < 0.05$ was selected as the standard for significant difference.

3. Results

3.1 Dilation rate of the abdominal aorta

The mean dilation rates of abdominal aortic diameter after perfusion are shown in Figure 2. There were approximately 50% dilation rates in each group immediately after perfusion, and the rates were not significantly different among the groups ($F=1.401$, $P=0.257$). In the two-day groups (A2, B2, C2), the aortic diameters in each group decreased two days after perfusion compared with those immediately after perfusion, but still increased compared with the

diameters before perfusion. The mean dilation rates of group A2, B2, and C2 were 19.18%, 29.58%, and 24.94%, respectively, and there was no significant difference among the three groups ($F=5.330$, $P=0.055$). In the seven-day groups (A7, B7, C7), the mean abdominal aortic dilation rates were 7.50%, 120.62%, and 39.20%, respectively. The mean aortic dilation rate of group B7 at the time of sampling was statistically different than those of A7 and C7 ($P < 0.01$). An abdominal aortic dilation rate of more than 100% was selected as the diagnostic criteria for AAA. Accordingly, all the rabbits in group B7 had AAA, while there was no AAA formation in rabbits in group C7.

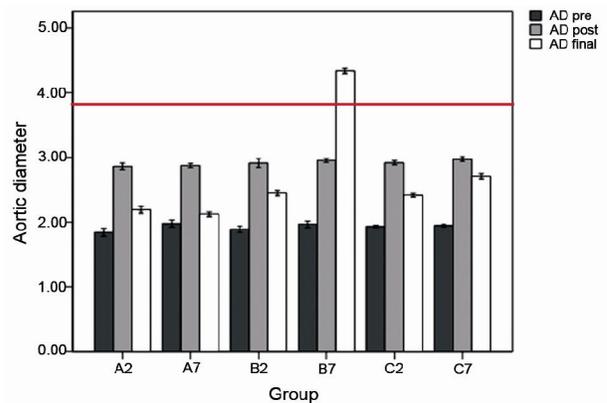


Figure 2. The aortic diameters

The aortic diameters at different times in each group are shown as the mean \pm standard error by bar graph. The red line represented the mean preoperative aortic diameter of all experimental rabbits* (1 +100%), which was also the criteria for AAA in this study.

3.2 Changes in arterial pressure

The rabbit blood pressure was monitored to find out whether the antihypertensive effect of benazepril was involved in the inhibiting of benazepril of AAA formation. Our results showed that there was no significant difference in BP pre between each group of rabbits ($F=0.164$, $P=0.974$, Table 1). Paired *t* test showed that BP final was not significantly different from BP pre in each group ($P > 0.05$). Person correlation analysis showed that BP final was not obviously correlated with the final abdominal aortic dilation rates ($r=-0.137$, $P=0.456$).

The systolic pressure and diastolic pressure were measured directly by a catheter puncturing into the iliac artery before first surgical perfusion. Mean arterial pressure = (systolic pressure + 2*diastolic pressure)/3. Mean value of three times of measurement was calculated in three rabbits from each group. Data were shown as mean \pm SD.

3.3 Histological staining

In the saline infusion groups (groups A2 and A7), there was no significant inflammatory cell

infiltration, and the structures of elastic fibers were continuous and intact. In groups B and C, there was significant inflammatory cell infiltration of the arterial walls after elastase perfusion. However, there was no significant difference in the degree of inflammation between group B2 and group C2 ($P=0.297$), while there was a significant difference between group B7 and group C7 ($P=0.028$). The content of elastic fibers in group B2 decreased after elastase perfusion and significantly decreased in group B7, characterized by

elastic fiber rupture and degradation and vacuole formation. In the benazepril intervention groups (group C2 and C7), the content of elastic fibers also decreased in group C2, and there was no significant difference compared with group B2 ($P=0.176$). The content of elastic fibers in group C7 decreased further; however, when compared with group B7, the content and structural integrity of elastic fibers in group C7 were superior ($P<0.01$). (Figure 3)

Table 1. Measurement of mean arterial pressure of rabbits of each group.

Mean BP (mmHg)	Group A2	Group A7	Group B2	Group B7	Group C2	Group C7
BP pre	88.9±3.0	89.4±4.7	89.5±3.5	90.8±3.3	89.4±3.1	90.1±3.6
BP final	86.8±2.8	89.0±2.9	88.3±3.3	87.5±2.6	88.4±3.6	86.8±2.0

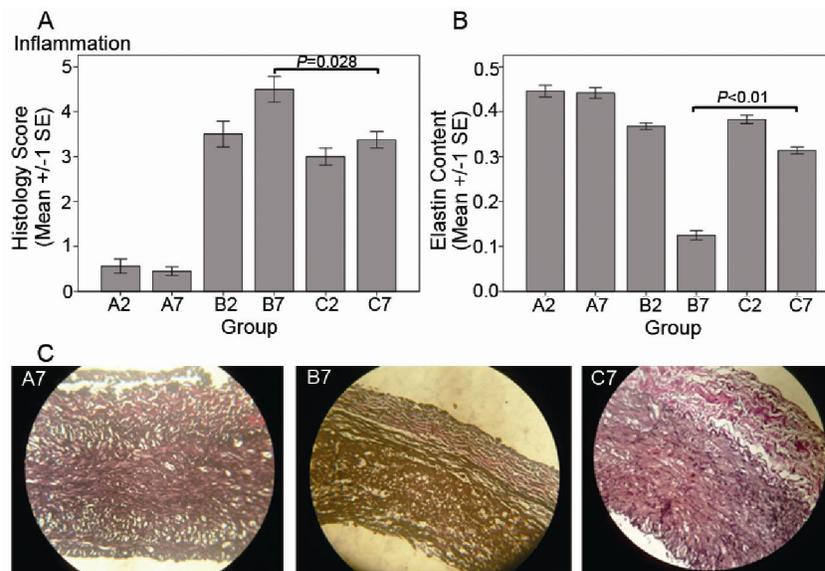


Figure 3. the degree of inflammation

A. There was no significant difference in the degree of inflammation between group B2 and group C2 ($P=0.297$), while there was a significant difference between group B7 and group C7 ($P=0.028$). B. The content of elastic fibers decreased in group C2, and there was no significant difference compared with group B2 ($P=0.176$). The content of elastic fibers in group C7 decreased further, and there was a significant difference between group B7 and group C7 ($P<0.01$). C. VG staining in each group (VG*100): Group A7- The elastic fibers were continuous and intact. Group B7- Elastic fibers in the medial arterial wall were significantly injured, noncontinuous and had a large amount of vacuole formation. Group C7- Continuous elastic fibers can be seen in the medial arterial wall. The content and structural integrity were both significantly better than those of group B7.

3.4 Western blotting

The results of MMP-9 expression in each group are shown in Figure 4. There was no MMP-9 protein expression in either of the two saline perfusion groups. The expression of MMP-9 in elastase perfusion groups increased two days after surgery (group B2) and increased significantly seven days after surgery (group B7). This was significantly different compared with group B2 ($P<0.01$). After benazepril intervention, MMP-9 expression in each time group (group C2 and C7) showed a significant decrease ($P<0.01$).

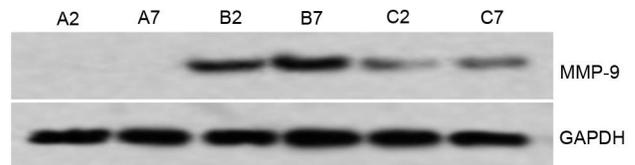


Figure 4. Expression of MMP-9 in each group

The intranuclear expression of NF- κ B p65 was also detected using western blot analysis. Non-activated NF- κ B p65 is bound in the cytoplasm by its inhibitor

I B . After phosphorylation and subsequent degradation of I B , NF- B p65 is activated and then translocates to the nucleus. Therefore, the detection of intranuclear expression of NF- B p65 is equivalent to detection of its activity. The results are shown in Figure 5. There was no intranuclear expression of NF- B p65 in either of the two saline perfusion groups. In the elastase perfusion groups, the intranuclear expression of NF- B p65 after perfusion was increased in both the two-day group (B2) and the seven-day group (B7); however, there was no significant difference between the two groups ($P=0.092$). In the benazepril intervention groups, the intranuclear expression of NF- B p65 in the two-day group (C2) and the seven-day group (C7) both decreased compared with those in group B, and there were significant differences between groups B2 and C2 ($P<0.01$) and between group B7 and C7 ($P<0.01$). These results suggested that the activation of NF- B was inhibited after intervention with benazepril.

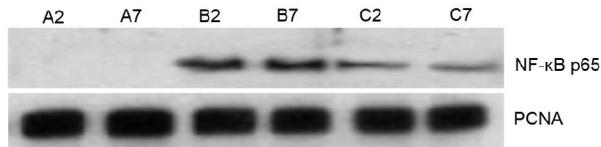


Figure 5. Intranuclear expression of NF- B p65 in each group

4. Discussions

Abdominal aortic aneurysm (AAA) is a common life-threatening disease. Currently, chronic transmural inflammation and destructive remodeling of structural proteins in the medial layer of the aorta are considered to be the most significant pathophysiological changes associated with AAA. Some studies indicate that ACE-Is may inhibit the formation and development of AAA, but there have been no further studies on its mechanism. In this study, we focused on dynamic changes of MMP-9 and NF- B as indicators, and for the first time, we found that benazepril could protect the medial aortic structural proteins and partially inhibit the infiltration of inflammatory cells by inhibiting the expression of MMP-9 and the activity of NF- B, thereby inhibiting AAA formation.

The results of this study showed that the content of elastic fibers started to decrease two days after elastase perfusion, and a large amount of inflammatory cells infiltrated the arterial wall, accompanied by increased MMP-9 expression and NF- B activation. However, there was no significant change in aortic diameter. This may be due to the considerable amount of elastic fibers that can resist arterial pressure. ACE-Is could partially inhibit MMP-9 expression and NF- B activation but could not prevent the decrease of elastic fiber content and inflammatory cell infiltration

completely. In the later stage of this study, in groups without benazepril intervention, the expression of MMP-9 in the abdominal aorta continued to increase, NF- B remained in an activated state, and the number of infiltrated inflammatory cells increased further. Additionally, the degraded fragments of elastic fibers could further induce inflammatory cell infiltration and increase the release of MMP-9, thereby causing a cascade that would significantly decrease aortic elastic fibers and lead to an AAA. ACE-Is could inhibit the continued expression of MMP-9, provide more protection to elastic fibers, inhibit activation of NF- B, reduce inflammatory cell infiltration, and block this cascade effect.

The current study shows that benazepril did not lower the rabbit blood pressure while inhibiting AAA formation, suggesting AAA suppression may not correlate with blood pressure decrease. However, no blood pressure reduction after a relatively short period of drug treatment in this study can not exclude the possibility of lowering blood pressure by long-term application of benazepril in rabbits. Besides, the dosage of benazepril required to suppress tumor, determined by preliminary experiments, may be lower than that decreasing blood pressure. Rabbits with normal blood pressure rather than those with hypertension were used in this study. Suguru^[19] found that AAA diameter in rats with hypertension increased more rapidly than that in rats with normal blood pressure after perfusion with elastase. In any case, these results at least indicate that the function of benazepril in lowering blood pressure is not the main mechanism of its effect in AAA inhibition in rabbits.

The inhibitory effect of ACE-I on MMP-9 may be through the following mechanisms. (1) Inhibition of MMP-9 activity by direct binding. Angiotensin-converting enzymes (ACE) are zinc-dependent endopeptidases produced by macrophages and smooth muscle cells. Some researches found that ACE-I inhibits MMP-9 activation in rat kidney and human myocardium in in vitro experiments^{20, 21}. Another study found that, because there was a zinc binding structure (histidine side chain) in the activation center of MMP-9, which was similar to ACE, ACE-I could bind MMP-9 via two pathways to inhibit MMP-9²². (2) Inhibition of the expression of MMP-9 through inhibiting NF- B. Nakashima et al.²³ found that formation of AAA and expression of MMP-9 in aortic tissues in rats could be effectively inhibited by inhibiting the activity of NF- B using oligodeoxynucleotide. A study of Lawrence et al.²⁴ used the immunosuppressant rapamycin to inhibit the formation of AAA in experimental rats and found a similar effect. In this study, the results showed that inhibition of NF- B activation was accompanied by down regulation of MMP-9 expression levels after

benazepril intervention. NF- κ B is an important component of Ras and vascular inflammation. Angiotensin II activates the nuclear factors in macrophages and smooth muscle cells through one subtype of AT-1 receptor. After dephosphorylation, these activated nuclear factors translocate into the nucleus and up-regulate the expression of cytokines, adhesive molecules, and other inflammatory mediators, such as MCP-1, IL-6, and a variety of growth factors²⁵. In a rabbit model of atherosclerosis, quinapril was found to reduce NF- κ B-mediated expression of pro-inflammatory response factors²⁶. (3) Direct inhibition of inflammatory cell infiltration to reduce the synthesis and release of MMP-9. (4) A possible direct protective effect on elastic fibers. An experimental animal model developed by Isenburg²⁷ was used to show that the protective agent of elastic fibers could inhibit protease-mediated elastolysis simply through binding the hydrophobic area in elastic fibers, thus inhibiting formation of AAA without downregulation of MMP-9 and inflammation inhibition. However, further research is needed to explore whether ACE-I has this effect.

Acknowledgments

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Competing interests

The author declare that they have no competing interests.

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