The active participation of p22^{phox}-214T/C in the formation of intracranial aneurysm and the suppressive potential of edaravone

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Abstract. Oxidative stress reactions play an important role in the pathogenesis of intracranial aneurysm (IA). p22^{phox} is involved in the oxidative stress reaction, and it is a critical subunit of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. The present study investigated the association of genetic variants within the gene encoding p22^{phox}-214T/C with IA. The p22^{phox}-214T/C gene polymorphisms in 192 cases of IA and 112 controls were analyzed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The mRNA expression of NADPH oxidase was also analyzed by RT-PCR. The results of RT-PCR were validated by ELISA. In a rabbit model of elastase-induced aneurysm, we used edaravone for anti-oxidative stress treatment to observe the curative effects. In the clinical cases, a significant difference in p22^{phox}-214T/C allele frequencies in the IA group was observed compared with the control group (P<0.001). The expression level of NADPH oxidase was differed significantly between the IA group and the control group. In the rabbit model of elastase-induced aneurysm, the success rate of the aneurysmal model in the edaravone group and the wound ulcer rate were lower than those in the control group. In addition, the diameter of the aneurysm was smaller than in the edaravone group than in the control group (3.26±0.13 mm vs. 3.85±0.07 mm), and the expression of matrix metalloproteinase-9 (MMP-9) was significantly lower than that in the control group (P<0.0001). Thus, these data suggest the active participation of p22^{phox}-214T/C in the formation of IA and the suppressive potential of edaravone against IA formation.

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Introduction

Intracranial aneurysm (IA) is a common cerebrovascular disease, and ruptured IA remains life-threatening. With the improvement of surgical and intervention techniques, the treatment of IA has achieved superior results (1-3). However, difficulties still exist in the treatment of complex IAs (4-7). In the treatment of complex IA that is complicated by severe atherosclerosis, surgical clipping may lead to fatal bleeding, causing the failed blockage of the internal carotid artery temporarily. The passage of the guide wire through the internal carotid artery remains difficult to be passed and thus, the embolization cannot be completed. This type of aneurysm requires novel technical treatment; however, to date, and at least to the best of our knowledge, no practical reports of drug treatment for IA are available. Thus, the understanding of the biological characteristics of IA development and the identification of molecular markers that can help pinpoint people at high risk are crucial to the development of novel therapeutic strategies.

It is now widely accepted that oxygen free radicals contribute to the pathogenesis of ischemic cerebrovascular disease (8-10). The nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system is an important enzymatic source of oxygen free radicals (11). p22^{phox} is an important component of both phagocytic and nonphagocytic NADPH oxidase (12-14).

It has been demonstrated that the NADPH oxidase system is involved in the pathogenesis of atherosclerotic vascular disease (15). However, previous studies have mainly focused on the association of p22^{phox} in abdominal aortic aneurysm, thoracic aortic aneurysm and ischemic disease (16-18), while fewer scholars have investigated the association between p22^{phox} single nucleotide polymorphisms (SNPs) and IA (19).

A few scholars have emphasized the role of oxidative stress in abdominal aortic aneurysmal (AAA), suggesting that antioxidant therapy may be a novel therapeutic strategy with which to delay AAA progression (20). Some antioxidant treatments, such as apocynin and vitamin E (21,22) have been reported to prevent the formation of AAA. However, some other researchers have presented significantly lower plasmatic levels of vitamin A and vitamin E in patients suffering

from subarachnoid hemorrhage (SAH) than in the controls, speculating that the NADPH oxidase system may influence the rupture of IA (23).

Theoretically, IA formation can be inhibited by antioxidants. In the clinical setting, although low circulating vitamins, such as B6/C/D/E (not B12) levels are associated with the presence of AAA, the supplementation of vitamins B6/B12/E may not reduce the incidence of AAA (24). Previous studies have demonstrated that matrix metalloproteinase (MMP)-2 and MMP-9 are required for the formation of AAA (25,26) and that reactive oxygen species (ROS) induce the apoptosis of vascular smooth muscle cells (VSMCs) (27). Edaravone is a widely used free radical scavenger in patients with acute cerebral infarction, and its administration has been shown to inhibit the production of ROS, preventing aneurysm formation and expansion in a rat model of AAA (28). The free-radical scavenger, edaravone, may thus be an effective pharmaceutical agent for use in the treatment of IA in clinical practice.

The association between p22^{phox} and the occurrence and development of IA has not been reported to date, at least to the best of our knowledge, and there is still no ideal pharmaceutical treatment for IA. We hypothesized the active participation of p22^{phox}-214T/C gene (rs4673) polymorphisms in IA formation and the suppressive potential of edaravone against IA formation. Hence, this study aimed to explore the role and mechanisms of action of p22^{phox} in the pathogenesis of IA, and to examine the inhibitory effects on oxidative stress and the curative effects in an effort to provide a novel strategy for the treatment of IA.

Materials and methods

Study population. This case-control study was approved by the Research Ethics Committee of Taihe Hospital, Hubei University of Medicine, Shiyan, China. A total of 192 patients (77 males and 115 females; age range, 15-76 years; median age, 54±6.6 years) with IA and 112 healthy volunteers (61 males and 51 females; age range, 14-65 years; median age, 45±4.8 years) from Taihe Hospital (between March, 2013 to October, 2016) participated in this study. The specific inclusion criteria and personnel structure were consistent with those previously reported (29). Informed consent was obtained from all the patients. All specimens were handled and made anonymous according to the ethical and legal standards.

Amplification of the p22^{phox}-214T/C (rs4673) locus and DNA sequencing. According to previous reports (30-32), venous blood was collected from patients with IA on an empty stomach and from healthy volunteers and was used for the isolation of genomic DNA according to the kit instructions (MBI Fermentas, Burlington, ON, Canada). The following primers were used: 5'-TGCTTGTGGGTAAACCAAGG-3' and 5'-GGAAAAACACTGAGGTAAGTG-3'. The amplification conditions were consistent with those of a previous study (29).

Analysis of NADPH oxidase expression by RT-PCR. According to a previous study (18), total RNA was randomly extracted from the vein samples of 3 patients with IA and from 4 anonymous blood donors using regular TRIzol reagent (15596-026, Thermo Fisher Scientific, Inc., Waltham, MA, USA). The concentrations, as well as purity were assayed using an ultraviolet spectrometer (NanoDrop One, Thermo Fisher Scientific, Waltham, MA, USA). A reverse transcription kit (KR116, Tiangen Biotech, Beijing, China) was used for cDNA synthesis, and the primer sequences were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) according to the sequences of GenBank: 5'-GTTTTGAAAGCTACC GCAGAAC-3', and 5'-GGAACCTTTTGTCTTCCTG ATG-3'. GAPDH was used as an internal control. According to the sequences of GenBank: 5'-CCATGTTCGTCATGGGTG TGAACCA-3', and 5'-GCCAGTAGAGGCAGGGATGTTC-3'. The reaction system was set consistent with that of a previous study (18).

Animals. This study was approved and conducted according to the guidelines set by the Experimental Animal Center of the Hubei University of Medicine (Shiyan, China). All animal experiments were performed in the Experimental Animal Center according to the protocols approved by the Animal Ethics Committee of the Hubei University of Medicine. Adult male Japanese white rabbits (weighing, 2.32±0.25 kg; >5 months of age, ordinary food rearing) were provided by the Experimental Animal Center of Hubei University of Medicine. Those rabbits were randomly divided into 3 experimental groups as follows: The edaravone group (edaravone 2 mg/kg/day, n=18), the control group (saline, 4 ml/day, n=18) and the normal group (normal rabbits, n=10). Edaravone (Simcere Pharmaceutical Co., Ltd., Nanjing, China) or saline (0.9% NaCl, Taihe Hospital, Shiyan, China) was intravenously injected into the mice twice daily, beginning from 7 days after aneurysm preparation to 21 days. Edaravone was intravenously administered for 14 days twice daily respectively. The dose and the route of administration of edaravone were according to previous reports (28,33).

Aneurysm creation. The animals were sedated by an auricular vein intravenous injection of 3% pentobarbital sodium (30 mg/kg). For the rabbits in the edaravone group and the control group, the middle of the right common carotid artery (RCCA), approximately 2 cm long, was temporarily occluded with two aneurysm clips. Subsequently, 20 units of pancreatic elastase was injected into the clipped RCCA, and pancreatic elastase leakage was prevented. In order to minimize post-operative infection in the rabbits with aneurysm, the animals were administered penicillin (North China Pharmaceutical Group Co., Ltd., Shijiazhuang, China) intramuscularly at 0.1 million units per kilogram for 7 days after surgery. The specific surgical procedures and subsequent infection prevention measures were as previously described (34).

ELISA. The plasma from 10 random patients with IA and 10 donors using EDTA as an anticoagulant was collected. The samples were centrifuged for 15 min at 1,000 x g at 2-8˚C within 30 min of collection. The plasma was then removed and assayed immediately or sampled in aliquots at -20˚C. The detection of NADPH oxidase in plasma was achieved by ELISA following the manufacturer's instructions (15272, Amplite Colorimetric NADPH Assay kit; AAT Bioquest, Sunnyvale, CA, USA). Each sample was assayed in triplicate.

Table I. Allele frequencies of $p22^{phox}$-214T>C gene polymorphism in patients with IA and controls.

	No.	T allele	C allele	P-value
Total numbers	303	101	202	
Controls	112	86	26	
Aneurysmal SAH	191	25	166	<0.001
IA single	147	8	139	
IA multiple	44	3	41	0.731
IA anterior	177	9	170	
IA posterior	13	2	9	0.070
IA anterior+posterior	1	0	1	0.818

P-value indicates the differences between 2 groups (such as cases and controls or IA single and IA multiple). SAH, subarachnoid hemorrhage; IA, intracranial aneurysm. There were patients with 192 IA in this study. For one patient with IA, no results in the blood specimen were available which may be due to the improper handling of the specimen.

Table II. Results obtained from the rabbit model of elastase-induced aneurysms.

Group	Control	Edaravone
Total numbers	18	18
Intraoperative death	1	2
Postoperative death	0	0
Aneurysm	14	10
Aneurysm rate	82.3%	62.5%
Wound ulcer rate	35.3%	12.5%
Ear vein	5	2
Operative incision	2	1
Ear vein + incision	1	1
Mean diameter (mm)	3.85±0.07	3.26±0.13

Intraoperative death in the rabbits was caused by the anesthesia or the loss of blood during the surgery. The aneurysm rate and wound ulcer rate values did not include the rabbits lost during the anesthesia and the surgery. The mean diameter is the mean the average value of the maximal fusiform aneurysm diameter or maximum diameter of the common carotid artery.

The plasma of rabbits was also collected using use EDTA as an anticoagulant. The detection of NADPH, MMP-2 and MMP-9 in the plasma was achieved by ELISA according to the manufacturer's instructions (15272, Amplite Colorimetric NADPH Assay kit; AAT Bioquest, Sunnyvale, CA, USA, E-EL-RB1540c, rabbit MMP-2 ELISA kit and E-EL-RB1997c, rabbit MMP-9 ELISA kit; Elabscience, Wuhan, China). Each sample was assayed in triplicate.

Histological analysis of elastase-induced aneurysms. The experimental animals were sacrificed after 3 weeks. Each rabbit was humanely euthanized through transcardial perfusion while under deep anesthesia [auricular vein intravenous injection of 3% pentobarbital sodium (30 mg/kg)]. The diameter of the elastase-induced aneurysms was measured, and the aneurysms or the common carotid artery samples were fixed in 4% paraformaldehyde and then embedded in paraffin for histopathological examination. The sections (5-μm-thick) were cut and mounted on saline-coated slides. The slides were stained with hematoxylin and eosin (H&E) and CD34 (ZM-0046, 1:200, ZSGB-BIO, Beijing, China) for histopathological and immunohistochemical analyses using standard techniques and reagents. The sample was exposed to an immunospecific primary antibody diluted in 5% goat serum overnight at 4˚C, then to a goat-anti-mouse secondary antibody (PV-6002, ZSGB-BIO) for 1 h at room temperature (35,36). Measurements were performed with an optical microscope (DP73; Olympus, Tokyo, Japan; x4 to x40 magnification) that was attached to a video camera and connected to a computer equipped with image analysis software (cellSens Standard software, Olympus).

In order to determine whether the rabbit aneurysm models were successfully modeled, we selected typical human aneurysm specimens as a control group. The control group was a specimen of a large middle cerebral artery aneurysm in a 53-year-old female hospitalized in May, 2017 and a renal artery specimen of a 50-year-old male renal cell carcinoma admitted to the hospital at the same time. Informed consent was obtained from all the patients. CD34 antibody (ZA-0550, 1:200, ZSGB-BIO) was used for histopathological and immunohistochemical analysis using standard techniques and reagents. The sample was exposed to an immunospecific primary antibody diluted in 5% goat serum overnight at 4˚C, then to a goat-anti-rabbit secondary antibody (PV-6001, ZSGB-BIO) for 1 h at room temperature. All specimens were handled and made anonymous according to the ethical and legal standards. This case-control study was approved by the Research Ethics Committee of Taihe Hospital, Hubei University of Medicine.

CD34 is a known marker of circulating progenitor cells. Studies have examined the role of CD34 cells in AAA and peripheral vascular disease (PVD) (37,38). The number of CD34-positive cells was counted using an optical microscope (DP73; Olympus) within 3 separate fields.

Study course and statistical methods. SPSS software version 17.0 for Windows (SPSS Inc., Chicago, IL, USA) and GraphPad Prism software 6.07 for Windows (GraphPad Software, Inc., La Jolla, CA, USA) were used for statistical analysis. Data are presented as the means ± standard deviation (SD). A P-value <0.05 was considered to indicate a statistically significant difference. Deviation from the Hardy-Weinberg equilibrium was evaluated by comparing the observed and expected genotype frequencies by an exact goodness-of-fit test separately in the IA and control groups. The influencing factors of IA, genotype and allele frequencies were analyzed using the χ^2 test. Differences in the ELISA results were compared using the t-test (for 2 groups). One-way ANOVA was performed to compare the multiple independent variables (3 or more groups). Post hoc adjusted comparisons were performed timely with Bonferroni correction and were considered significant at P<0.05.

Results

Increased IA formation with p22^{phox}-214T>C single nucleotide variation. Genotype distributions were consistent with the Hardy-Weinberg equilibrium in both the patient and control groups. The genotype and allele counts in patients and controls, as well as the χ^2 and P-value are presented below. The frequencies of p22^{phox}-214T>C (rs4673) genotypes in the patients with IA differed significantly from those of the control group (P<0.001). In the IA group, the p22^{phox}-214T>C allele frequencies were 86.9% (166 IA patients with the C allele divided by 191 total IA patients), while they were 23.21% (26 healthy volunteers with the C allele divided by 112 total healthy volunteers) in the control group. The differences between the frequencies of the allele in patients with IA and the controls were statistically significant. Our data indicated that the C allele single nucleotide variation increased IA formation in the Chinese population examined (Table I). Statistical analysis revealed that the C allele was not associated with the IA number (single or multiple) and IA position (anterior or posterior circulation), suggesting no statistical significance.

Increased NADPH oxidase content in the plasma of patients with IA and in an animal model of elastase-induced aneurysm. p22^{phox} is a subunit of NADPH oxidase that is expressed in VSMCs and serves as an important component of superoxide-generating vascular NADPH oxidase (12). According to the p22^{phox}-214T>C single nucleotide variation result, the mRNA expression of NADPH oxidase in the IA group was significantly higher than that in the control group (Fig. 1). The results of ELISA demonstrated that the serum level of NADPH oxidase in the IA group was significantly higher compared with that in the control group (P<0.0001; Fig. 2).

In the animal model of elastase-induced aneurysm, the results revealed that the serum levels of NADPH oxidase in the control group were significantly higher compared with those in the normal group, as shown by ELISA (P<0.05). However, no significant difference was observed between the edaravone group and the control group (P=0.8382; Fig. 3). These results suggest that NADPH oxidase contributes to the pathogenesis of IA and that drug therapy for IA warrants further investigation.

Suppression of aneurysm development due to the inhibition of ROS by edaravone in an animal model of elastase-induced aneurysm. In the model of elastase-induced aneurysm, the success rate of the model in the edaravone group was 62.5%, which was lower than that in the control group (82.3%), and the diameter of the aneurysm was smaller than that of the control group (3.26±0.13 mm vs. 3.85±0.07 mm). In the edaravone group, there were 2 rabbits with peripheral ulcers following the injection of edaravone into the auricular vein, and there was 1 rabbit with a combination of ulcers around the incision site. However, in the control group, the wound ulcer rate was higher than that in the edaravone group (Table II). As shown in Table II, there was 1 intraoperative death in the control group, and in the edavarone group, there were 2 intraoperative deaths. Thus, in total, 3 rabbits died during surgery. This was due to the anesthesia or the loss of blood. Our aneurysm modeling results are consistent with those of previous studies. In a previous study, there were 121 (17%) deaths among 700 subjects. In that

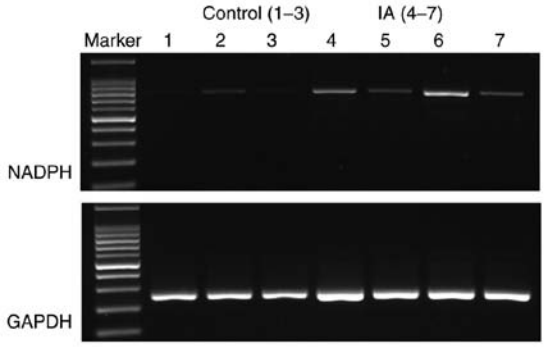

Figure 1. Results of RT-PCR. The mRNA expression levels of NADPH oxidase and GADPH were detected by RT-PCR. Lanes 1-3, control group; lanes 4-7, IA group. GADPH was used as the loading control (lanes 1-7). The expression of NADPH oxidase in the IA group was higher than that in the control group. IA, intracranial aneurysm.

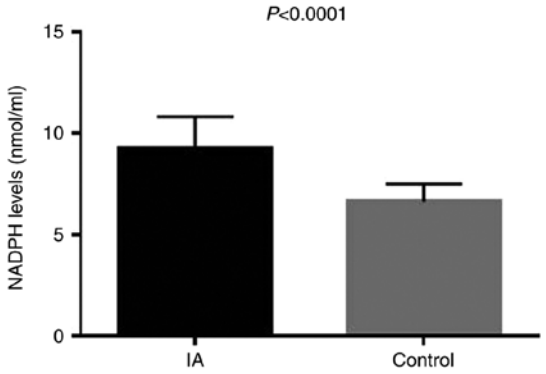

Figure 2. The expression of NADPH oxidase in patients in the IA and control groups. The expression of NADPH oxidase were random from 10 IA patients and 10 donors plasma. The expression of NADPH oxidase differed significantly between the IA group (n=10) and the control group (n=10), as determined by ELISA using SPSS software (Student's t-test, P<0.0001), suggesting a significantly higher expression of NADPH oxidase in the IA group. IA, intracranial aneurysm.

study, it was determined that the causes of death were related to the anesthesia, device failure, failure to thrive (FTT), etc. (39). The forms of aneurysm in the edaravone group and the control group were very similar, although the size of the fusiform aneurysms differed (Fig. 4).

General views of fusiform aneurysm at day 21 or the maximum diameter of the common carotid artery differed significantly among the 3 groups. The mean diameter demonstrated the average value of the maximal fusiform aneurysm diameter at day 21 or the maximum diameter of the common carotid artery in normal rabbits, suggesting significant differences among the 3 groups (P<0.05; Fig. 5). The fusiform aneurysms in the control group were larger in size and more typical than those in the edaravone group. The histologic findings from H&E staining confirmed the general views. The vascular smooth muscle of the control group was fractured and the distribution remained inhomogeneous, which conforms to the morphological characteristics of the aneurysm. The vascular smooth muscle of the edaravone group was similar to the characteristics of the normal vascular intima (Fig. 6).

Cell apoptosis and inflammatory cell infiltration are closely related to oxidative stress. The patients with IA had a

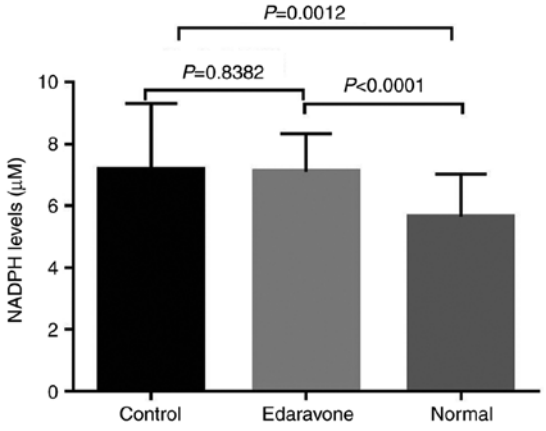

Figure 3. The expression of NADPH oxidase in the 3 groups of rabbits. The expression of NADPH oxidase in plasma at day 21 in each group. All data are expressed as the means ± SD for rabbits in each group. Statistical analysis was carried out using one-way ANOVA (post hoc adjusted comparisons were performed with Bonferroni correction) and were considered significant at P<0.05. Control group (n=17), the rabbits received saline; edaravone group (n=16), the rabbits received 2 mg/kg/day edaravone; normal group (n=10), the rabbits were left untreated.

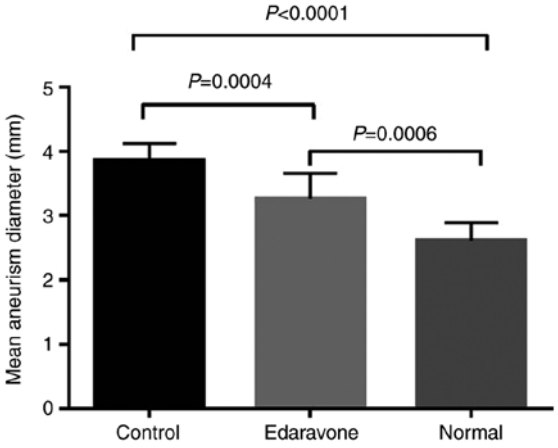

Figure 5. Mean diameter of the aneurisms in the 3 groups. The mean diameter demonstrated the average value of the maximal fusiform aneurysmal diameter at day 21 or the maximum diameter of the common carotid artery in rabbits in the normal group. All data are expressed as the means ± SD for rabbits in each group. Statistical analysis was carried out using one-way ANOVA (post hoc adjusted comparisons were performed with the Bonferroni correction) and were considered significant at P<0.05. Control group (n=17), the rabbits received saline; edaravone group (n=16), the rabbits received 2 mg/kg/day edaravone; normal group (n=10), the rabbits were left untreated.

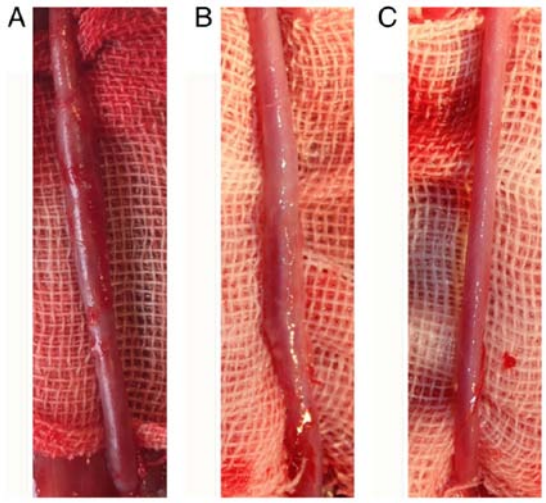

Figure 4. The aneurisms in the 3 groups. The figure represents aneurysmal formation of the common carotid artery at 21 days in each group. (A) Control group rabbits received saline; (B) edaravone group rabbits received 2 mg/kg/day edaravone; and (C) normal group rabbits were left untreated. The average diameter of the aneurysm in the edaravone group was smaller than that in the aneurysm control group, but it was thicker than that of the normal group.

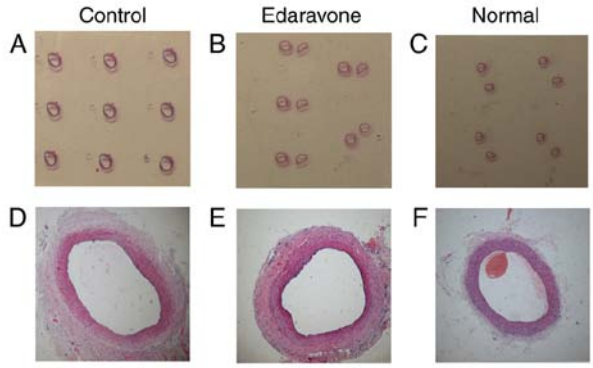

Figure 6. Histological findings of the fusiform aneurysm and the common carotid artery. Histological findings of the fusiform aneurysm and the common carotid artery at day 21 stained with hematoxylin and eosin (H&E) [magnification; (A-C), x1 and (D-F), x10]. The average diameter of the aneurysm in the edaravone group was smaller than that in the aneurysm control group, but it was thicker than that of the normal group. (A and D) Control group, rabbits received saline; (D) is an enlargement of (A). (B and E) edaravone group, rabbits received 2 mg/kg/day edaravone; (E) is an enlargement of (B). (C and F) Normal group, rabbits were untreated; (F) is an enlargement of (C).

higher proportion of $CD34^+$ cells than the patients with renal cell carcinoma in our control group (Fig. 7). However, there were no obvious positive findings observed in the 3 groups of tissue with CD34 staining, although the internal diameter of the 3 groups exhibited similar results with the H&E findings (Fig. 8).

Effects of edaravone on MMP-2 and MMP-9 expression. Since aneurysmal degeneration is involved in the destructive remodeling of the connective tissue of the aortic wall, this structure is associated with the excessive production of local matrix-degrading proteases and chronic inflammation (40); thus, in this study, we evaluated the expression of MMP-2 and MMP-9 in the serum of rabbits with elastase-induced aneurysm at day 21.

The results revealed that the serum levels of MMP-9 in the edaravone group were significantly lower compared with those in the control group, as shown by ELISA (P<0.05). However, as regards the serum levels of MMP-2, no significant differences were observed between the edaravone group and the control group (P=0.7544; Fig. 9).

Discussion

Although many researchers believe that IA is caused by the interaction of a number of factors, such as genetic factors and

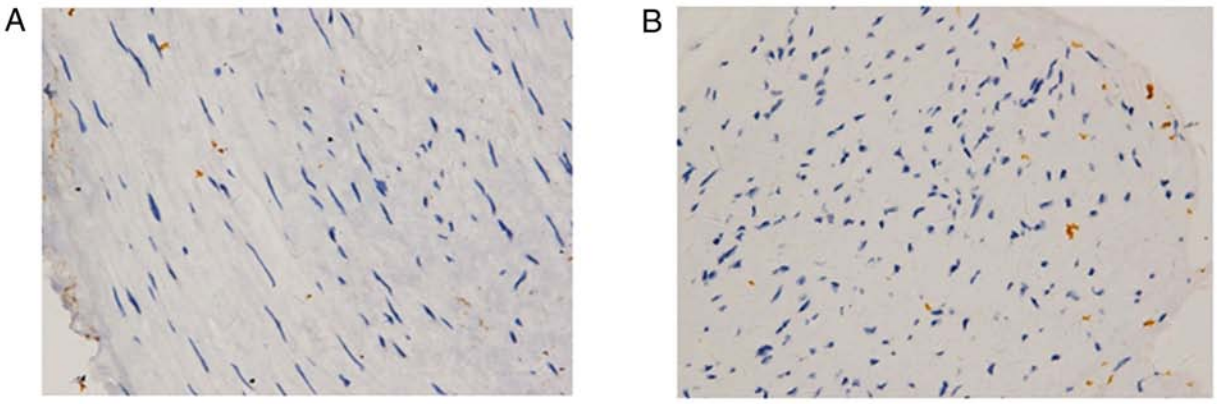

Figure 7. CD34$^+$ cells in a large middle cerebral artery aneurysm vs. renal artery. Histological findings of middle cerebral artery aneurysm and renal artery stained with CD34 antibody (x40 magnification). There is a little infiltration on CD34$^+$ cells in aneurysms, and the number of CD34$^+$ cells in renal arteries was lower than that in the aneurysms. (A) Renal artery sample stained with CD34; (B) middle cerebral artery aneurysm sample stained with CD34 antibody.

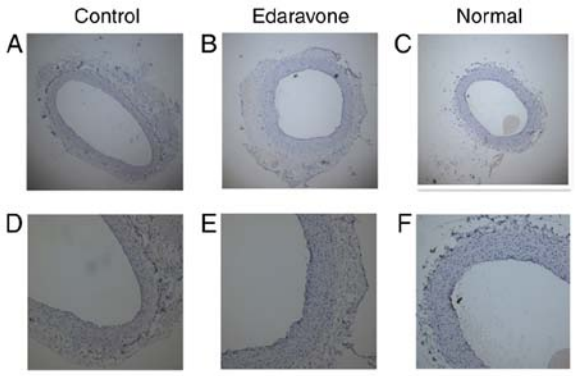

Figure 8. CD34$^+$ cells in fusiform aneurysm vs. common carotid artery. Histological findings of fusiform aneurysm and common carotid artery at day 21 stained with CD34 [magnification: (A-C) x4 and (D-F) x10]. There were no obvious positive findings observed in the 3 groups of tissue CD34 staining, although the internal diameter of the aneurisms in the 3 groups was smaller in turn. (A and D) Control group, rabbits received saline; (D) is an enlargement of (A). (B and E) Edaravone group, rabbits received 2 mg/kg/day edaravone; (E) is an enlargement of (B). (C and F) Normal group, rabbits were left untreated; (F) is an enlargement of (C).

environmental factors (41-44), little is known about its specific biological mechanisms. Genetic studies on IA have indicated that the onset of IA is associated with a few specific single nucleotide mutations, such as the tumor necrosis factor (*TNF-α*) gene (45), the interleukin (*IL)-6* gene (46), the *IL-12* gene (47), and the *NADPH oxidase p22*^{phox} gene (48,49), which suggests that there is a positive association between susceptibility genes and IA. The genetic background of these findings however, remains unclear. Currently, treatment options for complex IA include conservative observations, microsurgical clipping and endovascular embolization. Currently, there are no other effective options for the treatment of IA. Very little is known about the mechanisms of IA formation and progression. We have only a restricted knowledge regarding the mechanisms of IA formation and progression, which is the main reason for the difficulties in achieving breakthroughs in current complex IA treatment situation.

With the development of molecular biotechnology and the increasing understanding of IA, the importance of genetic factors related to IA development has gained increasing attention, and the family genetic predisposition of IA in different races and different populations has been confirmed (20). p22^{phox} is an important component of the NADPH oxidase system and is closely related to the development of atherosclerosis and ischemic cerebrovascular disease. In a previous study, in a UK population group, a significant correlation was demonstrated between the 214C>T polymorphism and cardiovascular disease (CVD) (30). However, some studies provide evidence that these polymorphisms are not associated with the occurrence of IA in Caucasians (50). In this study, we found that the p22^{phox}-214T/C was associated with IA in a Chinese population. In the IA group, the mRNA expression of NADPH oxidase was significantly higher than that of the control group. The plasma NADPH oxidase levels were also significantly higher in the IA group than in the control group ($P<0.001$). The high expression of NADPH oxidase is controlled by the p22^{phox} polymorphism. However, through statistical analysis, we found that the C allele was not associated with the IA number (single or multiple) or IA position (anterior or posterior circulation). These conflicting results may be explained by ethnic-related differences, in addition to the differences in study design, and the selection and size of the study samples.

For clinical applications, as IA is always found in a pre-existing form, it must be regressed by medical treatment. In this study, we clarified the key role of ROS in the formation of IA. ROS are the major mediators of various inflammatory cascades and are associated with the onset of various diseases, including arteriosclerosis and AAA (51,52).

The role of ROS activation by nuclear factor (NF)-κB in the aneurysm wall in the formation of IA plays a key role. It has been demonstrated that the c-Jun N-terminal kinase inhibitor, SP600125, can promote the degradation of aneurysms in a model of AAA (53); however, the drug has never been used in the clinical. Clinically, vitamin E supplementation may not reduce the incidence of AAA (54), and thus these drugs may not be a suitable choice for clinical use. Edaravone has been shown to effectively inhibit the activation of NF-κB through the ROS system, resulting in decreased expression of MCP-1, VCAM-1 and MMP-2 (25). The inhibition of MCP-1 and VCAM-1 has been shown to significantly prevent macrophage infiltration (25). These findings indicate that edaravone can improve the oxidative degeneration of the aortic wall and

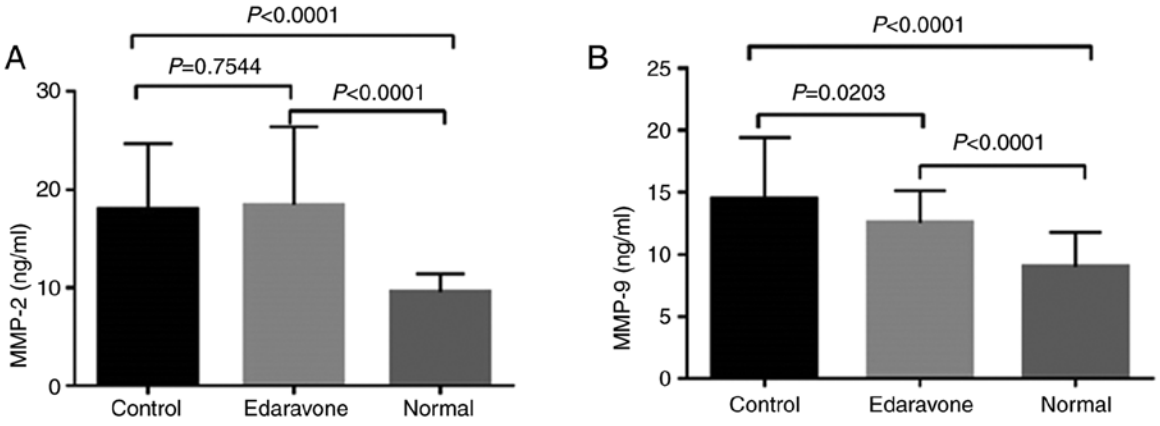

Figure 9. Expression of (A) MMP-2 and (B) MMP-9. Graphs show the expression of MMP-2 and MMP-9 in the plasma at day 21 in each group. All data are expressed as the means ± SD for rabbits in each group. Statistical analysis was carried out using one-way ANOVA (post hoc adjusted comparisons were performed with Bonferroni correction) and were considered significant at P<0.05. Control group (n=17), the rabbits received saline; edaravone group (n=16), the rabbits received 2 mg/kg/day edaravone; normal group (n=10), the rabbits were left untreated. MMP, matrix metalloproteinase.

prevent the development of aneurysms. We thus hypothesized that edaravone, as an oxygen free radical scavenger, may have the potential to inhibit the growth of aneurysms.

In this study, in our model of elastase-induced aneurysm, after observing the development of aneurysms, treatment with edaravone began 7 days after the aneurysm was observed. Edaravone at 2 mg/kg/day, twice daily, effectively reduced the success rate of large auricular aneurysm models and the wound healing rate. In the edaravone group, there were 2 rabbits with peripheral ulcers following the injection of edaravone into the auricular vein, and there was 1 rabbit with a combination of ulcers around the incision site. However, in the control group, there are 5 rabbits with peripheral ulcers following the injection of saline into the auricular vein, and 2 rabbits with ulcers around the incision site, resulting in a higher wound ulcer rate than the edaravone group. In theory, the expression of NADPH oxidase should be significantly altered following oxidative treatment with edaravone; however, the actual results are shown in Fig. 3. There is a significant difference in the NADPH oxidase levels between the edaravone group and the normal group; however, there was no significant difference between the edaravone group and the aneurysm control group. The diameter of the aneurysms in the edaravone group was lower than that of those in the control group (3.26±0.13 mm vs. 3.85±0.07 mm), and the expression of MMP-9 was lower in the edaravone group than in the control group (P<0.0001). The major finding of this study was that edaravone was beneficial for wound healing and suppressed the development of aneurysms. Effective IA drug therapy may have significant benefits for many patients with complex IA. The results of this study provide new insight into the mechanisms of IA formation.

However, this study has some limitations. Firstly, we only analyzed some of the genes involved. In addition to $p22^{phox}$, genetic variations in other components of the NADPH oxidase complex may also result in alterations in superoxide in the blood vessel wall and, as a consequence, aneurysm formation. Secondly, in this study, we used only one dose of edaravone. In order to evaluate the dose-dependent effects of edaravone on IA prevention, it is necessary to further examine the effects of edaravone on IA at other concentrations. Thirdly, there is no better method to accurately measure the diameter of aneurysms and blood vessels. The measurement results from our direct method are prone to certain errors. Fourthly, in theory, MMP-2 and MMP-9 are required for aneurysm formation, and edaravone can reduce the expression of MMP-2 and MMP-9, thereby preventing the development of aneurysms (26,27). However, in our actual study, MMP-9 was inhibited; however, MMP-2 expression did not differ significantly between the edaravone group and the control group (P=0.7544). Fifthly, CD34 is an important pro-inflammatory and pro-angiogenic cell in chronic inflammatory vascular diseases. Previous studies have indicated that CD34 cells are closely related to the development of aneurysms. Patients with AAA have a higher proportion of $CD34^+$ cells than patients with peripheral vascular disease (PVD) (55,56). However, in our study, $CD34^+$ cells were difficult to find. The abnormal expression of MMP-2 and the low expression of CD34 cells may be related to the short time of our aneurysm modeling. In future studies, long-term and large-scale aneurysm model samples are required to arrive at more accurate conclusions.

In addition, IA as a complex disease affected by multiple genes, is also greatly affected by environmental factors, which has brought difficulties to molecular biology research. At present, researchers have conducted a large number of basic and clinical studies on the NADPH oxidase $p22^{phox}$. The results of this study, indicate that the *p22*^{phox} gene polymorphism is associated with vascular disease, at least in Chinese populations. ROS was inhibited and the potential of edaravone to inhibit the formation of IA was shown. These results may provide new strategies for the treatment of IA.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

JH, JL and QC made substantial contributions to the conception and design of the present study. JH, HW, CW, RL, AL, YZ and ZF performed the experiments. JH wrote the manuscript. HW and RL edited and revised the manuscript critically for important intellectual content. All authors have read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

This case-control study was approved by the Research Ethics Committee of Taihe Hospital, Hubei University of Medicine, Shiyan, China. Informed consent was obtained from all the patients. The animal experiments were approved and conducted according to the guidelines set by the Experimental Animal Center of the Hubei University of Medicine (Shiyan, China). All animal experiments were performed in the Experimental Animal Center according to the protocols approved by the Animal Ethics Committee of the Hubei University of Medicine.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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