Pharmaceutical stabilization of abdominal aortic aneurysms : changing its natural history

Kokje, V.B.C.

Chapter 7

COLCHICINE INHIBITS AORTIC ANEURYSM FORMATION IN A RODENT MODEL

Vivianne B.C. Kokje, Arend J. Nieuwland,
Jaap F. Hamming, Jan H.N. Lindeman

Department of Vascular Surgery, Leiden University Medical Center,
Leiden, The Netherlands

ABSTRACT

Abdominal aortic aneurysms (AAA) are characterized by chronic trans mural inflammation. Neutrophils, in particular, have been described to be pivotal in this process. Colchicine is known to diminish neutrophil chemotaxis and therefore might be beneficial for stabilizing AAA growth. The aim of this study is to test the effect of Colchicine in a murine model of AAA disease and to explore the mechanisms by which Colchicine might engage in the chronic inflammation seen in the human AAA wall.

We identified the effect of Colchicine on AAA formation in an established murine model of AAA disease. Male 8-10 week old, wild type C57/BL6 mice underwent aortic perfusion with porcine pancrease elastase. The mice were treated either with Colchicine or as a control group with saline. At baseline, day 7 and day 14 the aortic diameter was measured by ultrasound. 14 days after the elastase perfusion aortas were harvested for immunohistochemical analysis.

Experimentally treated animals had significantly less growth in aortic diameter (31.71%±14.97%) compared to the control animals (79.25%±34.88%) ($p<0.01$). Quantitative immunohistochemical staining indicated that the number of tissue leukocytes were significantly decreased in Colchicine treated animals (15 vs 44 cells per aorta, $p< 0.05$).

We propose two mechanisms for Colchicine to engage in human AAA growth. The first mechanism that is proposed states that Colchicine might directly reduce neutrophil chemotaxis in the AAA wall. The second mechanism describes that Colcicine might indirectly interfere with neutrophils via the NALP3 inflammasome. Here we find the NALP3 inflammasome and its effector interleukins IL1 and IL18 prominently present in the human AAA wall.

This, and the finding that Colchicine reduces murine AAA formation after elastase perfusion indicate that Colchicine might be a promising means to stabilize human AAAs.

INTRODUCTION

Excess protease activity caused by chronic inflammation is thought to be a critical factor in abdominal aortic aneurysm (AAA) growth by destructing structural proteins as elastin and collagen, which ultimately leads to AAA rupture[32]. Currently, pharmaceutical therapies that interfere with chronic inflammation are widely investigated in order to reduce the growth of small AAAs and thereby to reduce the need for surgical repair[3; 13].

To date however, no pharmaceutical intervention has been shown to effectively interfere with AAA progression[31]. Therefore further investigation of the mechanisms of chronic inflammation, observed during AAA progression, is needed. Recently, it has been described that neutrophils are one of the key elements of chronic inflammation. They are responsible for orchestrating an ongoing inflammatory immune response in the human AAA wall[5]. Additionally, neutrophils appear to be an important source of cytokines found in the aneurysm wall, e.g. CXCL8[20]. Previous research including our own has indicated that interfering with neutrophil chemotaxis, by inhibiting of the neutrophil receptors or by the using of L-selectin knockout mice reduces or even abrogates aneurysm formation[15].

The immune modulatory drug Colchicine interferes with several aspects of neutrophil activation.

Besides the direct effect of Colchicine on neutrophils, an indirect effect of Colchicine on neutrophils has been described via the NALP3 inflammasome[26]. It has been described that the NALP3-inflammasome via caspase-1 activates IL1β and IL18. This induces endothelial cell and fibroblast IL6 and CXCL8 production thereby causing neutrophil chemotaxis and preservation[11; 26; 29].

The aim of this study is to test the effect of Colchicine in a murine model of AAA disease and to explore the mechanisms by which Colchicine might engage in the chronic inflammation seen in the human AAA wall.

METHODS

Elastase model

All murine investigations were approved by the Leiden University Medical Center animal welfare committee and were in compliance with the Dutch government guidelines. Eight-to-ten week old, male, C57/BL6 mice were obtained from Charles River, France. The aneurysms were created via porcine pancreatic elastase (PPE) infusion as previously described[1]. After the elastase infusion 0.05-0.1 mg/kg/12hrs buprenorfine was given and the mice recovered with free access to food and water. Colchicine (1mg/L) (Sigma-Aldrich, st Louis, MO, USA), was daily provided by drinking water. Treatment was started the day before the elastase infusion and the mice were sacrificed 14 days after the infusion. Control animals received plain drinking water daily for 15 days. To compare the aortic growth rates of the different groups one observer blindly measured the maximum axial diameter of the aorta by means of ultrasound one day prior to elastase infusion, after one week and two weeks after infusion by means of the Vevo 770 Imaging system using RMV 704 microvisualization scan head (Visualsonics, Ontario, CA). At day 14 after the elastase infusion, the mice were sacrificed and the aorta was removed and embedded in paraffin for later analysis. Immunohistochemical sections were deparaffinized and incubated overnight at room temperature with the primary antibody diluted in 1% PBSA. The sections were incubated with CD45 (clone 30-F11,

BD Pharmingen, USA), MAC3 (clone M3/84, BD Pharmingen,USA) and MMP9 (C-20, Santa Cruz Biotechnology, USA). Additional sections were stained with Weigert's elastin stain to visualize elastic laminae. Staining with haematoxyline-phloxine and saffron (HPS) staining was performed to provide an overview of the murine aortic wall. Eight slides per animal were used per staining for analysis and only moderate or strongly reactive cells were counted as positive. The slides were blindly evaluated. The mean value for positive staining cells on eights slides was calculated for each animal.

Human Samples

The investigation conforms to the principles outlined in the Declaration of Helsinki (59th, October 2008). Sample collection and handling was performed in accordance with the guidelines of the medical ethical committee of the Leiden University Medical Center. For the control samples; all human aortic arterial wall samples were provided by the Vascular Tissue bank (Department of Vascular Surgery, Leiden, The Netherlands). None of the patients in the study had a history of diabetic or chronic inflammatory disease. The primary cause of death in the control group was fatal brain injury due to a major head trauma or subarachnoidal bleeding.

The abdominal aortic aneurysm samples were obtained from the anterior-lateral aneurysm wall during elective surgery for asymptomatic AAA (>5.5 cm or larger).

Both control and aneurysm samples were cut in half. One half was immediately snap frozen in liquid nitrogen and stored at -80°C for mRNA (real time PCR analysis) and protein (multiplex analysis). The snap-frozen samples were partly used for total RNA extraction, which was performed according to manufacturer's instructions. Subsequently, cDNA was prepared and the RT-PCR for IL1β and IL18 (Life technologies, Paisley, UK) was conducted as previously described[21]. Another part of the snap-frozen human tissue samples was used for multiplex assay using a Bio-Plex 17 panel for multiple cytokines (Bio-Rad Laboratories, Hercules, CA, USA) as previously described[20].

The other half of the control and aneurysm samples was fixed in 4% formalin for 12 hours and decalcified. Afterwards the fixed segments were paraffin embedded and 4 µm sections were processed into slices. Immunohistochemical sections were deparaffinized, treated for 10 minutes with H_2O_2 to block endogenous peroxidase activity and incubated overnight at room temperature with the primary antibody diluted in PBS-1% albumin. The following primary antibody was used: NALP3 (Abcam, Cambridge, UK). Envision mouse (Dako, Glostrup, Denmark) was used as secondary antibody. Sections were stained with DAB (Dako, Glostrup, Denmark).

Statistical Analysis

All values are shown as mean (SD) and probability values of $P<0.05$ were considered statistically significant. The Mann-Whitney U test was used to detect significant difference in aortic diameter and in cell count between the two groups of mice. All analysis were performed using SPSS 20.0 (SPSS Inc. Chicago).

RESULTS

Aortic dilatation after Colchicine treatment in the murine elastase AAA model

Aneurysm formation in control mice 14 days after elastase perfusion was found in 8 out of 9 mice (mean diameter increase 86.76% ± 28.39%) and 1 revealed an increase in diameter of 19.93%. Colchicine treatment had profound effects an aneurysm was growing in only 1 of the 10 animals (mean diameter increase of 31.71% ±14.97%, p<0.01) (Fig. 1AB). Clear morphological differences between both groups were visualized with an overview HPS staining, the control animals revealed thickened, fibrotic aortic wall as the Colchicine treated animals had a preserved aortic structure (Fig. 1C).

Histological and immunohistochemical analysis of the murine aortas

Colchicine treatment preserved the elastin lamellae as the control aneurysmal aortas revealed loss of normal arterial architecture, with an increase in elastic lamellae breaks (Fig. 2).

Quantitative immunohistochemical analysis showed that tissue leukocytes 14 days after elastase perfusion were significantly decreased in the Colchicine treated group as compared with the controls, 15 vs. 44 cells per aorta (p=0.01) (Fig. 3). The cells did localize mostly within the adventitia of the aortic wall. Colchicine did not significantly reduce the macrophage content

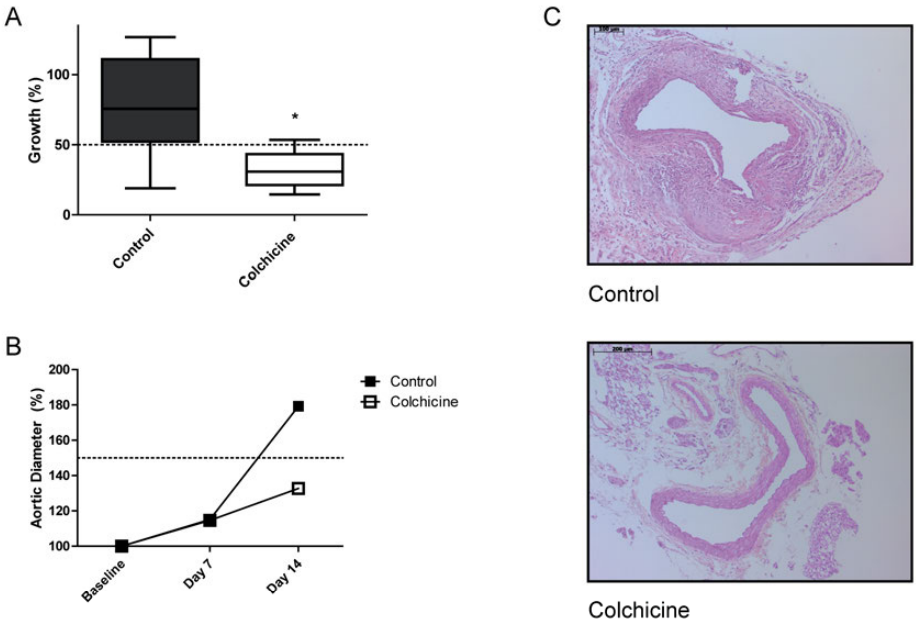

Figure 1. (A) Percentage aortic diameter growth measured by ultrasound in colchicine treated animals (colchicine) and in animals treated with saline (control) at day 14 compared with baseline. The data is presented as median range ± minimal and maximum values. (B) Abdominal aortic development in colchine treated animals and controls at the day before elastase perfusion (baseline), day 7 and day 14 after elastase perfusion. (C) Haematoxyline-phloxine and saffron (HPS) staining in saline treated animals (control) and colchicine treated animals (colchicine) 14 days after elastase perfusion.

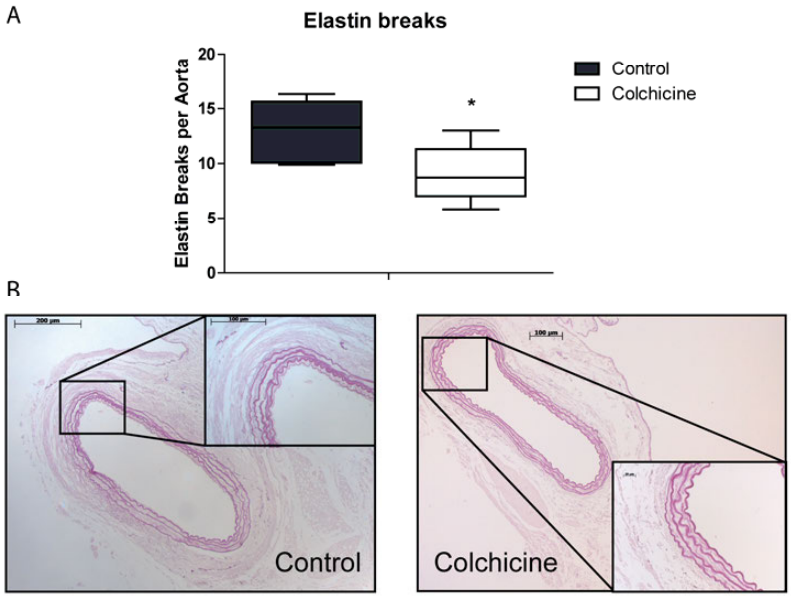

Figure 2. (A) Elastin breaks per aorta measured in saline treated (control) and colchicine treated animals at day 14 post-elastase perfusion (p=0.01) The data is presented as median range ± minimal and maximum values. (B) Control and colchicine treated animals day 14 after elastase perfusion stained with Weigert's elastin stain to visualize elastic laminae.

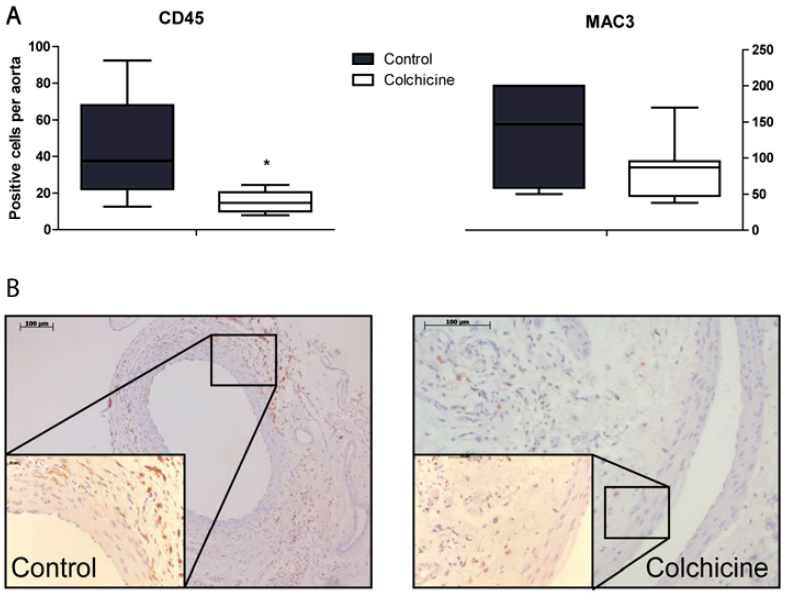

Figure 3. (A) Leucocytes stained by CD45 in colchicine treated animals vs controls (p<0.01) and macrophages visualized with MAC3 staining were similarly expressed in colchine treated and control animals (p>0.05). The data is presented as median range ± minimal and maximum values. (B) Control and colchicine treated animals day 14 after elastase perfusion stained with CD45 stain to visualize leucocytes.

(Fig. 3), yet we found a significant decrease in neutrophil and neutrophil elastase (MMP9) expression in the Colchicine treated animals ($p<0.01$) (Fig. 4).

Potential targets of Colchicine treatment in human aneurysms

To evaluate the potential NALP3-route of neutrophil activation in human AAAs, we performed immunohistochemical staining for the NALP3 inflammasome on human AAA samples. By means of this staining, we revealed prominent expression of the NALP3-inflammasome in human abdominal aortic aneurysm tissue samples (Fig. 5). IL1β and IL18 were also observed to be strongly expressed in the AAA tissue. Compared with the control aortic tissue, the IL18 mRNA level was significantly higher in AAA (-1.75 ± 0.50 in control aorta, -1.03 ± 0.32 in AAA, $p<0.01$), IL1β expression was borderline significant ($p=0.09$). Yet, IL1β aortic protein content was significantly higher in the AAAs (0.67 ± 0.72 in the controls and 5.27 ± 4.80 in the AAAs) (Fig. 6). These findings confirm presence of different Colchicine targets in AAA disease.

DISCUSSION

Neutrophils are thought to play a critical role in AAA disease. The immune modulatory compound Colchicine quenches neutrophil involvement through multiple and distinct mechanisms. In this study we show that Colchicine prevents AAA formation in an established murine model of elastase induced aneurysm formation. In this model, Colchicine protected the vascular wall from loss of elastin and preserved the structure of the aortic wall. Moreover, there were fewer leucocytes and neutrophils in the murine aortic wall in Colchicine treated mice compared with the controls.

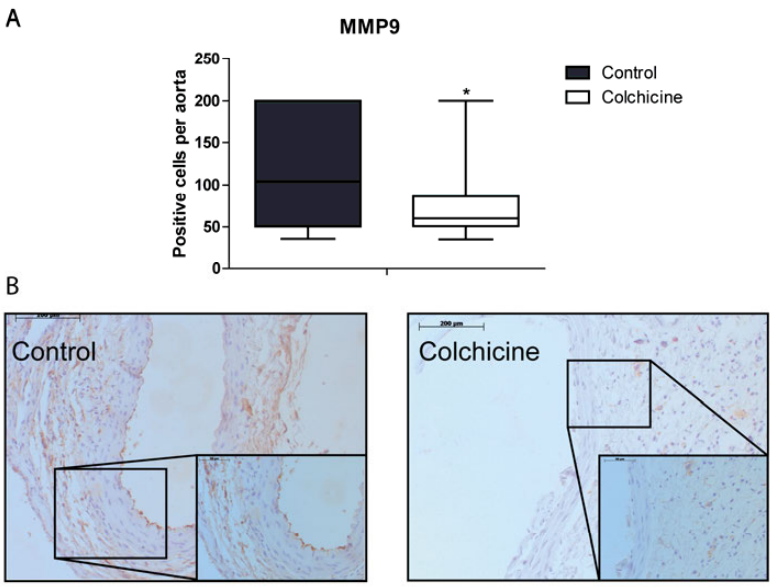

Figure 4. (A) Matrix metallo protease 9 (MMP9) was significantly lower expressed in the colchicine treated animals ($p<0.01$) The data is presented as median range ± minimal and maximum values. (B) Control and colchicine treated animals day 14 after elastase perfusion stained with MMP9.

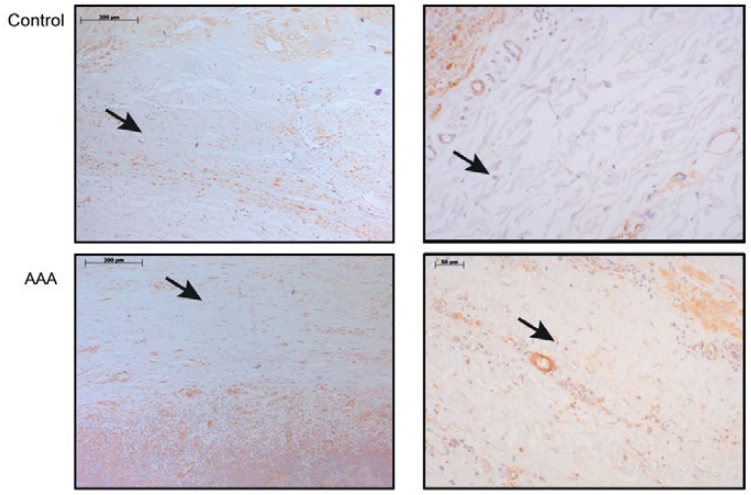

Figure 5. NALP3-inflammasome stained in human abdominal aortic aneurysm and aortic atherosclerotic tissue (left 10x and right 20x magnification).

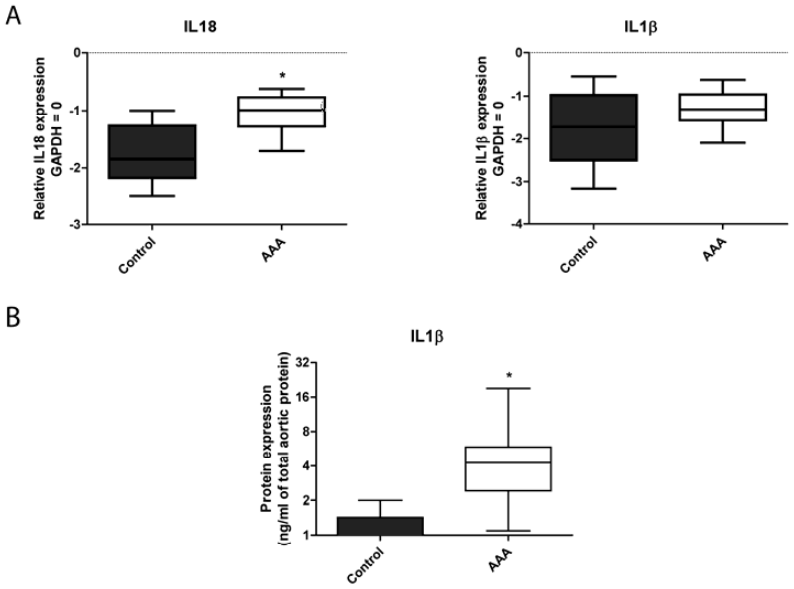

Figure 6. (A) mRNA expression of IL1β (-1.76 ± 0.84 in control aorta, -1.2 ±0.41 in AAA, p=0.09) and IL18 (p<0.01) was analyzed via RT-PCR. (B) Differences in the protein expression level of IL1β was determined via a Bio-plex assay (p<0.01).

AAAs are characterized by localized infiltration of inflammatory cells. The large amount of interleukins, TNFα and other pro-inflammatory cytokines released by this infiltration leads to an increase in expression of matrix metalloproteases and cathepsins, which in their turn digest

the extracellular matrix of the aortic wall and eventually thereby cause aneurysm rupture[33]. The activation of neutrophils, in particular, has been described to be pivotal in the process of AAA formation[5]. Interference with neutrophil influx or activation in the aortic wall in the elastase model has been found to completely abrogate the aneurysm formation[10; 28]. Also the activation of neutrophils and the abundant release of its enzymes throughout the vascular wall cause an ongoing inflammatory immune respons, which leads to the chronic inflammatory state of human AAAs[24]. Previously we and others revealed abundant expression of CXCL8 in human AAA wall[20]. CXCL8 is known to be a strong chemo-attracted for neutrophils and it also prevents apoptosis, which prolongs the neutrophil survival and thereby further contributes to neutrophil abundance in the aneurysm wall[16].

Colchicine directly interferes with neutrophil chemotaxis and thus provides a promising drug to alter the chronic inflammatory state of AAAs. There are two mechanisms that explain the effect of Colchicine on neutrophils. Firstly, Colchicine inhibits the priming effect of tumor necrosis factor α (TNFα) on neutrophils by reducing the synthesis of TNFα and by down regulating its receptors on macrophages and endothelial cells. Secondly, in low concentrations, Colchicine reduces adhesion of the neutrophils to endothelium[7; 26].

It is now apparent that Colchicine may also influence NALP3-inflammasome mediated inflammation[26]. This inflammasome is known to activate the NFkB-cascade and inducing indirectly neutrophil activation[2]. If and how the NALP3-inflammasome is involved in human aneurysm disease is unknown. Yet, recently it has been described that a part of the gene encoding for the NALP3-inflammasome, the NLRP3 gene, alters patients' susceptibility to AAA[30]. We here show prominent expression of the NALP3 inflammasome in human AAA samples, as well as up regulation of interleukins IL1β and IL18, which are activated by this inflammasome. Therefore, the NALP3-inflammasome may be involved in AAA disease.

There are several mechanisms probable to contribute to the prominent involvement of this inflammasome in human aneurysms. One of the proposed mechanisms is, as within gout, activation of the via uric acid, which is significantly increased in the wall of aortic aneurysms[29]. Current studies reveal that in emphysema, a disease with several pathophysiological parallels with AAA, uric acid form dying cells can activate the inflammasome[6; 8]. Another mechanism for activation of this inflammasome is smoking. It is known that mitochondrial reactive oxygen species (ROS) induced by cigarette smoking can activate the NALP3 inflammasome[9]. Smoking is one of the key risk factors for aneurysm development and when the incidence of smoking is reduced, the prevalence of AAA drops equally[19]. Therefore, ROS induced by cigarette smoking might be a likely explanation for inflammasome activation in human AAA walls.

A further possible explanation for the activation of the inflammasome might be induction via cholesterol crystals. High cholesterol is a well-recognized risk factor for AAA disease and cholesterol crystals have been frequently reported in the AAA wall. In atherosclerotic disease they were found to trigger the inflammasome by entering macrophages and rupturing lysosomes[14; 27]. Additionally, a recent study suggests activation of the inflammasome contributes to mechanical stretch-induced lung inflammation, a factor we know is important in AAA formation[34]. Although there are ongoing concerns about the narrow therapeutic margin between the gastrointestinal side-effects of Colchicine and its therapeutic efficacy, Colchicine has been recently shown to be

safe and efficacious in preventing the post-pericardiotomy syndrome after cardiac surgery[17] and in the prevention of recurrent pericarditis[22]. No differences in side effects were reported between placebo and Colchicine. Moreover, a recent study, in which the patients received Colchicine in a low dose for a minimum of two years, concluded that Colchicine is an attractive therapy for secondary prevention of cardiovascular events[25]. This, and our findings that Colchicine in the murine AAA model reduces aneurysm growth, indicate that Colchicine might be a cost-effective option for prevention of aneurysm growth and rupture in clinical practice.

Before the drug is considered for prevention of AAA growth in aneurysm patients, however, more research has to be done to investigate the effect of Colchicine on human abdominal aortic aneurysms.

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