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Original Article

Suramin combined with ginsenoside Rg3 alleviates cascular lesions and adventitial inflammation in venous allografts

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Abstract: Purpose: The current study aimed to investigate the effects of suramin combined with ginsenoside Rg3 on vascular lesions and adventitia inflammation in venous allografts. Methods: Thirty New Zealand rabbits were divided into five groups, including the Sham group, Negative control (NC) group, Suramin group, Rg3 group, and Suramin + Rg3 group. The experimental animals were transplanted with carotid arteries of external jugular veins using normal blood vessel donors (obtained from another six New Zealand white rabbits). Serum samples were collected 2 months after the operation. CRP, IL-6, and TNF-α serum levels were detected by ELISA. Graft vessels were obtained. Morphological changes were observed by H&E staining and Masson's staining. Thickening of intima and media was measured. Apoptosis of vascular smooth muscle cells was detected by TUNEL. Expression of CD31, CD34, and α-SMA was detected by immunohistochemistry. Results: Grafts in the NC group showed typical characteristics of chronic graft rejection, while grafts in the Suramin + Rg3 group showed a normal vascular structure. Compared with the NC group, the Suramin group, Rg3 group, and Suramin + Rg3 group exhibited decreased CRP, IL-6, and TNF-α levels, intimal thickening degrees, and number of positive CD31, CD34, and α-SMA cells, as well as an increased number of TUNEL positive cells (all $P < 0.05$). Comparing the Suramin group and Rg3 group, the Suramin + Rg3 group had significantly lower CRP, IL-6, and TNF-α levels, intimal thickening degrees, and numbers of positive CD31, CD34, and α-SMA cells, as well as higher TUNEL positive cell numbers (all $P < 0.05$). Conclusion: Suramin combined with ginsenoside Rg3 may alleviate vascular lesions and adventitial inflammation in venous allografts.

Keywords: Vein allografts, suramin, ginsenoside Rg3, vascular lesions, adventitial inflammation

Introduction

Coronary Artery Bypass Grafting (CABG) is a procedure used to replace obstructed coronary arteries. This method could improve myocardial blood supply and angina, as well as reduce risks of death from coronary heart disease [1]. Vascular transplantation is a common surgical method when blood vessels are defective and cannot be directly sutured. Autologous or allogeneic blood vessels and veins, as well as artificial blood vessels, could be used for vascular grafting. Vascular transplantation is suitable for patients with arterial lesions or vascular ruptures that are difficult to repair. These patients are often associated with infections, vascular obstruction, and other complications that could easily lead to atherosclerosis. Atherosclerosis is an inflammatory disease [2]. Inflammatory reactions could be caused by vascular transplantation [3]. In fact, due to vascular inflammation, intimal hyperplasia, and accelerated development of atherosclerosis, the therapeutic effects of the grafts are often unsatisfactory [4]. Moreover, drug therapy is necessary after surgery, aiming to decrease vascular inflammation caused by intimal hyperplasia and vascular injuries of vein grafts [5, 6].

Suramin is a urea derivative that inhibits intimal thickening and intimal smooth muscle cell proliferation after rabbit aortic intimal injuries [7]. Moreover, a variety of growth factor-mediated signal transduction pathways could alleviate vascular damage and reduce inflammatory response [8]. Ginsenoside Rg3 is a sterol com-

pound considered to be an active ingredient in ginseng [9]. It could induce macrophage M2 polarization and promote inflammation regression. Thus, it could be used as a therapeutic drug improving allergic inflammatory disease. The slow rejection reaction is due to long-term slow damage of vascular endothelial cells, resulting in a gradual occlusion of the lumen [10]. Allogeneic renal transplantation rejection is the main obstacle to the long-term survival of grafts after renal transplantation [11]. Similarly, slow rejection of transplanted blood vessels is also an important factor affecting graft survival. Of these, inflammation of the outer membrane is the infiltration of more inflammatory cells in the adventitia. With the inflammation of the external model aggravated, obvious arteriosclerosis is observed in the adventitia and intima of the graft. The intima is the unit membrane structure located in the inner layer of the outer membrane. Intimal thickening is mainly caused by the accumulation of smooth muscle cells [12]. The angiogenesis of transplanted cells is inhibited by ginsenoside Rg3, thus vascular thickening is reduced [13]. Suramin could reduce inflammation and inhibit arteriosclerosis caused by vascular smooth muscle proliferation [14]. However, there are few studies concerning the treatment of intimal hyperplasia and inflammation of transplanted vessels by suramin combined with ginsenoside Rg3. Therefore, it is necessary to conduct in-depth research concerning the specific effects of suramin combined with ginsenoside Rg3.

The current study examined the effects of the combined use of suramin and ginsenoside Rg3 on vascular graft lesions and adventitial inflammation. Findings of this study may provide a theoretical basis for clinical treatment of vascular transplantation diseases.

Methods

Experimental animals

Thirty-six healthy New Zealand white rabbits (male, weighing 2-2.5 kg) were purchased from Shandong Lukang Pharmaceutical Co., Ltd., China. The feeding environment was in line with the two grade standards of national laboratory animals. Rabbits were euthanized by intravenous injections of KCL solution (2 mg/kg) under anesthetic conditions. This study was approved by the Animal Ethics Committee.

Grouping of animals and establishment of models

Suramin (Sigma, USA) and ginsenoside Rg3 (A0239, Chengdu Manst Biotechnology Co., Ltd., China) were dissolved in DMSO to a final concentration of 3 mg/mL. F-127 polygel (Sigma, USA) was dissolved in normal saline with a concentration of 20%. Thirty experimental animals were randomly divided into the Sham group, Negative control (NC) group, suramin group, ginsenoside Rg3 group, and Suramin + Rg3 group. Rabbits without any treatment were served as the Sham group. In the NC group, F-127 polyhydrogel, containing 20% DMSO, was applied around the graft vein and around the anastomosis. Suramin, ginsenoside Rg3, and Suramin + Rg3 groups were coated with F-127 polyhydrogels containing the corresponding drugs. The experimental animals were transplanted with carotid arteries of external jugular veins using normal blood vessel donors (obtained from the other six New Zealand white rabbits). The graft arteries were examined by histopathology examinations 2 months after the operation. The external jugular vein preparation process is described as follows: Auricular veins (1 mg/kg) were injected with 3% pentobarbital for anesthesia. After the skin was sterilized, the rabbits were given heparin (1 mg/kg) intravenously for systemic heparinization. The external jugular veins were exposed by making an incision at the midline of the neck, followed by the branches being ligated. Then 2.5-3 cm venous segment was harvested and flushed with heparin saline solution. The proximal end of the venous segment was clamped with a microvascular clamp, as a marker, and placed in the heparin saline solution for storage. The other thirty New Zealand white rabbits were treated in the same manner. Their common carotid arteries were isolated and exposed for about 1.5-2 cm. After blocking the blood flow at the upper and lower ends of the common carotid arteries with an artery clip, a circular orifice with a diameter of 1 mm was cut in the anterior wall of the artery with microsurgical scissors. Disconnected veins and the arterial anastomosis were sutured continuously with 8-0 sutures. The same method was used to treat the other end. After anastomosis, the blood vessels were subjected to venting, knotting, and blood flow recovery. No bleeding and anastomosis were found. The graft veins were treated differently

according to the groups mentioned above. After the gel was naturally solidified, the subcutaneous tissues and skin were sutured layer by layer.

Detection of serum CRP, IL-6, and TNF-α levels by ELISA

Blood samples were collected before the rabbits were sacrificed and stored at -80°C. Before detection, all blood samples were reconstituted at room temperature. Serum CRP (Shanghai Yuanmu Biotechnology Co., Ltd., YN-539J, China), IL-6 (Shanghai Yuanmu Biotechnology Co., Ltd., YN-KL2441, China), and TNF-α (Shanghai Xitang Biotechnology Co., Ltd., F2041, China) were detected by ELISA, according to kit instructions. A BIO-RAD450 microplate reader was used to automatically read optical density values of different concentrations of standard samples at a 450 nm wavelength. Serum levels of CRP, IL-6, and TNF-α were calculated using the optical density-concentration curve.

H&E staining

Vascular grafts were fixed in 4% neutral formalin solution and embedded in conventional paraffin. Tissue sections were dried at 60°C for 2 hours, then all sections were subjected to H&E staining. The staining steps were as follows: Dewaxed tissue sections of vascular transplantation were dehydrated with gradient ethanol. They were then they were stained with hematoxylin. After being rinsed with pure water, all tissue slices were immersed in PBS solution for 1 minute. This was followed by rinsing with pure water. Tissue sections were then stained with eosin solution and dehydrated using 95% ethanol, 100% ethanol, and xylene, in sequence. Finally, the tissue sections were sealed with neutral resin. Histopathological changes were observed under an optical microscope.

Masson's staining

Paraffin sections of vascular transplantation were dewaxed with xylene and soaked in lodine liquor and sodium thiosulfate solution. They were then stained with Weiger's hematoxylin for 5-10 minutes and differentiated by 1% hydrochloric acid alcohol for 1-2 seconds. Subsequently, the sections were stained with vermilion acid fuchsin for 5-10 minutes and treated with 1% phosphomolybdic acid solution for 5 minutes. This was followed by re-staining with toluidine blue solution. After treatment with 1% glacial acetic acid for 1-2 minutes, all sections were placed in 95% alcohol, 100% alcohol, and xylene for dehydration. Finally, the sections were sealed with neutral resin. Intimal and medial thickness levels of the transplanted vessels were measure using Image Pro plus 6.0 professional image analysis software.

Immunohistochemistry

Paraffin sections of vascular transplantation were dewaxed with xylene and rehydrated with gradient alcohol. All sections were placed in boiling 0.01 M citrate buffer for antigen repair. After the sections were cleaned with PBS, hydrogen peroxide (3%) was added for 15 minutes of incubation at room temperature. Normal goat serum was then added for incubation for 15 minutes at room temperature. A total of 50 uL of primary antibodies (Rabbit Anti-CD31 antibody, bs-0195R, 1:400, Rabbit Anti-CD34 antibody, bs-0646R, 1:400, Rabbit Anti-alpha smooth muscle Actin antibody, bs-10196R, 1:400, Beijing Boaosen Biotechnology Co., Ltd., China) was added into the sections. The sections were then placed at 4°C overnight. HRP-labeled Mouse Anti-Rabbit IgG (bs-0295M-HRP, 1:500, Beijing Boaosen Biotechnology Co., Ltd., China) was added for 15 minutes of incubation at 37°C. The sections of vascular transplantation were washed 3 times with PBS and DAB color reaction was carried out. The sections were then washed with distilled water and counterstained with hematoxylin for 30 seconds. This was followed by sealing with neutral balsam. Positive staining results were observed under light microscopy and positive cells were counted using Image Pro plus 6.0 professional image analysis software.

Detection of vascular smooth muscle cell apoptosis by TUNEL staining

TUNEL Apoptosis Assay kit (Roche) was used to detect apoptosis levels of vascular smooth muscle cells. Briefly, the transplanted blood vessels were fixed with 10% formaldehyde and embedded in paraffin, followed by sectioning. The slices underwent conventional dewaxing and hydration. They were then incubated in 20% normal bovine serum at room temperature for 30 minutes. The TUNEL reaction mixture was added to the slices and incubated at 37°C for 90 minutes. This was followed by re-activation of color reaction with substrate DAB. Fi-

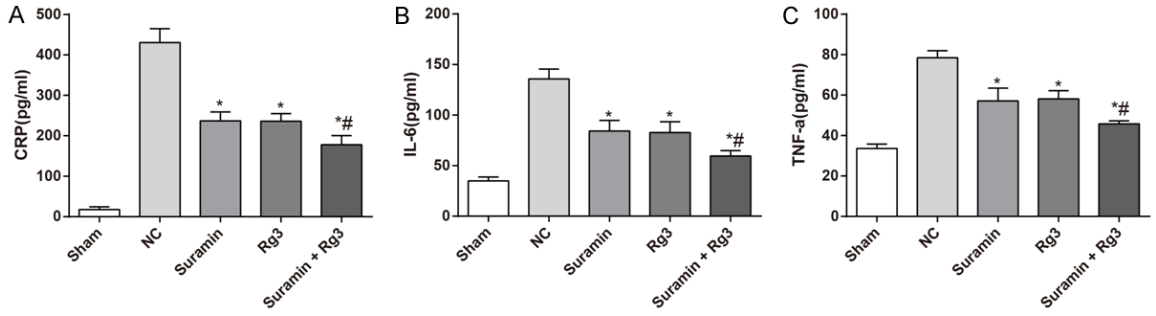

Figure 1. Serum concentrations of inflammatory factors in each group were detected by ELSIA. Note: A. Serum CRP concentration; B. Serum IL-6 concentration; C. Serum TNF-α concentration. **P* < 0.05 (versus NC group). #*P* < 0.05 (versus the Suramin group or Rg3 group).

nally, each section was photographed under a high-power field fluorescence microscope and the number of TUNEL positive cells was counted.

Statistical analysis

Measurement data are expressed as mean ± SD and were processed with SPSS 21.0 (SPSS Inc., Chicago, IL, USA) statistical software. Single-factor analysis of variance (ANOVA) was used to assess the statistical significance of results. *P*-values less than 0.05 are considered statistically significant.

Results

Suramin combined with ginsenoside Rg3 inhibited the inflammatory response of venous allografts

Compared with the NC group, serum concentrations of CRP, IL-6, and TNF-α in the Suramin group, Rg3 group, and Suramin + Rg3 group were obviously decreased ($P < 0.05$). Serum levels of CRP, IL-6, and TNF-α in the Suramin + Rg3 group were significantly lower than those of the Suramin group and Rg3 group ($P < 0.05$). There were no obvious differences in the above serum inflammatory factors between the Suramin group and Rg3 group ($P > 0.05$) (**Figure 1A-C**).

Suramin combined with ginsenoside Rg3 alleviated morphology changes of venous allografts

Two months after surgery, H&E staining and Masson's staining results were assessed, as shown in **Figure 2A**, **2B**. Collagen fibers were blue and the smooth muscle cells were red. In the Sham group, the structure of each layer was clear with flat and smooth intima. There was no inflammatory cell infiltration and the smooth muscle cells were arranged neatly. However, there were high levels of inflammatory cell infiltration and smooth muscle cell migration in the NC group. Furthermore, collagen fiber deposition and mesangial smooth muscle disorders were observed. Only a small amount of inflammatory cell infiltration, migrating smooth muscle cells, and deposition of collagen fibers was found in the Suramin + Rg3 group. In addition, the Suramin + Rg3 group presented more uniform arrangement of smooth muscles. Morphological changes of transplanted vessels in the Suramin group and Rg3 group were between the NC group and Suramin + Rg3 group.

As shown in **Figure 2C**, **2D**. Compared with the NC group, graft intima thickness and media thickness levels were significantly decreased in the Suramin group, Rg3 group, and Suramin + Rg3 group ($P < 0.05$). Compared with the Suramin group or Rg3 group, intima-media thickness and median thickness levels of the Suramin + Rg3 group were significantly decreased ($P < 0.05$). Suramin group and Rg3 groups showed no statistical differences in graft intima thickness and media thickness ($P > 0.05$).

Suramin combined with ginsenoside Rg3 inhibited intimal hyperplasia of venous allografts

Positive expression of CD31 and CD34 was localized in the cell membrane. Positive expression of α-SMA was localized in the cytoplasm. Cells that exhibited brownish yellow particles were considered positively expressing cells. As

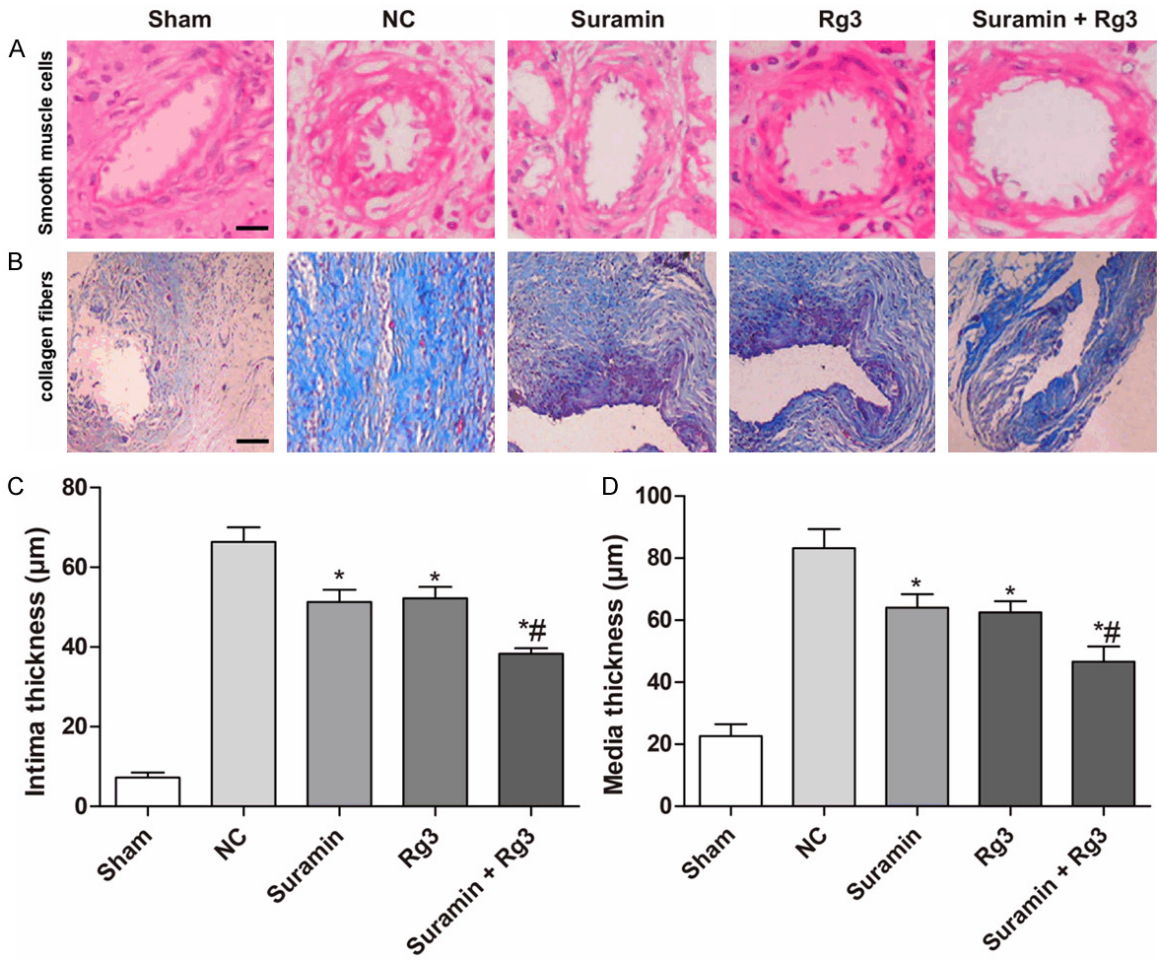

Figure 2. H&E staining and Masson's staining were used to observe the morphology of grafts. Note: A. H&E staining for smooth muscle cells; B. Masson's staining for collagen fibers; C. Intima thickness (μm); D. Mesenteric thickness (μm). Scar bar = 50 μm. *P < 0.05 (versus NC group). #P < 0.05 (versus the Suramin group or Rg3 group).

shown in **Figure 3A-C**, the number of CD31, CD34, and α-SMA positive cells in the Suramin group, Rg3 group, and Suramin + Rg3 group was significantly decreased, compared with the NC group (P < 0.05). The Suramin + Rg3 group still exhibited much lower CD31, CD34, and α-SMA positive cell numbers than the Suramin group or Rg3 group (P < 0.05). There were no obvious differences between the Suramin group and Rg3 group (P > 0.05).

Suramin combined with ginsenoside Rg3 promoted vascular smooth muscle cell apoptosis in venous allografts

According to TUNEL staining, cells with brown-yellow nuclei were positive cells, considered to be apoptotic cells. Results showed that, compared with the NC group, the number of positive cells in the Suramin group, Rg3 group, and Suramin + Rg3 group was significantly increased (P < 0.05). More importantly, compared with the Suramin group and Rg3 group, the Suramin + Rg3 group showed significantly increased numbers of TUNEL positive cells (P < 0.05) (**Figure 4**).

Discussion

In the current study, the combination of suramin and ginsenoside Rg3 significantly reduced concentrations of serum inflammatory factors, including CRP, IL-6, and TNF-α. At the same time, postoperative inflammatory cell infiltration and thickness of intima and media levels of the grafts were reduced. CRP is a biomarker of inflammation and cardiovascular disease [15]. Inflammatory status and therapeutic ef-

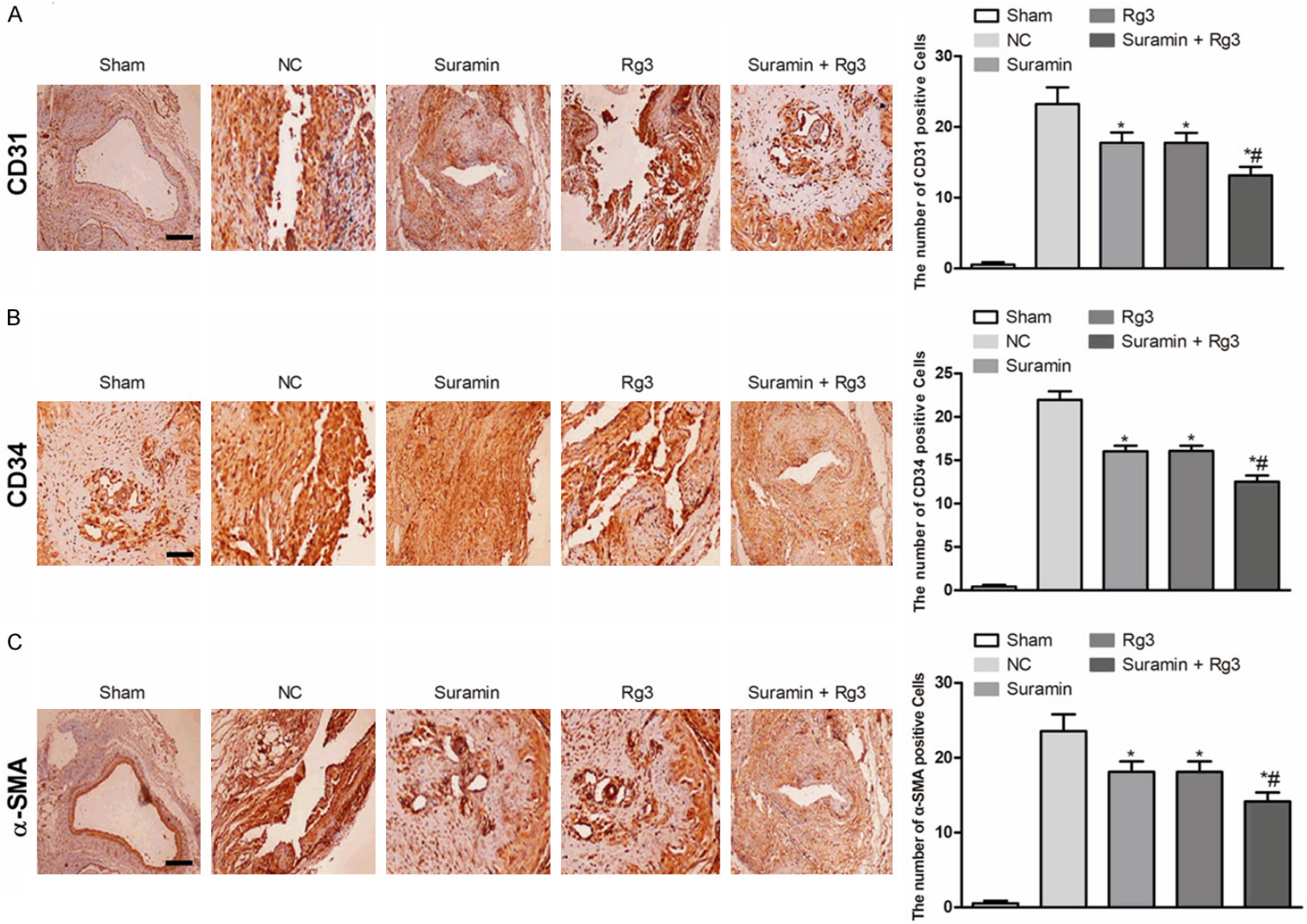

Figure 3. Immunohistochemical detection of intimal hyperplasia-associated protein expression in grafts. Note: Immunohistochemistry was used to detect expression of (A) CD31; (B) CD34; and (C) α-SMA in the grafts of each group. The experiment was repeated 5 times. Scar bar = 50 μm. *$P < 0.05$ (versus the NC group). #$P < 0.05$ (versus the Suramin group or Rg3 group).

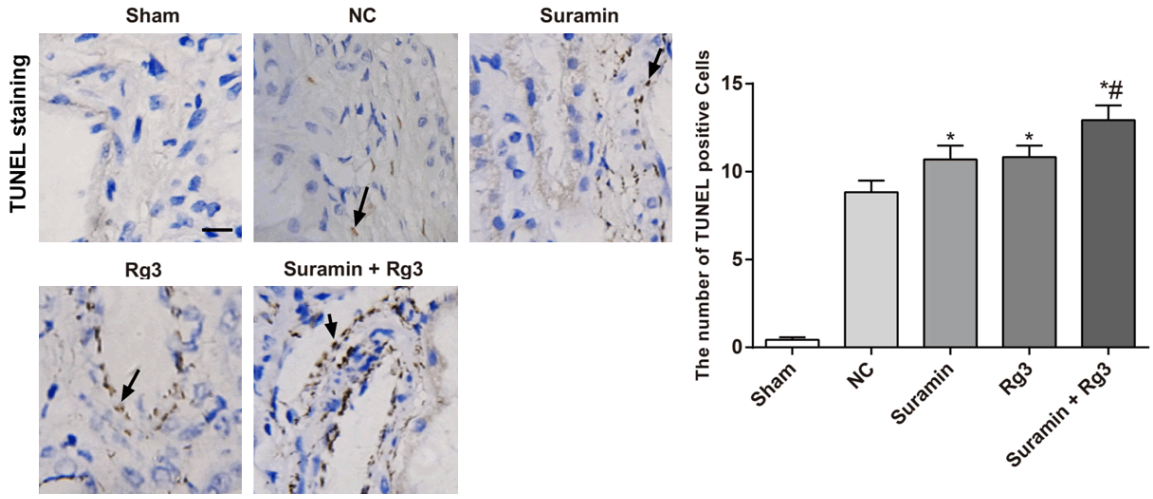

Figure 4. Detection of vascular smooth muscle cell apoptosis by TUNEL staining. Scar bar = 100 μm. Black arrows indicate positive cells. Note: *$P < 0.05$ (versus the NC group). #$P < 0.05$ (versus the Suramin group or Rg3 group).

fects could be monitored by CRP. Local inflammatory cancer could be amplified by pro-inflammatory cytokine IL-6 [16]. In addition, endothelial cell inflammation and atherosclerosis are mediated by TNF-α. [17]. Suramin is a compound that could inhibit the interaction between many cytokines/growth factors and their receptors. It is a blocker of transforming factor β1 receptor and a powerful competitive inhibitor of reverse transcriptase, which could block infection and cytopathy [18, 19]. Suramin has been shown to inactivate myofibroblasts and inhibit the expression of inflammatory factors in an animal model of renal ischemia-reperfusion injury [20], while ginsenoside Rg3 may rely on its anti-apoptotic and anti-inflammatory pharmacological effects to improve myocardial ischemia-reperfusion damage [21]. In addition, ginsenoside Rg3 pretreatment attenuates acetaminophen (APAP)-induced apoptosis and inflammatory infiltration of liver tissues [22]. This suggests that suramin combined with ginsenoside Rg3 provides significant effects in reducing vascular inflammatory response.

Reduced numbers of CD31, CD34, and α-SMA positive cells in transplanted blood vessels of rabbits have been observed by the combination of suramin and ginsenoside Rg3. CD31 is a member of the immunoglobulin superfamily. Its expression is an important factor confirming the integrity of endothelial cells and smooth muscle cells [23]. Moreover, the number of CD31 cells is increased by the injection of suramin within two weeks after muscle injuries. Neovascularization is also increased [24]. CD-34, a highly glycosylated type-I transmembrane glycoprotein, is involved in cell adhesion and inflammatory response. CD34 in the blood circulation is accumulated after vascular injuries [25]. Moreover, α-SMA is encoded in vascular smooth muscle cells. Its expression is changed in the pathology of atherosclerosis. Moreover, expression of endothelial cell markers (CD31 and CD34) in normal human tissues is specific [26]. Expression levels of vascular markers CD31 and CD34 are reduced and angiogenesis is inhibited by ginsenoside Rg3 [27, 28]. After vascular injuries, intimal hyperplasia development would be promoted if α-SMA and CD31 subpopulations are accumulated in the injured site and neointima [29]. Neointimal cells of mice with mechanical intimal injuries are subpopulations of fibroblasts expressing SMA and CD31. Moreover, intimal hyperplasia cannot occur when CD31+ fibroblasts are inhibited from proliferating [30]. This suggests that suramin and ginsenoside Rg3 administration is effective in inhibiting intimal hyperplasia of transplanted blood vessels.

Suramin combined with ginsenoside Rg3 could significantly increase apoptosis of smooth muscle cells. Vascular smooth muscle cells are the tissue structures constituting the blood vessel wall. Cell accumulation is a marker of atherosclerosis and vascular damage [31]. In addition, arteriosclerosis caused by vascular grafting may result in damage to the arterial wall and dysfunction of endothelial cells and smooth

muscle cells by the recipient's immune system. Main manifestations include thickening of the intima and occlusion of the lumen [32]. Moreover, inflammation is the core of the pathogenesis of transplanted arteriosclerosis [33]. The underlying cause of intimal hyperplasia is the proliferation and migration of vascular smooth muscle cells caused by injuries, inflammation, and stretching [34]. Furthermore, intimal hyperplasia is the main cause of restenosis or occlusion after vascular surgery [35]. In the case of restenosis after carotid injuries, inhibition of proliferation and migration of vascular smooth muscle cells could suppress intimal hyperplasia [36]. In addition, the formation of neointima in the graft veins could also be inhibited by regulating the proliferation and migration of vascular smooth muscle cells and the signal of inflammatory cytokine secretion [37]. Angiogenesis may be inhibited by suramin via targeting fibroblast growth factor and vascular endothelial cells [38]. This suggests that suramin combined with ginsenoside Rg3 inhibits intimal hyperplasia of vascular grafts by promoting smooth muscle cell apoptosis.

In the current study, the chronic rejection of rabbit external carotid artery-common carotid arteries was studied. It was found that suramin combined with ginsenoside Rg3 could alleviate graft vascular disease and adventitial inflammation, providing a theoretical basis for clinically successful vascular grafting.

Disclosure of conflict of interest

None.

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