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*Original Article*

# The role of sildenafil in the development of transplant arteriosclerosis in rat aortic grafts

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**Abstract:** Chronic rejection (CR), which is characterized histologically by progressive graft arteriosclerosis, remains a significant barrier to the long-term survival of a graft. Sildenafil has been shown to protect vascular endothelial cells. In this study, we found that sildenafil significantly reduces the thickness of transplant vascular intima in a rat aortic transplant model. Moreover, sildenafil dramatically decreased the expression of transforming growth factor-β1 (TGF-β1), vascular endothelial growth factor (VEGF), and α-smooth muscle actin (α-SMA) in the grafted aortas and increased the concentrations of cyclic guanosine monophosphate (cGMP) and endothelial nitric oxide synthase (eNOS) in serum. Furthermore, the ratio of regulatory T (Treg) cells and the expression of FoxP3 were increased, and the ratio of Th17 cells was decreased in the sildenafil-treated group. These results demonstrate that sildenafil enhances nitric oxide (NO) signaling by increasing the availability of cGMP, leading to an increase in the ratio of Treg/Th17 cells to attenuate transplant arteriosclerosis in a rat aortic transplant model.

**Keywords:** Chronic rejection, graft arteriosclerosis, sildenafil, eNOS, cGMP, Th17/Treg

## Introduction

Although advances in surgery and usage of immunosuppressive drugs have occurred in recent years, chronic rejection (CR) remains a significant barrier to the long-term survival of a graft. After the first year following kidney transplantation, 3-5% of grafts fail each year [1]. CR is characterized histologically by progressive graft arteriosclerosis, a vasculopathy that consists of diffuse concentric intimal thickening and adventitial fibrosis [2]. Sildenafil is a selective inhibitor of cyclic guanosine monophosphate (cGMP)-specific phosphodiesterase (PDE) type 5 and is known for the significant curative effect on erectile dysfunction in patients [3].

Previous studies [4, 5] demonstrated that sildenafil restores endothelial function and improves vascular remodeling in patients with refractory secondary Raynaud's phenomenon and pulmonary artery hypertension. Furthermore, PDE inhibitors also restrain T-cell activity by different mechanisms [6, 7] and increase the expression of cGMP to decrease the activity of pro-inflammatory Th1 cells in experimental arthritis and experimental autoimmune encephalomyelitis [8, 9]. No previous studies, however, have evaluated the use of PDE inhibitors for the treatment of transplant arteriosclerosis. In this study, we investigated the effect of sildenafil on graft arteriosclerosis in a rat model of aortic transplantation and examined the possible molecular mechanisms that underlie these effects.

## Materials and methods

### *Animals*

Twenty-four healthy male Wistar rats (200-300 g) were used as donor animals. Another 6 healthy Wistar rats and 18 healthy SD rats (200-300 g) served as recipient animals. All animals were provided by the Slack Laboratory Animal Company, LTD (Shanghai, China). All animal protocols were approved by Wenzhou

Medical University Animal Policy and Welfare Committee.

*Aortic transplantation*

Aortic transplantations were performed as previously described [10]. After transplantation, the animals were divided into four groups: isograft control (saline as placebo) group (n=6), allograft control (saline as placebo) group (n=6), prednisone-treated group (n=6), and sildenafil-treated group (n=6). In the isograft control group, the thoracic aorta of a Wistar rat was transplanted into the abdominal cavity of another Wistar rat and anastomosed to the abdominal aorta of the recipient. In the allograft control group, the prednisone-treated group, and sildenafil-treated group, the thoracic aorta of a Wistar rat was transplanted into the abdominal cavity of a SD rat and anastomosed to the abdominal aorta of the recipient. After transplantation, in the isograft control group and allograft control group, saline was administered by lavage to the recipient animals at a dosage of 1 ml daily. In the prednisone-treated group, recipient animals were treated with prednisone at a dosage of 3.5 mg/kg daily. In the sildenafil-treated group, sildenafil was administered to the recipient animals at a dosage of 5.8 mg/kg daily. At the end of the study, 8 weeks after transplantation, the rats were humanly sacrificed, and the grafts were harvested for analysis.

*Histopathology and immunohistochemistry*

Graft aorta segments were fixed in 10% formalin for 2 d and embedded in paraffin. Then, the tissues were cut into 4-μm slices and stained with hematoxylin-eosin-saffron (HES) for general morphological examination following deparaffinization and rehydration. Immunohistochemistry was performed to localize the expression of transforming growth factor-β1 (TGF-β1), α-smooth muscle actin (α-SMA), and vascular endothelial growth factor (VEGF). All antibodies were purchased from Abcam Biotechnology (Cambridge, MA). The protein was quantified by scanning densitometry using Image Pro Plus.

*Endothelial nitric oxide synthase (eNOS) and cGMP assay*

Blood samples from each recipient were collected by cardiac puncture and used for determining the production of eNOS and cGMP in serum using an enzyme-linked immunosorbent assay (ELISA) in accordance with the manufacturer's instructions. The ELISA kits were purchased from Westang (Shanghai, China).

*Western blotting*

Western blotting was performed to evaluate the expression of TGF-β1, α-SMA, and VEGF in the graft vessel tissues. The harvested graft vessel tissues were homogenized in lysis buffer, and the total protein concentrations were determined. Isolated proteins (20 μg per specimen) were separated on a 10% SDS polyacrylamide gel, blotted onto nitrocellulose membranes, and incubated with primary antibodies against TGF-β1, α-SMA, VEGF, and β-actin (Abcam Biotechnology, Cambridge, MA) overnight at 4°C. Then, the membranes were incubated with a secondary horseradish peroxidase-conjugated antibody for 2 h at room temperature. The signal was detected with an enhanced chemiluminescence kit (Westang) according to the manufacturer's instructions. The intensities of the protein bands were quantified using the Quantity One software (Version 4.6.2, Bio-Rad, Hercules, CA).

*Flow cytometry*

Lymph cell suspensions were prepared from the collected blood specimens, and the concentration of lymphocytes in each sample was adjusted to $5\times10^6$ cells/ml. Then FITC-labeled CD4 and PE-labeled interleukin (IL)-17 antibodies were added. Cell membranes were permeabilized to allow detection, and the ratio of Th17 cells to $CD4^+$ cells was determined. The concentration of lymphocytes was adjusted to $5\times10^8$ cells/ml, and then FITC-labeled CD4 and PE-labeled CD25 antibodies were added. Following permeabilization, the cells were incubated with PE-labeled Foxp3 antibodies overnight. The ratio of $CD4^+CD25^+Foxp3^+$ T cells to $CD4^+$ cells was detected. Flow cytometry was performed using FACS Calibur (Becton Dickinson, San Jose, CA), and FACS data were analyzed using CellQuest software (Becton Dickinson, Rutherford, NJ). All antibodies were purchased from eBiosciene (San Diego, CA).

*Statistical analysis*

All data are presented as the mean values ± standard error of the mean. Statistical analysis was performed using one-way ANOVA and

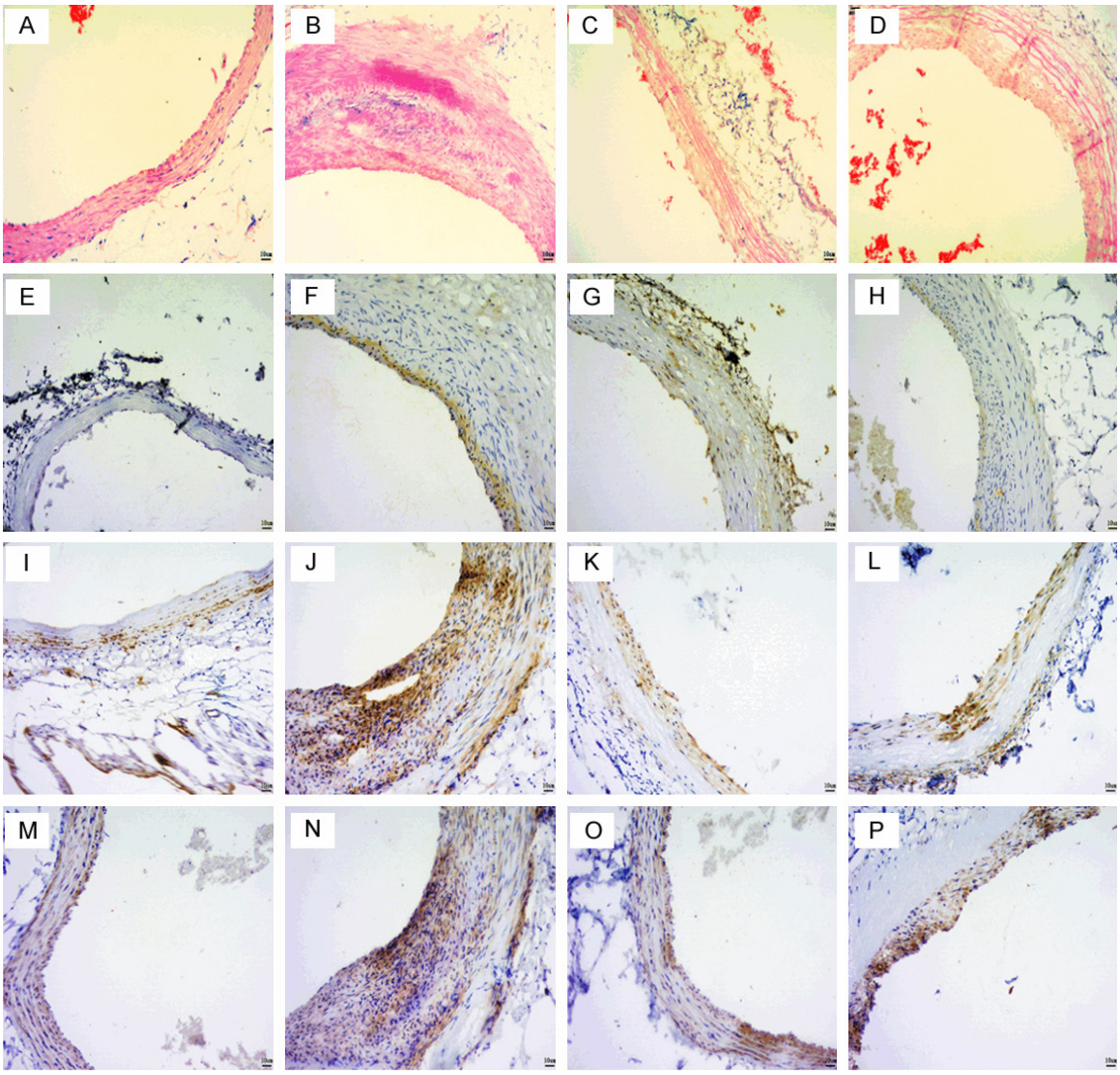


**Figure 1.** The remodeling of arterial wall and the expression of VEGF, α-SMA and TGF-β1 in the transplanted arterial segment at 8 weeks after transplantation. (A-D) Illustrating microscopic finding (100×) of HES staining for identification of the proliferations of intimal and medial layer of transplanted aortas. (E-H) Immunohistochemical staining for VEGF (original magnification, 100×). (I-L) Immunohistochemical staining for α-SMA (original magnification, 100×). (M-P) Immunohistochemical staining for TGF-β1 (original magnification, 100×). In transplanted aortas of the (A, E, I, M) is isograft, (B, F, J, N) is allograft, (C, G, K, O) is Prednisone treatment and (D, H, L, P) is Sildenafil treatment.

q-test to compare the differences between the two groups. All *p*-values <0.05 were considered significant. The data were analyzed using SPSS software (version 13; SPSS).

## Results

### *Neointimal thickness*

HES staining results showed marked proliferation of intimal cells in the control group and a significant reduction in neointimal thickness in the prednisone-treated and sildenafil-treated groups compared with the control group (0.32±0.06 μm [isograft]; 10.38±1.97 μm [allograft]; 2.15±0.26 μm [prednisone]; 3.97±0.31 μm [sildenafil]; $P<0.05$; **Figure 1**). These results demonstrate that prednisone and sildenafil alleviate transplant arteriosclerosis; however, neointimal thickness was not significantly decreased in the animals treated with sildenafil compared to those treated with prednisone.

### *Inhibition of TGF-β1, α-SMA, and VEGF expression in aortic allografts by prednisone and sildenafil*

Immunohistochemical staining revealed that TGF-β1, α-SMA, and VEGF were expressed in the vascular wall in the allograft control group,

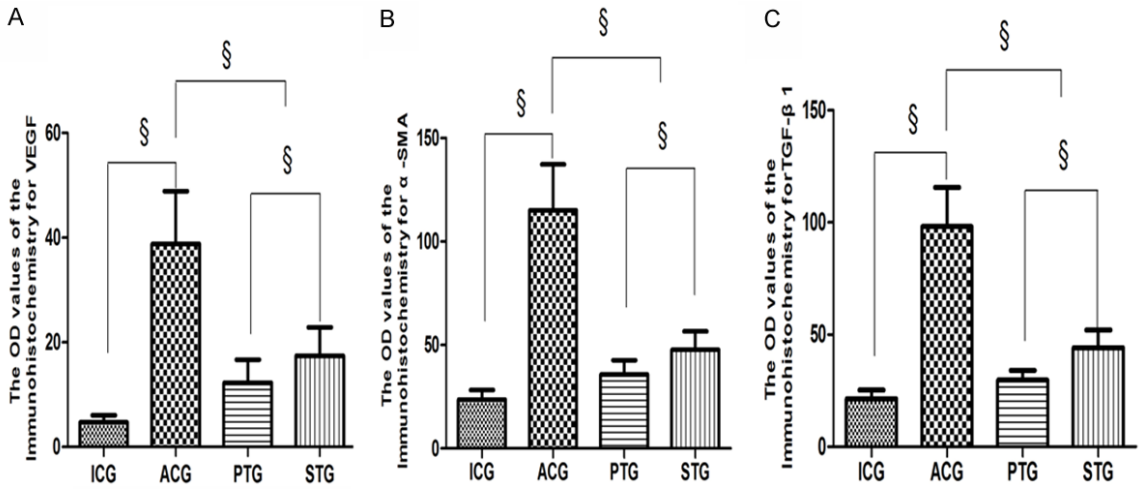


**Figure 2.** Comparison of the expression for VEGF, α-SMA, and TGF-β1 expression in the aortic grafts. The protein was quantified by scanning densitometry. A. The OD values of the Immunohistochemistry for VEGF, versus other groups with symbol §; P<0.05. B. The OD values of the Immunohistochemistry for α-SMA, versus other groups with symbol §; P<0.05. C. The OD values of the Immunohistochemistry for TGF-β1, versus other groups with symbol §; P<0.05. ICG = isograft control group; ACG = allograft control group; PTG = Prednisone treated group; STG = Sildenafil treated group.

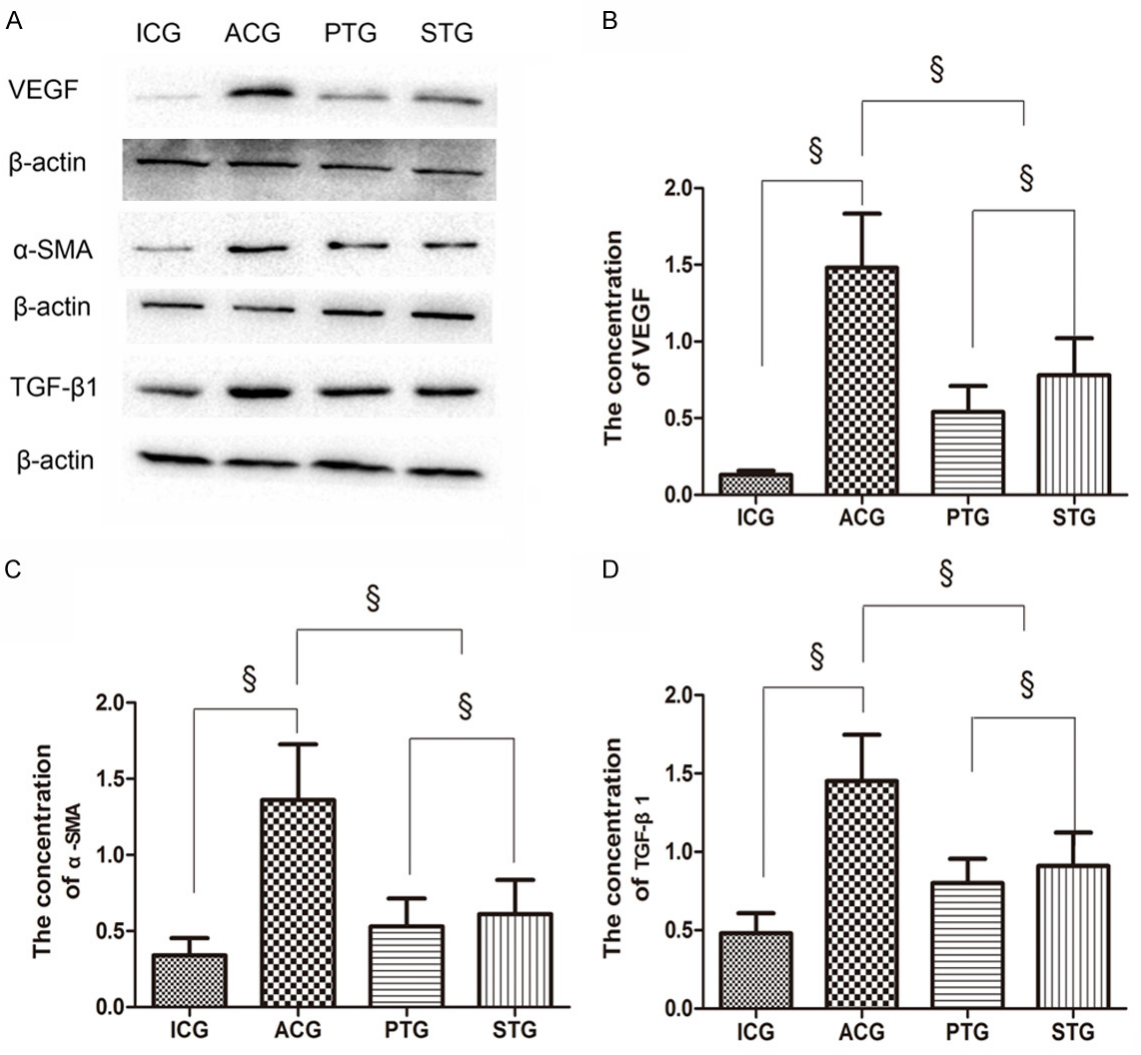

**Figure 3.** Comparison of the expression for VEGF, α-SMA, and TGF-β1 in the aortic grafts. A. Western blotting showed the expression of VEGF, α-SMA, and TGF-β1 in the different groups. The same blot was stripped and reprobed with actin to confirm equal loading. B. The ratio of VEGF/β-actin, versus other groups with symbol §; P<0.05. C. The ratio of α-SMA/β-actin, versus other groups with symbol §; P<0.05. D. The ratio of TGF-β1/β-actin, versus other groups with symbol §; P<0.05. ICG = isograft control group; ACG = allograft control group; PTG = Prednisone treated group; STG = Sildenafil treated group.

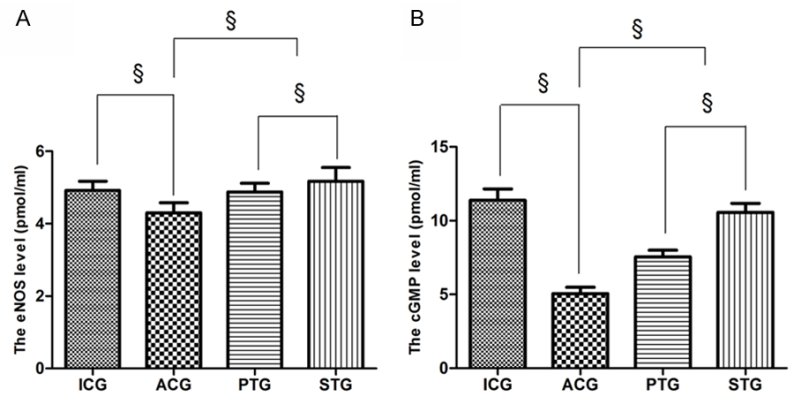


**Figure 4.** The plasma eNOS and cGMP levels in different groups after eight weeks of treatment. A. The plasma eNOS level, versus other groups with symbol §; P<0.05. B. The plasma cGMP levels, versus other groups with symbol §; P<0.05. ICG = isograft control group; ACG = allograft control group; PTG = Prednisone treated group; STG = Sildenafil treated group.

while no significant expression was observed in the prednisone-treated and sildenafil-treated groups (**Figure 1**). Semi-quantitative score revealed the OD values of TGF-β1, α-SMA, and VEGF decreased in the prednisone-treated and sildenafil-treated group compared with the control group; the OD values of TGF-β1, α-SMA, and VEGF in sildenafil-treated group was higher than the prednisone-treated group (*P*<0.05; **Figure 2**). Western blot analysis revealed the TGF-β1, α-SMA, and VEGF expression in the prednisone-treated group and in the sildenafil-treated group was significantly lower than that in the control group; expression of TGF-β1, α-SMA, and VEGF in sildenafil-treated group was higher than the prednisone-treated group (*P*<0.05; **Figure 3**). These results indicate that treatment with prednisone and sildenafil reduce the expression of TGF-β1, α-SMA, and VEGF in aortic allografts in rats.

*Effects of prednisone and sildenafil treatment on the concentration of eNOS and cGMP in serum*

The ELISA assay showed that prednisone and sildenafil significantly enhanced the production of cGMP and eNOS in the serum of treated aortic allograft recipient rats compared to control recipients. Furthermore, compared with the levels in the prednisone-treated group, the production of cGMP and eNOS was significantly enhanced in the serum from sildenafil-treated animals (*P*<0.05, **Figure 4**).

*Effect of prednisone and sildenafil treatment on the distribution of the $CD4^+CD25^+FoxP3^+$ regulatory T (Treg) and $CD4^+$ Th17 cells in the peripheral blood*

As shown in **Figures 5** and **6**, flow cytometry analysis demonstrated that the number of $CD4^+CD25^+$ T cells and expression of Foxp3 in the allograft control group were markedly lower than those in the isograft control group (*P*<0.05). Meanwhile, prednisone and sildenafil significantly increased these values compared to those in the allograft control group (*P*<0.05); however, the number of Th17 cells in the prednisone and the sildenafil treatment groups were markedly lower than that in the allograft control group (*P*<0.05). The ratios of $CD4^+CD25^+FoxP3^+$ T cells to Th17 cells in the prednisone and sildenafil treatment groups were significantly higher (*P*<0.05) than that in the allograft control group but significantly lower than that in the isograft control group (*P*<0.05).

## Discussion

The development of CR remains the leading cause of late allograft dysfunction and allograft failure following solid organ transplantation. CR is an insidious process that is characterized histologically by progressive graft arteriosclerosis, an obliterative vasculopathy that consists of diffuse concentric intimal thickening and adventitial fibrosis. The pathogenesis of CR, which is a multi-factorial process that includes both immunological and nonimmunological risk

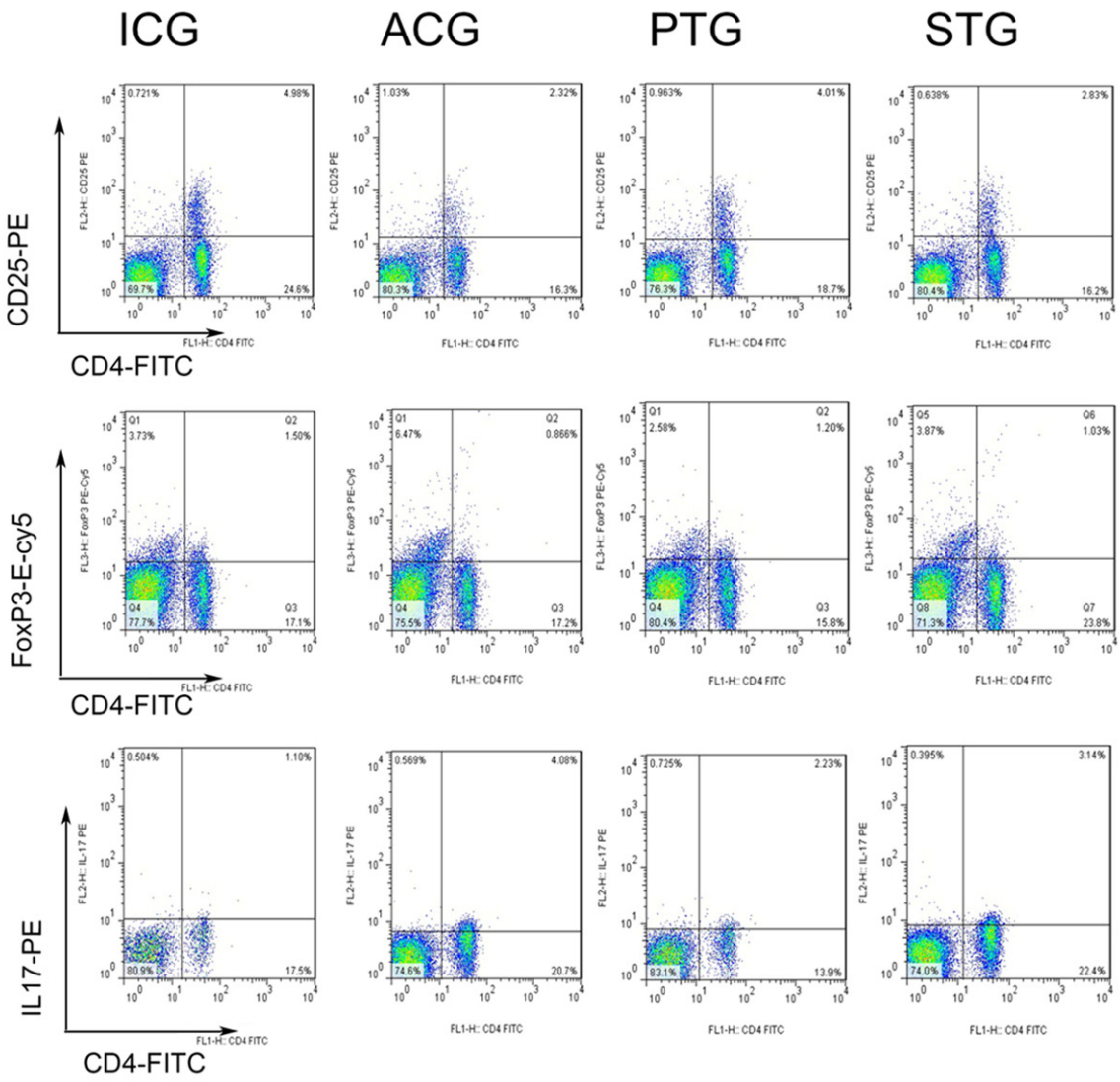


**Figure 5.** The proportion of the $CD4^+CD25^+$ Treg, FoxP3 and $CD4^+$ Th17 cells in rat peripheral blood in the different groups. The ratio of $CD4^+CD25^+FoxP3^+$ T cells/Th17 cells in PTG and STG was significantly higher. ICG = isograft control group; ACG = allograft control group; PTG = Prednisone treated group; STG = Sildenafil treated group.

factors [11, 12]; the present study revealed that the development of CR, characterized by transplant arteriosclerosis, involves growth factors, pro-inflammatory cytokines, and T cells [13-15].

TGF-β1, a fibrogenic growth factor, has been shown to promote the proliferation and migration of vascular smooth muscle cells (VSMCs) and potentiate the development of neointimal hyperplasia during vascular wall remodeling [16, 17]. Numerous studies have identified TGF-β1 as the key factor involved in allograft fibrosis induction and suggested that TGF-β1 plays an important role in CR [18-21]. Increased expression of VEGF has been shown to occur in transplanted hearts and accelerate the development of allograft arteriosclerosis by enhancing VSMC migration and recruiting macrophages [22-24]. Indeed, VEGF levels can be monitored as an index of the development of allograft vasculopathy [25, 26]. Furthermore, the extracellular matrix protein α-SMA is a specific molecular marker of activated myofibroblasts, and the expression of α-SMA is correlated with the fibrosis process [27, 28]. Nitric oxide (NO), a potent vasodilator that stimulates soluble guanylate cyclase to produce cGMP and is synthesized from the amino acid L-arginine by NOS, inhibits key processes in atherosclerosis and

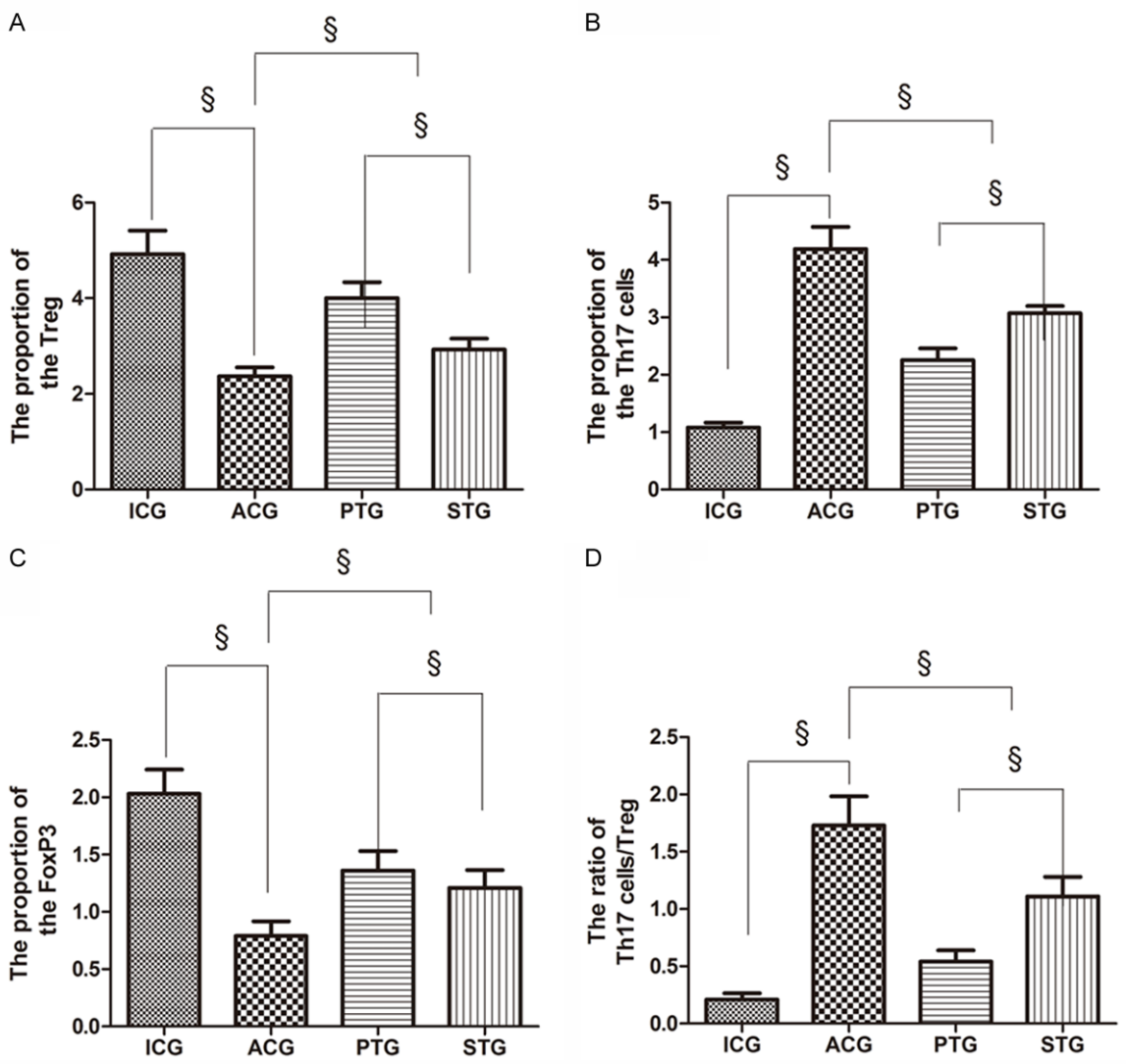


**Figure 6.** The proportion of the $CD4^+CD25^+$ Treg, FoxP3 and $CD4^+$ Th17 cells in rat peripheral blood in the different groups. A. The proportion of the Treg, versus other groups with symbol §; $P<0.05$. B. The proportion of the Th17 cells, versus other groups with symbol §; $P<0.05$. C. The proportion of the FoxP3, versus other groups with symbol §; $P<0.05$. D. The ratio of Th17 cells/Treg, versus other groups with symbol §; $P<0.05$. ICG = isograft control group; ACG = allograft control group; PTG = Prednisone treated group; STG = Sildenafil treated group.

vascular inflammation [29]. In addition, eNOS is constitutively expressed by endothelial cells [30], and one recent study found that eNOS deficiency plays a role in the progression of transplant vasculopathy [31].

Sildenafil is a selective inhibitor of PDE-5, and this drug was developed for the potential therapeutic effect in the treatment of cardiovascular diseases in the early stages. Sildenafil, however, was not promising as an antianginal drug in early human trials, because an erectogenic "adverse event" was discovered during clinical trials, and now, this drug is famous for its remarkable curative effect for the treatment of erectile dysfunction in patients [3, 32]. In recent years, sildenafil, which has been reported to restore endothelial function and reduce oxidative stress, has also been proposed as a potential therapeutic strategy for pulmonary arterial hypertension and atherosclerosis [4, 5, 33]. Of interest, sildenafil has been found to upregulate eNOS expression, enhance NO bioavailability, and play a role in treatment of erectile dysfunction via the NO-cGMP signaling pathway [6]. In addition, a report by Savvanis et al. demonstrated that the administration of sildenafil attenuates hepatocellular injury by

upregulating the expression of eNOS and increasing the intrahepatic NO production after liver ischemia reperfusion in rats [34]. Despite these studies of the effects of sildenafil, previous reports of the role of sildenafil in the progression of transplant vasculopathy are scarce or nonexistent.

Here, we designed our study to determine the effects of sildenafil on allograft arteriosclerosis in a rat model of aortic transplantation as well as to determine the molecular mechanism underlying these effects. In this study, we found that sildenafil reduced intimal thickening in a rat model of transplant arteriosclerosis. Moreover, immunohistochemistry and western blotting showed that sildenafil significantly inhibited the expression of TGF-β1, α-SMA, and VEGF, suggesting that sildenafil may alleviate allograft arteriosclerosis in rats. Although studies have demonstrated that the main mechanism of sildenafil on endothelial function occurs via stimulation of the expression of NO via the sGC-cGMP pathways [6, 7, 35], the mechanism of alleviating allograft arteriosclerosis has not been reported previously. Furthermore, we observed a significant increase in serum eNOS and cGMP levels in rats treated with sildenafil compared with those in the control group and those in the prednisone-treated group. These findings suggest that sildenafil alleviation of allograft arteriosclerosis may occur through enhancement of NO signaling by increasing the availability of cGMP.

It is well known that Treg cells play a key role in the control of reactivity to alloimmune response [36], and $CD4^+CD25^+$ T cells, a subset of Treg cells that constitutively coexpress CD4 and CD25 antigens, are thought to be the best-characterized population of Treg cells [37]. One study demonstrated, however, that $CD4^+CD25^+$ T cells differentiate into two distinct populations of cells: effecter $CD4^+CD25^+$ T cells and regulatory $CD4^+CD25^+$ T cells, which also have expression of the Treg cell-specific FoxP3 marker [38]. Treg cells have been regarded as a viable alternative to control immune reactivity of solid organ allografts and to induce immunological tolerance in clinical transplantation [39-42]. Th cells express the surface protein CD4 and modulate immune responses. Th17 cells comprise a Th cell subset that regulates inflammation via production of IL-17 and is distinct from Th1 and Th2 lineages [43]. Accumulating evidence indicates that the Th17 effector cells play a pivotal role in the development of human autoimmune diseases, such as psoriasis, rheumatoid arthritis (RA), Crohn's disease, and many others [44]. Interestingly, numerous studies, conducted with animal and human models, confirm that Th17 cells negatively mediate vascular inflammation and accelerate the process of graft vascular disease in a model of cardiac allograft vasculopathy (CAV) [45]. The interaction between Treg cells and Th17 cells is not well understood, but recent data suggest that the imbalance of Th17/Treg cells in the development of human autoimmune diseases and the process of organ transplantation rejection is essential [46, 47]. Autoimmune and pro-inflammatory effects appear to predominate following an increase in the ratio of Th17/Treg cells, and when this ratio is lowered, immune tolerance and anti-inflammatory effects predominate.

Sildenafil plays a role in the suppression of T effector cells (Teffs), downregulation of Th1/Th2/Th17 responses, and upregulation the Tregs [8, 48] in experimental autoimmune encephalomyelitis progression. Moreover, Gonzalez-Garcia et al. confirmed that PDE inhibitors restrain T-cell activity by multiple mechanisms in experimental autoimmune encephalomyelitis [9]. In the present study, the number of $CD4^+CD25^+FoxP3^+$ T cells in the prednisone-treated group and the sildenafil-treated group was significantly higher than that in the allograft control group, while the number of Th17 cells was markedly lower than that in the allograft control group ($P<0.05$). Therefore, the ratio of Th17/Treg cells in the prednisone and sildenafil treated groups was significantly lower than that in the allograft control group. These findings suggest that sildenafil may alleviate allograft arteriosclerosis via immune regulation, leading to a concomitant increase in the number of Treg cells and a decrease in the number of TH17 cells.

In conclusion, we have demonstrated that sildenafil attenuates graft arteriosclerosis via a complex mechanism that involves both immunological and nonimmunological pathways. In addition, sildenafil enhances NO signaling by increasing the availability of cGMP, leading to an increase in the number of Treg cells. Taken

together, these data suggest that sildenafil may be the drug of choice for the treatment of transplant arteriosclerosis.

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## Disclosure of conflict of interest

None.

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