

Tadalafil attenuates graft arteriosclerosis of aortic transplant in a rat model

Xu Ziqiang¹, Wang Jingjun¹, Zheng Jianjian¹, Liang Yong¹, Xia Peng^{1*}

¹ Transplantation Center, the First Affiliated Hospital of Wenzhou Medical University, Wenzhou, China

ARTICLE INFO

Article type:

Original article

Article history:

Received: Jan 3, 2015

Accepted: Jul 3, 2015

Keywords:

cGMP

Neointimal thickness

Tadalafil

Transplants

ABSTRACT

Objective(s): Tadalafil can restore endothelial function and treat atherosclerosis. However, the effect of tadalafil on transplant arteriosclerosis remains unclear. In this study, we explore the effects of tadalafil on allograft vasculopathy.

Materials and Methods: Male Brown-Norway rats supplied aorta grafts for Male Lewis rats. All recipients were divided into 3 groups: saline as placebo (control) treated group, low dose tadalafil (0.5 mg/kg/day) treated group, and high dose tadalafil (1.0 mg/kg/day) treated group. Eight weeks after transplantation, the grafts were harvested and analyzed by histological and Western blot analysis. An enzyme-linked immunosorbent assay (ELISA) was used for measure of plasma cyclic guanylate monophosphate (cGMP).

Results: the treatment with tadalafil significantly alleviated the neointimal thickness of aortas compared with the control group ($P<0.05$). Tadalafil also remarkably enhanced the production of cGMP in plasma and expression of cGMP-dependent kinase I (PKG-I) and RhoA compared with control group ($P<0.05$).

Conclusion: These results showed that tadalafil can attenuate graft arteriosclerosis by cGMP-PKG-I pathway.

► Please cite this article as:

Ziqiang X, Jingjun W, Jianjian Zh, Yong L, Peng X. Tadalafil attenuates graft arteriosclerosis of aortic transplant in a rat model. Iran J Basic Med Sci 2015; 18:927-931.

Introduction

In recent years, acute rejection inducing graft loss has been obviously reduced, but chronic rejection still limits the graft's long-term survival. It is well-known that the development of chronic rejection results in graft arteriosclerosis which is common in most cases, finally leading to short-term graft survival (1). This progressive graft arteriosclerosis is characterized by diffuse concentric intimal thickening, migration and proliferation of vascular smooth muscle cells (VSMC) from their normal position in the tunica media to the intimal region (2). Similar to native arteriosclerosis, endothelial dysfunction can accelerate allograft vascular remodeling and arteriosclerosis.

Nitric oxide (NO) has been regarded as a key mediator in the development of transplant arteriosclerosis (3), and causes vasodilation, protects the blood vessel from endogenous injury and inhibits VSMC proliferation and migration (4, 5).

Phosphodiesterase type 5 (PDE5) plays an important role in hydrolyzing cyclic guanylate monophosphate (cGMP), which can enhance vascular tone through regulation of NO activity and is expressed throughout various tissues, including the vascular, visceral, and pulmonary smooth muscles.

Activation of NO-cGMP pathway via inhibiting PDE5 activity could cause smooth muscle relaxation and VSMC proliferation inhibition. Thus, NO enhancing therapies are an attractive area to explore. To date, no studies have evaluated this strategy in transplant arteriosclerosis. Sildenafil, a kind of PDE5 inhibitor, improves brachial artery function in a small group of hypertensive male heart transplant recipients (6). In a recent study, Balarini confirmed that sildenafil could restore endothelial function, reduce oxidative stress and treat atherosclerosis in a murine model (7). Therefore, we investigated the effect of chronic low-dose tadalafil (a PDE5 inhibitor) administration on allograft arteriosclerosis in a rat model of aortic transplantation. Tadalafil has a unique characteristic, which is a long half-life of 17.5 hr when taken on a full stomach (8).

Materials and Methods

Animals

Twenty four male Brown-Norway (BN) rats and twenty four male Lewis rats (body weight, 220–250 g) were obtained from Vital River Laboratory Animal Technology Co, Ltd. (Beijing, China). All rats were housed in plastic cages and had free access to rodent chow and tap water. Animals'

*Corresponding author: Xia Peng, Transplantation Center, the First Affiliated Hospital of Wenzhou Medical University, Wenzhou, China. Tel: 86+0577-55579473; email: 372019615@qq.com

body weights and food intake were measured weekly. All animal procedures conformed to international guidelines and were approved by Wenzhou Medical University Animal Policy and Welfare Committee.

Aorta transplantation

Aortic transplantations were performed as previously described (9). Allogeneic aortic transplantation was performed using BN rats as donors and Lewis rats as recipients. Pentobarbital (60 mg/kg) was used to anesthetize these rats by intraperitoneal injection. The thoracic aorta was harvested from BN rats after intravenous injection of heparin. Then the aortic grafts were transplanted into recipients with an end-to-end anastomosis technique.

The recipients were randomized into three groups: one group received saline vehicle after an allograft for 8 weeks (control group); one group received low dose tadalafil (0.5 mg/kg/day), and one group received high dose tadalafil (1.0 mg/kg/day) after an allograft for 8 weeks. At the end of the study, the rats were anesthetized and the grafts were harvested for analysis.

Histopathology

Graft vessel tissues were fixed in 10% formalin for 48 hr and embedded in paraffin and cut into 4 μ m slices. Following deparaffinization and rehydration, every section of graft tissue was stained with hematoxylin and eosin for general morphological examination as described previously (10). The intima area was calculated by subtracting the lumen area from the area enclosed by the internal elastic lamina lining.

cGMP Assay

Rats were anesthetized and blood samples of each recipient were collected by cardiac puncture and centrifuged at 3000 rpm for 15 min at 4 °C to prepare plasma. Plasma blood sample of each recipient was collected after euthanasia and centrifuged for 15 min at 3000 rpm and stored at -80 °C. cGMP level in the plasma of all rats were measured by ELISA using the cGMP Complete Kit. Rat cGMP ELISA kit was purchased from Westang (Shanghai, China). Briefly, the plasma samples were initially precipitated, extracted, evaporated to dryness, and then reconstituted in an assay buffer. The samples and standards were acetylated to allow the detection of nucleotides in the picomolar range.

Western blot assay

Graft vessel tissues of all groups were homogenized in lysis buffer and the supernatants were collected by centrifugation at 12,000 \times g and 4 °C. After determining the total protein concentration, twenty micrograms of protein per specimen were run on 10% SDS-

polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes. After blocking at room temperature and incubation with different primary antibodies, the membranes were washed and incubated with secondary horseradish peroxidase-conjugated antibody at room temperature (11-13). An enhanced chemiluminescence kit (Amersham, USA) was used to visualize the antigen-antibody complexes, and the intensity of the protein bands was quantified using the Quantity one software (Version 4.6.2, Bio-Rad, USA). The primary antibodies against cGMP-dependent kinase I (PKG-I) and RhoA were purchased from Abcam (Cambridge, MA, USA), and β -actin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Statistical analysis

Data are presented as means \pm standard error of the mean (SEM). Statistical analyses were performed using one-way analysis of variance and $P < 0.05$ was considered significant. SPSS software (version 13; SPSS) was used for statistical analyses.

Results

Neointimal thickness

To determine whether allograft arteriosclerosis could be alleviated by tadalafil, we examined the neointimal hyperplasia of aortic grafts by histological examination at 8 weeks after transplantation. As shown in Figure 1, treatment with tadalafil decreased intimal thickening compared with saline controls and there was no significant difference in intimal thickening between low-dose and high-dose tadalafil groups. The results showed that tadalafil can alleviate transplant arteriosclerosis.

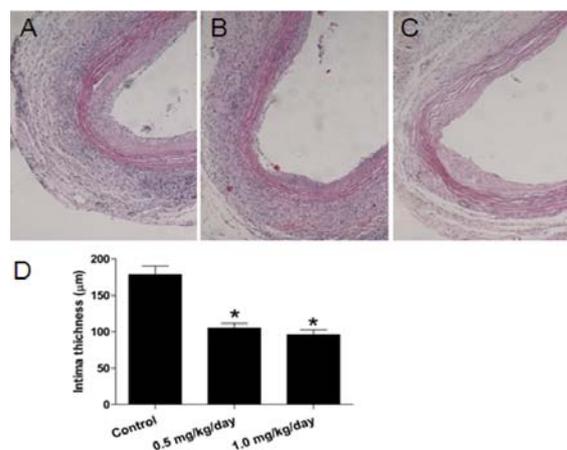


Figure 1. Histological findings (hematoxylin-eosin staining; magnification \times 200) in aortic graft specimens obtained 8 weeks after transplantation. Specimens are from rats given an allograft and saline vehicle (A), an allograft and 0.5 mg/kg/day (B), or an allograft and 1.0 mg/kg/day of tadalafil (C). The intimal thickening is thinner in the rats given tadalafil than the rats given saline (D). There was no significant difference between the tadalafil treatment groups (0.5 mg/kg/day vs. 1.0 mg/kg/day)

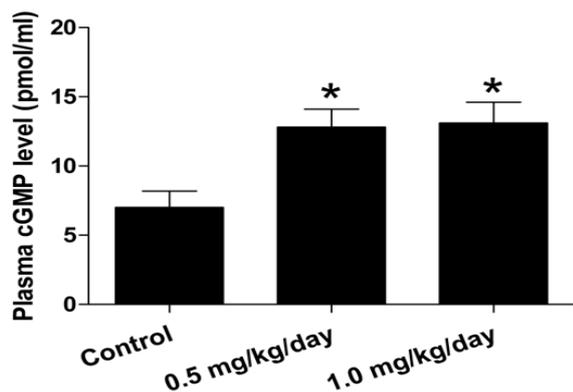


Figure 2. The plasma cyclic guanylate monophosphate levels in different groups after eight weeks of treatment with tadalafil

Plasma cGMP concentrations

A significant increase in plasma cGMP levels was observed in rats receiving tadalafil compared to saline controls ($P < 0.05$, Figure 2) and there was also no significant difference in plasma cGMP levels between the tadalafil treatment groups (0.5 mg/kg/day vs. 1.0 mg/kg/day).

PKG-I, RhoA expression in aortic allografts

We next examined expression of PKG-I and RhoA in aortic allografts in the 3 groups (Figure 3). The experimental groups receiving tadalafil demonstrated markedly upregulated expression of PKG-I compared to the saline controls ($P < 0.05$). As compared to saline controls, RhoA expression of the group treated with tadalafil was downregulated ($P < 0.05$). We did not detect that the expression of PKG-I and RhoA protein were remarkably different between the tadalafil treatment groups (0.5 mg/kg/day vs. 1.0 mg/kg/day).

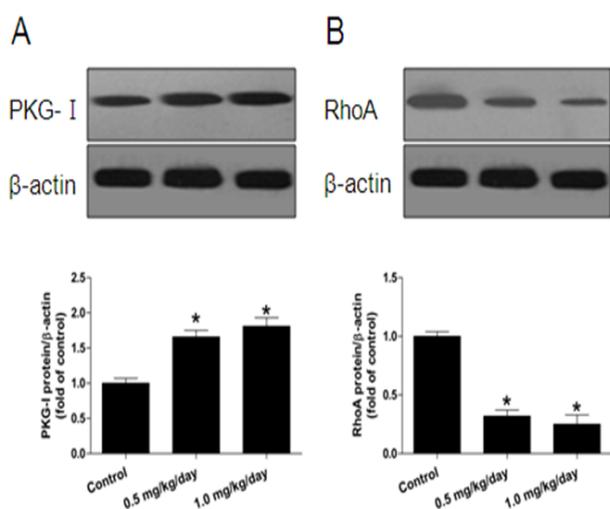


Figure 3. Western blotting showed the expression of cGMP-dependent kinase I (PKG-I) and RhoA in the different groups of tadalafil doses. * $P < 0.05$, compared with the control group

Discussion

Chronic rejection of vascular graft is a wholly peculiar arteriosclerotic entity. The pathogenesis of graft arteriosclerosis is a multi-factorial complex, associated with both immunologic and non-immunologic risk factors (14, 15). As a consequence of these multi-factorial injuries, the vessel tends to constrict, accompanied by platelet activation, proliferation, and vascular inflammation, leading to intimal thickening and VSMC proliferating (16). In our study, these features were also observed in the aortic allografts, suggesting that our rat model was similar to human allograft arteriosclerosis.

In this study, we found that intimal thickening in rat aortic allografts was remarkably reduced by administration of tadalafil compared to the saline controls. In addition, tadalafil could increase plasma cGMP level, up-regulate the expression of PKG-I and decline activation of RhoA. These findings suggest that tadalafil may inhibit allograft arteriosclerosis through NO-cGMP-PKG-I pathway. We did not find any significant difference between 0.5 and 1.0 mg/kg/day tadalafil. Maybe there will be differences between the two groups with prolonged treatment.

Ahmad *et al* (17) indicated that endothelium which regulates vascular tone and structure by releasing vasoactive substances, plays a crucial role in sensing changes in hemodynamic forces and blood-borne signals. It is reported that transplant recipient immune response to the donor endothelium may determine allograft adaptation and/or vascular disease, however the mechanisms remain unknown (18). Allograft vasculopathy development is generally considered to begin as a response to injury, and then endothelial dysfunction and intimal hyperplasia develop as a result of vascular remodeling in response to repetitive transplant-related endothelial injury. The continual vascular remodeling and fibroproliferative modifications of the vessel wall ultimately contribute to allograft arteriosclerosis. Modification of endothelial-derived NO availability is a feasible management strategy because the allograft arteriosclerosis is initiated by an endothelial dysfunction. NO regulates vessel tone, blood fluidity and vascular cell growth. It is well established that NO regulates vascular tone through activation of guanylyl cyclase, elevation of cGMP, and activation of PKG. The effects of NO-cGMP pathway on vascular contraction appears to be regulated specifically by PKG (19). The specific substrates for PKG in smooth muscle include the regulatory myosin-binding subunit of myosin phosphatase, calcium-activated maxi K^+ channels, and IRAG (IP3 receptor associated cGMP kinase substrate), which lead to a reduction of intracellular Ca^{2+} concentration or reduction in sensitivity to Ca^{2+} and thereby decreased smooth muscle tone (20). Besides, endothelial-derived NO regulates blood fluidity and vascular cell growth. Tomasoni *et al* (21) reported that

the correction of endothelial dysfunction reduces the progression of arteriosclerosis.

PDE5, as a major cGMP-hydrolyzing PDEs, could effectively control cGMP/PKG signaling pathway and exists in all types of VSMC (22). Inhibition of PDE5 causes a higher rate of accumulation of cGMP in response to the NO and restores endothelial function. After heart transplantation, treatment with the PDE5-inhibitor was able to reduce the contractility in cardiomyocytes by activation of the cGMP/PKG signaling pathway (23). Additionally, increased cGMP can inhibit NADPH oxidase expression/activity which is able to reduce $\cdot\text{O}_2^-$ production, and consequently restore NO bioavailability (24). Balarini *et al* (7) confirms that the treatment with chronic sildenafil restores endothelial function and alleviates oxidative stress in a mice atherosclerotic model based on reduction in plaque deposition in these mice. Another study reported that inhibitor of PDE5 treatment can enhance the expression and activity of eNOS which could also explain the reduction in allograft arteriosclerosis lesions (25).

We also found a significant down-regulation of RhoA expression in tadalafil treatment groups after transplantation. The RhoA/Rho-Kinases pathway plays an important role in many biological processes including VSMC proliferation and migration in vessels (26). This probably explains the role of RhoA/Rho-Kinases pathway in allograft arteriosclerosis. Activation of PKG-I can presumably lead to RhoA phosphorylation and inhibition of RhoA activity (27). Sildenafil increases cGMP levels in VSMC by reducing cGMP-hydrolyzing, and elevated cGMP activates PKG-I which will further inhibit RhoA/Rho-Kinases pathway (28). We speculated that cGMP-dependent regulation of RhoA expression might be a key component of the determinant function of the graft artery in normal and pathological conditions. Our study showed that chronic low tadalafil could diminish RhoA/Rho-Kinases activity and alleviate neointima formation.

Conclusion

In summary, tadalafil, a kind of PDE5 inhibitor, may play a positive role in the prevention of allograft vasculopathy in a rat aortic transplant model. The results show that PDE5 inhibitors hold promise to be a potential adjunctive therapy for reduction of the incidence of transplant vasculopathy.

Acknowledgment

This project was supported by Scientific Research Fund of Zhenjiang Provincial Educational Department (Y201326684), Science Technology Department of Zhejiang Province (2013C37006), and Science and Technology Department of Wenzhou (Y20120157).

References

1. Von Rossum A, Laher I, Choy JC. Immune-mediated vascular injury and dysfunction in transplant arteriosclerosis. *Front Immunol* 2015; 5:684.
2. Qin L, Yu L, Min W. Mouse models for graft arteriosclerosis. *J Vis Exp* 2013; e50290-e50290.
3. Tiefenbacher C, Kreuzer J. Nitric oxide-mediated endothelial dysfunction-Is there need to treat? *Curr Vasc Pharmacol* 2003; 1:123-133.
4. Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med* 2005; 352:1685-1695.
5. Cerrito MG, Scagliarini A, Froio A, Liloia A, Busnelli M, Giovannoni R, *et al*. Heme oxygenase-1 inhibition prevents intimal hyperplasia enhancing nitric oxide-dependent apoptosis of vascular smooth muscle cells. *Biol Pharm Bull* 2011; 34:1204-1214.
6. Schofield RS, Edwards DG, Schuler BT, Estrada J, Aranda JM, Pauly DF, *et al*. Vascular effects of sildenafil in hypertensive cardiac transplant recipients. *Am J Hyper* 2003; 16:874-877.
7. Balarini CM, Leal MA, Gomes IB, Pereira TM, Gava AL, Meyrelles SS, *et al*. Sildenafil restores endothelial function in the apolipoprotein E knockout mouse. *J Transl Med* 2013; 11:10.1186.
8. Mostafa ME, Senbel AM, Mostafa T. Effect of chronic low-dose tadalafil on penile cavernous tissues in diabetic rats. *Urology* 2013; 81:1253-1260.
9. Onuta G, Rienstra H, de Boer JF, Boer MW, Roks AJ, Klatter FA, *et al*. Rosiglitazone attenuates transplant arteriosclerosis after allogeneic aorta transplantation in rats. *Transplantation* 2007; 84:517-526.
10. Lee PC, Wang ZL, Qian S, Watkins SC, Lizonova A, Kovsdi I, *et al*. Endothelial nitric oxide synthase protects aortic allografts from the development of transplant arteriosclerosis. *Transplantation* 2000; 69:1186-1192.
11. Maimaitiyiming H, Li Y, Cui W, Tong X, Norman H, Qi X, *et al*. Increasing cGMP-dependent protein kinase I activity attenuates cisplatin-induced kidney injury through protection of mitochondria function. *Am J Physiol Renal Physiol* 2013; 305:F881-F890.
12. Tang J, Fitzgerald SM, Boughtman BN, Cole SW, Brands MW, Zhang JH. Decreased RhoA expression in myocardium of diabetic rats. *Canad J Physiol Pharmacol* 2005; 83:775-783.
13. Tang J, Liu J, Zhou C, Alexander JS, Nanda A, Granger DN, *et al*. Mmp-9 deficiency enhances collagenase-induced intracerebral hemorrhage and brain injury in mutant mice. *J Cereb Blood Flow Metab* 2004; 24:1133-1145.
14. Libby P, Pober JS. Chronic rejection. *Immunity* 2001; 14:387-397.
15. Osto E, Tona F, Bon ED, Iliceto S, Cella G. Endothelial dysfunction in cardiac allograft vasculopathy: potential pharmacological interventions. *Curr Vasc Pharmacol* 2010; 8:169-188.
16. Lietz K, Miller LW. Current understanding and management of allograft vasculopathy. *Semi Thorac Cardiovasc Surg* 2004; 16:386-394.
17. Ahmad A, Khan RM, Alkharfy KM. Effects of selected bioactive natural products on the vascular endothelium. *J Cardiovasc Pharmacol* 2003; 62:111-121.
18. Colvin-Adams M, Harcourt N, Duprez D. Endothelial dysfunction and cardiac allograft

- vasculopathy. *J Cardiovasc Transl Res* 2013; 6:263-277.
19. Chen J, Crossland RF, Noorani MM, Marrelli SP. Inhibition of TRPC1/TRPC3 by PKG contributes to NO-mediated vasorelaxation. *Am J Physiol Heart Circ Physiol* 2009; 297:417-424.
20. Schlossmann J, Ammendola A, Ashman K, Zong X, Huber A, Neubauer G, *et al.* Regulation of intracellular calcium by a signalling complex of IRAG, IP3 receptor and cGMP kinase β . *Nature* 2000; 404:197-201.
21. Tomasoni L, Sitia S, Borghi C, Cicero A, Ceconi C, Cecaro F, *et al.* Effects of treatment strategy on endothelial function. *Autoimmun Rev* 2010; 9:840-844.
22. Russo I, Del Mese P, Doronzo G, Mattiello L, Viretto M, Bosia A, *et al.* Resistance to the nitric oxide/cyclic guanosine 5'-monophosphate/protein kinase G pathway in vascular smooth muscle cells from the obese Zucker rat, a classical animal model of insulin resistance: role of oxidative stress. *Endocrinology* 2008; 149:1480-1489.
23. Loganathan S, Radovits T, Hirschberg K, Korkmaz S, Barnucz E, Karck M, *et al.* Effects of selective phosphodiesterase-5-inhibition on myocardial contractility and reperfusion injury after heart transplantation. *Transplantation* 2008; 86:1414-1418.
24. Koupparis AJ, Jeremy JY, Muzaffar S, Persad R, Shukla N. Sildenafil inhibits the formation of superoxide and the expression of gp47phox NAD [P] H oxidase induced by the thromboxane A2 mimetic, U46619, in corpus cavernosal smooth muscle cells. *BJU Int* 2005; 96:423-427.
25. Das A, Xi L, Kukreja RC. Phosphodiesterase-5 inhibitor sildenafil preconditions adult cardiac myocytes against necrosis and apoptosis Essential role of nitric oxide signaling. *J Biol Chem* 2005; 280:12944-12955.
26. Shimokawa H, Takeshita A. Rho-kinase is an important therapeutic target in cardiovascular medicine. *Arterioscler Thromb Vasc Biol* 2005; 25:1767-1775.
27. Kato M, Blanton R, Wang G-R, Judson TJ, Abe Y, Myoishi M, *et al.* Direct binding and regulation of RhoA protein by cyclic GMP-dependent protein kinase Ia. *J Biol Chem* 2012; 287:41342-41351.
28. Hrometz SL, Shields KM. Sildenafil citrate for the treatment of pulmonary hypertension. *Drugs Today* 2006; 42:771-784.