

Original Research Article

Influence of cilostazol on thromboangiitis obliterans in rats and the mechanism involved

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Abstract

Purpose: To study the effect of Cilostazol on rat thromboangiitis obliterans (TAO), and the mechanism of action involved.

Methods: Rats ($N = 45$) were injected with sodium laurate to establish the model of TAO, and then divided into Sham group ($n = 15$), TAO group ($n = 15$) and TAO + cilostazol group ($n = 15$). After administration of cilostazol, TAO lesions in the rats were graded, and the femoral arteries were stained by hematoxylin-eosin (H&E) to determine the degree of vascular lesions. The status of vascular endothelial cells was determined using transmission electron microscopy. Furthermore, the expression and transcription levels of hypoxia-inducible factor (HIF)-1 α and vascular endothelial growth factor (VEGF) proteins were evaluated by Western blotting and real-time polymerase chain reaction (RT-PCR) respectively.

Results: In contrast to Sham group, TAO group exhibited symptoms such as changes in skin temperature and color, and limb swelling and thanatosis, while in the TAO + cilostazol group, the damage was reversed, vascular and vascular endothelial cell lesions were significantly ameliorated ($p < 0.05$), and the transcription and translation levels of HIF-1 α and VEGF significantly suppressed ($p < 0.05$).

Conclusion: Cilostazol alleviates sodium laurate-induced TAO lesions in rats via HIF-1 α /VEGF pathway. This study may provide new insights for the treatment of TAO.

Keywords: Cilostazol, Hypoxia-inducible factor/vascular endothelial growth factor pathway, Thromboangiitis obliterans, Limb swelling, Thanatosis

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INTRODUCTION

Thromboangiitis obliterans (TAO) is a common peripheral vascular disease of unknown cause and pathogenesis, and it is characterized by a high risk of amputation as well as a high incidence rate. There is still a scarcity of definite therapies so far [1]. Thromboangiitis obliterans is

accompanied by recurrent superficial thrombophlebitis and upper limb involvement, and while smoking cessation can hinder the progression of this disease, it is unable to prevent it from worsening. Both the immune system and inflammations play a key role in the pathogenesis of TAO [2,3]. Although the reactants at an acute stage generally have

normal erythrocyte sedimentation rate and C-reactive protein, as well as the usually-measured autoantibodies, the abnormality of immunoreactivity is considered as a driver for the inflammatory processes. Compared with those with atherosclerosis, TAO patients have enhanced cellular immunity to type I and II collagen [4]. Moreover, high-titer anti-endothelial cell antibodies are seen in these patients [5].

In multiple randomized clinical trials, Cilostazol has proven to be able to reduce the incidence of coronary in-stent restenosis, and its pharmacological actions include vasodilation, inhibiting of thrombosis, increasing the blood flow of limbs, improving serum lipid, decreasing triglyceride, raising high-density lipoprotein cholesterol and suppressing the growth of vascular smooth muscle cells [6]. Cilostazol is used to treat patients with intermittent claudication and peripheral vascular disease worldwide, and while it is recommended for the prevention of secondary stroke in Asia [7], its effect on TAO has yet to be fully understood.

The present study explored the therapeutic effect of cilostazol on the recovery of affected limbs and the vascular lesions in the rat models of TAO, and elucidated on its mechanism of action in the occurrence and development of TAO.

EXPERIMENTAL

Materials

Sprague-Dawley (SD) rats were purchased from Beijing HFK Bioscience Co. Ltd (Beijing, China); Sodium laurate and reverse transcription (RT) kit from Sigma-Aldrich (St Louis, MO, USA); Cilostazol from Shenzhen Neptunus Pharmaceutical Co. Ltd (Shenzhen, China), and messenger ribonucleic acid (mRNA) primers and TRIZol reagent from Invitrogen (Carlsbad, CA, USA).

Ethical approval

This study was approved by the Animal Ethical Committee of the Animal Center of Zhejiang University School of Medicine. All procedures were conducted in accordance with the 'Animal Research: Reporting in vivo Experiments Guidelines 2.0' [8].

Modeling, grouping and treatments

The male SD rats were divided into Sham group (n = 15), TAO group (n = 15) and TAO + Cilostazol group (n = 15). The animals for modeling were injected with 0.2 mL 10 g/L

sodium laurate solution to establish the rat model of TAO [9], and the rats in Sham group were given 0.2 mL normal saline. The ischemic changes appearing in limbs on the next day indicated that the model was successfully established. On the next day after modeling, the rats were gavaged with 3 mg/kg cilostazol for 10 days.

Evaluation of degree of lesion on rat limb

At 1 h after the last administration, the status of the affected rat limbs (skin temperature and color, limb swelling, thanatosis and degree of mummification), and the degree of lesions were assessed. The following criteria are based on the existing literature [10]: Grade 0 (no change in the whole appearance), Grade I (changed toenail color), Grade II (changes in the color of the affected limbs), Grade III (thanatosis in the affected limbs) and Grade IV (mummification in the affected limbs).

Transmission electron microscopy

Fresh specimens were fixed in glutaraldehyde-osmic acid, dehydrated with gradient alcohol and coated with epoxy resin, and then they were cut into ultra-thin sections less than 0.1 μm thick. After being stained by uranyl acetate and lead citrate, the resulting specimens were observed under a transmission electron microscope (JEM1011, Tokyo, Japan).

Hematoxylin-eosin (H&E) staining

The rats were sacrificed by breaking their necks, in order to isolate the left lower extreme femoral artery and intercept a 2 - 3 cm long artery from below the puncture point. Then it was fixed in paraformaldehyde, dehydrated using gradient alcohol, embedded in paraffin and sliced. The paraffin-embedded sections were routinely deparaffinized using xylene twice (5 min/time) and dehydrated with ethanol at all levels of concentrations, and rinsed with distilled water (2 min/time). Finally, the sections were stained using H&E and observed for pathological changes.

Western blotting

After the thrombus in the left femoral artery of the rats were removed, the tissues were lysed using radioimmunoprecipitation assay (RIPA) (Beyotime, Shanghai, China) cell lysate containing 1 mmol/L protease inhibitor and centrifuged at 13,500 g for 15 min, and the supernatant was collected for proteins, followed by electrophoresis in 10 % sodium dodecyl

sulphate (SDS)-polyacrylamide gel. Hypoxia-inducible factor (HIF)-1 α and vascular endothelial growth factor (VEGF) antibodies were purchased from Abcam (Cambridge, MA, USA), and β -actin was taken as an internal reference. The immunoreactivity was detected using the Odyssey imaging system (Biosciences, San Jose, CA, USA). Finally, Western blotting bands were quantified using Image-Pro Plus 6.0 software (Silver Springs, MD, USA).

Quantitative real time-polymerase chain reaction (qRT-PCR)

RNAs were extracted from the tissues of the lower extremity femoral artery in the rat models using TRIzol reagent, and the concentration and purity were determined with NanoDrop 8000. The reaction system volume was in total 25 μ l, pre-denaturation at 95° for 5 min, denaturation at 95° for 30 sec, annealing at 60° for 45 sec, extension at 72° for 3 min, with 35 cycles, and then extension at 72° for 5 min. The relative expression level of mRNA was quantified via real time RT-PCR using SYBR Green I (Applied Biosystems, Foster City, CA, USA). Relative mRNA expressions were calculated by 2- $\Delta\Delta$ Ct method with β -actin as the endogenous control. The primers used were shown in Table 1.

Table 1: Primer sequences used

Gene	Primer sequence
HIF-1 α	Forward: 5'-GTTTACTAAAGGACAAGTCACC-3' Reverse: 5'-TCCTGTTTGTGAAGGGAG-3'
VEGF	Forward: 5'-CCGCCTGTGTGCCCTAATG-3' Reverse: 5'-CGCATGATCTGCATAGTGACGTTG-3'
β -actin	Forward: 5'-CATCCGTAAGACCTCTATGCCAAC-3' Reverse: 5'-ATGGAGCCACCGATCCACA-3'

Statistical analysis

Experimental data were processed using GraphPad (La Jolla, CA, USA). Analysis of variance was used for comparisons among groups, and the Kruskal-Wallis H test was adopted for the grading of lesions. $P < 0.05$ was considered statistically significant.

RESULTS

Degree of lesion on affected rat limbs

After 10 days of treatment, pathological evaluation was made for the affected limbs, and

the results showed that the TAO model was established successfully. Moreover, the degree of lesion was significantly lower, without grade IV lesions, after treatment with cilostazol (Table 2). H&E staining results indicate that the femoral artery of the TAO rat model exhibited luminal stenosis, thrombosis and inflammatory cell infiltration. However, treatment with cilostazol alleviated the inflammatory infiltration, restored narrowed vascular lumen and reduced thrombosis. The transmission electron microscopy results revealed that the endothelial cells of TAO rats had severe histological injuries as vacuolar degeneration of mitochondria, endoplasmic reticulum fracture and cytoplasmic loosening. Treatment with cilostazol reversed these histological changes and relieved TAO-induced endothelial cell injuries to some extent.

Table 2: Grade of lesion degree in the affected limbs of rats

Group	Grade of lesion					P-value
	0	I	II	III	IV	
Sham	12	0	0	0	0	
TAO	0	0	5	9	1	
TAO + cilostazol	0	8	6	1	0	<0.05

Protein expression levels in femoral artery

The HIF-1 α and VEGF proteins were highly expressed in TAO group, and at the early stage of TAO-induced ischemia, increased in the expressions of HIF-1 α and VEGF, and promoted the pathological regeneration of small blood vessels accompanied by thrombosis. However, administration of cilostazol lowered their expressions (Figure 1). This suggests that cilostazol is involved in the progression of TAO through its effect on angiogenesis and thrombosis.

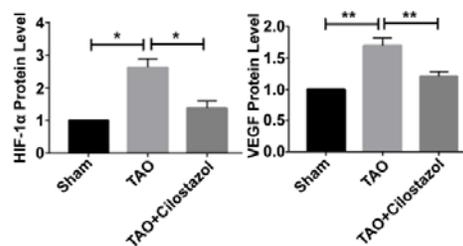


Figure 1: Effect of cilostazol on protein expression levels; * $p < 0.05$, ** $p < 0.01$

M-RNA levels in rat femoral artery

Following protein expression inhibition, HIF-1 α and VEGF mRNAs were highly expressed in TAO group, while their expressions were

significantly decreased in the TAO + cilostazol group (Figure 2).

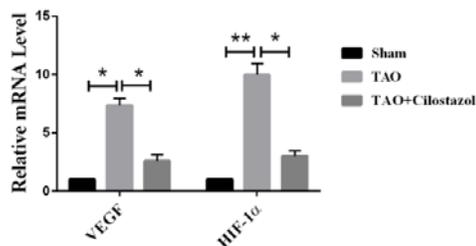


Figure 2: Effect of cilostazol on mRNA expression levels; * $p < 0.05$, ** $p < 0.01$

DISCUSSION

The TAO is a non-atherosclerotic inflammatory disease which affects small and medium blood vessels and is known to be closely associated with smoking [11,12]. With advances in immunochemical technology research, numerous factors including inflammation apoptosis, oxidative stress, macrophages and lymphocytes have been found to be related to the occurrence of TAO [13-15].

Cilostazol, a quinoline derivative, can be combined with aspirin to lower the incidence rate of cardiovascular diseases. It has been reported severally that it suppressed the degradation of cyclic adenosine monophosphate (cAMP), but increased cAMP in platelets and vascular endothelial cells. The increase in cAMP reduced the release of adenosine diphosphate and decreased platelet aggregation, thus avoiding thrombosis [16]. However, cilostazol dilates peripheral arteries and mitigates vascular obstruction, thereby lowering the incidence of atherosclerosis and TAO [16]. A study revealed that cilostazol substantially reduced TAO-induced abnormal rise in ICAM-1 and VCAM-1 and abnormal expressions of inflammatory factors [17]. However, TAO-related mechanisms and therapeutic strategies have yet to be fully elucidated. To further determine the effect of cilostazol in the development of TAO, the rat model of TAO was established by injecting sodium laurate. This induced injuries on femoral artery endothelial cells, and the model had similar symptoms to those of TAO patients. Therefore, this method of inducing TAO in rats has been well accepted and applied in the studies on this disease [18,19].

In this study, several typical symptoms of TAO were observed in the rat model. The treatment with sodium laurate caused vasculitis and local ischemic necrosis, infections and pus outflow, histological changes such as vascular stenosis

and thrombosis, and structural damage of endothelial cells in the hind legs. On the contrary, the administration with cilostazol significantly relieves wound infection, necrosis, thrombosis and endothelial cell damage. Moreover, another study reported that a rat model of TAO manifested hypercoagulation [20]. However, this was not observed in the present study due to limited experimental conditions. Overall, the typical symptoms in the model of TAO in this study were consistent with those reported in previous studies, thus laying a solid foundation for subsequent experiments. Furthermore, it has been demonstrated that cilostazol exerts some level of therapeutic effect on TAO. As an important nuclear transcription factor, HIF-1 α plays key roles in cell and tissue growth and even physiological stress processes in some diseases [21]. Research has shown that HIF-1 α is highly expressed at the early stage of TAO in rats and decreases with time, and that it probably serves as a key player in the diagnosis and treatment of TAO. Vascular endothelial growth factor (VEGF) is highly expressed in organisms under an ischemic state to promote angiogenesis. However, at the early stage of TAO, decreasing the level of VEGF reduced endometrial angiogenesis and the number of ectopic endometrial vessels [22].

In this study, compared with those in Sham group, rat tissues in TAO group exhibited raised mRNA and protein levels of HIF-1 α and VEGF, leading to high expression of multiple angiogenic factors, but excessive damage repair might result in the pathological regeneration of small blood vessels and thrombosis. In TAO + cilostazol group, cilostazol notably decreased the abnormally increased expression of HIF-1 α and VEGF expressions in the affected limbs of TAO rats due to the effect of sodium laurate. The above results indicate that cilostazol exerted a favorable anti-TAO effect, and also unravels the mechanism of action of cilostazol, thus providing a new, feasible and effective solution to the prevention and treatment of TAO in the future.

CONCLUSION

Cilostazol alleviates sodium laurate-induced TAO lesions in rats *via* HIF-1 α /VEGF pathway. Thus, these findings provide a theoretical basis for the development of cilostazol for the treatment of thromboangiitis obliterans.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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