Research Paper

The effects of Batroxobin on the intimal hyperplasia of graft veins

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KEYWORDS:
Autologous veins graft; Batroxobin; Intimal hyperplasia

Abstract

BACKGROUND: To investigate the effects of Batroxobin (BX) on the intimal hyperplasia of graft veins.

METHODS: Twenty dogs were evenly divided into 2 groups. The femoral veins were grafted into the femoral artery by microsurgery, and the experimental group was treated with BX (0.1 BU/kg/48 hours). The serum level of endothelin-1 (ET-1) was detected 2 weeks after operation. Computer image analysis system was performed to calculate the cross-sectional area of neointima and media in the vein grafts 8 weeks after operation. Immunohistochemistry method was performed to identify proliferating cell nuclear antigen (PCNA) and c-Myc.

RESULTS: The experimental group had a lower level of serum ET-1 than the control group \((P<.01)\), and both intimal hyperplasia and media thickness of graft veins were reduced by BX in comparison with the control group \((P<.05)\). C-Myc expression was higher in the control group than in the experimental group \((P<.01)\). The PCNA expression in the experimental group was significantly lower than the control group \((P<.05)\).

CONCLUSIONS: These findings suggested that BX could inhibit intimal hyperplasia through suppressing cell proliferation activity.

Vein grafting is widely used for the treatment of vascular reconstruction, especially in coronary artery bypass.\textsuperscript{1} However, its efficacy can be limited by late graft failure from either intimal hyperplasia or progression of the underlying atherosclerotic disease.\textsuperscript{2} The migration and proliferation of vascular smooth muscle cells (VSMCs) are thought to be central to the development of intimal hyperplasia.\textsuperscript{3}

Batroxobin (BX) is a thrombin-like enzyme derived from Bothrops atrox, moojeni venom. In contrast with thrombin, which converts fibrinogen into fibrin by cleavage of fibrinogen A and B chains, BX only splits off fibrinopeptide A. Additionally, thrombin activates various blood coagulation proteins such as factors V, VIII, and XIII, whereas BX has no direct effect on these factors.\textsuperscript{4,5} In some countries, not including United States, BX is clinically used for the treatment of various thrombotic diseases including deep vein thrombosis, myocardial infarction, pulmonary embolism, and acute ischemic stroke.\textsuperscript{6,7} New evidence revealed a relationship between BX and inhibited proliferation and migration of VSMCs in vitro.\textsuperscript{8,9} However, there are no precise experimental data regarding the effect of BX on vein graft intimal hyperplasia.

Therefore, this study was designed to examine whether BX could inhibit intimal hyperplasia after canine autologous vein grafts and the probable mechanism.

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Methods

Main reagents

The main reagents include batroxobin injection (Beijing Dangling Biomdica, Beijing), endothelin kit (ADR), monoclonal antibody against proliferating cell nuclear antigen (PCNA; Beijing Zhongshan Biotechnology), and monoclonal antibody against c-Myc (Beijing Zhongshan Biotechnology).

Animals

Twenty healthy mongrel dogs of either sex, weighing 10 to 15 kg, were divided randomly into 2 groups: the experimental group ($n = 10$) and the control group ($n = 10$). The dogs were fed a standard diet and random water. All animal procedures were performed in accordance with the National Research Council’s Guide for the Humane Care and Use of Laboratory Animals.

Vein graft

Each dog underwent anesthesia with peritoneal injection and intravenous injection of 3% napental (60 mg/kg). A 3-cm segment of left superficial femoral vein was dissected through a transversal incision in the groin. All side branches were ligated and divided with 7-0 polypropylene monofilament suture. The animals underwent heparinization (200 IU/kg) and their veins were clamped proximally and distally. A vein was harvested and rinsed with normal saline solution. The 3-cm segment was isolated and mobilized. The left superficial femoral artery was clamped just below the deep femoral artery and 2 cm below the proximal clamp. The superficial femoral artery was cut between the clamps and replaced with the vein. The vein graft was anastomosed in a reversed end-to-end fashion by using interrupted 7–0 polypropylene monofilament suture (Fig. 1). The clamps were removed, lidocaine 0.5% was applied locally to reduce spasm, and the incisions were closed in 2 layers. The experimental group was treated with BX (0.1 BU/kg intravenous every 48 hours) twice before the operation, once 1 hour later in the operation day, and then 8 times every 48 hours. The control group did not receive BX.

Serum endothelin-1

Venous blood samples of 3 mL were obtained 2 weeks after the operation. Blood plasma was separated by centrifuge and then freezed for testing. Endothelin-1 (ET-1) was measured by enzyme-linked immunosorbent assay in strict accordance with the kit description.

Harvest of implanted grafts

The vein grafts were harvested under general anesthesia 8 weeks after implantation. The graft was isolated and harvested, and the dogs were sacrificed with an overdose of pentobarbital. The harvested vein graft was fixed with 10% paraformaldehyde at 100 mm Hg for 30 minutes. The perfused vein graft was used for histologic study and immunohistochemistry. Each sample was embedded in paraffin and cut into 5-μm sections.

Histomorphologic analysis

Each section was deparaffinized in a xylene–ethanol series and stained with hematoxylin and eosin. Four sections were obtained from each vein graft. The cross-sectional areas of intima and media were measured using the Axioplan 2 imaging system (Carl Zeiss Far East), and the 4 values were averaged to represent the graft.

<table>
<thead>
<tr>
<th>Class</th>
<th>n</th>
<th>Intima (mm²)</th>
<th>Media (mm²)</th>
<th>Intima/media × 100 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experimental group</td>
<td>10</td>
<td>1.62 (.50 ~ 3.61)</td>
<td>1.89 (.93 ~ 2.94)</td>
<td>97.88 (16.89 ~ 228.76)</td>
</tr>
<tr>
<td>Control group</td>
<td>10</td>
<td>2.94 (1.13 ~ 10.92)</td>
<td>2.94 (1.63 ~ 4.72)</td>
<td>115.82 (63.52 ~ 231.53)</td>
</tr>
</tbody>
</table>

Table 1: Intima area, media area, and the ratio of intima–media area in the 2 groups

Value expressed with median (maximum, minimum).
Immunohistochemical staining and evaluation

For immunohistologic analysis, monoclonal antibodies against PCNA or c-Myc protein were used, strictly according to the kit description. The formalin-fixed and paraffin-embedded sections were deparaffinized, and each section was treated with 3% H₂O₂ for 10 minutes at room temperature to inhibit endogenous peroxidase activity. The sections were then incubated with normal horse serum for 30 minutes to reduce nonspecific binding. After washing with phosphate-buffered saline (PBS), the sections were incubated with antibody against PCNA or c-Myc protein for 60 minutes at room temperature and again washed with PBS. The sections were applied with peroxidase-labeled polymer conjugated to antimouse immunoglobulins for 30 minutes at room temperature, then washed with PBS for 25 minutes, incubated with 3,3′-diaminobenzidine plus substrate-chromogen solution for 10 minutes, and finally counterstained by hematoxylin solution.

Brown-stain cells were considered positive. The assessment of all the samples was conducted blindly by calculating the average fraction of positive cells in 10 random vision fields under a 100× microscope. If the average positive cell fraction was >10%, this sample was considered positive.

Statistical analysis

Values were expressed as mean plus or minus standard error of mean or median (maximum, minimum). Statistical analysis was performed with the Student t test or nonparametric Wilcoxon test where appropriate. A value of \( P < .05 \) was considered statistically significant.

Results

All animals survived. Two vein grafts were obstructed by thrombus (control group), and the other 18 vein grafts were patent until harvest.

Assessment of intimal hyperplasia

To evaluate the influence of BX-induced inhibition on intimal hyperplasia, we measured the cross-sectional areas of intima and media and the intima–media ratio with Axioplan 2 imaging system. For statistical analysis on the asymmetric intima and media in different dogs, we performed a nonparametric Wilcoxon test expressed as median (maximum, minimum).

Intimal hyperplasia developed in both groups, but the proliferation levels of intima and media in the experimental group were significantly suppressed in comparison with the control group (\( P < .05 \)). There was no statistical difference of the intima–media ratio between the experiment group and the control group (\( P > .05 \)) (Table 1 and Fig. 2).

Suppression of cell proliferative activity

The results of immunohistochemical analysis are showed in Table 2. Positive cells are buff colored (Figs. 3 and 4). The PCNA and c-Myc expressions were measured using vein grafts harvested 8 weeks after implantation. There was a significantly lower PCNA expression in the experimental group than the control group (\( X^2 = 5.05, P < .05 \)), as was c-Myc expression (\( X^2 = 8.143, P < .01 \)).

![Figure 2](image.png)  
**Figure 2** Representative photomicrographs of intima and media area of the cross-sections of vein grafts, harvested 8 weeks after implantation, hematoxylin and eosin staining. (A) control group magnification, 50×. (B) Experimental group magnification 50×. The arrows indicates intima and media area of the cross-sections of vein grafts.

### Table 2  Expression of proliferating cell nuclear antigen, c-Myc in experimental group and control group

<table>
<thead>
<tr>
<th>Class</th>
<th>n</th>
<th>PCNA</th>
<th>Positive</th>
<th>c-Myc</th>
<th>Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experimental</td>
<td>10</td>
<td>7</td>
<td>3</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td>2</td>
<td>8</td>
<td>0</td>
<td>10</td>
</tr>
</tbody>
</table>

**PCNA** = proliferating cell nuclear antigen.
Endothelin-1 levels

Fourteen days after the graft plantation, serum ET-1 level was significantly lower in the experimental group (1.2 ± 0.6 ng/L) than in the control group (3.5 ± 1.5 ng/L) \((F = 16.30, \ P < .01)\) (Table 3).

Comments

This study demonstrated that BX could reduce vein graft intimal hyperplasia through suppression of cell proliferative activity. We also found that BX could influence serum ET-1, which is considered contributive to intimal hyperplasia.

Intimal hyperplasia is the universal response of vessel injury. VSMCs migrate from the medial into the intimal layer and proliferate and synthesize extracellular matrix, thus playing a key role in the progression of intimal hyperplasia and angiostenosis. A number of vasoactive agents are involved and considered possible modulators in the proliferation and migration of VSMCs, but the definite mechanism is still obscure.

BX is a thrombin-like enzyme. Studies from Asia have demonstrated that BX has the ability to degrade fibrinogen, decrease aggregation of platelets, and induce the release of tissue plasminogen activator. Furthermore, experimental studies in vitro indicate that BX has a protective effect on vascular endothelium through endothelial nitric oxide synthase activation. BX could also attenuate cerebral edema in ischemic gerbils and inhibit c-fos expression in rat brains during ischemia and reperfusion. Clinical use has proved BX to be an effective drug for thrombosis, atherosclerosis, myocardial infarctions, cerebral infarction, and peripheral arterial ischemic diseases.

This study demonstrates that BX could reduce intimal hyperplasia. PCNA is an auxiliary protein of DNA polymerases and enzymes and is necessary for DNA synthesis. PCNA expression increases during the G1 phase, peaks at the S phase, and declines during the G2/M phases. C-Myc is another crucial promoter in human and animal cell proliferation. Its overexpression was detected in many types of tumor cells, and decreased expression could prevent the transition from the G1 to the S phase in a variety of cells. In this study, immunohistologic analysis showed that BX suppressed PCNA and C-Myc expressions efficiently, suggesting that BX could reduce vein graft intimal hyperplasia through suppressing cell proliferative activity.

We found that the intima–media ratio had no significant difference between the experimental group and the control group. Specifically BX appears to suppress both the intima and the media at equal amounts, suggesting that the ratio is actually not important. This may be a different view from other reports. Medial thickening together with intimal thickening is the hallmark of intimal hyperplasia. Therefore, the effects of BX on intima and media are both important.

Figure 3  Representative photomicrographs of proliferating cell nuclear antigen (PCNA) positive staining and negative staining in the intimal regions of vein grafts 8 weeks after implantation from the 2 groups. Positive cells are buff colored. (A) Positive PCNA expression in control group (>10% positive) 100×. (B) Negative PCNA expression in experimental group (<10% positive) 100×. The arrows indicates PCNA positive cells.

Figure 4  Representative photomicrographs of c-Myc positive staining and negative staining in the intimal regions of vein grafts 8 weeks after implantation from experimental group and control group. Positive cells are buffy. (A) Positive c-Myc expression in control group (>10% positive), 100×. (B) Negative c-Myc expression in experimental group (<10% positive), 100×. The arrows indicates c-Myc positive cells.
hyperplasia contribute to the vein graft failure, which also resulted from VSMCs proliferation. Both intimal hyperplasia and medial thickening were significantly decreased in vein grafts treated with BX in this study.

We also found that BX could decrease serum ET-1, which is a 21-amino acid peptide released from the endothelial cells and VSMC and is one of the most potent known vasoconstrictors. Recently, extensive studies have revealed that ET-1 promotes the proliferation of VSMCs. A variety of signaling systems have been involved in the proliferative effect of ET-1, including the individual mitogen-activated protein kinase pathways, reactive oxygen species, and calcium channels. This reflects that ET-1 is related to the inhibitory effect of BX on intimal hyperplasia of vein grafts. However, additional well-designed trials are needed to better define the optimum role of ET-1 in the inhibition effect of BX on intimal hyperplasia.

Drug concentration

In previous reports, the intravenous administration of BX in coronary and peripheral thrombosed dogs was at doses from .05 BU/kg to 1 BU/kg. In the clinic, the administration of BX in thrombosis patients is at a dose of 5 BU every 48 hours, approximately .1 BU/kg every 48 hours. We finally choose the clinical dosage, so this study indicates that the dose of BX to reduce intimal hyperplasia was almost the same as the clinically approved dose in thrombosis patients.

Conclusion

This study demonstrated that BX can slow the development of intimal hyperplasia of the vein graft through suppressing cell proliferative activity.

References