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Effects of Natural and Recombinant Hirudin on VEGF Expression and Random Skin Flap Survival in a Venous Congested Rat Model

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We aim to investigate the effects of locally injected natural and recombinant hirudin on vascular endothelial growth factor (VEGF) expression and flap survival in venous congested skin flaps using a rat model. A dorsal random skin flap (10 × 3 cm) was prepared on each of 30 Wistar rats to establish a venous congested model. The rats were randomly divided into 2 treatment groups [receiving subcutaneous injection of either natural hirudin (6 U) or recombinant hirudin (6 U)] and a control group, which received subcutaneous injection of physiologic saline. After treatment, skin flap survival rates were calculated. VEGF messenger RNA levels and VEGF-positive vessel density as a marker for VEGF levels were measured in the flaps during and after treatment. The skin flap VEGF messenger RNA levels increased in the natural hirudin-treated group. The VEGF-positive vessel density was increased in all 3 groups. Statistically significant increases of VEGF levels were observed in the natural and recombinant hirudin-treated groups compared with the control group ($P < 0.05$). The skin flap survival rates were improved in both hirudin treated groups. Natural and recombinant hirudin can increase VEGF expression in random skin flaps, which can potentially improve random skin flap survival in rats through angio genic mechanisms. Our results showed that hirudin treatment led to an increase in VEGF expression in the congested skin flaps. Natural hirudin demonstrated more pronounced effects than recombinant hirudin. Further studies are needed to understand the specific mechanisms.

Key words: Natural hirudin – Recombinant hirudin – VEGF – Random skin flap – Flap survival rate

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Flap venous congestion is a postoperative complication that can cause partial or full thickness skin flap necrosis and may lead to flap failure if not treated promptly. Partial necrosis of random pattern skin flaps is difficult to avoid. To improve the survival rate of skin flaps, considerable efforts have been made to increase blood flow in the flap and to enhance its tolerance to ischemia.

Leech therapy has been successfully applied clinically to treat venous congestion.[1–4] This therapy is a standard treatment for venous congestion in the United States as well as in some European countries. Medicinal leeches can rapidly enhance perfusion in the region by actively drawing blood from the tissue. In addition, the anticoagulation substances secreted in leech saliva prevent blood clotting, inhibiting venous congestion.

Hirudin is an anticoagulant substance secreted in the saliva of medicinal leaches. The effects of natural and recombinant hirudin on flap venous congestion have not been extensively studied. Previous studies suggest that hirudin can improve the survival rate of congested flaps because of its anticoagulant activity.[5] Multiple studies[6,7] have shown that a low dosage of hirudin (6 U/mL) is optimal for improving flap congestion. However, whether hirudin can induce the growth of new blood vessels is unknown.

The aim of this study was to examine the molecular effects of hirudin treatment by measuring vascular endothelial growth factor (VEGF) expression (*i.e.,* VEGF messenger RNA [mRNA] and VEGF-positive vessel density) after natural and recombinant hirudin treatment using a rat model. We also compared the effect of natural and recombinant hirudin on flap survival.

Materials and methods

The use of 30 Wistar rats (250 ± 50 g) was approved by the Animal Experimental Center. The animal protocol was in accordance with the Regulations of Laboratory Animal Care. Recombinant hirudin (catalogue no. 53289) was obtained from Sigma-Aldrich (Shanghai, China). Natural hirudin (patent no. ZL03113566.8) was purchased from Canton Xike Kang Biotechnology Co, Ltd (Guangxi, China). VEGF monoclonal antibody was purchased from Chemicon (Temecula, California). The immunohistochemical staining kit was purchased from Beijing Golden Bridge Biotechnology Co., Ltd (Beijing, China). PCR reagents, Real MasterMix (SYBR Green I) Fluorescence Assay Kit and DNA markers were purchased from Tiangen Biochemical Technology Co, Ltd (Shanghai, China). The RNA kit was purchased from Fermentas (Glen Burnie, Maryland). RNA extraction kit was purchased from Invitrogen (Carlsbad, California). Real-time PCR was performed on an Applied Biosystems 7500 Real-Time PCR System (Hercules, California). The Vertical Electrophoresis Tank was purchased from Bio-Rad (Shanghai, China). The Astra4700 Scanner was provided by UMAX (Hercules, California).

Surgical procedure

All rats were anesthetized by intraperitoneal injections of xylazine (10 mg/kg) and ketamine (50 mg/kg). The dorsal hair was removed with 8% sodium sulfide and the skin was sterilized with betadine. Caudally based dorsal flaps in size were raised under sterile conditions. The palpable hip joints were used as anatomic landmarks to define the base of the flap. The flap was dissected and detached from its panniculus carnosus and reattached in the native position with interrupted sutures (Fig. 1). The flaps were injected immediately after surgery and again on postoperative days 3 and 5. The injections were performed within each flap (1–3 cm proximal to the distal limit of the flap) at the hypodermic level (level of the deep dermis) (Fig. 2). The rats were randomly divided into 3 groups (10 in each group). In the natural hirudin group, 6 U (1 mL) of natural hirudin (Canton Xike Kang Biotechnology Co, Ltd) was injected into each flap. In the recombinant hirudin group, 6 U (1 mL) of recombinant hirudin (Sigma-Aldrich) was injected into each flap. In the control group, isotonic NaCl (1 mL) was injected into each flap.

Fig. 1 Flap was elevated during surgery.

Fig. 2 Injection of hirudin after surgery.

Skin flap survival

Skin flap color, edema, and necrosis in the rats were monitored daily after operation. Seven days after surgery, the rats were anesthetized and the skin flaps were photographed with a digital camera. Image analysis software was used to examine photographs of the flaps and measure the areas of viable and necrotic-appearing tissue. The flap survival rate was then calculated with the formula:

$$\text{Flap survival rate} = (\text{Viable flap area}/\text{total flap area}) \times 100.$$

VEGF mRNA expression

An approximately 0.5 × 0.5 cm section was harvested from the distal edge of each flap for analysis on postoperative days 3, 5, and 7. Each harvested section was divided in half. The half of each harvested section fixed in 4% paraformaldehyde (line 80) and sectioned for immunohistochemcial detection of VEGF was used to determine vessel density. The half stored at –70°C for RNA extraction was used for quantitative reverse transcriptase–polymerse chain reaction (RT-PCR) to determine the VEGF mRNA level. The PCR products were separated by electrophoresis to identify the specific bands corresponding to VEGF (164 bp) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 152 bp). The size of the PCR products was determined by comparing to the DNA markers.

Quantitative RT-PCR

To measure the VEGF mRNA level, total RNA was extracted from the flap tissue using Trizol reagent. A UV-VIS Spectrophotometer was used to determine the purity and concentration of the total RNA. Two micrograms of total RNA was reverse transcribed into complementary DNA.

The SYBR Green I dye was used for quantitative real-time PCR. The following PCR setting was used for all experiments: denaturation at 95°C for 2 minutes; 36 cycles of denaturation at 95°C for 10 second, annealing at 50°C for 10 seconds, and extension at 72°C for 45 seconds. RT-PCR was performed in the ABI prism 4700 sequence detection system (UMAX). The relative expression level of VEGF mRNA was calculated using the formula: $2^{-\Delta CT}$ ($\Delta CT = CT_{VEGF} - CT_{GAPDH}$). After gel electrophoresis of the amplified products, a fully automated Gel Imaging System was used to analyze mRNA expression by comparing the intensity between the groups. All results were normalized to GAPDH.

Statistical analysis

SPSS 16.0 statistical package was used for statistical analysis. The two groups were compared using one-way analysis of variance (ANOVA). SNK test was used to compare each groups. A P value < 0.05 was considered as significant.[8]

Results

Skin flap survival

All rats survived until the end of the study without infection. Seven days after surgery, the necrotic area had stabilized with clear boundaries between the viable and necrotic areas. In the natural and recombinant hirudin-treated groups, parts of the eschar of the flaps were inconsistent in depth and were gray or light gray in color. The soft scar tissue could be easily peeled off, revealing the wound and bleeding underneath. In contrast, the scabs in the control group were hard, deep dark brown in color, and contained local inflammation. These scabs could not be easily peeled off (Figs. 3 to 5).

Fig. 3 Skin flap of the natural hirudin group 7 days after surgery. Top arrow, viable; bottom arrow, nonviable.

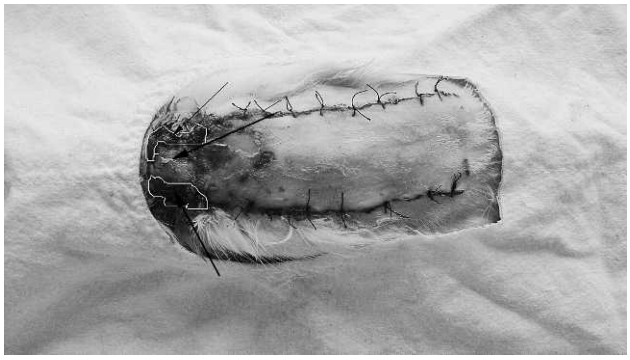

Fig. 4 Skin flap of the recombinant hirudin group 7 days after surgery. Lower arrow, viable; upper arrow, nonviable.

The flap survival rates of each group are listed in Table 1.

Table 1 Flap survival rate in hirudin-treated and control groups

Groups	Flap survival area (cm^2)	Total flap area (cm^2)	Flap survival rate (%)
Natural hirudin	26.61 ± 2.21	30	88.92 ± 2.21
Recombinant hirudin	23.62 ± 2.11	30	79.92 ± 2.01
Control	20.73 ± 1.44	20	69.12 ± 1.43

RNA, messenger RNA; VEGF. The survival rates are significantly different from each other ($P < 0.05$).

VEGF-positive vessel density

The VEGF-positive vessel density in flap reflects the VEGF levels in the skin flaps (Table 2).

VEGF mRNA expression

The expression levels of VEGF mRNA are listed in Table 3.

Discussion

Clinical manifestations of flap venous congestion include skin swelling and purple discoloration. If this condition is not treated in time, flap necrosis will occur. According to published reports, the incidence rate of venous crisis after flap surgery is much higher than that of arterial crisis.[9] Venous congestion contributes to several pathologic conditions in skin flaps, such as reduction in the number of capillaries, slowed microvascular blood flow, progressive increase in red blood cell aggregation, and the formation of white thrombus.[10] It is extremely difficult to improve microcirculation if new blood vessels are not developed (if angiogenesis does not occur) in the congested area.

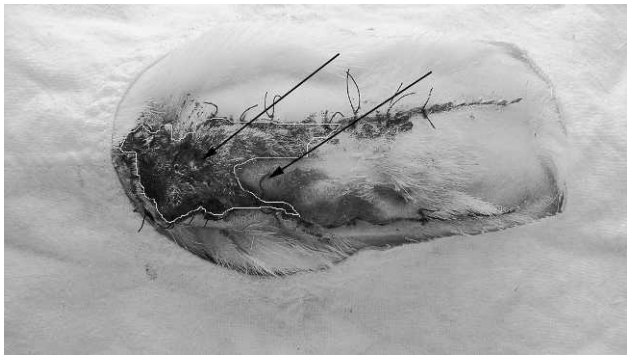

Fig. 5 Skin flap of the control group 7 days after surgery. Right arrow, viable; left arrow, nonviable.

Endogenous VEGF, a predominant regulator of angiogenesis, may play several important roles in flap survival.[11] Animal tests have shown that VEGF can promote endothelial cell proliferation and angiogenesis[12–14] by interacting with a combination of VEGF receptors on vascular endothelial cells. VEGF is also the most potent mitogen for endothelial cells.[14] Upon binding to its receptor, VEGF activates classic receptor tyrosine kinase signaling pathways to induce endothelial cell cycle progression and proliferation. VEGF also regulates the production of plasminogen activators and inhibitors in endothelial cells, which mediate basement membrane degradation during angiogenesis. In addition, VEGF is a potent stimulator of vascular permeability (*i.e.*, 5000 times more potent than histamine). However, unlike histamine, VEGF-induced vascular permeability is not associated with decreased mast cell granules and cannot be blocked by histamine inhibitors. Furthermore, VEGF can promote plasma extravasation and provisional matrix formation, which are conducive to angiogenesis. Finally, VEGF induces endothelial nitric oxide synthase expression in endothelial cells and thereby increases nitric oxide synthesis and release, leading to the dilation of blood vessels.[15]

Our quantitative RT-PCR results showed that VEGF mRNA levels in the control group subsequently decreased on day 5 and on day 7 from the level recorded on postoperative day 3. These results are consistent with previous reports by Han *et al*.[10] Compared with the control group, the VEGF mRNA levels in the natural and recombinant hirudin groups were significantly higher at all three time

Table 2 Effects of hirudin treatment on VEGF-positive vessel density in the skin flaps

Group	Time after injection (d)		
	3 (number/HPF)	5 (number/HPF)	7 (number/HPF)
Natural hirudin	41.72 ± 3.12^{a}	55.21 ± 3.92^{a1}	40.71 ± 3.63^{a2}
Recombinant hirudin	33.21 ± 2.23^{b}	41.61 ± 3.92^{b1}	32.93 ± 2.52^{b2}
Control	14.71 ± 2.62^{c}	8.63 ± 2.62^{c1}	3.61 ± 2.94^{c2}

HPF, high power field; VEGF, vascular endothelial growth factor.

1. Significant difference between [a] and [b] as well as [a] and [c] ($P < 0.05$).
2. Significant difference between [a1] and [b1] as well as [a1] and [c1] ($P < 0.05$); significant difference between [a2], [b2], and [c2] ($P < 0.05$).
3. Significant difference between [a] and [a1]; [b] and [b1]; [c] and [c1] as well as [c] and [c2] ($P < 0.05$).

points. The VEGF mRNA in the hirudin-treated groups peaked on day 5, suggesting a time lag for peak expression after treatment. The elevation of VEGF mRNA levels was prolonged in the treated groups. Among the 2 treatment groups, the natural hirudin group showed a more potent effect. The number of VEGF-positive vessel density was significant in the natural hirudin than in the recombinant hirudin group at all time points. Consistent with the RT-PCR results, immunohistochemical staining showed higher VEGF-positive vessel density in the natural and recombinant hirudin-treated groups.

An increase in the VEGF level in congested flaps is usually associated with an increase in new capillaries, thus promoting skin flap survival. Our results showed that both natural and recombinant hirudin significantly improved flap survival. The flap survival rate in the natural hirudin group was the highest among the three groups, followed by the recombinant hirudin group. The control group had the lowest flap survival rate.

Hirudin treatment may potentially improve flap survival through several mechanisms. First, hirudin temporarily increases skin blood flow by helping with discharge of venous pooling, providing more time for angiogenesis to occur. Second, hirudin can promote absorption of blood and exudate by alleviating arterial wall spasm, dilating blood vessels, and accelerating circulation. Third, hirudin has a high affinity for thrombin and can form a 1:1 complex with it through irreversible covalent bonds. The anticoagulation, antithrombotic, and antiplatelet aggregation effects of hirudin help VEGF to promote microvessel growth.[16] Finally, hirudin plays an anti-inflammatory role in a variety of cells.[16] It reduces free radicals and inflammatory factors in the damaged flaps, contributing to VEGF expression and angiogenesis. Our results showed that hirudin treatment led to an increase in VEGF expression in the congested skin flaps.

In our experiments we also found that natural hirudin surpassed recombinant hirudin in inducing VEGF expression at all time points tested. The only difference between natural and recombinant hirudin is that Tyr63 of the recombinant hirudin is unsulfated.[17] The unsulfated recombinant hirudin is known to have a 10 times lower affinity for thrombin. As a consequence, the effects of natural hirudin on VEFG expression and flap survival surpass those of recombinant hirudin. Determining the exact mechanism for these differences requires further study.

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Table 3 The expression levels of VEGF mRNA in the skin flaps at 3, 5, and 7 days after surgery

Group	3 days after surgery	5 days after surgery	7 days after surgery
Natural hirudin	28.73 ± 2.14^{a}	33.13 ± 4.12^{a1}	27.92 ± 2.63^{a2}
Recombinant hirudin	23.92 ± 3.11^{b}	29.53 ± 2.82^{b1}	22.93 ± 3.43^{b2}
Control	19.63 ± 1.91^{c}	15.52 ± 3.54^{c1}	10.54 ± 2.94^{c2}

mRNA, messenger RNA; VEGF, vascular endothelial growth factor.

1. Significant difference between [a] and [b] as well as [a] and [c] ($P < 0.05$).
2. Significant difference between [a1] and [b1] as well as [a1] and [c1] ($P < 0.05$); significant difference between [a2], [b2], and [c2] ($P < 0.05$).
3. Significant difference between [a] and [a1]; [b] and [b1]; [c] and [c1] as well as [c] and [c2] ($P < 0.05$).

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