Effect of pingyangmycin emulsion on the microenvironment of infantile proliferating capillary hemangioma

Wei-Li Xu, Ai-Guo Niu, Zhen-Dong Li, Suo-Lin Li, Bao-Jun Shi, Yu-Bin Zhang and Zhan-Tao Ye
Shijiazhuang, China

**Background:** Capillary hemangioma in infants has to be treated because of its damage to figuration. Injection of pingyangmycin (PYM) into the tumor is a commonly used method, but often rejected by patients because of complications such as skin fibrosis and skin necrosis. PYM is made into emulsion and smeared on the surface of the tumor. This study was undertaken to explore the effect of PYM emulsion on the microenvironment of the hemangioma so as to find an optimal method for the treatment of hemangioma.

**Methods:** Thirty infants with proliferating hemangioma were divided into control group (10) and medication group (20). PYM made into emulsion was smeared on the surfaces of the tumor in the medication group 3 times a day and only matrix in the control group. Tumor specimens were taken from the two groups on day 7 for pathological and ultrastructural examination under an electron microscope. Vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and type IV collagen (Col-IV), laminin (LM), and fibronectin (FN) in hemangiomas were detected by immunohistochemistry. The apoptotic indexes of the two groups were tested by the molecular biological method (TUNEL test).

**Results:** The expressions of VEGF, Col-IV, LM, and FN in the two groups were significantly different, but not for bFGF. The number of apoptotic cells in the experimental group was more than that in the control group. The difference between the apoptotic indexes of the two groups was significant \((P<0.01)\).

**Conclusions:** PYM emulsion could probably accelerate the regression of proliferating capillary hemangioma by reducing VEGF expression, inhibiting synthesis and regeneration of extracellular matrix, and inducing apoptosis of tumor cells.

**Key words:** antitumor antibiotics; pingyangmycin; hemangioma; angiogenesis factor; extracellular matrix; apoptosis

**Introduction**
Capillary hemangioma is common in infants and children with a high incidence and a rapid growth speed. It has to be treated because of its damage to figuration especially for tumors on face although some of them may regress. The tumor is characterized by over-proliferation of the endothelial cells of blood capillaries. The proliferative endothelial cells or sinus refilled with blood, supported by fibrous and adipose tissues, form an elementary microenvironment.

Injection of pingyangmycin (PYM) into the tumor is a common method for the treatment of hemangioma, but it is often rejected by patients for its complications of skin fibrosis and necrosis.\(^{1,2}\) PYM is thus made into emulsion and smeared on the surface of the tumor in order to produce a gentle and continuous effect. This experiment aimed to explore the effect of PYM emulsion on the microenvironment of infantile proliferating capillary hemangioma and the mechanism of accelerating extinction of capillary hemangioma so as to find an optimal method for the treatment of hemangioma.

**Methods**

**Patients**
Thirty infants with capillary hemangioma at body surface aged less than 6 months were enrolled in
this study. The study was approved by the Ethics Committee of the hospital and signed patient consent was obtained from the parents or the relatives. These infants were divided into 2 groups at random: control group, 3 were boys and 7 girls, aged from 2 days to 6 months (mean 3.37±1.76 months); and medication group, 9 boys and 11 girls, aged from 2 months to 6 months (mean 3.70±1.33 months). In the 30 patients, the tumor was located at the parotid gland (1 patient), cervical region (3), limbs (7), and trunk (19). The specimens of the tumor were verified histologically as proliferative capillary hemangioma after operation in all patients.[3]

**Samples and markers**

**Light microscopic observation**

PYM (produced in Tianjin Taihe Pharmaceutical Factory) adding with a little bit of assist-permeating stroma was made into emulsion with a concentration of 2 mg/dl by the ultrasound-atomized technique. With the permission from the parents of infants, the emulsion was smeared on the surface of the tumors in the medication group once every 8 hours. At the same time, only matrix was smeared on the surface of the tumors in the control group. On the 7th day, the tumors of the two groups were all resected, and the tissue specimens 5 mm away from the epidermis were taken, doused by 0.9% saline. Fixed by 10% formaldehyde, they were embedded, sectioned, and stained for observation under a light microscope.

**Electron microscopic observation**

Two patients were selected randomly from the two groups and smeared with PYM emulsion. After 7 days, tissues of 1 mm³ at the growing parts of the tumor were taken and quickly put into 4% glutaral solution for fixation. The tissue was made into samples for electron microscopic observation.

**Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) in hemangioma detected by immunohistochemistry**

The vascular endothelial cells whose cell membrane and/or cell plasm dyed yellow-brown were positive cells. Five visual fields were observed randomly under a 400 times microscope and the number of positive cells were counted in 500 endothelial cells and classified according to the proportion of positive cells and the criteria formulated at Beijing Seminar on Standardization of Immunohistochemistry Techniques and Diagnosis: 0%-25% (−); 26%-50% (+); 51%-75% (++); and 76%-100% (+++).

**Type IV collagen (Col-IV), laminin (LM) and fibronectin (FN) in hemangioma detected by immunohistochemistry**

Every marker was classified into three grades under a microscope: strongly positive samples (+++), the markers expressed clearly by a deep brown continuous thick line at the basilar membrane of the capillary; weakly positive samples (+) that the markers expressed by a brown discontinuous thin line at the basilar membrane of the capillary; feeble positive samples (+) that the markers expressed by a very superficial brown fragment at the basilar membrane of the capillary and a negative continuous staining.

**Apoptotic indexes**

The apoptotic indexes of the two groups were tested molecular biologically (TUNEL test) in accordance with the specifications of the apoptosis test kit of the BOSTER Corporation, USA. The cells with yellow-brown particles in the nuclei were defined as positive cells. Ten visual fields were observed in each section under a microscope and 100 cells were counted in each field as positive cells. Apoptotic indexes = number of positive cells (10 HPF) / total number of counted cells (100 × 10) × 100%.

**Statistical analysis**

The expressions of VEGF, bFGF and Col-IV, LM, FN of the two groups were compared with Wilcoxon's rank-sum test and the apoptotic indexes of the two groups by Student's \( t \) test.

**Results**

In the medication group, the tumor color of 14 patients turned darker and their superficial skin shrank lightly after drug smear for 7 days, and 6 patients showed no changes. In this group, no hemorrhage, necrosis and inflammation, or complications such as fever and digestive tract response were found. In the control group, no changes took place at all.

Under the light microscope the endothelial cells of the tumor proliferated to form a mass in the control group. The fibrous tissue of blood vessels in tumors began to proliferate in the medication group. Electron microscopic examination showed the endothelial cells of tumors in the control group were shaped oblong and arrayed tightly. The outline and junctions of cells or organelle were normal, with evenly distributed nucleus
Effect of pingyangmycin emulsion on the microenvironment of infantile proliferating capillary hemangioma

heterochromatin and less apoptosis. However, most of the endothelial cells in the medication group shrank with a small volume, concentrated cell plasm and organelle, and even nucleus heterochromatin gathered beneath the nuclear membrane, forming a crescent structure (Fig. 1). Part of endothelial cells showed pyknosis and disruption of nuclei into apoptotic bodies (Fig. 2).

The comparison of the two groups showed that the expression of VEGF in the medication group was lower than that in the control group ($P<0.05$) and that the expressions of bFGF were high in the two groups ($P>0.05$). The expressions of the three extracellular matrices were decreased significantly in the two groups ($P<0.01$) (Tables 1 and 2, Figs. 3-12).

A few yellow-brown positive apoptotic cells were distributed in blue normal cells in the sight of the control group after staining of these samples by the TUNEL method (Fig. 13). Nevertheless, a lot of yellow-brown positive cells were seen in the sight of the medication group. The number of apoptotic cells near the lumen of blood vessels was increased significantly than in other sites (Fig. 14). The apoptotic indexes in the two groups were not significantly different ($P<0.01$). In the control and medication groups, the apoptotic indexes were $9.600 \pm 5.397$ and $32.005 \pm 19.048$, respectively.

### Discussion

Capillary hemangioma is a kind of embryonal angiodysplasia with unknown etiopathogenesis. Vascularization is considered to involve proliferation and migration of endothelial cells, development of extracellular matrix, and formation and recanalization of blood vessel structure. Endothelial cells, vascularization factors, and extracellular matrix play important roles in the establishment of microenvironment in hemangioma. Therefore, a method influencing the microenvironment of the hemangioma and accelerating its extinction might be optimal for the treatment of the tumor.

PYM, an antitumor antibiotic, is derived from streptomyces and its major component is bleomycin A$_{5}$. PYM injection into the tumor has been commonly used

| Table 1. Comparison of VEGF and bFGF in the two groups |
|-----------------|-----------------|-----------------|-----------------|-----------------|---------------|
| Contents        | Grade | Control group | Medication group | Total | Range for order | Average order | Orders | Control group | Medication group | $u$ |
| VEGF ($P<0.05$) | – 1   | 8              | 9               | 1-9   | 5               | 5             | $T_1=204$ | 40             | 40             | 2.228 |
|                 | + 2   | 6              | 8               | 10-17 | 13.5             | 27            | 81         | 19            | 38             |                   |
|                 | ++ 1  | 2              | 3               | 18-20 | 19               | 19            | 38         | 153           | 102            |                   |
|                 | +++ 6 | 4              | 10              | 21-30 | 25.5             | 19            | 38         | 153           | 102            |                   |

| n$_1=10$ | n$_2=20$ | 30  |

| bFGF ($P>0.05$) | – 0   | 0              | 0               | 0     | 0               | 0             | 0         | 0             | 0              | 0.218 |
|                 | + 0   | 3              | 3               | 1-2   | 2               | 0             | 6         | 19.5          | 19.5           |                   |
|                 | ++ 3  | 3              | 6               | 4-9   | 6.5             | 19            | 19.5       | 19.5          | 19.5           |                   |
|                 | +++ 7 | 14             | 21              | 10-30 | 20               | 140           | 280       | $T_1=159.5$ | $T_2=299.5$ | |

| n$_1=10$ | n$_2=20$ | 30  |

| Table 2. Comparison of the three extracellular matrices in the two groups |
|-----------------|-----------------|-----------------|-----------------|---------------|
| Contents        | Grade | Control group | Medication group | Total | Range for order | Average order | Orders | Control group | Medication group | $u$ |
| Col-IV ($P<0.01$) | + 0   | 15             | 15              | 1-15  | 8               | 0             | 120       | 16.5          | 16.5           | 3.802 |
|                 | ++ 1  | 1              | 2               | 16-17 | 16.5             | 16            | 16.5      | 16.5          | 16.5           |                   |
|                 | +++ 9  | 4              | 13              | 18-30 | 24               | 216           | 96        | $T_1=232.5$ | $T_2=232.5$ |                   |
| n$_1=10$ | n$_2=20$ | 30  |

| LM ($P<0.01$) | + 2   | 3              | 5               | 17-21 | 19               | 0             | 136       | 38            | 57             | 4.404 |
|                 | ++ 8  | 1              | 9               | 22-30 | 26               | 0             | 38        | 57            | 57             |                   |
| n$_1=10$ | n$_2=20$ | 30  |

| FN ($P<0.01$) | + 0   | 14             | 14              | 1-14  | 7.5             | 0             | 105       | 0             | 66             | 4.315 |
|                 | ++ 10  | 2              | 12              | 19-30 | 24.5            | 245           | 49        | 245           | 49             |                   |
| n$_1=10$ | n$_2=20$ | 30  |
Fig. 1. Nucleus heterochromatin gathered at the nuclear membrane and formed a crescent structure (original magnification × 7000).

Fig. 2. Part of endothelial cells with pyknosis and nucleus disrupted into the apoptotic body (original magnification × 20 000).

Fig. 3. VEGF of the control group: strongly positive expression (original magnification × 400).

Fig. 4. VEGF of the medication group: weakly positive expression (original magnification × 400).

Fig. 5. bFGF of the control group: strongly positive expression (original magnification × 400).

Fig. 6. bFGF of the medication group: positive expression (original magnification × 400).

Fig. 7. Col-IV of the control group: strongly positive expression (original magnification × 400).

Fig. 8. Col-IV of the medication group: weakly positive expression (original magnification × 400).

Fig. 9. LM of the control group: strongly positive expression (original magnification × 400).

Fig. 10. LM of the medication group: weakly positive expression (original magnification × 400).

Fig. 11. FN of the control group: strongly positive expression (original magnification × 400).

Fig. 12. FN of the medication group: weakly positive expression (original magnification × 400).

Fig. 13. A few positive apoptotic cells among the normal cells in the control group.

Fig. 14. A lot of positive apoptotic cells in the medication group. The apoptotic cells near the lumen of blood vessels increased more significantly than those in the other sites.
to treat hemangioma with considerable results,[1,2] but it is often rejected by children and their parents because the dosage of the injection and the depth of injection are hard to control, which may result in complications such as baryodynia, skin fibrosis, skin necrosis, and scar contracture. PYM is made into emulsion and smeared on the surface of the lesion so that a gentle and continuous effect can be produced. In this investigation, two thirds of tumors turned darker from bright red and the superficial skin shrank lightly after drug smear. In contrast, the samples of the control group did not change at all, suggesting that PYM emulsion indeed penetrated into the tumor effectively.

Vascularization factors could induce or accelerate generation of blood capillaries, of which VEGF and bFGF are two kinds of different and important factors.[5-12] VEGF is known to be the most fierce factor which directly affects blood vessels and its expression is closely correlated to the density of capillaries and the quantity of new born blood vessels.[13] Chang et al.[14] found that the expressions of VEGF and VEGFmRNA in proliferative phase hemangioma were all higher than those in regressive phase hemangioma, suggesting that VEGF is closely correlated with hemangioma proliferation. In this study, the expression of VEGF in proliferative phase capillary hemangioma in the medication group decreased markedly than that in the control group (P<0.05), indicating the role of PYM emulsion in inhibiting proliferation of endothelial cells and accelerating regression of hemangioma is related to the reduced expression of VEGF protein. In another study,[15] the expression of bFGF in 38 capillary hemangiomas of different stages, overexpression of bFGF was observed in proliferative phase and early regressive phase but not in late regressive phase. This finding suggested that bFGF participated in the course of proliferation and acted as a major factor of hemangioma growth. In our study, no significant difference was seen between the two groups, as was found by Takahashi. Whether PYM emulsion influences the expression of bFGF in hemangiomas of different phases awaits further study.

Extracellular matrix (ECM) is the substance around the lower layer connective tissue of epithelial cells or endothelial cells, which provide mechanical support and physical intensity for the integrity of the whole body. ECM is one of the essential conditions for vascularization, of which Col-IV, LM and FN are most closely related to hemangioma.[16-21] In this study, the markers of the control group all strongly expressed positively by a deep brown continuous thick line, but the markers of the medication group expressed by a brown discontinuous thin line. The latter coincided with the expression of regressive hemangioma, showing that PYM emulsion produced a marked therapeutic effect that capillaries lost corresponding mechanical support, vessels atrophied, and tumors regressed progressively.

Hasan et al.[22] found the level of clusterin/apoJ was higher in regressive phase than in proliferative phase. It is shown that apoptosis played an important role in tumor regression.[21,23-26] In this study, increased apoptosis of endothelial cells in the medication group was confirmed by the TUNEL method, indicating that PYM emulsion accelerated apoptosis in hemangioma of proliferative phase. When the cells were closer to the structure of the lumen of blood vessel, the apoptotic rate was higher. We deduced that PYM affects the tumor through blood circulation, particularly the cells around the lumen of vessels, thus leading to the increasing or accelerating apoptosis of endothelial cells.

This study confirmed that microcosmic mechanism of PYM emulsion exerts effect on the microenvironment of infantile proliferating capillary hemangioma. PYM emulsion could accelerate regression of proliferating capillary hemangioma, whose mechanism is related to the reduction of VEGF expression and prevention of synthesis and regeneration of extracellular matrix and induction of cell apoptosis, which are mutually influenced and correlated in vivo. For example, VEGF accommodates cell cycle by autocrine and reduces apoptosis. VEGF also could alter extracellular matrix and make vascular growth easy, but extracellular matrix might accelerate adhesiveness, growth and differentiation of endothelial cells.[27] For this reason, PYM emulsion decreases the expression of VEGF and makes apoptosis superior to proliferation and synthesis of extracellular matrix reduction, which are beneficial to the regression of hemangioma. Presently, there are reports on successful treatment of condyloma acuminata in China, but reports on PYM emulsion for the treatment of hemangioma are rarely seen. Its absorptive concentration, medication course, and long-term effect of PYM emulsion should be further investigated.

**Funding:** This study was supported by grants from the Scientific Research Foundation of Hebei Province Health Bureau (No. 2002045).

**Ethical approval:** This study was approved by the Ethics Committee of the hospital and signed patient consent was obtained from their parents.

**Competing interest:** None declared.

**Contributors:** XWL proposed the study and wrote the main body of the article under the supervision of NAG and LZD. All authors contributed to the design and interpretation of the study. ZYB and YZT are the guarantors.
References


Received December 26, 2005
Accepted after revision June 2, 2006