

Effect of Kanglaite Injection on Angiogenesis

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Abstract

Objective: This study was designed to evaluate the effect of Kanglaite Injection(KLT) on angiogenesis. **Method:** The rings of rat aorta imbedded in gels of collagen were cultured in a 1 to 1 mixture of DMEM/HAM F12, a serum-free medium for 28 days. 24 culture flasks were divided into three groups. A group: control group, B group: 0.1mg/ml VitE and C group: 10 μ /ml KLT. Curves of microvascular growth were generated by counting the number of newly formed microvessels daily with inverted microscope. **Result:** Both 10 μ /ml KLT and 0.1mg/ml VitE significantly inhibited angiogenesis ($P<0.01$). Furthermore the inhibition action of 10 μ /ml KLT group was more intensive than that in 0.1mg/ml VitE group ($P<0.01$). **Conclusion:** KLT could significantly inhibit angiogenesis which formed an important mechanism of antitumor action of KLT.

Key words: Kanglaite, angiogenesis, Vitamin E

Malignant tumor is one of the major diseases which seriously threaten the human health. How to efficiently prevent and treat cancers has drawn much attention. Anti-tumor research has partly shifted from killing cancer cells (chemotherapy) to inhibiting angiogenesis. A few angiogenesis inhibitors have been isolated and purified but no suitable substance has been applied clinically. The antineoplastic agent KLT could enhance immune function and control tumor growth, however. However the relationship between its anti-tumor action and angiogenesis was still unclear. In order to provide experimental basis for KLT to extend its clinical application, the effect of KLT on angiogenesis was studied by using rat aortic models which were cultured in serum-free medium.

1. Materials and methods

1.1 Reagents and instruments

Kanglaite Injection (KLT) was supplied by Zhejiang Kanglaite Pharmaceutical Co., Ltd. (Lot No. 9808191-2). DMEM Medium and HAMF-12 Nutrient Mixture were GIBCO products and VitE was purchased from Shanghai Second Reagent Factory.

Inverted microscope: OLYMPUS product, 250ml culture flask: Greiner Labortechnik product, YJ-875 medical purified working table: Suzhou Purifying Instrument Company, Low temperature high speed centrifuger: SIGMA 3K30 B Braun Biotech International product.

1.2 Tissue culture

Thoracalis and abdominalis aortas were taken from 2-3 weeks male rats and cut into 1 mm length rings. Using the collagen preparation of modified Elsdale and Bard to purify collagen solution. Mix the solution in ratio of 7:2:1 with 11.7mg/ml NaHCO₃ and 10×MEM. At the bottom of agarose well (internal and external diameter was 10mm and 17mm respectively) 4 drops of collagen mixture were added and after the colloid formed added more collagen mixture to full well and aortas rings were placed into well. After it got fully condensed, culture medium, tested drugs, 200µg/ml gentamycin were added and incubated at 35.5 °C. Culture medium was changed every other day.

1.3 Grouping

A group: control group, B group: 0.1mg/mL VitE group and C group: 10 µl/ml of KLT group

1.4 Quantitative analysis of newly formed blood vessels

Counted number of newly formed microvessels on daily basis and drew growth curves of microvessels according to the numbers. Through the drawing contours of aortic ring on paper, different cultured substances in the same culture flask could be distinguished. The counting standard of microvessels by using inverted microscope was as follows.

(1) Distinguished from fibroblasts by the special morphology of microvessels buds (more thick, same cohesive and persistent growth pattern).

(2) One blood vessel bud contained two fork's branches which produced two new buds should be counted as two pieces.

(3) Each blood vessel limb was counted as two buds because they were anastomized by two polymerized microvessels.

To study the statistic difference of various experimental groups SPSS analytical software and student-Newman-Keuls were used.

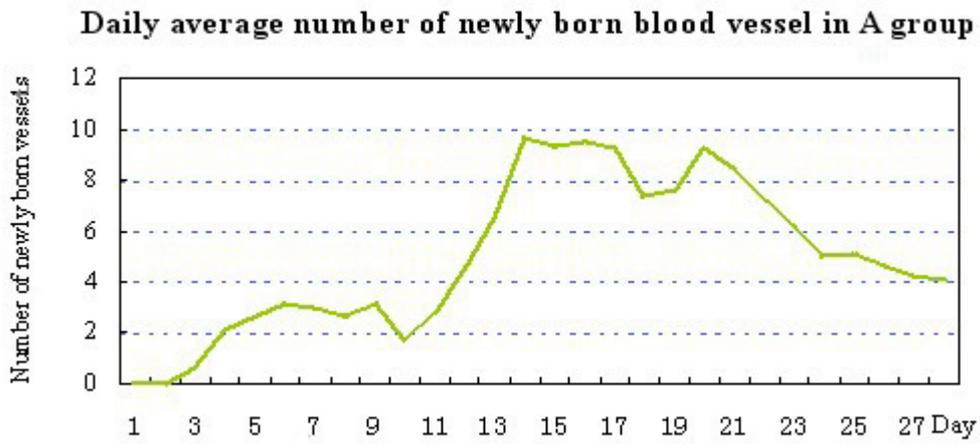
2. Results

2.1 The growth characteristics of blood vessels

The growth characteristics of blood vessels cultured in DMEM/HAMF12 medium was described as follows. Delayed phase > growth phase > decayed phase. The solid endothelial blood vessel bud grew up either from the terminal ends of the cut or from lateral side after being inoculated for 48 hrs. Then they started to grow rapidly at the end of first week and decayed quickly at third week. See Fig 1.

Day	1	2	3	4	5	6	7
Number	0±0	0±0	0.63±0.74	2.13±2.47	2.63±1.85	3.13±2.17	3±1.85
Day	8	9	10	11	12	13	14
Number	2.63±1.60	3.13±3.00	1.75±1.16	2.88±2.75	4.5±4.24	6.5±5.37	9.63±6.41
Day	15	16	17	18	19	20	21
Number	9.38±6.00	9.5±5.90	9.25±5.63	7.38±3.70	7.63±5.93	9.25±6.14	8.5±5.40
Day	22	23	24	25	26	27	28
Number	7.25±4.40	6.25±4.80	5±3.82	5.13±3.60	4.63±3.70	4.25±3.11	4.13±3.40

Fig1. Growth curve of blood vessels in A group (control group)

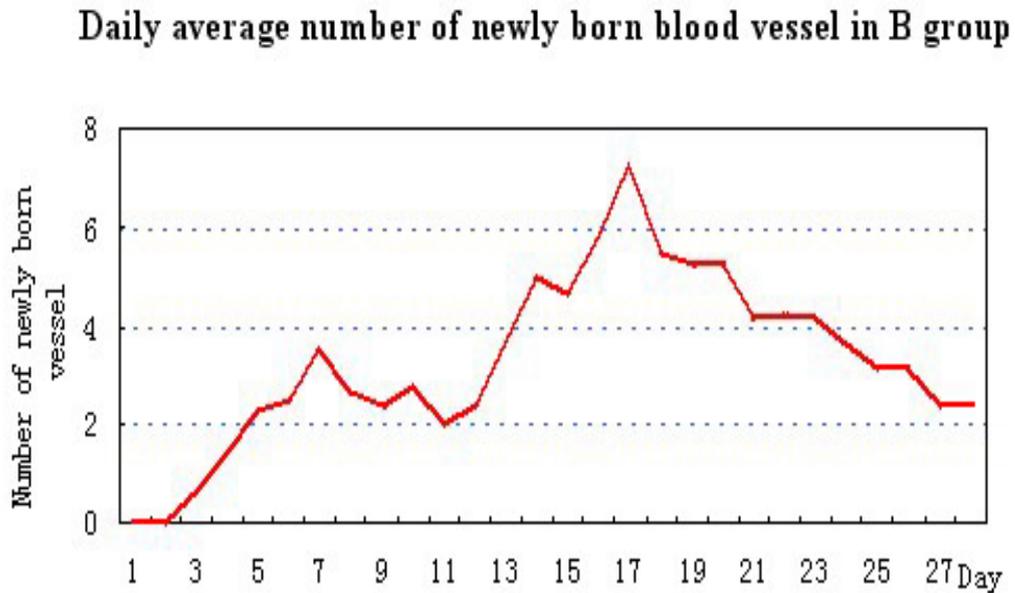


2.2 The effect of VitE on angiogenesis

Fig 2 demonstrated the growth curve of aortic ring treated with 0.1mg/ml of VitE. When the growth phase was over the number of newly formed blood vessels was less than that in control group and the growth curve was lower. The results of these two groups showed significant difference ($P<0.01$). See Fig 2.

Day	1	2	3	4	5	6	7
Number	0±0	0±0	0.63±0.52	1.38±1.06	2.25±1.16	2.5±0.76	3.5±1.69
Day	8	9	10	11	12	13	14
Number	2.63±1.60	2.38±1.30	2.75±1.91	2±1.41	2.38±1.19	3.63±1.85	5±2.33
Day	15	16	17	18	19	20	21
Number	4.63±2.56	5.88±2.53	7.25±2.76	5.5±2.67	5.25±2.82	5.25±2.05	4.13±2.03
Day	22	23	24	25	26	27	28
Number	4.25±1.83	4.13±2.30	3.63±1.41	3.13±1.73	3.13±1.73	2.38±1.06	2.38±1.06

Fig2. Growth curve of blood vessels in B group (0.1mg/ml VitE group)

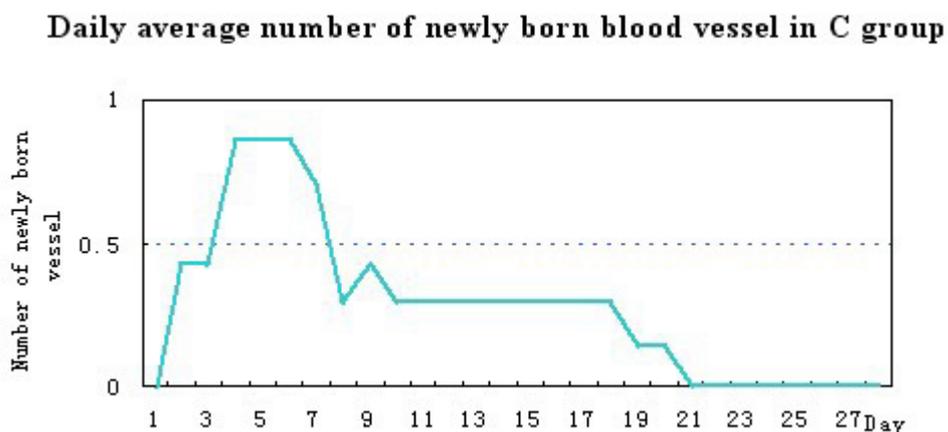


2.3 The effect of KLT on angiogenesis

Aortic ring treated with 10 μ l/ml KLT rarely produced new blood vessels and entered decayed phase at the end of first week. The growth curve became low and flat which indicated KLT could inhibit angiogenesis markedly. Compared with A or B group significant differences existed: C vs A: $P < 0.01$, C vs B: $P < 0.01$ See Fig 3.

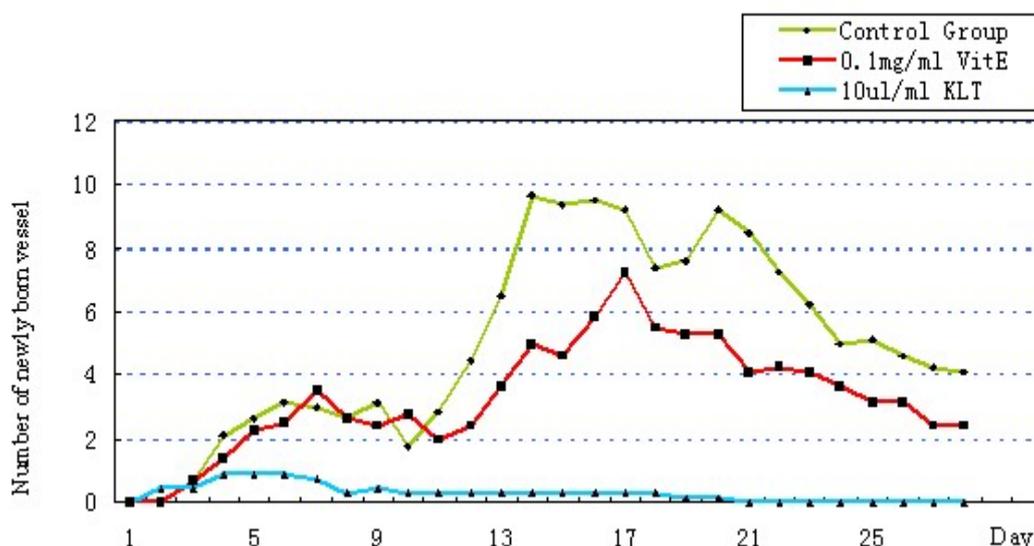
Day	1	2	3	4	5	6	7
Number	0 \pm 0	0.43 \pm 0.53	0.43 \pm 0.53	0.86 \pm 1.10	0.86 \pm 1.10	0.86 \pm 1.10	0.71 \pm 1.11
Day	8	9	10	11	12	13	14
Number	0.29 \pm 0.49	0.43 \pm 0.79	0.29 \pm 0.76				
Day	15	16	17	18	19	20	21
Number	0.29 \pm 0.76	0.29 \pm 0.76	0.29 \pm 0.49	0.29 \pm 0.49	0.14 \pm 0.38	0.14 \pm 0.38	0 \pm 0
Day	22	23	24	25	26	27	28
Number	0 \pm 0						

Fig3. The effect of 10ul/ml KLT group (C group) on angiogenesis



Daily average number of newly formed blood vessels of three groups (A, B, C groups) was illustrated in Fig 4.

Fig 4. Daily average number of newly formed blood vessel in 3 groups



3. Discussion

In our inhibitive angiogenesis study rat aorta was incubated in a three-dimension collagen gel with serum free medium which didn't restrict, inactivate or stimulate the tested substrate. Therefore this method had benefit of high sensitivity and reliability. It has become one of the advanced method in studying angiogenesis. In addition to blank control group the group of 0.1mg/ml VitE (positive group) was also set up in order to compare KLT inhibitive action on angiogenesis with other inhibitors. In 1994 Kelloff and associates had suggested that VitE inhibit activity of angiogenesis by clearing free oxygen radicals and recovering immune function for antiproliferative activity so that VitE had function of chemo-prevention against tumors. The animal experiments carried out by Shklar and Schwartz in 1996, who used VitE to treat the animal cancers induced by DMBA, showed that the tumor volume in VitE group was

much smaller than that in control group and endothelial cells of blood vessels and the expression of TGF- α were all inhibited. This suggested that the relationship among tumor, angiogenesis and VitE are existed. Our previous experiments also demonstrated that the concentration of 0.2 or 0.1mg/ml of VitE could inhibit angiogenesis and the number of newly-formed blood vessels was even less in the latter group. So we chose 0.1mg/ml of VitE in positive control group.

In this experiment after aorta ring was treated with 10 μ l/ml of KLT (the strongest anti-tumor concentration) few blood vessels was produced and they entered into decayed phase quite rapidly (See Fig.3). Therefore the inhibition effect of KLT was significant and much better than 0.1mg/ml VitE group ($P < 0.01$). This suggested that one of the anti-tumor channels of KLT is through inhibiting angiogenesis but its mechanism of anti-tumor action has been unclear.

Kanglaite Injection (KLT), an emulsive preparation is extracted from a traditional Chinese medicinal herb "semen coicis" through modern scientific process. It was awarded the Grade II National New Drug. KLT is a dual-function broad-spectrum antineoplastic agent which can kill tumor cells, promote immune function, provide high energy to patients, minimise toxic and adverse effect of chemotherapy and improve patients survival quality. It is the first time to study the effect of KLT on angiogenesis and the results indicated that the inhibition of neovascularization by KLT played another role in its anti-tumor action. The possible mechanism of KLT to inhibit angiogenesis could be: (1) To inhibit mitosis and migration of vascular endothelial cells. The results obtained from the research of cell cycle progression demonstrated that KLT could arrest tumor cells at G₂+M phase, reduce the proportion of cells at DNA synthesis phase (S phase); (2) To inhibit the tumor cells releasing up-regulating factors of angiogenesis; (3) To block the up-regulating factors of angiogenesis or its receptors in form of antibody pattern; (4) To interfere endothelial cells to differentiate into complete capillaries and to prevent newly formed blood vessels anastomized with host blood vessels. It needs further study to confirm through which channel KLT could inhibit angiogenesis and play anti-tumor action.

Angiogenesis is a complex biological process. The new blood vessels were generated from the existing blood vessels. In general they grow up from the original vascular endothelial cells by budding and influenced by soluble factors, endothelial cells and other extracellular substrates. The growth and metastasis of cancer cells were highly correlated with angiogenesis. Early in 1863 Virchow had noticed that the number of blood vessels in malignant tumor increased abruptly. The neovascularization concept was established by Algire in 1945. When solid tumor is formed it stays at prevascular phase and tumor cells survive mainly through the nutrition provided by diffusion. The diameter of tumor mass is less than 2-3mm with number of cells fewer than 10⁷ in general. Beyond that size if tumor mass still lacks newly formed capillaries and small blood vessels to provide nutrients, its growth would be arrested, cells died and tumor tissue degenerated. As newly formed capillaries infiltrate into tumor tissues which would grow rapidly, the growth rate of blood vessels was 50-100 times higher than normal tissues and easily lead to infiltrate and metastasis of cancer. So the neovascularization is one of the important condition for tumor proliferation and metastasis. It

gives a possible efficient approach to treat cancer by inhibiting angiogenesis. Taking blood vessels as a target to set up a cancer therapeutic regimen recommended by Folkman in 1971 has drawn much attention and the similar study in USA has been developed into clinical trials from 1998.

In conclusion, KLT could not only control tumor growth and metastasis, promote immune function, provide nutrition with high energy, control cancerous fluid, but also markedly inhibit angiogenesis which could further enhance the former action without obvious toxicity or side effect. It is confirmed that KLT is an ideal therapeutic drug among antineoplastic agents used today and it is valuable to promote its clinical application.

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