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Efficacy of transcatheter arterial chemoembolization combined with sorafenib in inhibiting tumor angiogenesis in a rabbit VX2 liver cancer model

WeiZhi Li [a], ShuZhen Kong [b], JingWen Su [a], Jin Huang [a], Hui Xue [a,*]

[a] Department of Gastroenterology, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, 710061, Shaanxi Province, China
[b] Department of Dermatology, The Second Affiliated Hospital of Xi'an Jiaotong University, Xi'an, 710004, Shaanxi Province, China

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ABSTRACT

Background: The aim of this study was to investigate the effects of transcatheter arterial chemoembolization (TACE) combined with sorafenib on tumor angiogenesis.
Materials and methods: Thirty New Zealand rabbit VX2 liver cancer model animals were divided into five groups, which received either normal saline (A), TACE (B), sorafenib (C), sorafenib followed by TACE (D), or TACE followed by sorafenib (E). Serum vascular endothelial growth factor (VEGF) levels were measured before and after TACE via ELISA. Immunohistochemistry for CD34 was performed to evaluate microvessel density (MVD), and ultrasonography was used to access tumor volume.
Results: VEGF levels declined in group C but increased significantly on the 3rd post-operative day in groups B, D, and E. Levels decreased after the 7th post-operative day. Peak levels were significantly lower in group D than in groups B and E. On the 14th post-operative day, VEGF levels were the lowest in group C, followed by those in groups D and B. MVD was the lowest in group C followed by that in group D and E, and was the highest in group B. Group D had the smallest tumor volume. HE staining of tumor tissues from group C showed apoptosis in a scattered patchy pattern, whereas in groups B, D, and E, large areas of tumor cell necrosis were visible.
Conclusion: TACE can up-regulate serum VEGF levels, which in turn accelerates the formation of new blood vessels. Thus, TACE combined with sorafenib inhibits VEGF and angiogenesis, and pre-operative administration of sorafenib has a more superior anti-angiogenic effect than post-operative administration.

Introduction

Surgical resection is currently the most effective treatment for hepatocellular carcinoma, however, it has many limitations.[1] Transcatheter arterial chemoembolization (TACE) has proven to be effective for the treatment of liver cancer. However, vascular growth factors such as vascular endothelial growth factor (VEGF) can promote angiogenesis and establish a collateral circulation following TACE, which could then result in tumor relapse/metastasis and poor long-term efficacy.[2] TACE combined with anti-angiogenesis therapy has been a popular topic of research in recent years. Sorafenib, an oral multi-kinase inhibitor, exerts anti-tumor and anti-angiogenesis effects by acting on multiple targets.[3] While some clinical trials have explored the role of TACE combined with sorafenib for treating liver cancer, the combination method, timing of medication (pre- or post-operative), and treatment cycle are not clearly specified in the guidelines.[3,4] In the present study, using a rabbit VX2 liver cancer model, we measured serum VEGF, intratumoral microvessel density (MVD), and tumor growth rate to determine the anti-angiogenic effect of TACE combined with sorafenib in the treatment of hepatocarcinoma. We also sought to determine the optimal timing of these treatments in order to provide evidence that could aid in the selection of an appropriate combination treatment for liver cancer.

Materials and methods

Materials

Thirty New Zealand rabbits (male and female) weighing 2.2–3.0 kg

* Corresponding author.
E-mail addresses: 309983251@qq.com (W. Li), 632482572@qq.com (S. Kong), 250094085@qq.com (J. Su), 949876510@qq.com (J. Huang), zj2@mail.xjtu.edu.cn (H. Xue).

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and bearing VX2 tumors were provided by the Experimental Animal Centre of Xi'an Jiaotong University, School of Medicine. The animals were randomized into five equal groups: group A (normal saline), group B (TACE only), group C (sorafenib only), group D (sorafenib followed by TACE), and group E (TACE followed by sorafenib). In group A, 0.2 mL/kg normal saline was injected via the hepatic artery. In group B (TACE only), hepatic artery catheterization was performed via the femoral artery using the Seldinger technique; after the catheter reached the target position, pirarubicin (2 mg/kg) and lipiodol suspension (0.2 mL/kg) were perfused via the SP catheter. In group C, sorafenib (20 mg/kg) was administered daily via gastric perfusion starting on the 7th pre-operative day until the 14th post-operative day. In group D, TACE was performed as in group B, and sorafenib (20 mg/kg) was administered daily via gastric perfusion starting on the 7th pre-operative day until the 14th post-operative day. In group E, TACE was performed as in group B, and sorafenib (20 mg/kg) was administered daily via gastric perfusion starting on the 3rd post-operative day until the 14th post-operative day.

VEGF levels were determined using enzyme-linked immunosorbent assay (ELISA) at 7 days and 1 day before TACE and 3, 7, and 14 days after TACE. All tumor-bearing rabbits were killed on the 14th post-operative day and immunohistochemistry for CD34 expression was performed to evaluate MVD. Sorafenib (200 mg/tablet) was obtained from Bayer, Germany. Rabbit VEGF ELISA kits were purchased from R&D Systems, USA. CD34 antibody was purchased from BioLegend, USA. Ready-to-use SABC immunohistochemical staining kits were purchased from Bioss, Beijing, China. The 3F SP micro-catheter was manufactured by Cook Medical Technologies LLC (Bloomington, IN, USA). A Philips Integris H5000 was used for digital subtraction angiography (DSA).

Methods

TACE

Tumor-bearing rabbits were anesthetized via an ear vein injection of pentobarbital sodium and then immobilized in the supine position using a bandage on a homemade wooden table. Skin preparation and disinfection were performed on the right groin. After the pulse and anatomy of the femoral artery were confirmed, the skin was cut open along the femoral artery and peeled off using hemostatic forceps. An oblique incision was then made above the femoral artery using eye scissors. The micro guidewire was delivered into the femoral artery via the oblique incision using eye scissors, then the guidewire was slowly deployed. A 3F-SP catheter was introduced into the artery at the level of T12 - L1, then the micro guidewire was withdrawn for angiography to view the celiac trunk. Under guidance of the micro guidewire, a second angiography was performed in the celiac trunk to identify the arterial anatomy and the tumor blood supply. Angiography showed a localized increase, thickening, and tortuosity of the tumor blood vessels, as well as staining of the tumor. After super selection of the artery or arteries feeding into the tumor, pirarubicin (2 mg/kg) and lipiodol ultra-fluid (0.2 mL/kg) were injected. Upon embolization, the catheter was rinsed with normal saline. X-ray examination showed homogeneous or heterogeneous deposition of iodized oil inside the tumor, and angiography showed tumor staining had mostly disappeared. The catheter was withdrawn and the proximal and distal ends of the femoral artery were ligated. The incision was sutured layer by layer to end the procedure. The total amount of contrast agent used in the procedure did not exceed 5 mL/kg. After surgery, penicillin (800,000 IU) was administered intramuscularly for 3 consecutive days.

Hematoxylin-eosin (HE) staining

Sections were dewaxed in xylene for 15 min, then repeated two more times with fresh xylene. Tissues were then rehydrated through a graded ethanol series: 100% ethanol for 2 min, 95%, 80%, and 75% ethanol for 1 min each, then deionized water for 2 min. Sections were stained with 30% hematoxylin for 8 min, rinsed three times with tap water, differentiated in 2% acid alcohol, rinsed three times in running water, placed in 1/500 ammonia water to allow the color change to blue, then rinsed three times with tap water. Sections were then stained with 1% eosin for 2 min, and dehydrated with 80% ethanol for 1 min, 95% ethanol for 2 min, and 100% ethanol for 2 min. Finally, tissues were subjected to two changes of xylene for 10 min each, mounted with neutral balsam, then observed under a light microscope.

Determination of serum VEGF levels

Blood samples (2 mL per rabbit) were collected from the marginal ear vein, and VEGF levels were determined using a double-antibody sandwich ELISA 7 days and 1 day before TACE and 3, 7, and 14 days after TACE. Step 1:Standard samples were prepared at different concentrations: 600 pg/mL, 300 pg/mL, 150 pg/mL, 75 pg/mL, 37.5 pg/mL, and 0 pg/mL. Step 2:Blank control wells, standard wells, and sample wells were set. Step 3:Plates were sealed with plate-sealing film and incubated for 30 min. Step 4:The 30-fold concentrated liquid was diluted with distilled water for further use. Step 5:The plate-sealing film was removed and the liquid was discarded. The wells were dried and then washing liquid was added for 30 s. This step was repeated five times, then the plates were dried. Step 6:ELISA reagents (50 μL) were added to each well (except the blank well). Steps 3 and 5 were repeated once more. Step 7:Color developers A and B (50 μL each) were added to each well. The plate was shaken gently to mix the solutions then placed in the dark and 37 °C for 10 min to allow the color to develop. Step 8:Termination solution was added (50 μL per well) and absorbance was measured at 450 nm within 15 min.

A standard curve was drawn, with standard sample concentration on the x-axis and measured OD on the y-axis. From this, the OD values of the samples at different concentrations were determined. Actual sample concentrations were then obtained after multiplying by the fold dilution.

Immunohistochemical staining to determine MVD (SABC method)

Streptavidin has a high affinity for biotin molecules, therefore we chose to use the SABC method due to its high sensitivity, low background, and simplicity. All tumor-bearing rabbits were killed on the 14th post-operative day and immunohistochemistry for CD34 was performed to evaluate microvessel density (MVD). Sections were baked at 58–60 °C for 30–60 min to ensure good attachment to the slides. They were dewaxed in xylene for 10 min, then rehydrated through a graded ethanol series: 100%, 95%, 80%, and 75% ethanol for 2 min each. After rinsing 3 times in phosphate buffer solution for 2 min each, sections were soaked in 3% H_2O_2 for 10 min at room temperature, then rinsed three times. The slides were then placed into PBS buffer (pH 6.0) and heated in a microwave oven at 92–98 °C for 15 min, then cooled to room temperature, and rinsed twice in PBS for 2 min each. Blocking solution was added and sections were incubated at 37 °C for 20 min to reduce non-specific antibody binding. Excess serum was wiped off and 50 μL rabbit anti-rabbit CD34 antibody was added. Sections were incubated for 1 h at 37 °C then rinsed 3 times in PBS for 2 min each. Fifty microliters of goat anti-rabbit Ig secondary antibody was then added and the sections were incubated for 30 min at 37 °C then rinsed 3 times in PBS for 2 min each. Sections were incubated in SABC reagent for 20 min at 37 °C then rinsed 4 times in PBS buffer for 5 min each. DAB was prepared by mixing 1 mL distilled water with one drop of DAB A (vortex), then one drop of DAB B (vortex), and finally, one drop of DAB C (vortex). Color was allowed to develop at room temperature for 5–30 min then sections were counterstained with Ehrlich's solution and rinsed thoroughly with tap water. The slides were then dipped in saturated lithium carbonate, rinsed with tap water, then dipped in 1% HCl/ethanol for 5 s, rinsed with tap water, and washed with distilled water. Finally sections were dehydrated through a graded ethanol series, cleared with two rinses in xylene and mounted with neutral balsam. Staining was observed under a light microscope.

Any single brown-stained cell or cluster of endothelial cells clearly separated from adjacent vessels was considered a microvessel. Vessels within necrotic and sclerotic areas were not counted. Also, larger vessels surrounded by smooth muscle cells were not included. Using the method described by Weidner,[5] stained sections were screened at ×40

magnification to identify regions with the highest vascular density. Vessels were then counted in the five regions with the highest vascular density at ×200 magnification. MVD was calculated as the mean number of vessels in these areas. An inter-group comparison was then performed.

Tumor growth rate

Ultrasonography was performed 7 days before surgery (at the time of grouping) and 14 days after surgery. Tumor volume was calculated using the following formula: tumor volume (mm^3) = $1/2\ ab^2$, where a is the largest diameter (mm) and b is the smallest diameter (mm). In addition, the tumor growth rate (TGR) was calculated for each group,[6,7] which was determined using the following formula: increase in tumor volume = tumor volume after treatment - tumor volume before treatment. Changes in tumor volume among groups was analyzed using one-way ANOVA.

Statistical analysis

All data were analyzed using the SPSS13.0 statistical package. t-test, Chi-square test, and one-way ANOVA (LSD test for homogeneous variances and Games-Howell post-hoc test for non-homogeneous variances) was performed. $P < 0.05$ was considered statistically significant.

Results

HE staining results

Fourteen days after treatment, HE staining was performed on tumor tissues from each of the five groups. In group A, which received normal saline, no obvious necrosis was observed in the tumor tissues. Animals in group C, which received sorafenib alone, showed obvious apoptosis in the tumor cells in a scattered patchy pattern. In groups B (TACE alone), D (sorafenib followed by TACE), and E (TACE followed by sorafenib), large areas of tumor cell necrosis were visible, in which nuclear pyknosis, karyorrhexia, or dissolution were common. Also, many red-stained tissues without specific morphology were visible around these necrotic cells, particularly in group D (Fig. 1).

Serum VEGF levels

Blood samples were collected from the marginal ear vein 7 days and 1 day before TACE and 3, 7, and 14 days after TACE, and VEGF levels were determined using ELISA. Paired comparisons showed no significant differences among the five groups either 7 days or 1 day before TACE ($P > 0.05$). However, in groups C and D, where sorafenib was administered before TACE, average VEGF levels were lower than those in the other three groups. After TACE, serum VEGF levels gradually increased in group A, but declined in group C; the difference between these two groups was statistically significant on the 14th post-operative day ($P < 0.05$). Serum VEGF levels significantly increased on the 3rd post-operative day in groups B, D, and E and showed significant differences when compared to the levels in group A ($P < 0.05$). However, peak serum VEGF levels were significantly lower in group D than in groups B and E ($P < 0.05$), but gradually decreased after the 7th post-operative day. On

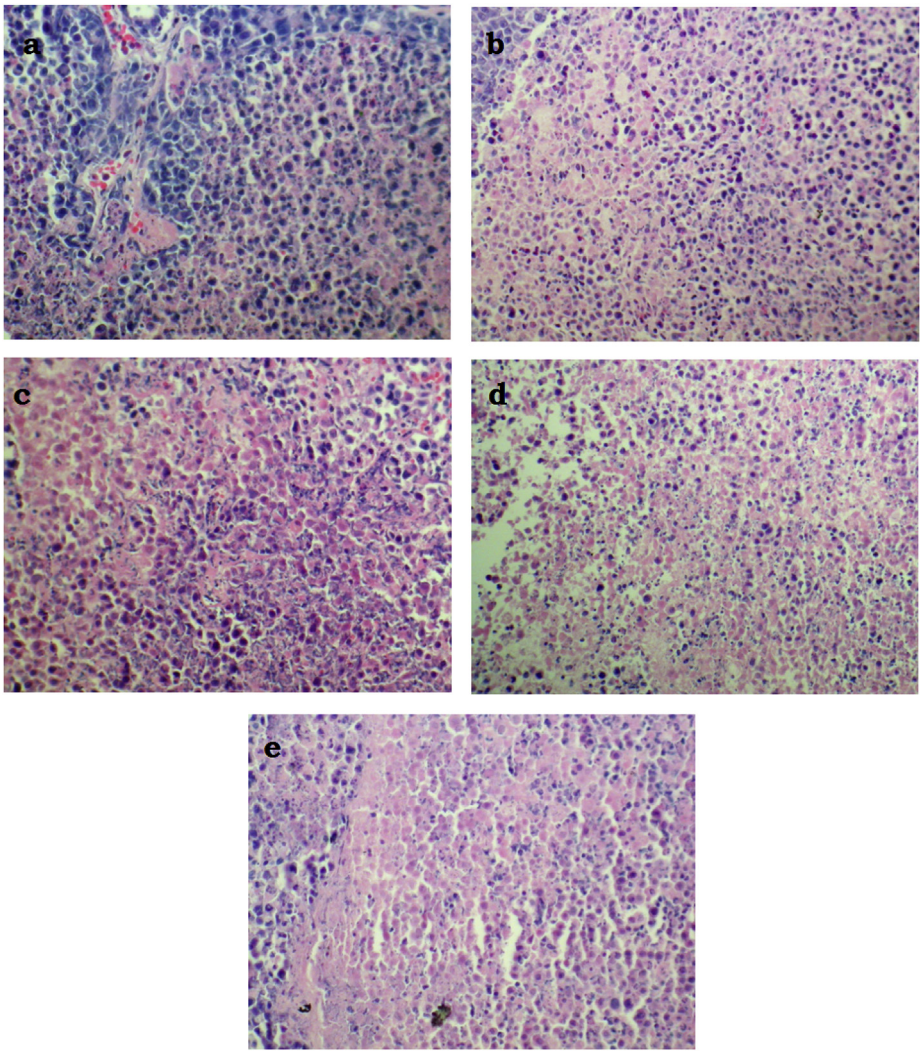

Fig. 1. HE staining of rabbit VX2 carcinoma tissue (14th post-operative day, 200 x). a) in group A, no obvious necrosis was observed; b) in groups B, D, and E, large areas of tumor cell necrosis were visible, in which nuclear pyknosis, karyorrhexia, or dissolution were common; many red-stained tissues without specific morphologies were also visible around these necrotic cells, particularly in group D; c) in group C, the tumor cells showed obvious apoptosis in a scattered patchy pattern. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

the 14th post-operative day, serum VEGF levels were the lowest in group C, followed by those in groups D and B (Table 1 and Fig. 2).

MVD in the tumors

Microvessel density (MVD) was 31.48 ± 5.72 in group A, 87.65 ± 6.99 in group B, 24.55 ± 3.65 in group C, 47.63 ± 5.60 in group D, and 67.39 ± 6.33 in group E, showing significant differences ($F = 121.775$, $P < 0.01$) (Table 2 and Fig. 3). LSD paired comparisons of MVD showed significant differences ($P < 0.05$). Any single brown-stained cell or cluster of endothelial cells clearly separated from adjacent vessels was considered a microvessel. From this figure you can see that MVD in groups D and E was significantly higher than in groups A and C, but lower than in group B ($P < 0.05$) (Fig. 4). The results showed that sorafenib could inhibit the formation of tumor neovascularization caused by the high expression of VEGF after TACE. The effect of combination of sorafenib before operation in group D was better than that of combination of sorafenib after operation in group E.

Tumor growth rate

Tumor volume on the 7th pre-operative day (at grouping) was 0.65 ± 0.09 cm^3 in group A, 0.63 ± 0.10 cm^3 in group B, 0.70 ± 0.12 cm^3 in group C, 0.63 ± 0.11 cm^3 in group D, and 0.70 ± 0.09 cm^3 in group E ($P > 0.05$, paired comparison). On the 14th post-operative day, tumor size and increase in tumor size were 3.43 ± 0.22 cm^3 and 2.78 ± 0.27 cm^3 in group A, 2.51 ± 0.21 cm^3 and 1.80 ± 0.11 cm^3 in group B, 2.78 ± 0.09 cm^3 and 2.08 ± 0.08 cm^3 in group C, 1.23 ± 0.15 cm^3 and 0.59 ± 0.12 cm^3 in group D, and 1.80 ± 0.16 cm^3 and 1.11 ± 0.21 cm^3 in group E, respectively. On the 14th post-operative day, tumor volume and increase in tumor size were significantly lower in groups B, C, D, and E than in group A ($P < 0.01$). Paired comparisons among groups B, C, D, and E showed that the comparison between group B and group C had a P value of 0.10, whereas the other P values were below 0.05, indicating significant differences. Group D had the smallest tumor volume and increase in tumor volume (Table 3).

Discussion

An imbalance between pro- and anti-angiogenic factors activates a tumor vessel switch, resulting in angiogenesis.[8] VEGF, the most important vascular growth factor, is not only involved in the formation and proliferation of tumor vessels, but also directly participates in tumor invasion and metastasis.[9] As one of the key tumor angiogenesis-promoting factors, it plays a key role in the occurrence, progression and prognosis of liver cancer. VEGFR-2 is the main receptor by which VEGF regulates the growth and proliferation of blood vessels. After specific binding of VEGF to its receptor, phosphorylation of the receptor activates intracellular signaling pathways. This not only increases the Ca2+ concentration in endothelial cells, but also initiates dimerization of the receptor. Autocrine and paracrine activity of endothelial cells then induces endothelial cell growth, increases vascular permeability, promotes angiogenesis and new stroma formation, and drives tumor cell infiltration/metastasis.[10] The expression profile of VEGF in serum and paracancerous tissues may be an independent indicator of distant metastasis of liver cancer and could be useful for determining prognosis.[11]

Table 1
Changes in VEGF levels before and after surgery (pg/mL).

VEGF	Pre-op 7th day	Pre-op 1st day	Post-op 3rd day	Post-op 7th day	Post-op 14th day
Group A	423.0 ± 58.2	458.3 ± 51.2	486.7 ± 40.8	516.7 ± 51.6	593.8 ± 42.5
Group B	414.2 ± 41.9	460.5 ± 44.2	1005.5 ± 84.3	854.3 ± 42.8	783.2 ± 36.7
Group C	432.7 ± 37.0	420.0 ± 29.8	408.0 ± 29.1	399.2 ± 22.1	388.5 ± 16.1
Group D	409.7 ± 52.8	418.0 ± 43.9	699.3 ± 51.3	593.3 ± 53.8	450.2 ± 37.3
Group E	421.3 ± 51.7	464.2 ± 53.0	991.7 ± 40.3	725.3 ± 57.6	536.3 ± 25.6
F value	0.189	1.512	166.256	85.910	125.537
P value	0.942	0.229	0.000	0.000	0.000

The current study showed that serum VEGF levels were not significantly different among the five groups 7 days or 1 day before TACE ($P > 0.05$). In addition, paired comparisons of serum VEGF levels on the 1st pre-operative day showed significant differences ($P < 0.05$); In groups C and D, where sorafenib was administered before surgery, average VEGF levels were lower than in the other three groups, suggesting sorafenib may reduce serum VEGF levels. However, the effectiveness of sorafenib may be reduced due to the short-term oral administration used here. In group A, serum VEGF levels increased gradually after surgery, which could be explained by the fact that tumors release a large number of cytokines, including VEGF, during the angiogenesis stage in order to maintain growth. Group C, however, showed a decrease in serum VEGF levels, which were significantly lower than in group A ($P < 0.05$) 14 days after TACE. This suggests that sorafenib can reduce serum VEGF levels. In tumor-bearing rabbits, serum VEGF is mainly secreted by VX2 carcinoma cells and stellate cells.[12] Hypoxia and activation of Ras, Raf, and other cancer genes can stimulate VEGF expression.[13,14] By suppressing Raf kinase in the Ras→Raf→MEK1, 2→ERK1, 2 signaling pathway, which is involved in cell proliferation, sorafenib can directly inhibit tumor growth, and can inhibit VEGF expression by blocking the MAPK pathway.[15] In addition, by preventing the formation of new vessels in tumors by blocking VEGFR-2, it can prevent the interaction between VEGF and VEGFR-2. Serum VEGF levels were significantly increased on the 3rd post-operative day in groups B, D, and E and showed significant differences when compared to levels in group A ($P < 0.05$). Therefore, ischemia and hypoxia in the tumor lesions after TACE turn on the angiogenic "switch" and up-regulate VEGF expression, resulting increased levels. Such up-regulation is manifested at the transcriptional and post-transcriptional levels; the former is associated with hypoxia-inducible factor-1, while the latter is achieved by increasing the stability of VEGF mRNA. However, the peak value of VEGF expression was significantly lower in group D than in groups B and E ($P < 0.05$), suggesting that early administration of sorafenib can lower the peak amplitude. Peak serum VEGF levels gradually decreased after the 7th post-operative day. On the 14th post-operative day, VEGF levels were the lowest in group C, followed by those in groups D and B ($P < 0.05$). Thus, TACE can up-regulate serum VEGF and accelerate the formation of new blood vessels that supply the tumor cells, leading to the progression and metastasis of residual tumors, and may even promote new tumor formation. Sorafenib can exert its anti-angiogenic effects by lowering VEGF expression and blocking binding to its receptors. Research has shown a positive correlation between VEGF levels in serum and tumor tissue.[16] Measuring serum VEGF levels can reveal dynamic changes in VEGF, suggesting that it could act as an independent and reliable indicator of distant metastasis of liver cancer, and could be used to more accurately determine prognosis.

MVD can be used to directly evaluate the degree of tumor angiogenesis, and can serve as an independent indicator to assess the prognosis of liver cancer.[17] Angiogenesis is a progressive process and MVD reflects the status at any specific time during this process, allowing the degree of angiogenesis to be quantified.[18] Martin et al. demonstrated that CD34 is one of the best markers of vascular endothelial cells.[19] Thus, in the present study, we used CD34 to mark tumor vascular endothelial cells in order to calculate MVD values.

We found no significant differences in tumor MVD between the five groups ($P < 0.01$). LSD paired comparisons did reveal significant differences ($P < 0.05$), where group B had the highest MVD. As TACE blocks the tumor-feeding arteries, resulting in ischemia, hypoxia and necrosis of the tumor cells, there is an up-regulation of VEGF expression and tumor

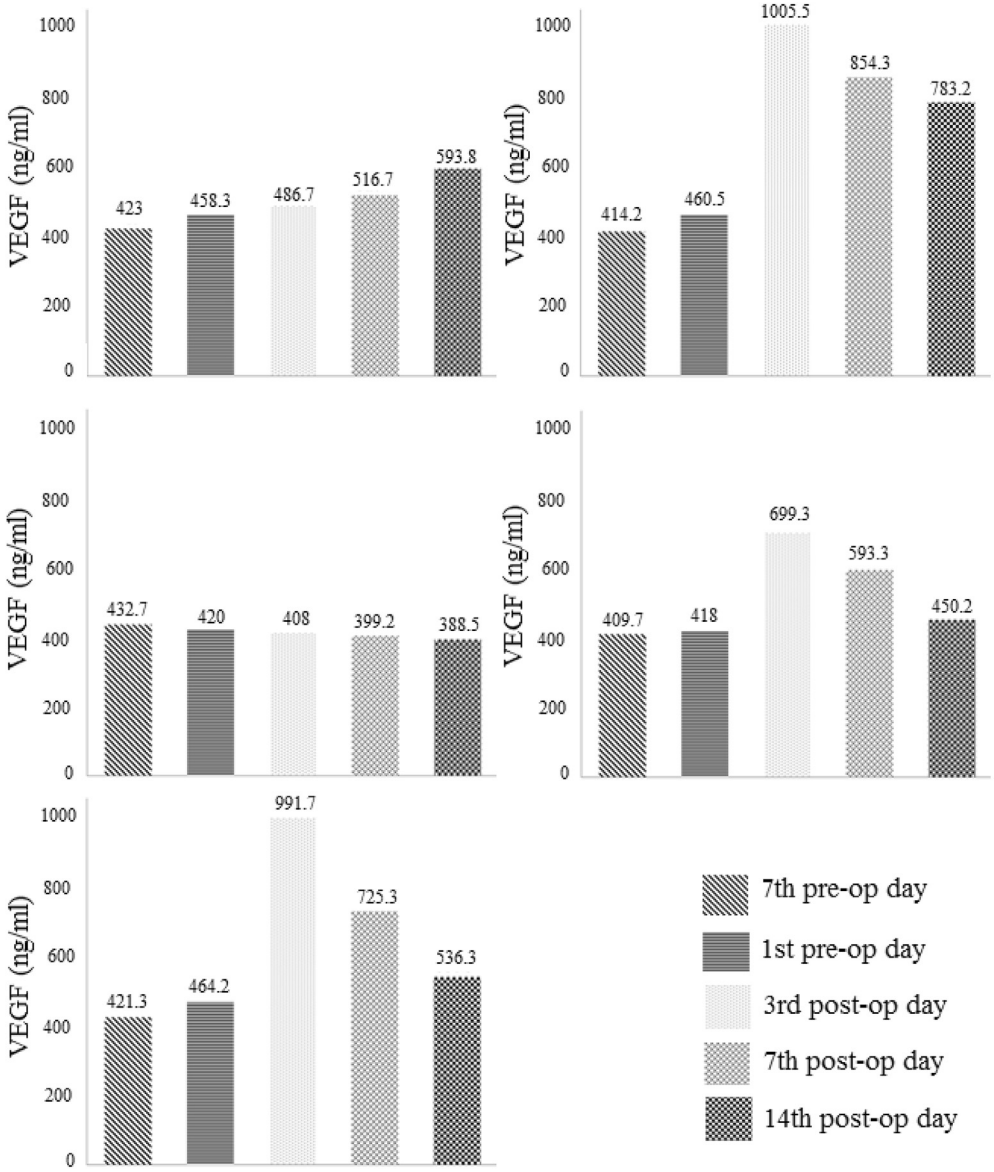

Fig. 2. Changes in serum VEGF levels before and after treatment. a) group A (normal saline), b) group B (TACE alone); c) group C (sorafenib alone); d) group D (sorafenib followed by TACE); e) group E (TACE followed by sorafenib).

Table 2
Comparison of MVD values among the five groups.

Group	MVD value	F value	P value
Group A	31.48 ± 5.72	121.775	0.000
Group B	87.65 ± 6.99		
Group C	24.55 ± 3.65		
Group D	47.63 ± 5.60		
Group E	67.39 ± 6.33		

angiogenesis, and thus an increase in MVD. Sorafenib has dual anti-tumor effects, both blocking tumor cell proliferation and inhibiting angiogenesis, and can also suppress tumor angiogenesis due to high VEGF levels after TACE. MVD was significantly higher in groups D and E than in groups A and C, and group D had a lower MVD value than group E. This indicates that: a) TACE in combination with an anti-angiogenic drug can inhibit the formation of tumor blood vessels and establish a collateral circulation after TACE; and b) pre-operative administration of sorafenib has more superior anti-angiogenic effects than post-operative administration.

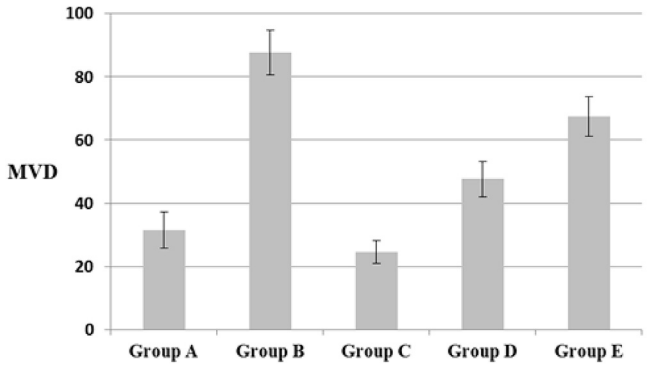

Fig. 3. MVD values in all groups.

Tumor growth is a dynamic process, involving continuous tumor cell formation and death. Growth rate depends on the balance between proliferation and death.[20] Thus, changes in tumor volume and tumor

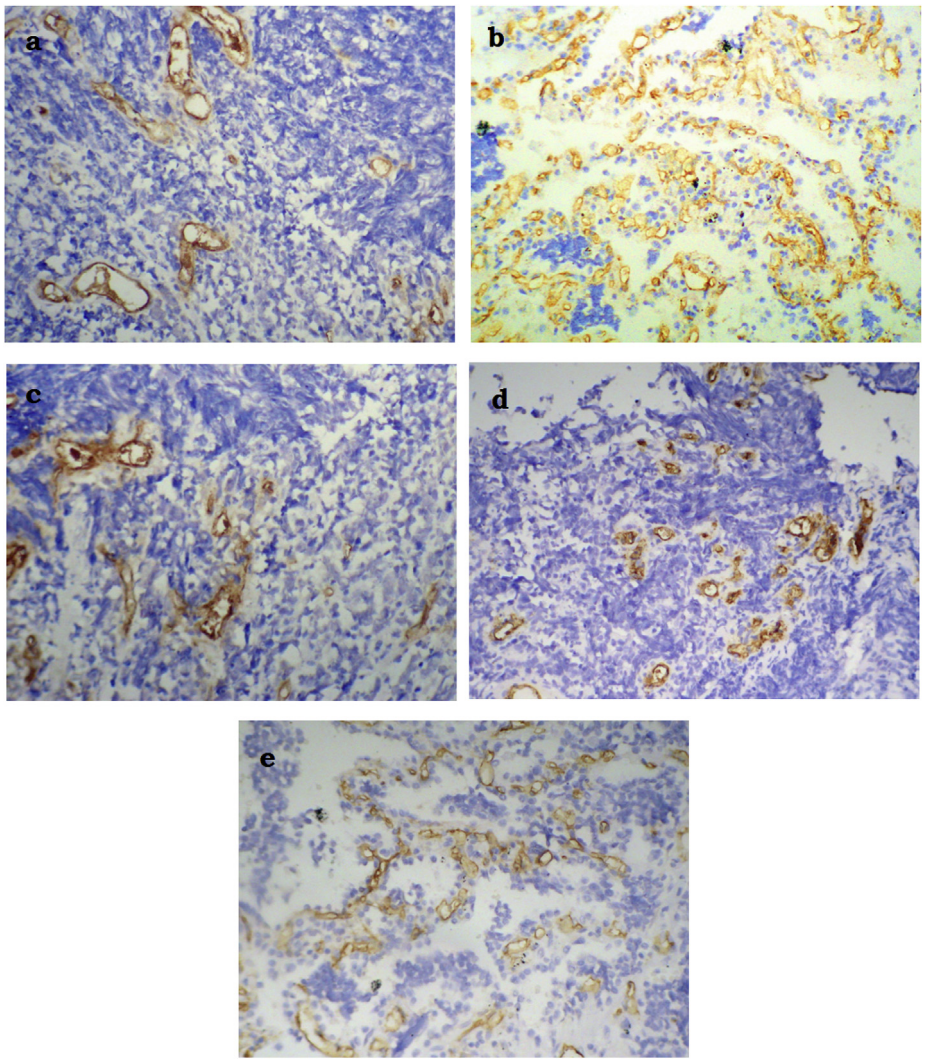

Fig. 4. CD34 staining of endothelial cells in all groups. a) MVD in group A (SABC, ×200); b) MVD in group B (SABC, ×200); c) MVD in group C (SABC, ×200); d) MVD in group D (SABC, ×200); e) MVD in group E (SABC, ×200).

Table 3
Changes in tumor volume before and after treatment ($\overline{X} \pm s$).

Group	Tumor volume (V1) on the 7th pre-operative day (cm^3)	Tumor volume (V1) on the 14th post-operative day (cm^3)	Increase in tumor volume (cm^3)
Group A	0.65 ± 0.09	3.43 ± 0.22	2.78 ± 0.27
Group B	0.63 ± 0.10	2.51 ± 0.21	1.80 ± 0.11
Group C	0.70 ± 0.12	2.78 ± 0.09	2.08 ± 0.08
Group D	0.63 ± 0.11	1.23 ± 0.15	0.59 ± 0.12
Group E	0.70 ± 0.09	1.80 ± 0.16	1.11 ± 0.21
F value	0.561	149.334	147.923
P value	0.693	0.000	0.000

Note: As shown by LSD paired comparisons, the P value was greater than 0.05 between group B and C (P = 0.10) and was less than 0.05 among the other groups, with significant differences.

necrosis can be used to assess the efficacy of a specific anti-tumor therapy.

In the current study, tumor volumes were not significantly different among the tumor-bearing rabbits at the time of grouping (on the 7th pre-operative day) (P > 0.05). However, on the 14th post-operative day, paired comparisons showed significant differences among the groups (P < 0.05), except for the difference between groups B and C (P > 0.05). A comparison of tumor volumes between groups A and B indicated that TACE not only destroyed tumor cells via chemotherapy, but also suppressed tumor growth by blocking tumor-feeding arteries. A comparison of tumor volumes between groups A and C indicated that sorafenib has high anti-tumor efficacy. Among the five groups, group D had the smallest tumor size and increase in tumor size, followed by group E. However, tumor size and increase in tumor size were smaller in these two groups (D and E) than in groups B and C. Therefore, TACE alone and sorafenib alone have limited anti-tumor efficacy. The anti-angiogenesis efficacy of TACE and sorafenib is based on different principles, and the combination of these two strategies can improve anti-tumor efficacy. Administration of sorafenib 7 days before TACE had superior efficacy to administration 3 days after TACE, indicating that sorafenib requires time to exert its effect. Therefore, it is more beneficial to use anti-angiogenic drugs before TACE.

There were some limitations to the current study. The New Zealand rabbit VX2 liver cancer model does not represent “real-world” hepatocellular carcinoma, but rather were implanted tumors. They therefore lack common liver diseases such as hepatitis and cirrhosis found in hepatocellular carcinoma. The main diagnostic indicators in this study, serum VEGF and MVD, reflect the effect of TACE in combination with

sorafenib on VX2 tumors, and may be different to that in hepatocellular carcinoma, which represents a more common clinical setting. Large liver tumors are common in clinical practice. In this study, however, the tumors were relatively small when the interventional therapy was administered. These tumors were still in a period of vigorous growth, and their blood supply may also differ from those in a clinical setting. Thus, the actual effectiveness of TACE combined with sorafenib for large liver tumors (rather than small 1–2 cm in size in the present study) should be explored further. Furthermore, we investigated the general condition, liver function, and anti-tumor/anti-angiogenesis effectiveness of TACE with (or without) sorafenib in tumor-bearing rabbits two weeks after surgery, but we did not determine the mid- and long-term safety and effectiveness (e.g., in terms of survival) of these strategies. Whether this can dramatically improve prognosis requires further investigation. We were limited by available resources, therefore we did not adopt the modified Response Evaluation Criteria In Solid Tumors (mRECIST) to assess the efficacy of TACE in combination with sorafenib. The conventional RECIST determines efficacy based on maximal tumor diameter (including the residual viable tumor and necrotic regions), whereas the mRECIST is based on residual viable tumor. The latter is a more accurate reflection of the remaining tumor burden of hepatocellular carcinoma after interventional therapy and/or targeted drug therapy. We were also limited by the small sample size and short duration of observation, therefore the rate of tumor metastasis was not significantly different among the five groups using Fisher's exact test. For this reason, no definite conclusion could be made on the rate of tumor metastasis. Our study was a preliminary investigation into the role of TACE combined with sorafenib in a rabbit model. In the clinical setting, however, most liver cancer cannot be controlled after a single TACE, and multiple TACE procedures are required. Thus, the optimal long-term clinical protocol for the combination of these strategies warrants further exploration. In this study, sorafenib was administered via gastric perfusion rather than hepatic arterial infusion; any potential differences between these two administration routes will also require further study.

Declaration of competing interest

We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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