Changes of tumor microcirculation after transcatheter arterial chemoembolization: First pass perfusion MR imaging and Chinese ink casting in a rabbit model

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AIM: To observe the change of tumor microcirculation after transcatheter arterial chemoembolization (TACE) with bletilla microspheres by using first pass perfusion MR imaging (FP) and Chinese ink casting.

METHODS: VX2 carcinoma cells were surgically implanted into the left and right lobes of liver of 30 New Zealand white rabbits, which were divided into 3 groups at random. Emulsion of lipiodol mixed with mitomycin C, and 5-FU blelilla microspheres were injected into the hepatic artery respectively, and saline was used as control agent. MR imaging was performed with turbo-flash sequence 14 d after tumor implantation and 7 d after interventional therapy. The steepest slopes (SS) of the signal intensity versus time curves were created for quantitative analysis, 7.5% Chinese ink gelatin solution was injected through ascending artery (17 cases) or portal vein (2 cases) for lesion microvessel area (MVA) measurement after the last MRI examination. The correlation between perfusion imaging and MVA was studied blindly.

RESULTS: The SS values at the rim of tumor in lipiodol group (mean, 49% per second) and blelilla group (mean, 35% per second) were significantly decreased (P<0.05) as compared with control group (mean, 124% per second), no difference was found between lipiodol and blelilla groups (P>0.05). In lipiodol group, the MVAs (24 974±11 836 µm²) in the center of the tumor were significantly smaller than those of the control group (35 510±15 675 µm²) (P<0.05), while the MVAs (80 031±22 745 µm²) around the tumor were significantly increased because small and dense plexes appeared around the tumor which correlated to intense reaction of granulation tissue. None of the vessels was seen in the tumor in blelilla group, the peripheral MVAs of the tumor were significantly smaller than those of the control group (P<0.05) and lipiodol group (P<0.05). There was a good correlation between SS and MVAs in control group (rₓ, 0.985, P<0.0001) and blelilla group (rₓ, 0.743, P<0.05), the correlation was not significant in lipiodol group (rₓ, 0.527, P>0.05).

CONCLUSION: TACE with blelilla microspheres may enhance its anti-tumor effect by inhibiting the angiogenesis, and FP-MRI provides useful information to assess the TACE effect by depicting tumor vascularization and perfusion.

Zhao JG, Feng GS, Kong XQ, Li X, Li MH, Cheng YS. Changes of tumor microcirculation after transcatheter arterial chemoembolization: First pass perfusion MR imaging and Chinese ink casting in a rabbit model. World J Gastroenterol 2004; 10(10): 1415-1420
http://www.wjgnet.com/1007-9327/10/1415.asp
by performing midline laparotomy, VX2 tumor fragments of approximately 1 mm³ were injected intraparenchymally into the right and left lobes of the rabbit liver. Thirty New Zealand white rabbits with tumors were divided into three groups at random. They were group A (control group, 8 rabbits), group B (lipiodol group, 12 rabbits) and group C (bletilla group, 10 rabbits). All three groups received their interventional therapy 14-21 d after tumor inoculation when MRI confirmed the successful implantation.

**Interventional therapy**

A polyethylene catheter (inner diameter 0.3 mm, outer diameter 0.5 mm) was retrogradely inserted into the gastroduodenal artery, the following agents were manually injected into the gastroduodenal artery, namely, saline only into group A, 0.4-0.6 mL lipiodol (Aulnay Sous-Bios, France) emulsion (lipiodol mixed with MMC) into group B, 0.3 mL ultravist mixed with 10 mg bletilla microspheres (Department of Radiology, Union Hospital, Wuhan, China, 40-200 µm in diameter, combined with 5-FU) into group C.

**MR imaging and data analysis**

Fourteen days after tumor implantation and 7 d after interventional therapy. MR imaging was undertaken by using a 1.5 T system (Magnetom Vision, Siemens Medical Systems) with a head coil. Transverse spin-echo T1-weighted images (repetition time was 525 ms, the echo time was 14 ms) and HASTE T2-weighted images (repetition time was 4.4 ms, the echo time was 90 ms) were obtained using a 3 mm thick section, 0.5 mm intersection space, and 4 four signals were acquired.

For the FP, a strongly T1-weighted, turbo-FLASH sequence was used with a high temporal resolution of 1.196 s per section, a single image was acquired sequentially with 65 repetitions, which resulted in an acquisition time of 2 min. The repetition time was 3.3 ms, the echo time was 1.4 ms, and time interval was 300 ms. At the end of the fourth acquisition, a dose of 0.1 mmol/kg body weight of gadopentetate dimeglumine (Bellona, Beijing, China) was administered via a marginal ear vein.

To quantitatively analyze FP, four circular ROIs were hand drawn, covering the center and rim of the lesion, signal intensity time curve was obtained over ROIs, and the steepest slope of the curve (SS) was calculated according to the previously described method 15.

**Evaluation of anti-tumor effect**

Tumor size was measured with calipers. The size of each tumor on the liver surface was measured immediately before treatment and seven days after treatment on MR imaging. We evaluated the anti-tumor effect based on the tumor volume, which was measured with calipers.

**Chinese ink casting and histological evaluation**

The tumor microvessels were demonstrated by perfusion of 75 g/L Chinese ink (Beijing Chinese Ink Company) gelatin solution into the ascending artery (17 cases) or portal vein (2 cases) after the last MR imaging examination. Before perfused with 75 g/L Chinese ink gelatin solution, 500 units of heparin was administered intravenously. After the rabbits were killed, the livers were removed and stored at -20°C for 24 h. The specimens were immersed in 250-995 mL/L ethyl alcohol at increasing concentrations, and finally in a solution of methyl salicylate. When the oil penetrated the tissue, the specimens became transparent. The sections (50 µm) were observed first under a high power (x200). Micro-vessels filled with Chinese ink gelatin were evaluated under a microscope, micro-vessel areas (MVAs) in the center and rim of the lesion were calculated by an imaging analysis system (Beijing Aeronautic University). A 5 µm sections of the same specimens were stained with hematoxylin and eosin for light microscopy.

**Statistical analysis**

All data were expressed as mean±SD, the statistical differences between different groups were analyzed by ANOVA, and the correlation between SS and MVA was assessed by Spearman correlation analysis. Significance was accepted when P<0.05.

**RESULTS**

**MR imaging**

MR imaging depicted all tumors on pretreatment images. All untreated tumors had a low signal intensity on T1-weighted images and an intermediate signal intensity on T2-weighted images. Central areas of high signal intensity on T2-weighted images and low signal intensity on T1-weighted images were compatible with the central necrosis when tumors were larger than 1 cm. T1-weighted images after injection of gadolinium showed a slight enhancement in the center of lesion and a rim enhancement around the center of lesion in all tumors (Figure 1). No significant difference in tumor volume was observed among the three groups before therapy.

Tumor volumes in the lipiodol group and bletilla group after TACE were significantly decreased compared with the control group on d 7 after treatment, no significant difference in tumor volumes was observed between lipiodol group and bletilla group (Figure 2, Table 1). The central slight enhancement area was larger, and the peripheral arterial phase rim enhancement in the two groups was thinner. The SS values at the rim of tumor in lipiodol group (mean, 49% per second) and bletilla group (mean, 35% per second) were significantly decreased (P<0.05), as compared with the control group (mean, 124% per second), no difference was found between lipiodol group and bletilla one (P>0.05) (Table 2).

**Table 1**

| Group        | Pre-treatment (cm³) | Post treatment (cm³) |
|--------------|---------------------|----------------------|
| Control      | 0.430±0.067         | 1.620±0.327          |
| Lipiodol     | 0.465±0.120         | 0.971±0.285*         |
| Bletilla     | 0.402±0.171         | 0.736±0.145*         |

*P<0.05 vs control.

**Table 2**

| Group        | SS(%·s⁻¹) | F     | P      |
|--------------|-----------|-------|--------|
| Control      | 124±62    |       |        |
| Lipiodol     | 49±15     | 11.08 | 0.004* |
| Bletilla     | 35±9      | 6.88  | 0.019* |

*P<0.05 vs control.

**Table 3**

| Group        | SS(%·s⁻¹) | MVA (µm²) | r | P  |
|--------------|-----------|-----------|---|----|
| Control      | 124±62    | 35±10±3±15 675 | 0.985 | <0.05 |
| Lipiodol     | 49±15    | 80.03±122 745 | 0.527 | >0.05 |
| Bletilla     | 35±9     | 15 530±17 973 | 0.743 | <0.05 |
Chinese ink casting
Investigation under microscope (×40) of the livers filled with Chinese ink in control group revealed networks of vessels or plexuses of dilated and tortuous course around and within the tumor originated from the arterioles, some sinusoid vessels were observed in the tumors, all these vessels were clearly distinct from the lobular architecture. In lipiodol group after TACE, the MVAs within the tumor (24 974±11 836 µm$^2$) were significantly smaller than those of the control group (35 510±15 675 µm$^2$) ($P<0.05$), while the MVAs around the tumor (80 031±22 745 µm$^2$) were significantly increased, as compared with the control group (35 510±15 675 µm$^2$) ($P<0.05$). Small and dense plexuses appeared around the tumor, with unknown origin. None of the vessels was seen in the tumor in bletilla group after TACE (Figure 3). The peripheral MVAs of the tumor (15 530±7 973 µm$^2$) in bletilla group were significantly smaller than those of the control group (35 510±15 675 µm$^2$) ($P<0.05$) and lipiodol group (80 031±22 745 µm$^2$) ($P<0.05$). There was a good correlation between SS and MVA at the rim of tumor in control group ($r_s$, 0.985, $P<0.0001$) and bletilla group ($r_s$, 0.743, $P<0.05$), the correlation was not significant in lipiodol group ($r_s$, 0.527, $P>0.05$) (Table 3).

**Figure 1** Images obtained before treatment in control group. A: T1WI shows hypointense lesions in left lobe of the liver. B: T2WI shows homogeneous hyperintense lesions in left lobe of the liver. C: FP T1-weighted image with gadolinium shows rim enhancement and no enhancement in the center of lesion. D: Signal intensity time curve derived from FP, SS of the curve in the border of the lesion is steeper than that of the normal liver.

**Figure 2** Images obtained after treatment in lipiodol group. Inhomogeneous hyperintense lesions with intermediate intense rim (arrow head) can be seen on T1WI (A) and T2WI (B), indicating the necrosis of the lesion and intratumor retention of lipiodol. (C) FP T1-weighted image with gadolinium shows thinner rim enhancement and no enhancement in the center of the lesion compared with control group although the lesion volume is increased. (D) SS of the curve in center of the lesion is decreased compared with those of the border.
Pathologic correlation

The specimens did not show necrosis or infarct when the lesion was less than 1 cm in control group. In lipiodol group none lesions underwent complete necrosis, coagulation necrosis was demonstrated at the center of all lesions. Viable or residual cancerous tissues as well as an intense reaction of granulation tissue were seen at the periphery of all lesions in lipiodol groups after TACE, small and dense vessels were scattered at the border of the lesions, correlating with the irregular thick rim enhancement on MR image. In bletilla group after TACE, all lesions demonstrated complete coagulation necrosis, large infarcts involving multiple adjacent lobules were seen as well, few vessels were seen at the border of the lesion, no granulation tissue was seen (Figure 4).

DISCUSSION

Bletilla inhibition of tumor micro-vessels

A previous study reported an intense reaction of granulation tissue at the periphery of all lesions in lipiodol group[16], the dense angiogenic plexus in the granulation tissue is, to our knowledge, a new finding. Granulation tissue which resulted in capillary proliferation and permeability increase was correlated to arterial phase rim enhancement on MR image. Angiogenesis was central to tumor stroma formation and offered nutrition to the remaining viable tumor cells, which limited lipiodal embolization and anti-tumor effect. As a result, local tumor recurrence would occur sooner or later[17,9,16].

TNP-470, cyanoacrylate and Plcg-mitomycin-microsphere have been used to inhibit capillary networks or to prolong
first-pass effect and to enhance the anti-tumor effect of TACE\cite{17,19}. Our experimental study indicated that *bletilla* could enhance the anti-tumor effect of TACE as well. *Bletilla*, as an anti-tumor and anti-inflammatory agent, is a Chinese medicine\cite{23,24}. Its microspheres (mixed with 5-FU, 40-200 µm in diameter) can result in dual embolization (embolization of hepatic arterial and portal venous blood supply) and inhibit the microvessels around and in the tumor completely. The reason why no microvessels developed after TACE was partly due to its anti-tumor and anti-inflammatory effect, partly through inhibition of the binding of vascular endothelial growth factor to its receptor\cite{25}. As a result, treatment with *bletilla* could necrose the tumor thoroughly. This was why *bletilla* could enhance the anti-tumor effect of TACE even the lesion was very large\cite{26}.

**VX2 microcirculation morphology**

Many materials, including Chinese ink and microfild, have been used to demonstrate the vascular morphology of tumors\cite{23,27}. Among them, microfild is commonly used because of its different colors, which could identify the origin of vessels. But microfild was too viscous to get into the sinusoid, no vessel was visible inside the tumors less than 0.25 mm in diameter\cite{28}. So we selected less viscous materials to reveal microvessel morphology of VX2. Three percent of Chinese ink gelatin solution was used to explore the vascular architecture of cheek pouch carcinoma\cite{29}. To avoid overfilling the microvessels, we added gelatin to increase the viscosity of the solution to obtain 7.5% solution. As a result, all the microvessels of normal liver including arterioles and sinusoid were filled with black Chinese ink, so did those of tumor. Moreover, the pathologic change of the other part of the same specimens stained with HE could be observed. So microvessel casting correlating with pathologic change could assess the changes of tumor microcirculation at the same time. We believe that Chinese ink gelatin solution casting may be used as the golden standard to evaluate the microvessels in perfusion study with CT and MR imaging.

**Value of FP-MRI**

Although power Doppler US has been used to assess vascularity of tumors\cite{30,31}, before contrast enhanced harmonic power Doppler US has widely been used, the major problems in the use of power Doppler are as follows. The detected velocities were too slow in the tumor, there were too many blooming artifacts associated with micro-bubble injection, the duration of enhancement was short and there were artifacts resulted from respiration\cite{32}. So the vascularity of tumors could not be evaluated in detail by power Doppler US. FP-MRI, with a high time resolution, can monitor the contrast agent first passing the target tissue by using signal intensity time curve, the steepest slope of which (SS, i.e. the maximum upward slope of the curve) correlates linearly with flow velocity and angiogenesis, and has been successfully used to quantify the myocardial perfusion reserve and to depict the tumor vascularization\cite{33,34}, but no study has assessed the tumor microcirculation after TACE by FP\cite{35,36}. Our experimental result revealed that areas with the fastest contrast medium uptake (SS) colocalized significantly with focal hot spots of MVA in control group and *bletilla* group, but no significant correlation was observed between SS and MVA in lipiodol group, suggesting that factors beyond the MVA are involved in the gadopentetate dimeglumine-related signals enhanced in microvessels. We believed that the discrepancy could reflect the difference between small dense vessels newly developed at the border of the lesion after lipiodol treatment and tumor microvessels before treatment. The new vessels were hyperpermeable\cite{37}, part of the small molecular contrast media (gadopentetate dimeglumine) might leak out of microvessels even at first pass course. On the other hand, the new vessels might be too small to have the same flow velocity like those before treatment. So we believed that when the same categoric vessels were assessed by FP, the flow velocity might be constant, the SS was correlated linearly with MVA. When different categoric vessels were evaluated by FP, the flow velocity and permeability were different, and not dependent only on MVA.

In this study, *bletilla* microspheres were administered for 7 d only, and further studies of extended duration are needed. We conclude that tumor microvessels can be markedly inhibited by using *bletilla* microspheres in combination with 5-FU, which can explain and confirm its effectiveness in clinic. The SS derived from FP has a good correlation with MVA and may be used as a noninvasive method to quantitatively evaluate tumor angiogenesis after TACE.

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Edited by Wang XL and Zhu LH  Proofread by Xu FM