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The effect of sequential transcatheter arterial chemoembolization (TACE) and portal venous embolizations (PVE) *vs.* TACE or PVE alone on rabbit VX2 liver carcinoma and on liver regeneration

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Abstract. – OBJECTIVE: This study aimed to build VX2 liver tumor model in rabbits and to investigate the sequential transcatheter arterial chemoembolization (TACE) and portal vein embolizations (PVE) *vs.* TACE or PVE alone on rabbit VX2 liver carcinoma and liver regeneration.

MATERIALS AND METHODS: VX2 liver tumor models were built in the rabbit. Rabbits carrying VX2 liver tumors were divided into four groups, including TACE+PVE, TACE, PVE and Sham groups respectively. Hematoxylin and eosin (HE) staining was performed to visualize the structures of tumor tissues. The volume data of caudal liver on day 3 and day 7 was measured by CT. Western blot analysis of active caspase-3 was performed to examine cell apoptosis. Immunohistochemical (IHC) staining of Ki-67 was performed to visualize hepatocyte regeneration. Serum IL-6, TNF-alpha, HGF and TGF-beta1 on 6th h, 24th h, day 3 and day 7 were measured by ELISA assay.

RESULTS: The TACE+PVE group had the strongest suppressive effect on tumor growth and induced the highest level of tumor cell apoptosis. TACE+PVE can induce evident liver regeneration, which is reflected by the largest caudal liver volume increase and the highest ratio of Ki-67 positive cells. ELISA assay showed that during the first 7 days since day 0, TACE+PVE group had the highest level of HGF, IL-6 and TNF-alpha.

CONCLUSIONS: TACE+PVE can significantly inhibit VX2 tumor growth, induce tumor cell apoptosis and liver regeneration, the effects of which are stronger than TACE or PVE alone. In the first 7 days since day 0, TACE+PVE group had the highest level of IL-6, TNF-alpha and HGF. This might be the reason why TACE+PVE induced the strongest liver regeneration.

Key Words
TACE, PVE, Metastatic hepatic carcinoma, Liver regeneration.

Introduction

The liver is one of the most common sites for malignant tumor metastasis[1-3]. In China, the occurrence rate of metastatic hepatic carcinoma (MHC) is similar to that of primary hepatic carcinoma (PHC)[4]. The prognosis of the patients with MHC is poor[5,6]. Currently, surgical resection is still the most effective strategy to improve the survival of the patients[3]. However, a large proportion of the patients suffered unresectable liver metastases due to late diagnosis and small future liver remnant (FLR)[7]. Therefore, how to increase FLR and to make the unresectable MHC resectable is one of the focuses of MHC therapy.

Sequential transcatheter arterial chemoembolization (TACE) and portal venous embolizations (PVE) in patients with hepatocellular carcinoma could significantly inhibit tumor growth and promote the increase in the percentage of the future liver remnant (FLR) volume, which were beneficial for following tumor resection[8]. However, only limited reports compared the effect of TACE+PVE *vs.* PVE alone on liver regeneration. Two previous studies[9,10] found that TACE+PVE had a better effect on increasing FLR volume than PVE alone. However, the underlying mechanisms are still not quite clear.

After a liver injury such as resection, the initiation and termination of liver regeneration were regulated by multiple genes and signaling pathways[11,12]. A series of cytokines and growth factors are involved in the regulation, such as TGF-α, aEGF, HGF, TNF-α and IL-6[13,14]. These factors cooperate to activate hepatocellular cells in G0 phase and initiate mitosis of the cells. After one or two cycles of replication, some other

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factors are involved in the termination of liver regeneration, such as TGF-β1[14]. However, how TACE+PVE affects the expression of these important factors has not been reported yet.

This study aimed to build VX2 liver tumor model in rabbits and to investigate the sequential TACE and PVE *vs.* TACE or PVE alone on rabbit VX2 liver carcinoma and liver regeneration.

Materials and Methods

Establishment of the VX2 Tumor Model

The protocol was approved by the Ethics Committee of Nanfang Hospital, Southern Medical University. Adult New Zealand White rabbits with weight around 2-2.5kg were purchased from the Experimental Animal Center of Sichuan University. Tumor implantation was performed according to the method introduced in one previous study[15]. In brief, VX2 tumor cell suspension was injected into thigh muscles of a carrier rabbit. Three weeks later, the solid tumor was harvested from the donor rabbit. Then, three tumor fragments around 0.5 mm^3 were injected superficially in the subcapsular area of the left medial liver lobe using a 16-gauge angiocatheter. 18 days after implantation, the tumor size was measured by CT scan and the rabbits carrying tumors around 15-30 mm in diameter were used for following experiments. The images of gross specimens of the VX2 liver tumor were captured after euthanization of the rabbits.

Experimental Design

Forty rabbits with VX2 tumors were divided into four experimental groups, including TACE+PVE, TACE, PVE and Sham groups respectively (n=10 in each group). PVE was performed with polyvinyl alcohol (PVA) particles 90-180 μm in combination with 300-500 μm particles and three or four platinum coils. TACE was performed by infusion of a suspension of 1 mg/kg 10-hydroxycamptothecin and 0.4 ml iodized oil into the left hepatic artery and subsequent embolization was performed with PVA particles 150-250 μm in diameter. In PVE and TACE groups, PVE or TACE was performed on day 0. In TACE+PVE groups, TACE was performed on day 7, PVE was performed on day 0. The sham group received sterile physiological saline as placebo embolization material. On day 3, 3 rabbits in each group were sacrificed to examine cell proliferation in the caudal liver by using immunohistochemical (IHC) staining of Ki-67. On day 7, the rest rabbits were sacrificed after CT scan of caudal liver volume.

Hematoxylin and Eosin (HE) Staining of Liver Specimen

HE staining was performed to visualize the structures of the tumor and normal tissues. In brief, the VX2 tumor and normal liver tissues after resection were fixed in 15% formalin. Then the samples were embedded in paraffin wax and were further sectioned into 4 μm slices. Then tissue silences were stained with hematoxylin and eosin (HE).

IHC Staining of Ki-67

Tissues for IHC staining were prepared following the methods introduced in one previous study[16]. The tissue sections were incubated with primary antibody against Ki-67 (ab15580, 1:500, Abcam, Cambridge, MA, USA) at 4°C in a humidified chamber overnight. Then the slides were incubated with biotinylated anti-rabbit secondary antibody for 30 minutes and then washed with PBS for 5 minutes. After that, the slides were incubated with streptavidin-horseradish peroxidase (HRP) solution for another 30 minutes. The HRP activity was detected using DAB as substrate. Counterstaining was performed using Harris hematoxylin. Negative controls were performed by incubation in PBS without the presence of primary antibody. Then, the slides were examined under a transmission light microscope.

Western Blot Analysis of Active Caspase-3

On day 7, the tumor tissues were obtained from the rabbit liver and the protein used for Western blot analysis was prepared by using a lysis buffer (Beyotime, Shanghai, China). 30 μg of protein were loaded in each lane and separated on 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. The proteins after separation were transferred to nitrocellulose membranes. The membranes were then blocked, washed and then incubated with anti-active caspase-3 (1:1000, ab2302, Abcam) or anti-GAPDH (1:1000, Abcam, ab181602) overnight at 4°C. After washing, the membranes were incubated with HRP conjugated secondary antibodies for another 1 hour at room temperature. The protein band signals were visualized using the ECL Western blotting substrate (Promega, Madison, WI, USA) and the relative gray-scale value was quantified using ImageJ software.

CT-scan

The volume data of caudal liver before the intervention, on day 3 and on day 7 after the intervention was obtained by using a multiphasic CT scan with a 64-slice CT scanner (Brilliance 64; Philips, Eindhoven, The Netherlands) according to the methods described in one previous study[17]. The total liver and the caudal liver lobe were manually delineated, and the total liver volume (TLV) and caudal liver volume (CLV) were calculated. The percentage of CLV and the degree of hypertrophy were calculated by the following formula:

$\%CLV_{pre\text{-}intervention} = (CLV_{pre\text{-}intervention}/TLV_{pre\text{-}intervention})\times 100\%$;

$\%CLV_{post\text{-}intervention} = (CLV_{post\text{-}intervention}/TLV_{pre\text{-}intervention})\times 100\%$;

Degree of hypertrophy = $\%CLV_{post\text{-}intervention}$-$\%CLV_{pre\text{-}intervention}$.

ELISA Assay

Serum level of IL-6, HGF, TNF-α and TGF-β1 on the 6th h, 24th h, day 3 and day 7 were measured using ELISA assay with the Kits purchased from Boster (Wuhan, China) according to manufacturer's instruction.

Statistical Analysis

Data were reported as means ± standard deviation (SD). Comparison between groups was performed using unpaired Student's *t*-test in Graphpad Prism 5.0. *p*-value of <0.05 was considered as statistically significant.

Results

Development of Rabbit VX2 Tumor Liver Carcinoma Model

18 days after VX2 tumor tissue implantation, the images of a gross specimen of VX2 liver tumor were captured after euthanization of the rabbits with confirmed tumor formation according to CT scan (Figure 1A). Compared with the normal liver tissue, the gross specimen of VX2 tumor was hard and with a round and clear edge (Figure 1A and B). HE staining showed that the tumor tissues have abundant cellular components, with little intercellular substance (Figure 1C). In addition, compared with the normal liver cells, the cancerous cells are with an irregular size and shape, had larger and prominent nucleus and less cytoplasm which is pale or intensely colored (Figure 1C and D).

The Therapeutic Effect of TACE+PVE vs. TACE or PVE Alone on the VX2 Liver Carcinoma

To assess the tumor suppressive effect of TACE+PVE, TACE or PVE on the VX2 liver carcinoma, four intervention groups were designed (Figure 2A). The rabbits in TACE+PVE group firstly had TACE and 7 days later had PVE administration. The rabbits in TACE and PVE groups firstly had a sham operation and then received TACE or PVE operation 7 days later (Figure 2A). On day 7, the rabbits were sacrificed. By examining the tumor volume of the tumors, we found that TACE+PVE showed the best inhibiting effect on VX2 tumor growth. The tumor diameter in this group was 2.03±0.14 cm, which was significantly smaller than that in TACE, PVE and sham groups (2.49±0.25 cm, 2.68±0.31 cm and 3.32±0.42 cm, respectively) (Figure 2B). HE staining showed that structural characteristics of tumor tissues in TACE group were similar to that of TACE+PVE groups. However, TACE group had more centralized tumor necrosis area. Fibrosis was quite evident in PVE groups, with necrosis tissues around (Figure 2C). By performing Western blot of active caspase-3, we confirmed that TACE+PVE group had the highest rate of apoptosis. The expression of active caspase-3 in

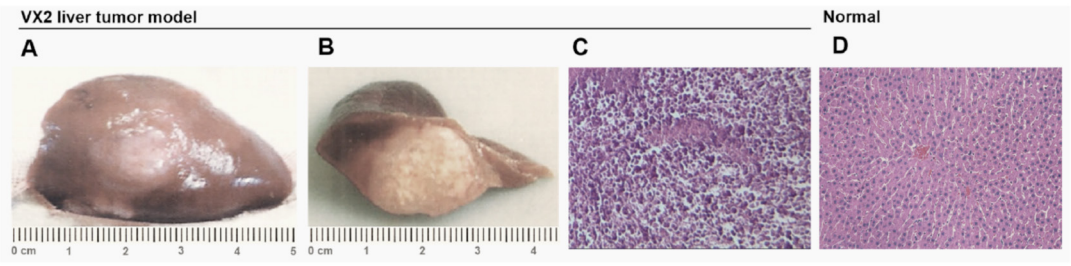

Figure 1. Development of rabbit VX2 tumor liver carcinoma model. **A** and **B**. The Gross specimen (**A**) and cross section (**B**) of rabbit VX2 liver tumor. **C** and **D**. HE staining of section of VX2 liver tumor (**C**) and normal rabbit liver section (**D**).

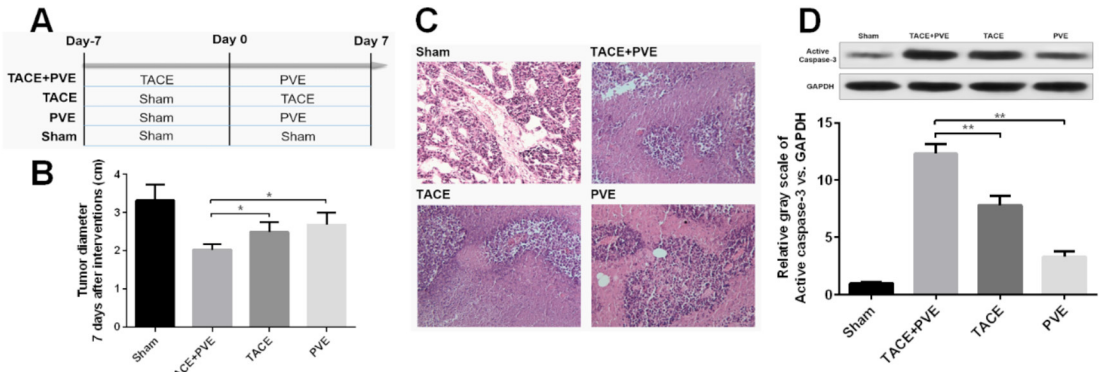

Figure 2. The therapeutic effect of TACE+PVE vs. TACE or PVE alone on the VX2 liver carcinoma. **A**, The experimental design of four intervention groups. In PVE and TACE groups, Sham operation was performed from day -7 to day 0. PVE or TACE was performed on day 0. In TACE+PVE groups, TACE was performed on day -7, PVE was performed on day 0. The sham group received sterile physiological saline as placebo embolization material. **B**, The average tumor diameter in the four groups on day 7. **C**, HE staining of tumor sections in the four groups on day 7. **D**, Western blot analysis (up: band images; down: quantitation data) of active caspase-3 expression in the tumor tissues in the four groups on day 7. $^{**}p<0.01$

the TACE+PVE group was about 1.5 times higher than in the TACE group and 4 times higher than in PVE groups (Figure 2D).

The effect of TACE+PVE vs. TACE or PVE alone on liver regeneration

Then, we examined how these interventions affected liver hypertrophy. After the interventions, the caudal liver in TACE+PVE, TACE and PVE groups all had hypertrophy to some extent (Table I). The TACE+PVE groups had the highest level of liver hypertrophy, with 7% and 14.3% increase on day 3 and day 7 respectively (Table I). The hypertrophy degree in TACE group were 2% and 5.1% and in PVE group were 5% and 12.4% (Table I). By performing IHC staining of Ki-67, we observed that the increase of Ki-67 positive cells was quite significant in both TACE+PVE and PVE groups on day 3 and on day 7 (Figure 3A and B). TACE+PVE group had a significantly higher proportion of Ki-67 positive cells than PVE group (Day 3: TACE+PVE *vs.* PVE: 64%±7% *vs.* 42%±4%; day 7: TACE+PVE *vs.* PVE: 53%±6% *vs.* 29%±6%) (Figure 3A and B).

Table I. CT volumetry data.

Group	Index	Time points		
		Before Interventions	Day 3	Day 7
Sham	CLV, cm3	18.2 ± 1.2	17.6 ± 0.9	17.2± 1.1
	CLV%	25.1 ± 1.3	23.6 ± 1.5	23.4 ± 1.7
	Hypertrophy level	-	-1%	-1.0%
TACE+PVE	CLV, cm3	17.1 ± 1.7	24.3 ± 2.9	31.4 ± 3.6
	CLV%	24.4 ± 2.3	29.8 ± 2.2	40.3 ± 2.2
	Hypertrophy level	-	7%	14.3%
TACE	CLV, cm3	17.6 ± 2.8	19.4 ± 2.4	22.7 ± 2.1
	CLV%	24.9 ± 2.5	26.3 ± 2.7	29.6 ± 2.6
	Hypertrophy level	-	2%	5.1%
PVE	CLV, cm3	17.9 ± 1.8	22.9 ± 1.3	30.3 ± 2.6
	CLV%	24.8 ± 2.1	29.8 ± 2.7	39.9 ± 3.8
	Hypertrophy level	-	5%	12.4%

Abbreviation: CLV, caudal liver volume; TACE: transcatheter arterial chemoembolization; PVE, portal vein embolization; N/A, not applicable.

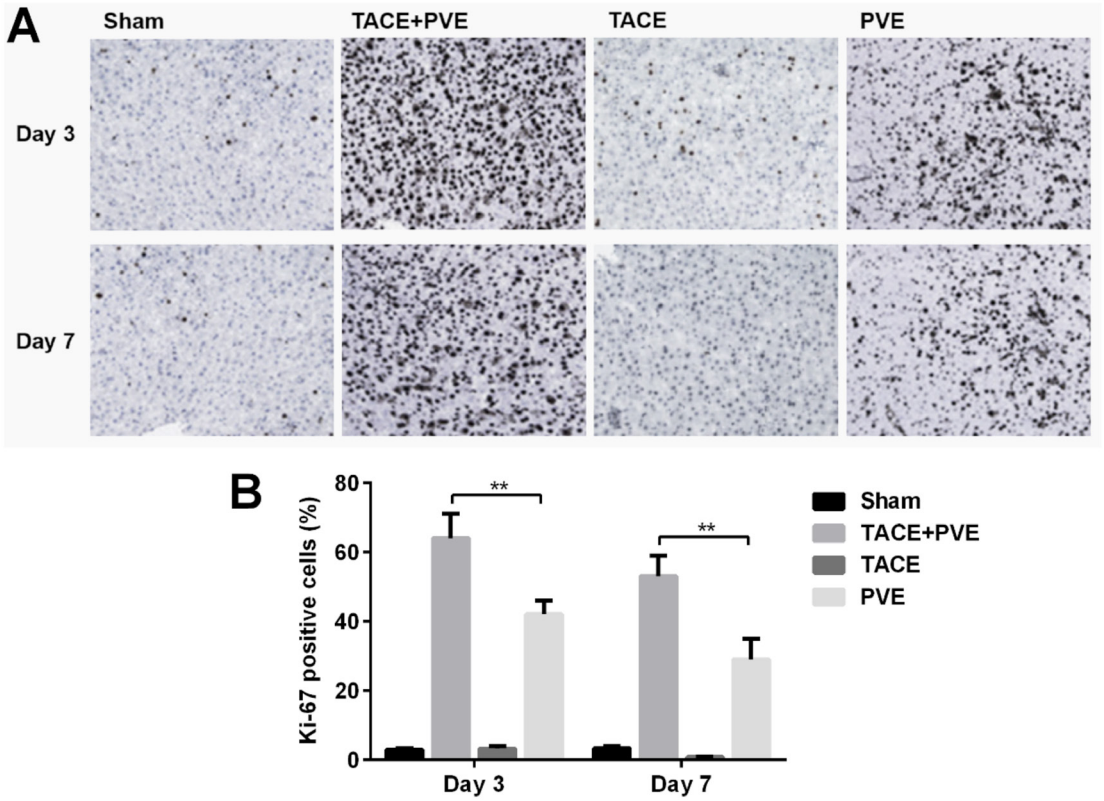

Figure 3. The effect of TACE+PVE vs. TACE or PVE alone on liver hypertrophy. **A**, Representative images of IHC staining of Ki-67 in caudal liver section of each groups on day 3 and on day 7. **B**, Quantitation of the ratio of Ki-67 positive cells showed in figure A.

The effect of TACE+PVE vs. TACE or PVE alone on the expression of liver regeneration related factors

To further explore why TACE+PVE is associated with higher level of liver hypertrophy, we measured serum IL-6, TNF-α, HGF and TGF-β1 on the 6th h, 24th h, day 3 and day 7 by ELISA assay. The results showed that both TACE+PVE and PVE groups had significantly increased HGF, IL-6 and TNF-α after the intervention.

At the 6th h, serum IL-6 in the TACE+PVE group was 685±28 pg/ml, in PVE group was 615±22 pg/ml (Figure 4A). Then, they substantially increased to 1538±113 pg/ml and 1338±101 pg/ml, respectively at the 24th h (p<0.01). TACE group only had minor increase. Serum IL-6 in TACE+PVE and PVE groups showed a significant decrease from the 24th h to day 7 (Figure 4A). Both TACE+PVE and PVE groups had TNF-α peak at the 24th h (6th h, TACE+PVE group: 685±28 pg/ml; PVE group: 615±22 pg/ml; 24th h: TACE+PVE group: 1538±113 pg/ml; PVE group: 1338±101 pg/ml) (Figure 4B).

TACE and PVE group had HGF peak at the 24th h (Figure 4C). HGF level in the TACE+PVE group increased from 392 pg/ml from the 6th h to 633 pg/ml at the 24th h. PVE group showed a similar trend. But the peak HGF level at the 24th h was about 583±24 pg/ml, which was significantly lower than that in the TACE+PVE group (p<0.01). On day 7, both TACE+PVE and PVE groups showed declined HGF level, which were 405±19 pg/ml and 378±27 pg/ml, respectively (Figure 4C).

TGF-β1 increased gradually after the intervention in TACE+PVE and PVE groups and reached the peak on day 7. However, the difference of TGF-β1 between the two groups was not significant. The increase was not evident in TACE and Sham group (Figure 4D).

Discussion

PVE can redirect portal vein flow toward specific hepatic segments and is a method currently used for FLR increase before surgical

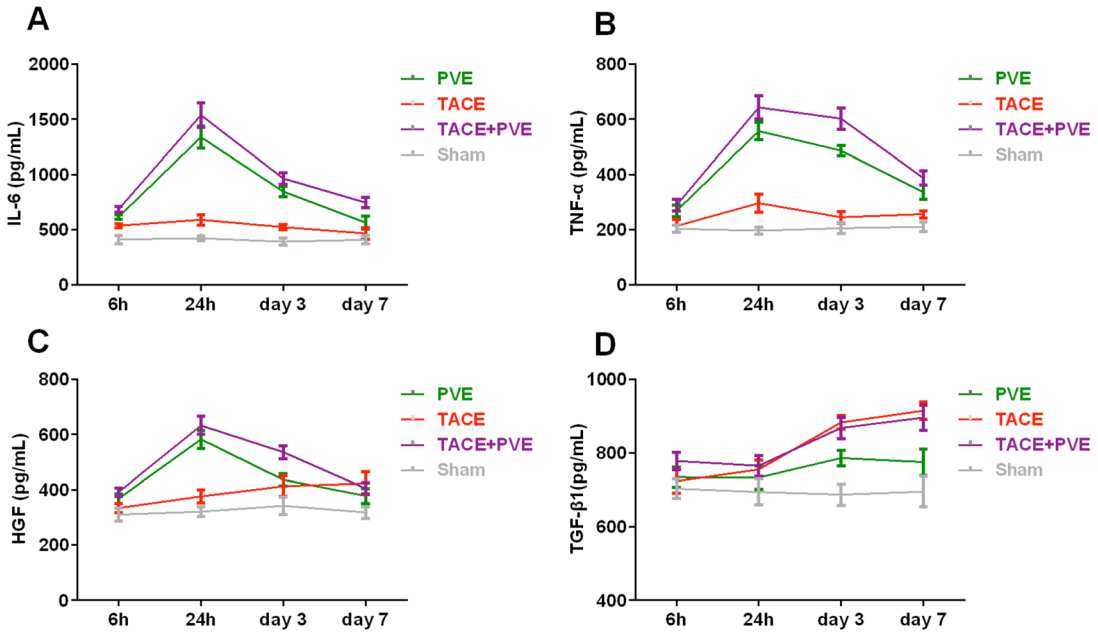

Figure 4 . The effect of TACE+PVE vs. TACE or PVE alone on the expression of liver regeneration related factors. **A-D**. The serum level of IL-6 (**A**), TNF-α (**B**), HGF (**C**) and TGF-β1 (**D**) in the four groups on the 6th h, 24th h, day 3 and day 7.

resection[18,19]. However, multiple studies found that PVE might be associated with higher disease recurrence and tumor growth acceleration in both embolized and non-embolized liver lobes[20-22]. The possible accelerated tumor growth and progression after PVE are one of the major obstacles for successful therapy. Therefore, how to limit tumor progression post-PVE is one of the current research focuses.

Several studies[23-25] evaluated the safety and efficacy of sequential TACE and PVE before major hepatectomy for patients with hepatocellular carcinoma (HCC). The results showed that the FLR after sequential TACE and PVE increased from 32.3-71.4% (mean 55.4%). In addition, no patients had intro- or extrahepatic metastasis[26]. Another work in Korea compared the prognostic impact of preoperative sequential TACE and PVE or PVE alone in patients received right liver resection for solitary HCC. The results showed that the 1-, 3-, 5-, and 10-year overall survival rates in the TACE+PVE group were 96.3%, 83.4%, 83.4% and 47.6%, respectively and in PVE alone group were 84.6%, 76.9%, 57.7% and 19.2%, respectively[27]. One recent retrospective research based on 116 Chinese patients with primary HCC showed that the 1-, 3- and 5-year overall survival rates for the TACE and TACE + PVE groups were 39/64, 16/64, 0/64 and 42/52, 19/52, 6/52 respectively (p = 0.015, 0.046 and 0.002, respectively)[28]. These studies suggest that TACE+PVE might be an effective strategy to improve patients' survival. However, the effect of TACE+PVE on MHC is still not quite clear. In this study, we firstly assessed the therapeutic effect of TACE+PVE, TACE or PVE alone on rabbit VX2 liver carcinoma. The results showed that TACE+PVE had the strongest suppressive effect on tumor growth and induced the highest level of tumor cell apoptosis.

In addition, in this work, we also observed that TACE+PVE can induce evident liver regeneration, the effect of which was stronger than PVE alone. This triggered our interest to explore further the underlying possible mechanisms. Liver regeneration can be divided into three stages, including priming, proliferation and growth termination[29]. In the priming phase, IL-6 and TNF-α are the two major signal modulators and their signaling duration is very tightly controlled[13]. The hepatocytes stimulated by these cytokines then become responsive to growth factors and then enter the second proliferation phase. Multiple growth factors are involved in this stage such as HGF, EGF receptor ligands such as EGF and TGF-α[29]. Among the growth factors, HGF is the strongest mitogen, which induces cell proliferation[30]. The metabolic demand increase in the FLR may trigger the signaling related to termination onset[29]. The proliferation inhibiting factors such as TGF-β1, 2, and 3, activins, and inhibins, are all

involved in the termination phase of liver regeneration[31-33]. Therefore, in this study, we measured the change of serum IL-6, TNF-α, HGF and TGF-β1, the four key players in the three phases respectively on the 6th h, 24th h, day 3 and day 7 by ELISA assay. The results showed that during the first 7 days since day 0, TACE+PVE group had the highest level of HGF, IL-6 and TNF-α. This might be the reason why TACE+PVE induced the strongest liver regeneration.

Conclusions

TACE+PVE can significantly inhibit VX2 tumor growth, induce tumor cell apoptosis and liver regeneration, the effects of which are stronger than TACE or PVE alone. In the first 7 days since day 0, TACE+PVE group had the highest level of IL-6, TNF-α and HGF. This might be the reason why TACE+PVE induced the strongest liver regeneration.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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