

# Cell apoptosis and regeneration of hepatocellular carcinoma after transarterial chemoembolization

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## Abstract

**AIM:** To evaluate whether cell apoptosis and regeneration were existed in normal liver cells adjacent to carcinoma after transarterial chemoembolization (TACE).

**METHODS:** Fifty rabbits with hepatic carcinoma were divided into 5 groups at random: group A (control group), groups B and C (TACE treatment groups), groups D and E (partial hepatectomy groups). There were 10 rabbits in each group. Rabbits in groups B-E were treated by transarterial chemoembolization (TACE) and partial hepatectomy (PH) respectively. The changes of S-phase cell fraction (SPF), proliferation index (PI) and cell apoptosis in the normal liver tissue were determined with flow cytometry (FCM) after operations on the first and third days. We determined the mitosis index (MI) with histo-pathological method and the apoptosis index (AI) with TUNEL method at the same time.

**RESULTS:** Twenty-four hours after operations, compared with control group, the rabbits in TACE group had much higher index of SPF, PI and MI (MI:  $t=4.89$ ,  $P<0.001$ ; SPF:  $t=5.27$ ,  $P<0.001$ ; PI:  $t=4.87$ ,  $P<0.001$ ). Moreover, the proliferation of liver cells in TACE group was much weaker than that of the cells treated by partial hepatectomy, and the differences were significant (MI:  $t=7.02$ ,  $P<0.001$ ; SPF:  $t=4.06$ ,  $P<0.001$ ; PI:  $t=2.70$ ,  $P<0.05$ ). Seventy-two h after operations, FCM showed a small sub-G1 peak in TACE group and PH group, compared with the control group, but there was no difference between them ( $t=0.41$ ,  $P>0.05$ ). TACE showed that AI in the treated rabbits was higher than that in control group ( $t=3.07$ ,  $P<0.05$ ), and there were no differences between TACE group and PH group, either ( $t=0.93$ ,  $P>0.05$ ).

**CONCLUSION:** Cell apoptosis and regeneration exist in rabbit liver tissues after TACE in some degree, which may be associated with the selective embolization of iodised oil, chemotherapeutic drug and free radical damage.

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## INTRODUCTION

The efficacy of transarterial chemoembolization (TACE) is greatly determined by the liver function reserve of liver cancer patients. More than 40% patients died of liver failure after TACE, in which most of them were accompanied by poor liver function and damage due to chemotherapy. Therefore, it is important to study the effects of TACE on non-neoplastic liver tissue to decrease the death rate. We have established the animal model of rabbit VX2 hepatic carcinoma. All the rabbits were treated by TACE to detect the effect on cell cycle of non-neoplastic liver tissues after operations<sup>[1]</sup>.

## MATERIALS AND METHODS

### *Establishment of the animal model*

Fifty Japanese alpine hares, weighing 2.5-3.3 kg, provided by the Experiment Animal Center of Tongji Medical College were used in this experiment. The rabbits were implanted hepatocellular carcinoma cell VX2, and observed for 3 wk before experiment<sup>[2]</sup>.

### *Treatment and radiological examination*<sup>[3,4]</sup>

Fifty rabbits with VX2 hepatic carcinoma were randomly divided into 5 groups: Group A (control group), groups B and C (TACE treatment groups), groups D and E (partial hepatectomy groups), 10 rabbits in each group. Three weeks later, at laparotomy of the rabbits, the feeding artery of liver carcinoma was punctured and arteriography was made<sup>[5]</sup>. A 1 mL of physiological saline was administered to group A. Groups B and C were treated with 0.6-0.8 mL of a mixture of iodine oil (10 mL) and mitomycin (10 mg)<sup>[6,7]</sup>. Groups D and E were treated by hepatectomy. Groups B and C were performed CT and MRI after TACE<sup>[8,9]</sup>.

### *Flow cytometry analysis*

Rabbits in groups A, B and C were killed on the first day after the procedure, and those in groups D and E were killed on the third day after the procedure. As soon as the rabbits died, a piece of non-neoplastic liver tissue was cut from each rabbit and digested with enzymes. The cells were fixed by 800 mL/L ice ethanol, hatched by 40  $\mu$ L PC on ice for 30 min, and washed in 1 g/L PBS. At last, they were stained by at 100  $\mu$ L PI 10 mg/L and 50  $\mu$ L 1 g/L RNA enzyme for 20 min, then flow cytometry was performed. The cells were detected by FAC Sort FCM machine (Becton Dickson Co, USA). The fluorescence of cells was analyzed with Cellquest software and the cell cycle was studied. The other liver tissues were photographed, fixed with 40 g/L formaldehyde, and stained by HE<sup>[10]</sup>.

Groups B and C were performed CT after TACE, and accumulation of iodized oil was observed. General pathological changes of carcinoma were detected in all the rabbits. Under the microscope, we calculated the number of liver cells in the mitosis

phase per 1 000 liver cells, which was called mitosis index (MI).

### FCM analysis

S phase cell fraction (SPF) was calculated according to the equation:  $SPF = S / (S + G1 / G0 + G2 / M) \times 100\%$ . Proliferation index (PI) was calculated according to the equation:  $PI = (S + G2 / M) / (S + G1 / G0 + G2 / M) \times 100\%$ . Apoptosis index (AI) was analyzed by TUNEL method<sup>[11]</sup>, the number of apoptotic cells was calculated under microscope<sup>[12]</sup>.

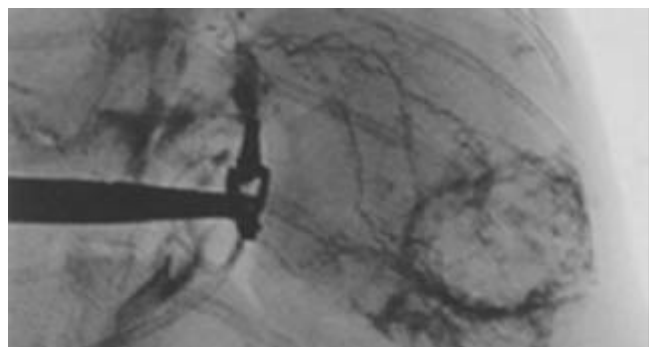
### Statistic analysis

All the data were analyzed by *t* test.

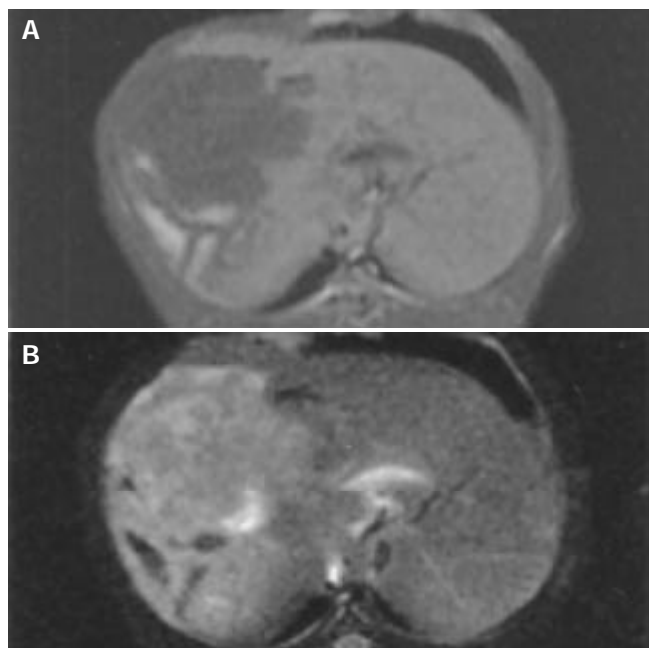
## RESULTS

### General pathological and radiological findings

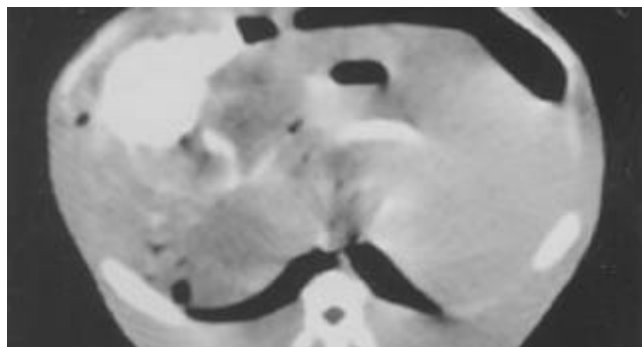
Three wk after tumor implantation, the size of VX2 liver carcinoma was  $3.5 \pm 1.6$  cm on average, and appeared as white nodules near the liver surface. There were no definite margins between carcinomas and normal liver tissues. On DSA, there was increased and irregular angiogenesis of the tumor vessels, with tumor stained mainly at the periphery (Figure 1). On MR, the signal of cancer was slightly lower than that of normal liver tissue on T1WI and slightly higher on T2WI (Figure 2). The iodized oil deposited well after TACE on CT<sup>[13]</sup> (Figure 3).



**Figure 1** Hepatic arteriography of rabbits. There was increased angiogenesis with thickening and irregularity of the vessels. There was also tumor staining mainly in the periphery of the tumor.



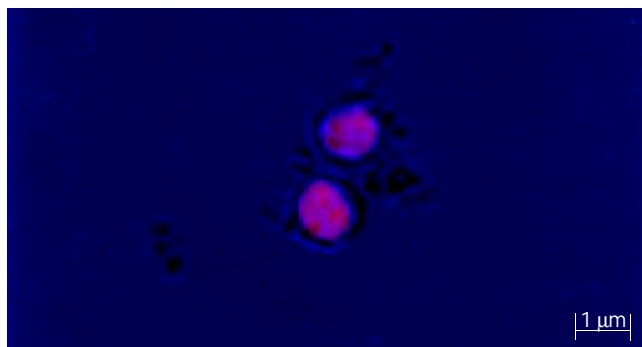
**Figure 2** MRI of rabbit liver. The tumor appeared hypointense on T1WI (A) and hyperintense on T2WI (B).



**Figure 3** CT scan shows massive retention of iodized oil in tumor after TACE.

### Cytological changes

Red fluorescent light could be seen in the cells under laser confocal microscope (Figure 4). Under light microscope (Olympus), we could find cell mitosis clearly, and mitosis index (MI) could be calculated. Flow cytometry showed that S phase and G2/M phase cells in TACE groups and partial hepatectomy groups increased much more on the first day after operations than that in the control group (Figure 5). On the third day after operations, the amount of S-phase and G2/M phase cells in TACE group and partial hepatectomy group was about the same as control group (Figure 6). In the cell apoptosis index (AI) determined by TUNEL method, no significant difference existed between TACE group (Figure 7) and partial hepatectomy group.



**Figure 4** Cells giving out red fluorescent light under laser confocal microscope.

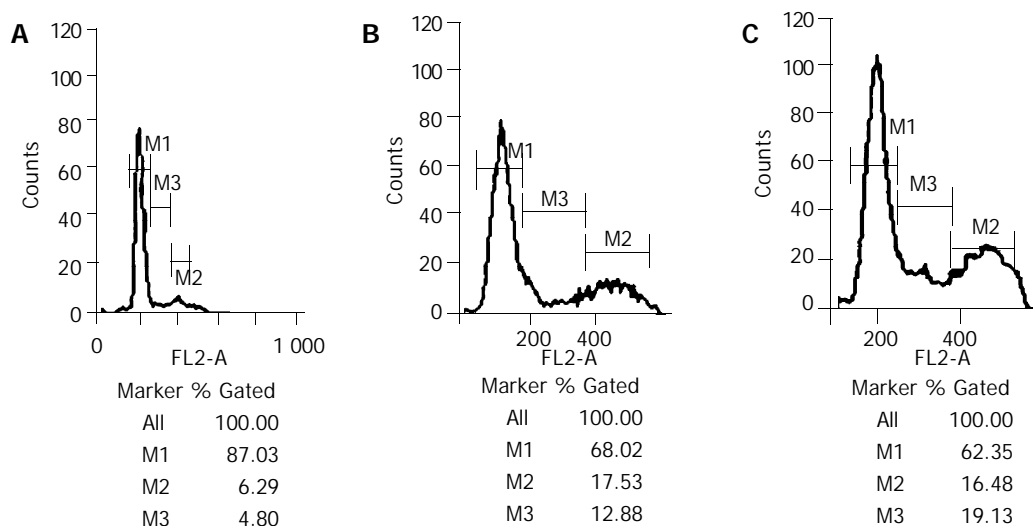
The *t* test values of MI, SPF and PI of TACE groups and control group are listed in Table 1. The *t* test values of MI, SPF and PI of TACE groups and partial hepatectomy groups are listed in Table 2.

**Table 1** *t* test value of MI, SPF and PI of TACE groups and control group on the first day after operations

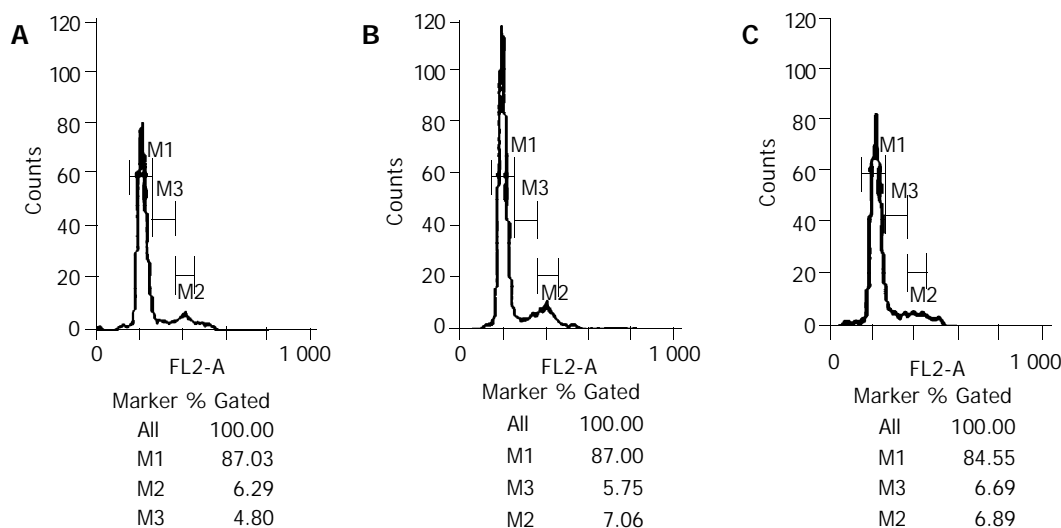
Index	TACE group	Control group	<i>t</i> value	<i>P</i> value
MI (%)	1.42±0.55	0.51±0.21	4.89	<i>P</i> <0.001
SPF (%)	12.46±4.18	4.90±1.76	5.27	<i>P</i> <0.001
PI (%)	23.38±8.31	9.93±2.68	4.87	<i>P</i> <0.001

**Table 2** *t* test value of MI, SPF and PI of TACE groups and partial hepatectomy group on the first day after operations

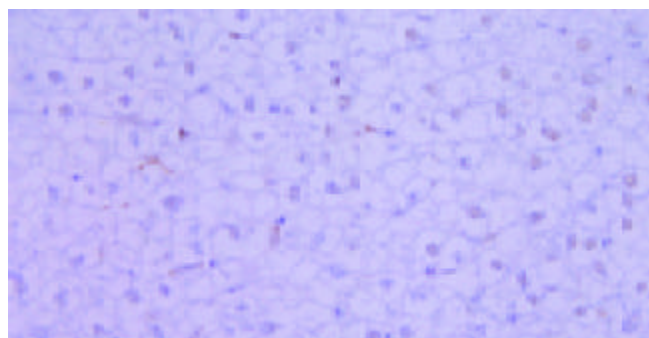
Index	TACE group	Partial hepatectomy group	<i>t</i> value	<i>P</i> value
MI (%)	1.42±0.55	3.13±0.54	7.02	<i>P</i> <0.001
SPF (%)	12.46±4.18	21.76±5.92	4.06	<i>P</i> <0.001
PI (%)	23.38±8.31	32.51±6.75	2.70	<i>P</i> <0.05



**Figure 5** A: Shows the S phase and G2/M phase cells were small in proportion on the first day after operations (4.80%, 6.29%) in control group. B: Shows the S phase and G2/M phase cells grew much more on the first day after operations (17.53%, 12.88%) in TACE group. C: Shows the S phase and G2/M phase cells grew much more on the first day after operations (16.48%, 19.13%) in PH group. \*M1 is G0/G1 phase cell ratio/M2 is G2/M phase cell ratio, M3 is S phase cell ratio.



**Figure 6** A: Shows the S phase and G2/M phase cells were small in proportion on the third day after operations (4.80%, 6.29%) in control group. B and C: Show the S phase and G2/M phase cells had slight changes on the third day after operations in TACE group and PH group.



**Figure 7** Analysis of apoptosis cells by TUNEL method in TACE group. The nuclei of apoptosis cells appeared brown while those of normal cells appeared blue. It was found that part of the cells were apoptotic cells.

The *t* test values of MI, SPF of TACE groups and control group are listed in Table 3. There was no significant difference between the two groups ( $P > 0.05$ ). However, the *P* value of AI and sub-G1 was less than 0.05. The *t* test values of MI, SPF, AI and PI of TACE groups and partial hepatectomy groups are listed in Table 4.

**Table 3** *t* test value of MI, SPF, AI, Sub-G1 and PI of TACE groups and control group on the third day after operations

Index	TACE group	Control group	<i>t</i> value	<i>P</i> value
MI (%)	0.62±0.33	0.51±0.21	0.88	$P > 0.05$
SPF (%)	5.70±1.93	4.90±1.76	0.95	$P > 0.05$
PI (%)	11.69±1.74	9.93±2.68	1.68	$P > 0.05$
Sub-G1 (%)	2.31±1.57	1.18±0.45	2.18	$P < 0.05$
AI (%)	20.33±2.36	13.68±1.97	3.07	$P < 0.05$

**Table 4** *t* test value of MI, SPF, sub-G1 and PI of TACE groups and partial hepatectomy group on the third day after operations

Index	TACE group	Partial hepatectomy group	<i>t</i> value	<i>P</i> value
MI(%)	0.62±0.33	0.81±0.30	1.28	$P > 0.05$
SPF(%)	5.70±1.93	6.62±1.56	1.34	$P > 0.05$
PI(%)	11.69±1.74	11.85±2.00	0.17	$P > 0.05$
Sub-G1 (%)	2.31±1.57	2.05±1.07	0.41	$P > 0.05$
AI(%)	20.33±2.36	22.69±3.79	0.93	$P > 0.05$

## DISCUSSION

Transarterial chemoembolization (TACE) has been one of the most important and commonly used methods for treatment of hepatocarcinoma<sup>[14]</sup>. Because most patients died of liver failure after TACE<sup>[15,16]</sup>, it is important to study the condition of postoperative normal liver tissues<sup>[17]</sup>. The most precise method to evaluate hepatocyte reproduction and apoptosis has been the study of cell cycle<sup>[18]</sup>. In our research, normal hepatocytes were isolated, fixed and stained, flow cytometry (FCM) was performed and DNA was analyzed. Results showed SPF, PI and MI value of TACE group was much higher than that of control group ( $P < 0.001$ , Table 1). We could conclude that there existed liver regeneration after TACE. From Table 2 at the same time, we could find that hepatocyte regeneration in PH group was stronger than that in TACE group on the first day ( $P < 0.05$ ). On the third day after TACE, the difference between TACE group and control group was not significant in all indexes. Furthermore, the difference between TACE group and PH group was not significant either on the third day. From these data, we could conclude that the regeneration process stopped, most of the cells entered the dormancy phase ( $G_0$  phase). However, the value of hypodiploid peak (sub-G1) increased on the third day after TACE ( $t = 2.18$ ,  $P < 0.05$ ). The difference of sub-G1 between TACE group and PH group was obvious ( $P > 0.05$ , Tables 3, 4). The apoptosis index (AI) became greater on the third day after TACE.

Cell apoptosis (programmed cell death) means the maintenance of stability of internal environment, i.e. the process of autonomic programmed cell death has been controlled by gene<sup>[19]</sup>. After partial hepatectomy, the hepatocytes would regenerate soon. At the peak of regeneration, cell apoptosis begins. Accompanying cell reproduction, cell apoptosis would eliminate the overgrown cells, and rebuilding of the tissue constitution is achieved. Some authors<sup>[20]</sup> pointed out that the process was mitogen  $\rightarrow$  cell reproduction  $\rightarrow$  regeneration  $\rightarrow$  cell apoptosis. It was different from the necrosis caused by liver cytotoxic material, which was in the order of cytotoxic material  $\rightarrow$  necrosis  $\rightarrow$  compensatory hyperplasia  $\rightarrow$  cell regeneration. Liver tissue regeneration after PH has been studied comprehensively. When the liver is resected less than 70%, the regeneration is proportional to the volume of the resected portion. Mitosis reaches the peak in 24 h, and completes in 72 h. Liver tissue regeneration exists also after TACE, this is due to the effect of super-selective embolization with iodized oil. The liver regeneration in TACE group was weaker than that in PH group, the reason was that the extent of "medical hepatectomy" was much smaller<sup>[21]</sup>. However, from Tables 3 and 4, we could find the apoptosis levels between TACE and hepatectomy groups. The apoptosis level following liver regeneration was directly proportional to the regeneration level. It is easy to draw the conclusion that there must exist a new mechanism, which promotes cell apoptosis after TACE. The mechanism might be that mitomycin depressed DNA synthesis and promoted cell apoptosis<sup>[22]</sup>. Some authors<sup>[23]</sup> pointed out that mitomycin (5 mg/mL) could cause cell apoptosis after 24 h. Almost all chemotherapeutic drugs could induce cell apoptosis by different ways<sup>[24]</sup>. For example, vincristine (VCR) and colchicine could induce cell apoptosis by disturbance of microtubule function  $\rightarrow$  loss of hepatocyte function  $\rightarrow$  identification of the injury by hepatocytes  $\rightarrow$  decrease of protein synthesis  $\rightarrow$  increase of  $[Ca^{2+}]$  activation of endonuclease and protein kinase  $\rightarrow$  DNA split  $\rightarrow$  cell apoptosis<sup>[25]</sup>. On the other hand, there were ischemia and hypoxia of locally embolized tissue, and release of a large amount of free radicals and cell toxins<sup>[26,27]</sup>. They could induce cell apoptosis<sup>[28,29]</sup>. For example, free radicals could injure DNA which would lead to activation of polydiadenosine phosphate pibottransferase<sup>[30]</sup> and accumulation of p53. A cell calcium ion would cause cell apoptosis. It could activate nucleus transcription factors such

as NF- $\kappa$ B which would induce cell apoptosis<sup>[31]</sup>. Normal hepatocyte apoptosis was mainly resulted from the effects of chemotherapeutic drugs and free radical injury rather than from normal tissue repair<sup>[32,33]</sup>.

The regeneration and apoptosis level of normal hepatocytes usually determine the patients' prognosis after transarterial chemoembolization. The obvious apoptosis after TACE was due to chemotherapeutic drugs and free radicals, which made the normal process of apoptosis more rapid. Therefore, excessive dosage of chemotherapeutic drugs may promote apoptosis and injury of normal hepatocytes in non-neoplastic area of the liver, and deteriorate the liver function of patients. We can draw the conclusion that it is important to use chemotherapeutic drugs, because they can protect the liver function of patients and improve their survival rate after treatment.

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