

Percutaneous intratumoral injection of traditional Chinese herbal compound medicine Star-99 in treatment of hepatocellular carcinoma of mice

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BACKGROUND: Unresectable hepatocellular carcinoma (HCC) lacks effective therapy and entails very poor progress. In 1991, we found that Chinese herbal compound Star-99 has potentially effect on HCC. The purpose of this study was to probe the anti-cancer effect and the mechanism of focal injection of Chinese herbal compound Star-99 into HCC of mice.

METHODS: In 32 nude mice transplanted with human hepatocellular carcinoma SMMC-7721, 16 received hypodermic implant and the other 16 orthotopic liver transplant. They were randomly divided into three groups: Star-99 group (Chinese herbal compound, 16 mice), alcohol group (8) and saline group (8), respectively. Intratumoral injection of Star-99, alcohol and saline was carried out 10 days after transplantation of HCC. Twenty days after the first injection, the nude mice were killed after being injected every 5 days with a total of 4 injections in each mouse. Tumor tissues were examined pathologically or via an electron microscope and flow cytometrical (FCM) DNA analysis. The three diameters of the tumor were measured with high-frequency ultrasound before and after injection, and the growth index was calculated with the following formula: volume of tumor (after treatment-before treatment)/volume of tumor (before treatment). Double-blind method was applied in the experiment.

RESULTS: The growth index of the Star-99 group (0.068 ± 0.022) and the alcohol group (0.079 ± 0.024) was markedly lower than that of the saline group (4.345 ± 1.453 , $P < 0.01$), but there was no significant difference between the Star-99 and alcohol groups. Coagulation (8/8) was the

major pathological change in the alcohol group. In the Star-99 group, however, the phenomenon of lymphocytes attacking cancer cells could even be seen under the electron microscope. The typical apoptosis cells and apoptosis bodies as well as the collagen fibrae lined in mass could also be seen in the group (14/16). FCM DNA analysis showed that the rate of apoptosis in the Star-99 group (93.8%) was significantly higher than that in the alcohol (12.5%) and saline groups (12.5%) ($P < 0.01$).

CONCLUSIONS: This study shows that Star-99 markedly inhibits and destructs hepatocellular cancer cells. Star-99 is effective to directly destroy the membrane, cytoplasm and nuclei of tumor cells, causing their crumbling, activate the immune function and inflammatory reaction of nude mice, and induce the apoptosis of cancer cells. The effect of Star-99 is significantly different from that of alcohol that mainly causes coagulation of cancer cells. Star-99 is feasible in the treatment of HCC.

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KEY WORDS: hepatocellular carcinoma; intratumoral injection; Chinese herbal medicine

Introduction

Ultrasound-guided percutaneous intratumoral injection of chemicals, especially alcohol, has been widely used in the treatment of hepatocellular carcinoma (HCC).^[1-5] However, the curative effect is not satisfactory because of regalgia and damage to the liver caused by the leaked alcohol.^[6] Therefore, searching for a better therapeutic agent for the treatment of HCC is of paramount importance.

In 1999, we found that Star-99, a Chinese herbal compound, is potentially effective in cancer treatment. To elucidate this effect, we studied experimentally the use of Star-99 in mice with HCC.

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Methods

Animals

BALB/CA nude mice (SPF, female and male) of 5–8 weeks old provided by the Medical Experimental Animal Unit, Anti-cancer Center of Xiamen University, Xiamen, China. The average weight of the mice was 18 ± 2.1 g. They were raised in the layer drift under condition of no pathogens. The cages, cushions, drinking water and standard forge (BiCai Company, Shanghai, China) were changed periodically.

The centrifugal cellular cultural suspension of human hepatocellular carcinoma was taken after the upper liquid was removed. The cellular cultural liquid was added to 5×10^7 cellular/ml, 0.2 ml of which was injected into the back of nude mouse. Part of the tumor was removed under aseptic conditions when the diameter of the tumor was about 1 cm. After being cut into 0.2 cm^3 , the pieces of the tumor were transplanted subcutaneously with a trochar to the back of another mouse to generate. Under general anesthesia induced by abdominal injection of sodium pentothal, the transplanted mouse was sterilized for incision of the skin and peritoneum at the linea alba about 1 cm below the xiphoid. After the liver was pushed out and a piece of the tumor was placed into it with a trochar, while blocking bleeding from the needle aperture with gelatin sponge. Finally, the liver was taken back into the abdominal cavity, followed by suturing of the peritoneum and skin. The orthotopic liver transplantation was accomplished.^[7]

Experimental method

Thirty-two HCC transplanted nude mice (hypodermic implantation and liver orthotopic transplantation, 16 mice each) were divided into three groups randomly. Star-99 group consisted of 16 mice (Star-99 group), alcohol group 8 mice (alcohol group) and saline group 8 mice (saline group), respectively. Hypodermic implantation and liver transplantation occupied 50% respectively in each group. The length, width and thickness of the tumor were measured to calculate its volume with high frequency ultrasound (Aloka-5500, Japan; probe frequency 10 MHz) 10 days after transplantation. The center of tumor was stabbed with a number-5 needle, followed by injection of 0.1 ml of Star-99, alcohol and saline respectively every 5 days for 4 times. Five days after the last injection, the length, width and thickness of the tumor were measured again before the mice were killed. The tissues of the central tumor were taken for pathological, electron microscopic and flow cytometrical (FCM) DNA analyses. Double-blind method was applied in the experiment.

Tumor growth index (TGI) was calculated by the following formula; volume of tumor (after treatment-before treatment)/volume of tumor (before treatment). Pathologically, the degree of degeneration and the nec-

rosis and reaction of histopathological cells were observed in addition to ultramicrostructural changes in tumor tissue seen under an electron microscope. FCM was used to analyze the apoptosis and heteroploidy of DNA.

Statistical analysis

The average value and standard deviation of TGI in each group were calculated, and the occurrence rates of heteroploid and apoptotic peaks of each group were also calculated using DNA analysis after each treatment. Subsequently significance test of TGI was performed in each group. The occurrence rates of apoptotic peaks were compared in the Star-99, ethanol and saline groups. A *P* value less than 0.05 was considered significant.

Results

Tumor growth index

Comparison of TGI of the 32 HCC transplanted mice after 20-day therapy (Table 1) showed that TGI of the Star-99 group was lower than that of the saline group ($P < 0.01$). It was also lower than that of the ethanol group, but there was no significant difference between them ($P > 0.05$).

Pathohistological changes

The three groups showed degeneration and necrosis in various degrees after therapy. In the saline group, slight change or few cellular degeneration (8 mice) and no obvious necrosis were seen in contrast to coagulative necrosis in the ethanol group. The Star-99 group mainly showed swelling, degeneration and necrosis of cells. In the necrotic area, the contour of cancerous tissue was visible, the nucleus dissolved and even disappeared, the cytoplasm was stained red, and swelling, degeneration, nuclear concentration could be seen in the periphery. Vaculation in the nucleus was its significant trait. It should be emphasized that infiltration of various numbers of lymphocytes were seen in the degenerative tumor tissue in the Star-99 group (16/16), in which 56.3% (9/16) even had lymphocytes accumulated in masses or sheets (Fig. 1).

Ultramicrostructure under electron microscope

Saline group

Ultramicrostructurally, hepatic cancer cells were not obviously changed. There were intact nuclear membrane, nuclei in different size, rich euchromatin but

Table 1. TGI of the Star-99, ethanol, and saline groups

Groups	Patterns	Mean \pm SD	<i>P</i> value
Star-99 (A)	16	0.068 \pm 0.022	A,B >0.05
Ethanol (B)	8	0.079 \pm 0.024	B,C <0.01
Saline (C)	8	4.354 \pm 1.453	A,C <0.01

poor heterchromation, rich cytoplasm, and intact cell membrane. No obvious changes were observed in mitochondria and endoplasmic reticulum.

Ethanol group

Electron microscopy showed that most abnormal chromatin nuclei became concentrated and agglutinated below the nuclear membrane. Nuclear membrane dissolved partly with the appearance of karyorrhexis and necrosis.

Star-99 group

A series of phenomena were observed under an electron microscope. They included various degrees of swelling and destruction in the endoplasmic reticulum and mitochondria of cancer cells, agglutinated heterchromatin in the nucleus, degenerated nucleus, and ruptured nuclear and cell membrane (Fig. 2). More interestingly, lymphocytes infiltrated into cancer cells in 16 mice, 9 of which even had the microvilli on the surface of lymphocytes or their deep filtration into cancer cells (Fig. 3). 87.5% (14/16) of tumor tissues in the Star-99 group showed global or crescent apoptosis cells and/or apoptosis bodies for heterchromatin accumulation

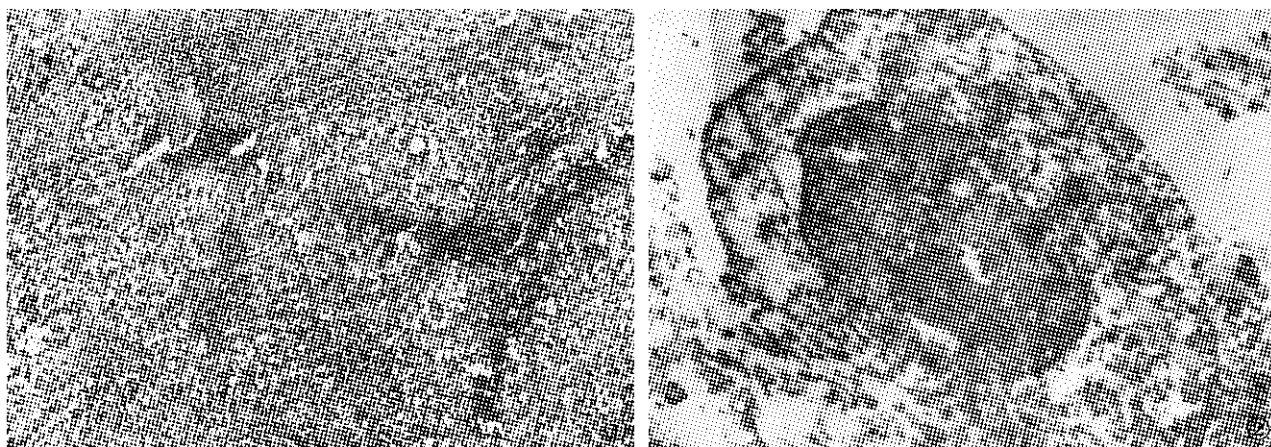


Fig. 1. Many lymphocytes seen in the interface of tissues and tumor of the nude mice transplanted with HCC and given Star-99 for 20 days.

Fig. 2. Electron microscopy showing swollen cells, ruptured and depleted nuclear membrane, severely destroyed organelles, damaged nuclear membrane, enlarged perinuclear space in hepatic cancer cells of nude mice after injection of Star-99.

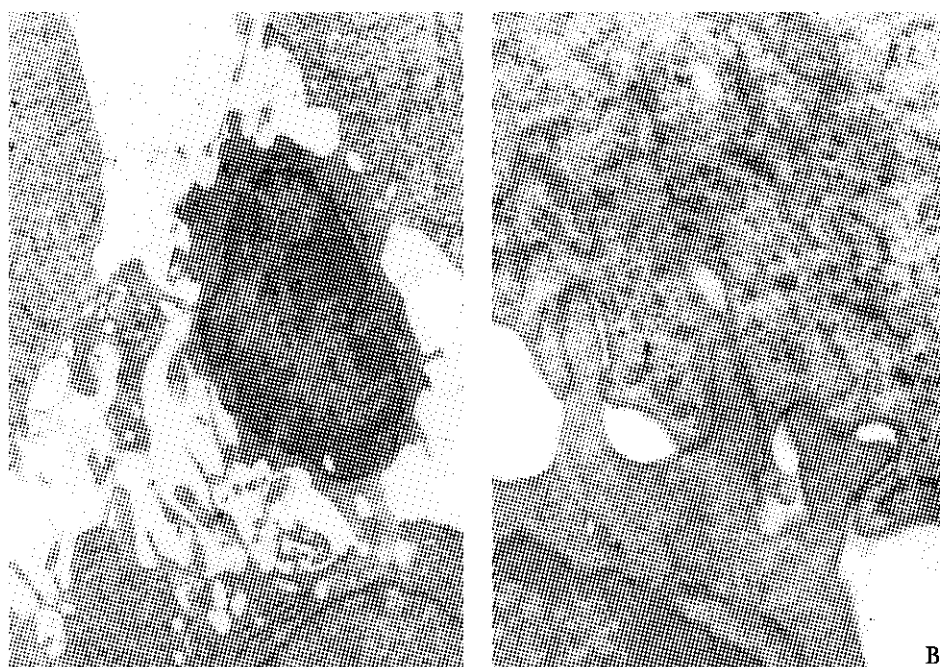


Fig. 3. Lymphocytes infiltration and the insertion of microvilli of lymphocytes into cancer cells (B: enlarged image).

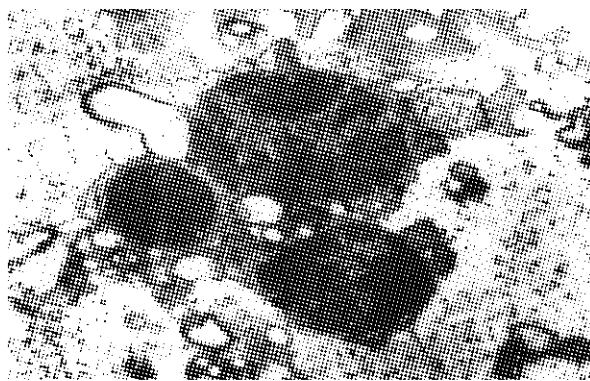


Fig. 4. Electron microscopy showing increased nuclear heterochromatin forming apoptotic cells and petaloid apoptotic bodies.

Table 2. The occurrence rate of peaked heteroploidy and DNA analysis of tumor tissues after treatment in the three groups

Groups	Cases	Occurrence rate of peaked heteroploidy (%)	Occurrence rate of peaked apoptosis (%)
Star-99	16	1/16 (6.3)	15/16 (93.8)
Ethanol	8	1/8 (12.5)	1/8 (12.5)
Saline	8	5/8 (62.5)	1/8 (12.5)

(Fig. 4). Moreover, macrophages, monocytes, eosinophil polymorphs and/or Kupffer's cells were visualized in the tumor stroma.

FCM DNA analysis

The results of DNA analysis of tumor tissues in the three groups are shown in Fig. 2. The occurrence rate of heteroploidy peak after treatment was 62.5% (5/8) in the saline group, 12.5% (1/8) in ethanol group and 6.3% (11/16) in the Star-99 group respectively. In the Star-99 group, 93.8% (15/16) had apoptosis peaked by the cells in the sub G1 period, which was higher than that in the saline and ethanol groups ($P < 0.01$, Table 2).

Discussion

Many kinds of ultrasound-guided interventional therapy for HCC have been accepted clinically. In 1983, Japanese researchers used successfully ultrasound-guided percutaneous injection of ethanol in treatment of liver cancer.^[8-10] Since the 1990s, chemicals and various laser, microwave, radio-frequency and high-energy focused ultrasound have been widely used with satisfactory results comparable to those of surgical resection.^[11-15] However, they have certain limits in practice.^[16] Using percutaneous ethanol injection (PEI) in the treatment of hepatic carcinoma,^[17] many patients especially elderly patients ceased the therapy because of intolerable

acute pain induced by ethanol or other chemicals. In some patients, the effect of treatment is the least after repeated injection for a long period at the peripheral infiltrative focus, and they had to stop the therapy because of destruction of hepatic function. Searching for a high efficacious, safe, non-toxic or less toxic medicine is of paramount significance.^[18,19] In this study, we found that Star-99 is powerful in inhibiting and destructing cancer cells, and its therapeutic effect is not inferior to that of ethanol. Pathohistological study showed swelling, degenerative necrosis and programmed death (PCD) of tumour cells in the Star-99 group, which was not less evident than in the ethanol group with coagulation of tumor tissues. More importantly, many lymphocytes in masses were seen in degeneratively swollen tumor tissues of the Star-99 group, but they were not observed in the ethanol group. This finding indicates that Star-99 could actively elicits the cellular immune function of lymphocytes and local inflammatory reaction. Its function of restraining or killing tumor cells is probably relevant to enhanced cellular immune function. The mechanism is markedly different from that of ethanol, which only kills tumor cells while lacking tissue reaction.^[20] This point is worthy of further study.

Electron microscopy confirmed the pathological finding and our hypothesis. In the Star-99 group, we not only found nucleus dissolution and degeneration, mitochondrial swelling, and rupture of cell and nuclear membrane, but also lymphocytes attacking cancer cells by their microvilli. Under an electron microscope, when the sensitized T lymphocytes contacted with the projection of cancer cells, the membrane of cancer cells at interface ruptured and disappeared. Consequently, cytoplasmic organelles and nuclei of tumor cells dissolved with vaculation in cytoplasm.^[21-24] Ours were similar to these findings, indicating that Star-99 could stimulate or induce cellular immune function of organisms. In the Star-99 group many apoptosis cells and bodies were observed under an electron microscope, indicating an important morphological symbol for tumor cell apoptosis or PCD.^[25-30] The apoptotic bodies rarely appeared in the saline group, in which tumor cells grew actively, but no apoptotic bodies were found in the ethanol group. This proved again that the effective mechanism of Star-99 is different from that of ethanol. In addition, macrophages, eosinophil polymorphs and/or Kupffer's cells and clustered collagen fibers could be seen in most interstitial tissues or necrotic area of tumor. It is worth further elucidation of immune function or tissue repairment after tumor cell necrosis.

The results of FCM DNA analysis of the three groups indicated that the occurrence rate of the heteroploidy peak in the Star-99 group was merely 6.3%, markedly lower than that of the saline group (62.5%, $P < 0.01$) and that of common digestive tumor (50%–60%).^[31,32] In contrast, the cell apoptosis peak of the

Star-99 group in the G1 period was much higher than that of the ethanol and saline groups ($P < 0.01$). It is indicated that Star-99 is effective in inducing tumor cell apoptosis. This biological change is consistent with morphological change of typical apoptotic cells and bodies shown by electron microscopy.

To sum up, the results of this study proved that Chinese herbal compound Star-99 is strongly effective in inhibiting or destroying tumor growth. The mechanisms of the effect include direct destroy of membrane, cytoplasm and nuclei of tumor cells, causing their crumbling, activation of immune function and inflammatory reaction of the organism, and induction of apoptosis and PCD of cancer cells. In this study, the anti-cancer effect of Star-99 was not lower than that of alcohol because of different mechanisms. It is of great significance in anti-cancer treatment by activating immune function and inflammatory reaction of the organism and inducing cell apoptosis or PCD. Therefore, ultrasound-guided percutaneous intratumoral Star-99 injection in treatment of HCC is promising. This study provides an experimental basis for ultrasound-guided injection of Star-99 in treatment of HCC and a new way for the combined use of traditional Chinese and Western medicine in the future.

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Competing interest

The author or authors do not choose to response to the statements listed in Instructions for Authors.

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