

Efficacy of intra-tumor injection of Xiao-Zhi-Ling on transplanted hepatoma in rats

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Abstract

AIM: To study the therapeutic effectiveness of intra-tumor injection of Xiao-Zhi-Ling (XZL) on transplanted hepatoma in rats.

METHODS: Sixty rats were divided into 3 groups (groups S, X and E), 20 in each. Different drugs were injected into the implanted hepatoma (Group S with 0.2 ml saline as control, group X with 0.2 ml XZL, group E with 0.2 ml ethanol). After 3 days and 8 days respectively, we detected the hepatoma volume (HV), the level of albumin, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) in serum, and the expression of proliferating cell nuclear antigen (PCNA) in hepatoma.

RESULTS: The results were obtained after 3 days, the HVs in groups X and E were smaller than those in group S (group X vs S $P=0.010^*$, group E vs S $P=0.002^*$, $P<0.05$). The levels of ALT and AST in group S and X were lower than those in group E (ALT Group S vs E $P=0.019^*$, group X vs E $P=0.003^*$, $P<0.05$; AST group X vs E $P=0.002^*$, $P<0.05$). The levels of ALP and PCNA labeling index in group X were lower than those in group S and E (ALP group X vs S $P=0.000^*$, group X vs E $P=0.000^*$, $P<0.05$; PCNA group X vs S $P=0.008^*$, group X vs E $P=0.048^*$, $P<0.05$). The levels of creatinine in group S were lower than those in group E (group S vs E $P=0.017$, $P<0.05$). The degree of tumor necrosis in group S was lower than those in groups X and E (group S vs X $P=0.006^*$, group S vs E $P=0.006^*$, $P<0.05$). After 8 days, the HVs in groups X and E were smaller than those in group S (group X vs S $P=0.007^*$, group E vs S $P=0.004^*$, $P<0.05$). The difference of HVs between groups X and E was not significant. The levels of albumin, ALT, AST and creatinine in group X were not higher than those in other groups, the levels of ALP and PCNA in group X were lower than those in groups S and E (ALP group X vs E $P=0.006^*$ $P<0.05$; PCNA group X vs S $P=0.044^*$, group X vs E $P=0.021^*$, $P<0.05$). The degree of tumor necrosis in group S was lower than that in groups X and E (group S vs X $P=0.001^*$, group S vs E $P=0.002^*$, $P<0.05$).

CONCLUSION: The therapeutic effectiveness of intra-tumor injection of XZL and ethanol on implanted hepatoma is obvious, but the toxicity of XZL on liver function is markedly lower than that of group E, at the same time XZL can inhibit the growth of tumor. XZL is relatively better and safer than ethanol in intra-tumor injection therapy.

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INTRODUCTION

The resection rate of hepatocellular carcinoma (HCC) is only about 20 %. Percutaneous ethanol injection therapy (PEIT) and transcatheter arterial chemoembolization (TACE) can be used in patients who have lost the chance of surgical operation. Both could be used easily and widely. But to small HCC, they cannot treat it thoroughly. To huge HCC, repeated use can cause liver function failure and biliary tract injury, so the application in clinic is limited. Xiao-Zhi-Ling (XZL) is a traditional Chinese drug, it can cause vascular embolization and tissue fibrosis. In this research XZL was used in the treatment of implanted hepatoma in rats in order to find its therapeutic effectiveness.

MATERIALS AND METHODS

Tumor implantation

Walker-256 tumor cells were injected into the abdominal cavity of Wistar rats, tumor ascites were removed from the abdominal cavity 7 days later. After diluting them subcutaneous vaccination was given in right forefoot axillary fossa of healthy Wistar rats^[1]. 8 days later we obtained the subcutaneous tumor which grew quickly, then cut it into pieces of $1\times 1\times 1$ mm³, put them into RPMI medium 1640^[2,3]. A small subcapsular incision on the left lateral lobe of the liver of healthy Wistar rats was made. Tumor fragments were gently put into the pocket and abdominal wall was then closed. After that rats were maintained on the previous diet^[4,5].

Experimental procedures

8 days after tumor cells were implanted, 60 rats were divided into 3 groups (groups S, X and E), 20 rats in each group. Different drugs were injected into the implanted hepatoma of each rat, group S with 0.2 ml saline as control, group X with 0.2 ml XZL (obtained from Ji-an Pharm Corp in Jilin province, China), group E with 0.2 ml ethanol.

After 3 days and 8 days respectively, 10 rats were sacrificed in each group at random. Tumors in the left lobe of liver were removed and fixed in 10 % formalin. The respective blood samples were centrifuged, then the level of albumin, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) in serum were detected^[6,7].

Immunohistochemistry analysis

The implanted liver tumor tissues were paraffin-embedded and sectioned, sections were made for standard histological detection using hematoxylin and eosin^[8,9]. The expression of proliferating cell nuclear antigen (PCNA) in hepatoma was detected in each case^[10,11].

Statistical analysis

The data were expressed as $\bar{x}\pm s$. Statistical differences were

analyzed by *t*-test, a value of $P < 0.05$ was considered significant. The difference between groups was compared by LAD method.

RESULTS

Comparison of hepatoma volume (HV) between different groups (Table 1 and 2)

After 3 and 8 days, the HVs in group X and E were smaller than those in group S ($P < 0.05$).

By the 3rd day, the differences of hepatoma volume between different groups were as follows: group S vs X $P = 0.010^*$, group S vs E $P = 0.002^*$, group X vs E $P = 0.443$.

By the 8th day, the differences of hepatoma volume between different groups were as follows: group S vs X $P = 0.007^*$, group S vs E $P = 0.004^*$, group X vs E $P = 0.771$.

Comparison of hepatoma necrosis between different groups

After 3 days the difference of hepatoma necrosis between groups X and S, and groups E and S was significant ($P < 0.01$).

After 8 days group S was significantly different from groups X and E. But there was no difference between groups X and E. By the 3rd day, the differences of hepatoma necrosis between different groups were as follows: group S vs X $P = 0.006^*$, group S vs E $P = 0.006^*$, group X vs E $P = 1.000$.

By the 8th day, the differences of hepatoma necrosis between different groups were as follows: group S vs X $P = 0.001^*$, group S vs E $P = 0.002^*$, group X vs E $P = 0.690$.

Rate of tumor inhibition (RTI)

Tumor volume was calculated by the formula ($\pi/6 \times \text{length} \times \text{width} \times \text{height}$). The rate of tumor inhibition (RTI) could be calculated by the formula: (volume of control group - volume of test group)/volume of control group $\times 100\%$; 3 and 8 days after injecting drug, RTIs in groups X and E were 45.86%, 57.73% and 62.47%, 68.24%. MDDT stands for

multiplication diameter time, which indicates the growth rate of tumor. Table 3 shows different growth rates in the 3 groups.

Table 3 MDDT of the tumor in groups S, X and E

Group	MD (3 days)	MD (8 days)	MDDT
Group S	4.56	9.62	4.51
Group X	4.6	6.74	10.75
Group E	3.54	6.5	5.9

Comparison of hepatic and renal function (Table 1 and 2)

Serum albumin After 3 and 8 days the level of serum albumin in group X was higher than those in groups S and E, but there was no statistical significance ($P = 0.13$).

Serum ALT After 3 days, the level of serum ALT in groups S and X was lower than that in group E, there was statistical significance ($P < 0.01$). After 8 days, the level of serum ALT showed no statistical difference among three groups ($P > 0.05$). By the 3rd day, the differences between every two groups were as follows: group S vs X $P = 0.309$, group S vs E $P = 0.019^*$, group X vs E $P = 0.003^*$.

Serum AST After 3 days, the level of serum AST showed statistical difference among three groups ($P < 0.05$). After 8 days, the level of serum AST showed no statistical difference among three groups ($P > 0.05$). By the 3rd day, the differences between every two groups were as follows: group S vs X $P = 0.077$, group S vs E $P = 0.067$, group X vs E $P = 0.002^*$.

Serum ALP After 3 days the level of serum ALP in group X was lower than those in groups S and E, there was statistical significance ($P < 0.01$). After 8 days the level of serum ALP in group X was lower than that in group E, there was statistical significance ($P < 0.01$). By the 3rd day, the differences between every two groups were as follows: group S vs X $P = 0.000^*$, group S vs E $P = 0.649$, group X vs E $P = 0.000^*$. By the 8th day, the

Table 1 Change of the parameters 3 days after injection $\bar{x} \pm s$

Indexes	Saline	XZL	Ethanol	F	P
Tumor volume (mm ³)	48.02±14.01	26.00±5.41	20.30±12.66	8.356	0.005
Tumor necrosis (mm ³)	1.40±0.55	3.20±0.84	3.20±0.10	7.364	0.008
Albumin (g/L)	34.02±2.98	47.10±18.31	32.88±4.92	2.540	0.120
ALT (U/L)	96.80±28.45	75.60±17.87	150.60±43.05	7.520	0.008
AST (U/L)	305.00±117.72	209.00±47.89	404.00±45.50	7.778	0.007
ALP (U/L)	373.80±78.02	184.40±25.39	422.60±52.18	25.119	0.000
Creatinine (μmol/L)	34.60±1.95	38.60±1.82	39.80±4.38	4.228	0.041
PCNA	62.80±12.62	40.60±7.89	56.00±12.06	5.288	0.023

Table 2 Change of the parameters 8 days after injection $\bar{x} \pm s$

Indexes	Saline	XZL	Ethanol	F	P
Tumor Volume (mm ³)	464.62±212.00	174.38±109.46	147.58±60.17	7.658	0.007
Tumor Necrosis (mm ³)	1.60±0.89	3.8±0.45	3.6±0.89	12.333	0.001
Albumin (g/L)	33.68±5.66	36.88±5.78	30.01±2.67	2.447	0.128
ALT (U/L)	104.00±43.39	68.80±13.81	118.60±42.72	2.522	0.122
AST (U/L)	341.00±84.63	233.14±52.28	328.20±64.27	3.714	0.056
ALP (U/L)	237.40±80.63	250.80±75.24	474.0±148.15	7.767	0.007
Creatinine (μmol/L)	38.40±6.02	37.80±6.38	36.60±4.0	0.135	0.875
PCNA	56.20±8.67	41.40±10.00	58.80±12.10	4.063	0.045

differences between every two groups were as follows: group S vs X, $P=0.846$, group S vs E, $P=0.004^*$, group X vs E, $P=0.006^*$. **Serum creatinine** After 3 days the level of serum creatinine in group E was higher than that in group S. There was statistical significance among three groups ($P<0.05$). After 8 days there was no statistical difference among three groups ($P>0.05$). By the 3rd day, the differences between every two groups were as follows: group S vs X $P=0.054$, group S vs E $P=0.017^*$, group X vs E $P=0.543$.

PCNA expression in tumor tissue (Table 1 and 2)

After 3 and 8 days fine positive expression existed in the remnant tumor tissue in every group. PCNA labeling index among 3 groups was statistically different ($P<0.05$). PCNA labeling index in group X was lower than that in groups S and E, but there was no statistical significance between groups S and E ($P>0.05$). By the 3rd day, the differences between every two groups were as follows: group S vs X $P=0.008^*$, group S vs E $P=0.350$, group X vs E $P=0.048^*$. By the 8th day, the differences between every two groups were as follows: group S vs X $P=0.044^*$, group S vs E $P=0.700$, group X vs E $P=0.021^*$.

DISCUSSION

Primary liver carcinoma is one of the most popular malignancies in China, and its incidence has been increasing in recent years. The reported mortality of primary liver carcinoma in China is higher than that of other malignant tumors. Because of difficulty in early diagnosis, 75 % of the clinically diagnosed are large ones when the patients lose the opportunity of operation. Non-operation therapy becomes more and more important, and many basic researches have been done^[12,13]. In some experiments, massive proliferation of donor-derived normal hepatocytes was observed in the liver of rats previously given retrorsine (RS). Suicide gene therapy based on ganciclovir (GCV) metabolism by transgene herpes simplex thymidine kinase (HSV-1 TK) has been used to selectively kill proliferating cells in hepatic tumor. Effect of Avemar and Avemar + vitamin C on hepatic tumor growth was observed in experimental animals. Through various experiments, some encouraging results have been reported^[14,15]. In the nude mice hepatoma model, the antitumor effects of pDR2-TK/GCV system on tumor growth were evaluated. Through MTT method, they found that the pDR2-TK/GCV had cytotoxic effect and about 70 % SMMC-7721 cells were killed when GCV was at 1 000 $\mu\text{mol/L}$. *In vivo* experiment showed that the tumor size in nude mice with transferred pDR2-TK gene was significantly smaller than that in control group. PEIT was used clinically, because this method of intra-tumor injection was easy, cheap and could be used repeatedly, it has been widely used. But both of these methods cannot treat the patients thoroughly. Repeated use can cause liver function failure and biliary tract injury. Therefore researches on drugs which can be used in intra-tumor injection therapy become more and more important. Some kinds of traditional Chinese drugs were purified and the immunosuppressive function was examined^[16].

XZL is mainly composed of Wu-Bei-Zi extracts and alum, both of which have the function of astringing, antidiarrheal, hemostasis and antiseptis. The vascular vessels would constrict immediately after injection of XZL. Then inflammatory reaction and hyperplasia would occur in the local artery. Endoangiitis and intravascular coagulation would occur in arteries and veins. All the above finally lead to fibrinoid necrosis of local tissue. These changes perhaps are the reason why XZL has antineoplastic activity.

From this research we conclude that the therapeutic effects of intra-tumor injection of XZL on implanted hepatoma is obvious. XZL can evidently inhibit the growth of tumor. The

inhibition of XZL on implanted hepatoma is no better than that of PEIT according to statistical analyses. But in terms of MDDT, XZL is much better than PEIT, perhaps because the toxicity of XZL on liver function is markedly lower than that of ethanol.

Based on the results of liver and renal function, the toxicity of XZL on liver function is relatively less severe than that of ethanol. For patients with disorder of liver function, PEIT could not be used, we can try XZL instead. The dose of ethanol is very important in treatment, high-dose of ethanol can lead to liver function failure and systemic toxicity to the patient, but low-dose can not kill the edge cells of the tumor thoroughly. Many experiments have shown that high-dose therapy of common drugs can lead to hepatic function failure, but if low-dose of drug is given, tumor cells cannot be killed thoroughly, because the tumor cells are supplied by duplicated vessels (liver artery and portal vein) and grow rapidly than before^[17]. The patients' prognosis could be influenced and the tumor perhaps would recur and metastasize quickly. So we could use XZL to resolve this problem. We can inject XZL into the edge of the tumor to kill the tumor cells, thus to improve the therapeutic effectiveness, reduce recurrence and metastases of the tumor. This study revealed that 3 and 8 days after injection of drug (saline, XZL and ethanol), fine positive expression of PCNA occurred in the remnant tumor tissue of every group. PCNA labeling index among 3 groups was significantly different. PCNA labeling index in Group X was lower than that in groups S and E ($P<0.05$), but there was no statistical significance between groups S and E ($P>0.05$). This result implies although injection of ethanol can lead to necrosis of tumor tissue, it cannot influence proliferation activity of the remnant tumor tissues. Intra-tumor injection of XZL into implanted hepatoma can reduce the expression of PCNA labeling index, suggesting XZL can inhibit the proliferation activity of tumor cells. This might be the reason why XZL has the antineoplastic activity.

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