Chinese Journal of Cancer Research 8(3):168–173,1996.

THE ENHANCEMENT OF TWEEN-80 ON THE ANTITUMOR EFFECT OF THE HYPERTHERMIA 41 ℃ IN TUMOR-BEARING MICE

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B16 melanoma cells were inoculated into the BALB/C mice to establish melanoma-bearing models. The antitumor effect of Tween-80 in combination with hyperthermia 41 ℃ was studied. We observed the changes of the mortality of tumor-bearing mice, the tumor growth curves, activities of serum tumor necrosis factor (STNF) and the level of serum sialic acid (SSA) in tumor-bearing mice. The number of pulmonary metastatic tumor foci from blood flow was also detected. The results showed that combined with Tween-80, hyperthermia 41 ℃ could dramatically suppress the growth of the melanoma in the feet of mice, survive the tumor-bearing mice and decrease the number of pulmonary metastatic tumor foci but no significant effects were observed by treatment with Tween-80 or hyperthermia at 41 ℃ alone. The activities of STNF and the level of SSA of the melanoma-bearing mice kept at higher levels than those of normal BALB/C mice. Tween-80 combined with heating 41 ℃ significantly decreased activities of STNF, but the level of SSA still kept high level within 1 to 2 weeks and decreased 10 weeks later with the tumor regression. These results demonstrate that Tween-80 may make hyperthermia exert effective antitumor effect below the critical temperature and increase the safety of hyperthermia in treatment. It is one of the most ideal synergist with hyperthermia. The changes of STNF and SSA suggest that the synergetic effect could involve in facilitating the exposure of tumor antigen and activation of immune system.

Key words: Tween-80, Hyperthermia (41 ℃), Tumor growth, Metastasis TNF, Sialic acid.

Hyperthermia has therapeutic potential in the treatment of solid tumors, especially when used in combination with other treatment modalities, such as chemotherapy. The selection of optimal combined drug is important to enhance the antitumor effect of hyperthermia and meanwhile reduce the side effect of hyperthermia. Our past research work on cell level demonstrated that Tween-80 was an ideal medicine combined with hyperthermia to antitumor, it effectively killing tumor cells and being comparatively safe and low in toxicity to normal cells.[2] In this study, we further investigate the antitumor effect and synergistic action of Tween-80 in combination with hyperthermia at 41 ℃ on the melanoma-bearing mice.

MATERIALS AND METHODS

Experimental Animal

180 BALB/C mice 6–7 week-old, male, were used to establish melanoma-bearing models. All mice were provided by Animal Center of China Scientific Institute, Shanghai.

Accepted August 27, 1996
This work is supported by National Nature Science Fund (No. 39170825) and Shanghai Science Developing Fund.

Cell Culture

B16 mouse melanoma cell line was obtained from cell research unit of China Scientific Institute, Shanghai, and routinely grown in RPMI-1640 supplemented with 10% newborn calf serum, 0.25 mM Hepes, 100 μ/ml penicillin and 100 μg/ml streptomycin. L929 cells (provided by Microbiology Department of Shanghai Second Medical University) were cultured in media as above. Exponential cells were detached by trypsination and harvested in 1640 media. The cell growth and viability were assessed by using a Coulter counter and trypan blue dye exclusion respectively. The cells had a greater than 95% viability in all experiments.

Tumor Growth Curve

5×10^5 B16 cells were inoculated in the rear feet of mice. 10 days later, tumor born could reach mean size of 0.5×0.5 cm^2 and the mice inoculated were randomly divided into 4 groups: (1) for hyperthermia study, the mouse was placed over a water bath (41 ℃ ±0.1) and the tumor-bearing foot was inserted into the water bath through a 1.5 cm diameter padded opening to a depth that permitted complete submersion of tumor. The tumor was heated for 100 min. (The time required to reach 41 ℃ was within 5 min.). Rear feet bearing melanoma were put into water bath 41 ℃±0.1 for 60 min.; (2) for Tween-80 study, mouse was given Tween-80 200 μl intravenously with a final concentration of 75 mM. (Tween-80 was purchased from Shanghai Da Zhong pharmaceutical factory.); (3) for Tween-80 combined heating study, 15 min. after Tween-80 injection, mice were followed by heating at 41 ℃ as above; (4) the control group. The sizes of tumors were estimated by caliper measurement every other day. According to the three dimensions (volume) of tumor, the tumor growth curves were plotted.

Lethal Rate of Tumor-bearing Mice

3×10^4 B16 cells were inoculated to each mouse in traperitoneally. 10 days after inoculation, mice were divided into 4 groups as above. During heating, mouse was immersed vertically into a well stirred, heated water bath (41 ℃) until the water reached its chest. The time required to reach 41 ℃ was within the range of 5 to 10 min. The mice were fed continuously under normal condition and their mortality or survival was observed for 10 weeks after treatment. For serum tumor necrosis factor (STNF) and serum sialic acid (SSA) studies, sterile sera of mice were collected by cardiac punctures 1 week and 2 weeks after treatment, respectively.

Assay of STNF and SSA

The sera were added to 96-well plates at various dilutions, amounting 200 μl/well. The rTNF provided by Beijing Military Scientific Institute with its activity rate 4×10^7 U/mg protein was used as criteria positive control. Negative control was included, too. Exponential L929 cells were seeded at 3×10^4 cells, 75 μl/well and finally add 25 μl Actinomycin D (Act-D, Fluka. Co.) per well and made a final concentration of 1 μg/ml. The cells were cultured at 37 ℃ in 5% CO_2 for 20 h, fixed in 4% formalin, stained with 0.25% crystal purple dye and dissolved in 1% SDS. Assay of STNF activity was carried out by measuring the OD under the Multiplate Reader. One unit of STNF activity is defined as the dilution of serum, in which 50% target cells were killed. The criteria rTNF were used as calibration. For SSA assessment, CCM method was used as described by Svennerholm L.[3] CCM reagents were provided by Biochemistry Department of Shanghai Second Military Medical University.

Pulmonary Metastatic Foci of B16 Melanoma

B16 cells were previously treated with Tween-80, heating at 41 ℃ or both, respectively. Then 4×10^5 cells were injected into each mouse from tail vein. After 3 weeks, the mice were killed and the number of metastatic foci of melanoma in the lungs was counted.

RESULTS

Lethal Rate

In Tween-80 combined with heating at 41 ℃ group, mortality of mice was much lower than the other groups ($P<0.05$). No significant difference could be seen between heating group, Tween-80 group and control group ($P>0.05$). At the same time, we found the mice who had subjected to treatment of both

heating and Tween-80 lived much longer than the mice in other groups (Figure 1). 9 mice survived even over 120 days, which was considered as recovery. These results demonstrated that the treatment of Tween-80 in combination with heating at 41 ℃ could produce effective antitumor action and improve the long-term survival of tumor-bearing mice.

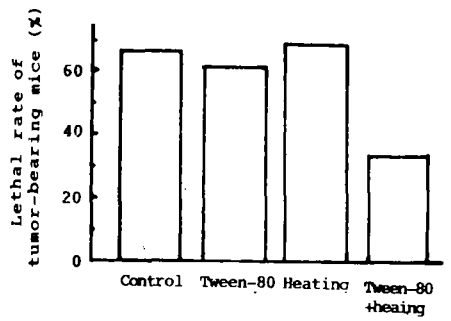

Fig. 1. The effect of Tween-80 or/and hyperthermia 41 ℃ on survival of tumor-bearing mice.

Pulmonary Metastatic Tumors

The number of pulmonary tumor foci migrated through blood flow was shown in Table 1. Under treatment of Tween-80 combined with hyperthermia 41 ℃, the number of metastatic foci in the lungs was significantly decreased ($P<0.01$). Whereas tumors seem to resist exposure to treatment of 41 ℃ for 100 min. or Tween-80 alone, the number of metastatic tumors in the lungs was not significantly decreased as compared with the control group. This demonstrated that Tween-80 combined with hyperthermia 41 ℃ could produce apparent inhibition on the tumor-producing ability of B16 cells.

Growth Curves

Growth curves of melanoma bearing in the mice's feet were illustrated in Figure 2. The tumors grew so fast that marcodiameter of tumors could reach 25 mm 4 weeks after inoculation in control group. Some tumors would present central necrosis for lack of blood flow and mice started dying 24 days after inoculation. Tween-80 treatment did not exhibit significant inhibition on tumor growth. The tumor growth curve was similar to that of control group. Local hyperthermia (41 ℃, 100 min.) produced slight temporary suppression on the growth of tumors. After 2 weeks, the tumor regrew as fast as before. Up to 28 days, the tumors could grow up to 1.6-fold of the initial tumor volume when treated, but still smaller than that of control group. When treated by Tween-80 combined with hyperthermia 41 ℃, the tumors first presented slight edema that subsided about 1 week later. Then tumor volume reduced gradually to 60% of its initial volume another 10 day later. Most mice could survive. The tumors disappeared in 8 mice, only some pigment remained at the former tumor site.

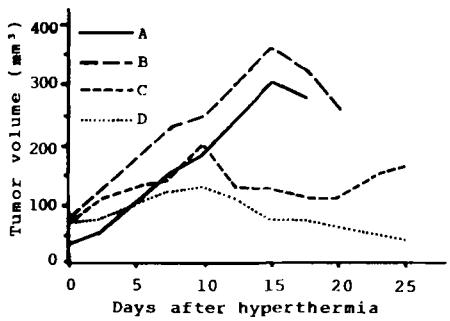

Fig. 2. Growth curves of B16 melanomas in the feet of mice after treatment of hyperthermia 41 ℃ for 100 min. or/and Tween-80. Each growth curve was plotted using mean values for tumor volume measured at two-day intervals in 15–20 tumors. A: Control; B: Heating alone; C: Tween-80 alone; D: Tween-80 combining with heating at 41 ℃ for 100.

sTNF and SSA

The levels of sTNF and SSA kept relatively steady in first 2 weeks after treatment. All sera in Table 2 were collected at the 2nd weekend except that with additional note. sTNF activity could not be detected in the normal mouse, but it was on high level in the tumor-bearing mice, 3.26±1.45 μ/ml. Hyperthermia 41 ℃ alone had no significant influence on sTNF level of tumor-bearing mouse. With treatment of Tween-80 or Tween-80 combined with hyperthermia, sTNF level could obviously decrease ($P<0.05$, $P<0.01$ respectively). The SSA, a special tumor marker, was very low in normal mouse (<50 mg/ml), whereas it was on high level in the melanoma-bearing

mice. Tween-80 combined with hyperthermia 41 ℃ had no significant influence on SSA first 2 week after treatment, but significantly decreased the level of SSA 10 weeks later with tumor regression. In the other three groups, SSA still kept on high level even 10 weeks later.

Table 1. The changes of B16 melanoma metastases in the lungs of mice after treatment Tween-80 or and hyperthermia 41 ℃

Group	1	2	3	4	5	6	7	8	9	10	11	$\bar{x}\pm s$
Control	49	147	127	220	214	180	107	295	390			192.11±92
Combining	7	7	12	5	11	56	24	20	8			16.78±16
Tween-80	120	135	20	30	346	204	450	150	289			193.78±143
Heating	308	168	146	196	51	98	204	362	330	170	317	213.64±101

Table 2. The effects of Tween-80 and hyperthermia 41 ℃ on serum tumor necrosis facter (STNF) and serum sialic acid (SSA) $\bar{x}\pm s$

Group (No.)	STNF (U/ml)	SSA (mg/ml)
Control (10)	3.26± 1.45	129.75±11.24
Tween-80 (8)	1.06± 0.72*	120.28±15.98
Heating 41 ℃(10)	1.93± 1.42	128.00±5.21
Combining (10)	0.35± 0.29**	149.50±22.50
		67.70±1.80 (10th week)***

*$P<0.05$ (t=3.91); **$P<0.01$ (t=5.56); ***$P<0.01$ (t=14.8)

All compared with control group; sera taken at 2nd week except special note.

DISCUSSION

B16 mouse melanoma cells are from a spontaneous melanoma arising in C57/B16 mice. In our experiments, it also showed high rate of producing melanoma in BALB/C mice (nearly 100%). The tumors implanted intraperitoneally grew fast and the mice without treatment would survive in average 59 days in our experiments. The tumors in the foot would enter logarithmic growth 10 days after inoculation. Neither Tween-80 nor heating at 41 ℃ could suppress tumor growth effectively and improve the long-term survival of tumor-bearing mice. But when treated by Tween-80 in combination with heating at 41 ℃, the tumor growth was apparently suppressed. The average survival time of the tumor-bearing mice exceeded 120 days. The tumor volume in the mouse feet reduced gradually after edema subsided, became much smaller than its initial volume before treatment and even disappeared in some mice. These results revealed that Tween-80 in combination with hyperthermia exerted a synergic effect on inhibiting tumor growth and killing tumor cells. Tween-80 or hyperthermia 41 ℃ alone did not present significant antitumor effect.

Tween-80 is polysorbate. As it contains double-bonds and belongs to unsaturated fatty acids, Tween-80 has a function to enhance cell membrane fluidity, which was similar to the plasma-membrane change caused by hyperthermia. Some reports pointed out that when cell membrane fluidity increased to a certain extent, hyperthermia could result in a serial changes of enzymes, proteins and charged-group integrated on the membrane and bring about the death of the cells.[4] Hence Tween-80 could enhance the cytotoxic effect of heating and decrease the critical temperature needed for hyperthermia killing tumor cells. In general, the critical temperature for most tumors was about 43 ℃.

Tween-80 was able to decrease the critical temperature about 2 ℃.[2,5] Thus, with the help of Tween-80, hyperthermia is able to kill tumor cells and block the tumor growth at 41 ℃ effectively.

The ability of tumor cells to form colonies in the lungs of recipient animals might assay the effect of tumor therapy *in vivo*. The advantage of this technique is that only the most important component of cell population, colongenic cells, is studied. The number and viability of colongenic cells were directly associated with the growth and metastasis of tumor. In the present study, the number of pulmonary metastatic foci of B16 melanoma in BALB/C mice was high up to 192.00±92 in average in the control group. Either Tween-80 or hyperthermia 41 ℃ for 100 min. alone was not strong enough to decrease significantly the metastatic foci in the lungs. However we found the metastatic tumors reduced dramatically to 16.44±16 when B16 cells were treated previously by Tween-80 combined with hyperthermia 41 ℃ for 100 min. ($P<0.01$). This indicated further that Tween-80 could enhance antitumor effect of hyperthermia. The proliferative tumor cells were sensitive to combined action. This also suggested that Tween-80 combined with hyperhermia might influence the expression and function of integrins, the cell adhesion molecules on cell membrane, which might be related to the metastatic potential of tumor.[6] This would need further studies.

TNF, a cytokine from macrophage, is able to regulate human immune function and exerts antitumor effect. This effect depends on its membrane receptors on the target cells. In general, there were about 1000–2000 TNF receptors on each tumor cell. The number of the receptors is closely correlated with cell killing effect on TNF. As cell membrane is one of the main targets attacked, hyperthermia and Tween-80 must affect the binding site of TNF on the surface of tumor cell membrane. It was reported that ^{125}I-labeled TNF could be engulfed rapidly by binding to its membrane receptors and degraded within the cell by lysosome. ^{125}I activity disappeared at about 60 min. later. This process was accompanied by a serial biochemical changes, such as the phosphorylation of membrane proteins, activation of enzymes, heat production and DNA degradation etc., resulting in the damage and death of tumor cells.[7] The increase of sTNF level of tumor-bearing mice could be due to the tumor cell's stimulation to mononuclear macrophage system, which might secrete TNF and other cytokines. It was estimated that within 1–2 weeks, Tween-80, especially combined with hyperthermia 41 ℃ could act on TNF receptor to accelerate TNF to get into tumor cell by affecting the biological structure of tumor cell membrane. As a result, a great amount of serum TNF would be engulfed into tumor cell and kill the target cells and reasonably sTNF level would decrease apparently at the same time. As SA often binds to surface of tumor cell membrane. Its dropping might facilitate the exposure of tumor antigen and activation of immune system. The treatments of Tween-80 and heating increased the dropping of SA. In the combined group, the tumor regression caused the apparent decrease of SSA level.

The normal tissues withstood heating more than tumor tissue. It might be due to the differences in the components of cytoplasm membrane between normal cell and tumor cell.[8] In our experiments, 41 ℃ and Tween-80 itself were safe to mice. We kept the cover of incubator open during heating process and dried the fur of the mice immediately by electric dryer after heating. No mice died of heating or Tween-80 treatment during experments.

These results demonstrate that Tween-80 may make hyperthermia exert effective antitumor effect below the critical temperature and increase the safety of hyperthermia in treatment. It is one of the most ideal synergist with hyperthermia. The changes of STNF and SSA suggest that the synergetic effect could involve in facilitating the exposure of tumor antigen and activation of immune system.

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A REPORT OF A CASE OF MALIGNANT INSULINOMA WITH LIVER METASTASIS

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A 47-year-old man was admitted to our department because of progresive cardiopalmus, trumor of hands and diaphoresis at hunger for two months. Cerebrovascular disease was diagnosed for two times because of coma at night, and every times, the patient recovered immediately following drip treatment. At the beginning, CT scan revealed a normal brain and a doubtful pancreatic lesion. In the period of hospitalization, the patient repeatedly developed the symptoms of hypoglycemia, which always relieved in 3–5 minutes after eating sugar. The blood glucose was 1.6 mmol/L and serum insulin was 160 μ u/ml when the hypoglycemia was developing. Ultrasonic examination displayed abnormal area (2 cm in diameter) in the head and the uncinate process of pancreas and an enlarged liver and multiple parenchymal lesions in the liver. Futher CT scan showed hepatic enlargement and a huge mass at the posterior segment of the hepatic right lober, and multiple parenchymal lesions in the liver. The CT also showed enlargement of the head of pancreas with disturbance of structure and a mass (2×2 cm in size) with uneven enhancement in the uncinat process. Laboratorial examination: serum AFP was 54 μ g/L, CEA 8.8 μ g/L, ferritin 222 μ g/L. Needle biopsy of the liver showed some acinous structures which were composed of polygonal cells, like the chrysanthemum. The edges of the cells were not clear, the cytoplasm was seldom and transparent or light pink color, the nucleus is circular or oviform with the same size, its chromatin was stained uniformly without nucleole. The nucleus of the tumor cells were low-grade heteromorphy, and the pathologic mitosis can be found occasionally. There were a small number of vessels in the tumor and some compression atrophy of the hepatic tissues around the tumor. The pathology diagnosis was metastatic malignant insulinoma of liver. The patient and his families refused the operation and left our hospital. Twelve days later, he died.

Discussion

Malignant insulinoma with liver metastasis is a rare disease. There was only one case in 47 cases of insulinoma that were reported by Ruijin Hospital. It was also the only one case to be reported in our country before. It is difficult to distinguish the malig-nant insulinoma from the benign one in morphologory. The most reliable difference between them is metas-tasis of other organ. Some authors thought that the tumor should be resected, even the metastasis is present, because the progress of the tumor is slow. Although the operation is not a radical one in this condition, it can prolong the patient's life.

(Accepted August 10, 1996)