



Thermoseed hyperthermia treatment of mammary orthotopic transplantation tumors in rats and impact on immune function

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Abstract. To evaluate the effect of thermoseed inductive heating on mammary orthotopic transplantation tumors and immunologic function in rats. Walker-256 tumor cells were inoculated subcutaneously into the mammary glands of Wistar rats. Rats were allocated to five treatment groups as follows: i) C group (control group); ii) M group (magnetic field group); iii) T group (thermoseed control group); iv) H₁ group (hyperthermia treatment, 45°C for 30 min); v) H₂ group (hyperthermia treatment, 50-55°C for 10 min). Immediately, 12 and 24 h after hyperthermia, two rats were sacrificed in each group for pathological and immunohistochemical examination of the expression of PCNA and HSP70. Tumor volume was measured and long-term survival was observed. The T lymphocyte subgroup IL-2 and IFN- γ levels were measured in C, H₁ and H₂ groups. Both types of hyperthermia induced necrosis and apoptosis in the tumor tissue, decreased tumor volume ($P < 0.05$), and increased survival time ($P < 0.01$). The expression of PCNA and HSP70 in hyperthermia group was significantly different compared to the C, M and T groups ($P < 0.05$). Hyperthermia increased CD4⁺ T lymphocytes and the levels of IL-2 and IFN- γ ($P < 0.05$). Both types of hyperthermia can suppress the growth of mammary tumors and improve immunological function of rats.

Introduction

Thermoseed inductive heating arose from radiofrequency ablation (RF) technology but has advantages over radio frequency, microwave and ultrasonic wave methods. In this method, thermoseeds are implanted into tumors and then

hyperthermia is produced under an alternating magnetic field. The focused thermal energy on the tumor prevents damage to normal tissue, reduces heating time, shows uniform heat distribution and is minimally invasive (1,2). A single thermoseed can be heated repeatedly to produce therapeutic efficiency similar to surgery (3), with good efficacy and safety in animal experiments (4,5) and clinical research in brain tumors (6) and prostatic cancer (7,8).

Hyperthermia has been used for many years to treat a wide variety of tumors in both experimental animals and patients (9-11). It is reported that hyperthermia can directly induce cell damage, necrosis or apoptosis (11-13) as well as activate immunological function (10,11). Hyperthermia methods include conventional hyperthermia (42-45°C) (14), sub-hyperthermia (39.5-41°C) (15) and thermal ablation (>50°C) (16). Conventional hyperthermia and thermal ablation are useful for treating regional tumors. Ferromagnetic thermoseed hyperthermia has not been reported in mammary cancer treatment. In this study, we orthotopically transplanted Walker-256 mammary cancer cells to induce tumors in the rat mammary gland. Ferromagnetic thermoseeds at a Curie point of 61°C may reach 45°C for conventional hyperthermia or a Curie point of 78°C may reach 50-55°C for thermal ablation. We treated the mammary tumors with conventional hyperthermia or thermal ablation to evaluate the effect of heating on tumor growth and immunologic function of rats.

Materials and methods

Establishment of the animal tumor model. One hundred female Wistar rats, 6-7 weeks old, were provided by the Laboratory Animal Center of the Chinese Academy of Medical Sciences. The use of animals was conducted in compliance with the laws and regulations as well as the principles expressed in Guide for the Care and Use of Laboratory Animals of China. The Walker-256 tumor cell line was purchased from the Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College. Walker-256 cells were injected into the abdominal cavity, and one week later, the abdominal cavity was full of ascitic fluid. The ascitic fluid was centrifuged (3-18 K, Sigma) at 1200 rpm for 7 min and the tumor cells were diluted with physiological saline. The cell suspension (1×10^7 /ml, 0.2 ml)

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was subcutaneously injected into mammary glands of the right anterior part of chest. Seven days later, 89 rats whose tumors had reached 1.5-2.0 cm were randomly divided into 5 groups: C group (control group, $n=19$); M group (magnetic field group, $n=14$): rats was placed in a magnetic field (magnetic-induction hyper-thermal machine, frequency, 100 kHz; magnetic induction intensity, 130 Gs, Tsinghua University) for 30 min; T group (thermoseed control group, $n=14$): 2 thermoseeds (gold-plated, nickel-copper alloy; diameter, 0.8 mm; length, 0.6 cm, spaced at 1 cm; Institute of Metal Physics at the University of Science and Technology, Beijing) were implanted into tumors; H group (hyperthermia group; H_1 , $n=21$ and H_2 , $n=21$) had 2 subgroups: H_1 group received 3-4 thermoseeds (spaced 1 cm) with a Curie point of 61°C , and in H_2 group thermoseeds had Curie points of 78°C . The heating time was 30 min for H_1 group and 10 min for H_2 group.

Effect of hyperthermia on tumor-bearing rats. The influence of hyperthermia on tumor volume changes and survival time of rats was observed. The C, M and T groups had 8 rats each, and the H_1 and H_2 groups have 10 each. In H_1 and H_2 , the long axis of the implanted thermoseeds was parallel with the direction of the alternating magnetic field; three thermocouples (It-18 copper-constantan thermocouple manufactured by Physitemp, 1 mm diameter and 0.1 sec temperature response time) connected to a 4-channel temperature indicator (XS01A-4, Beijing Kunlun Tianchen Instrument Science and Technology Co., Ltd.) were used to monitor temperature changes in the tumor center, tumor edge and body temperature (rectal temperature). Tumor volume, defined as $V = (axb^2)/2$, where a is the maximum diameter horizontally and b is the maximum diameter vertically, was measured 14 days after hyperthermia. Survival time was also measured.

Pathological examination and immunohistochemical analysis. Immediately, 12 and 24 h after thermotherapy respectively, 2 rats were sacrificed in each group by neck dislocation, and tumor tissue specimens were fixed in 10% neutrally buffered formalin, then embedded in paraffin for routine H&E staining, immunohistochemistry for PCNA (provided by Beijing Zhongshan Goldenbridge Biotechnology Co., Ltd.) and HSP70 (provided by Wuhan Boster Biological Technology Ltd.). PCNA and HSP70 staining were quantified by staining intensity and the percentage of positive cells. Five areas were randomly selected for counting under 400-fold magnification (17). Staining intensity scores: 0, no color; 1, yellow; 2, brown; 3, dark brown. The percentage of positive cells was counted: 0, 0% positive cell; 1, $\leq 25\%$ positive cells; 2, $\leq 50\%$ positive cells; 3, $> 50\%$ positive cells; minimum value = 0, maximum value = 6.

Flow cytometry and ELISA analysis. Five rats came from C, H_1 and H_2 group, respectively. Fourteen days after thermotherapy, rats were sacrificed and peripheral blood was collected. Heparin was used to anticoagulate peripheral blood (1 ml), and density gradient centrifugation was used to centrifugate lymphocytes from 0.2 ml serum. Cell density was adjusted to 1×10^6 cells/ml, and T lymphocyte subgroups, $CD4^+$ and $CD8^+$ (eBioscience, San Diego, USA), were

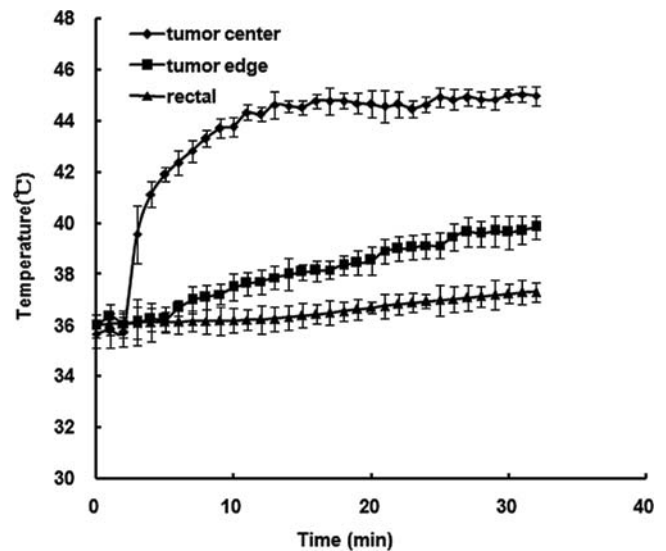


Figure 1. Temperature curve of H_1 group (thermoseeds with a Curie point of 61°C , heated for 30 min, heating operation began at the 2nd minute and ended at the 32nd minute), ($n=10$).

measured with a flow cytometer (Becton-Dickinson, USA). ELISA was used to measure the levels of IL-2 and IFN- γ in 0.2 ml serum (IL-2 and IFN- γ kits were purchased from R&D System). An ELISA reader was used to measure the OD450 (Bio-Rad 680, Hercules, CA) for calculation of sample concentration.

Statistical analysis. SPSS10.0 software was used for data analysis, and data were expressed as mean \pm SD. ANOVA analysis was used for multiple comparisons, but data with non-normal distribution and heteroschedasticity (e.g., a minimum-maximal value) were analyzed with the non-parametric rank-sum test. The Kaplan-Meier log-rank test was used to calculate survival rate. Significance levels were set at $P < 0.05$.

Results

Mammary cancer model in rats. Walker 256 cells produced large, homogeneous, invasive tumors in 89% of rats. Papillae was in the middle of the outstanding tumor, which was suitable for thermoseed implantation hyperthermia. Tumor cells invaded the breast tissue to produce hyperplasia and heteromorphic changes in breast epithelium (Fig. 5B).

Thermoseed temperature measurement after implantation. Tumor temperatures in H_1 started at $(35.7 \pm 0.6)^\circ\text{C}$, reached $(41.9 \pm 0.3)^\circ\text{C}$ after 3 min of heating, and then reached plateau at $44-45^\circ\text{C}$ (Fig. 1). Normal tissue in tumor edge heated slowly to $(39.8 \pm 0.4)^\circ\text{C}$, but rectal temperature remained at $36-37.3^\circ\text{C}$. The temperatures of tumor center in H_2 reached $51 \pm 0.5^\circ\text{C}$ after 5 min of heating and reached plateau at $54-55^\circ\text{C}$ (Fig. 2). The temperatures of normal tissue in tumor edge reached $39.4 \pm 0.4^\circ\text{C}$ at the end of heating and rectal temperature remained approximately at 36°C during hyperthermia.

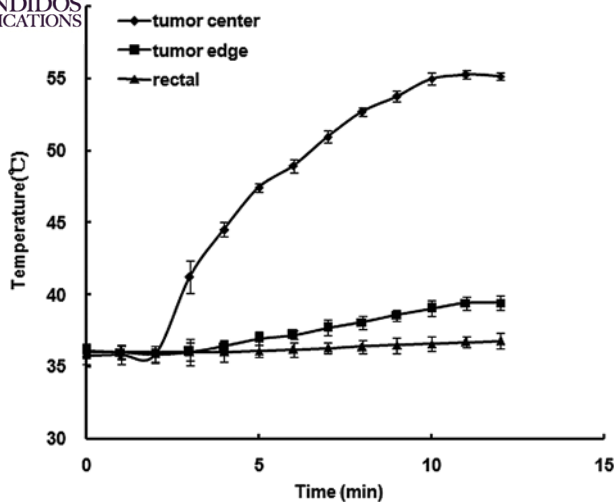


Figure 2. Temperature curve of H₂ group (thermoseeds with a Curie point of 78°C, heated for 10 min, heating operation began at the 2nd minute and ended at the 12th minute), (n=10).

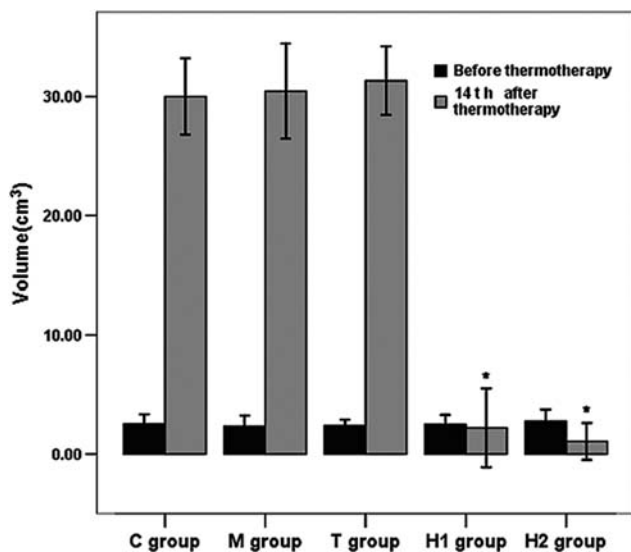


Figure 3. Tumor volumes before and after hyperthermia. *Compared with C, M and T group 14 days after the hyperthermia, $P<0.05$.

The effect of hyperthermia on tumors

Influence of hyperthermia on tumor volumes of rats. Tumor volumes were not different before magnetic-induction heating. Hyperthermia reduced tumor volume 14 days after heating, with H₁ (0-8.50 cm³), H₂ (0-4.37 cm³), C (26.25-35.52 cm³), M (25.04-36.53 cm³) and T (27.32-35.69 cm³) (H₁ and H₂ vs. C, M and T; $P<0.05$; Fig. 3).

Influence of hyperthermia on survival period of rats. As shown in Fig. 4, the survival period of tumor-bearing rats was observed for 390 days after tumor implantation. The tumor volumes of C, M and T groups rats grew rapidly and all the rats died by 47, 45 and 41 days, respectively. The survival period of C, M and T groups was (32.75±7.78), (32.00±7.39) and (29.88±5.82) days, respectively. Three rats

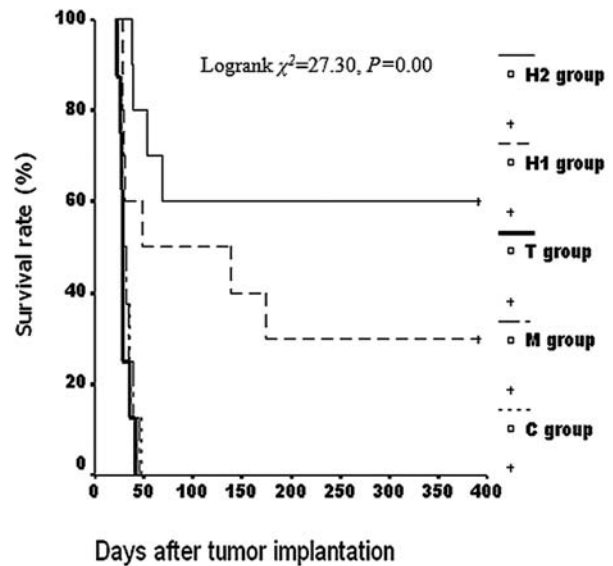


Figure 4. Survival rate after tumor implantation.

in H₁ group and 6 rats in H₂ group were cured and alive 390 days after tumor implantation ($P<0.01$ vs. C, M and T group, but no difference between H₁ and H₂ group).

Pathological observation and immunohistochemical analysis

Pathological observation. Cancer cells invaded mammary tissue and caused epithelial proliferation with multilayer, nuclear atypia changes (Fig. 5A, B). Tumor tissues in H₁ and H₂ group showed changes in coagulation and necrosis after heating, with acidophilic staining, loss of cytoarchitecture and nucleus, debris formation and morphologic variation such as nuclear pyknosis and chromatin agglutination and thickening (Fig. 5C). Tumor tissue showed high formation of blood vessels (Fig. 5D). A rat in H₁ group showed large amounts of tumor necrosis 24 h after hyperthermia, but still showed residual tumor cells at the tumor edge (Fig. 5E). One rat in H₂ group showed complete tumor necrosis 24 h after hyperthermia and necrotized mammary gland (Fig. 5F).

Immunohistochemical analysis. Nuclear and cytoplasmic HSP70 expression were high in all groups and increased immediately after heating, but decreased to lower levels than control groups ($P<0.05$) as tumor cells entered apoptosis and necrosis. Similarly, nuclear PCNA staining was initially high in tumor cells, but diminished immediately after heating to lower levels than control group ($P<0.05$) (Tables I and II).

Influence of hyperthermia on T lymphocyte subgroup. Heating increased CD4⁺ T lymphocytes more than C group ($P<0.05$), but the two hyperthermia groups were also different ($P<0.05$). The CD4⁺/CD8⁺ ratio for H₁ and H₂ group were higher than C group ($P<0.05$), and that of H₁ and H₂ group were also different ($P<0.05$; Table III).

Effect of hyperthermia on IL-2 and IFN- γ . Hyperthermia significantly increased IL-2 levels in serum, with H₁ group (26.27±2.80) pg/ml, H₂ group (23.38±3.67) pg/ml and C

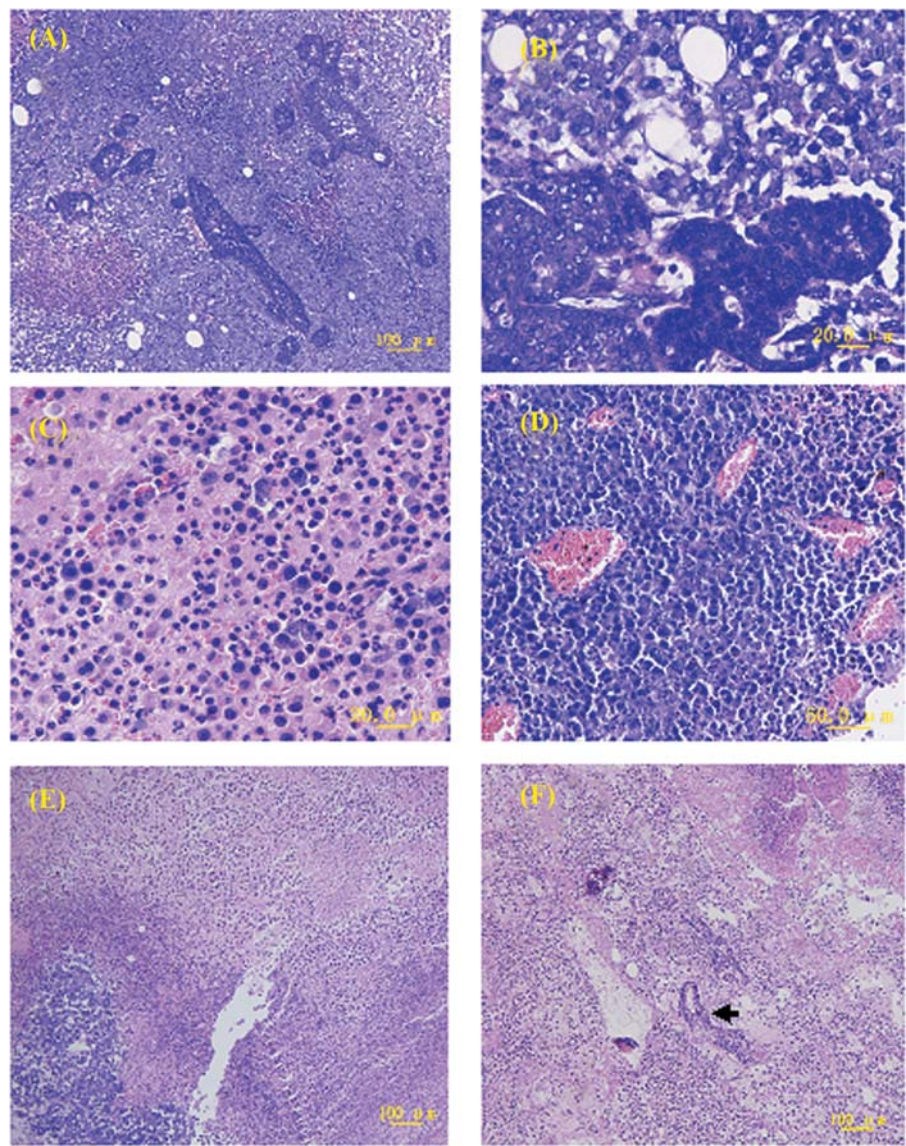


Figure 5. Pathologic changes in mammary tumor tissue. (A) Large tumor tissue invades the mammary gland; (B) Tumor tissue invades the mammary tissue, showing that mammary epithelia proliferate and nuclear atypia change; (C) Tumor tissue shows apoptosis and necrotizes immediately after heating treatment; (D) After 30 min of heating at 45°C, tumor tissue necrotizes in a large area and small vessels expand because of congestion; (E) Pathologic changes can be seen 24 h after heating at 45°C, the tumor necrotizes in a large area, leaving residual tumor tissue at the edge; (F) Complete tumor tissue necrosis at 24 h after treatment at 50-55°C, and necrotized mammary gland (arrow).

group (13.15 ± 3.11) pg/ml ($P<0.05$). Hyperthermia also

Table I. Expression of HSP70.

Groups	Immediately after thermotherapy	12 h after thermotherapy	24 h after thermotherapy
C	3.10 ± 0.88	2.90 ± 0.57	2.90 ± 0.57
M	3.10 ± 0.74	2.80 ± 0.42	2.80 ± 0.79
T	2.90 ± 0.88	3.10 ± 0.99	2.70 ± 0.82
H ₁	4.40 ± 0.97^a	1.50 ± 0.85^a	1.00 ± 0.67^a
H ₂	4.30 ± 0.95^a	1.10 ± 0.74^a	1.00 ± 0.82^a

^a $P<0.05$ compared with C, M and T groups in each time-point.

Table II. Expression of PCNA.

Groups	Immediately after thermotherapy	12 h after thermotherapy	24 h after thermotherapy
C	4.00 ± 0.82	4.20 ± 0.79	4.00 ± 0.94
M	4.20 ± 0.79	3.60 ± 0.70	3.80 ± 0.79
T	3.90 ± 0.99	3.70 ± 0.68	3.80 ± 0.92
H ₁	3.00 ± 0.82^a	1.10 ± 0.74^a	1.00 ± 0.67^a
H ₂	3.30 ± 0.95^b	1.00 ± 0.67^a	0.90 ± 0.74^a

^a $P<0.05$ compared with C, M and T groups in each time-point;

^b $P<0.05$ compared with M group immediately after thermotherapy.

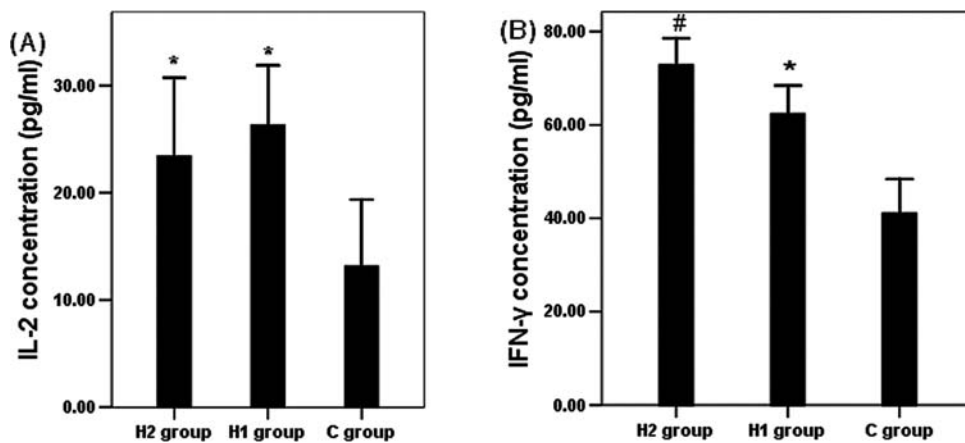


Figure 6. Cytokine levels in peripheral blood. (A) IL-2 levels in peripheral blood were detected by IL-2 ELISA kit. *P<0.05 compared with C group. (B) IFN-γ levels in peripheral blood were detected by IFN-γ ELISA kit. *P<0.05 compared with C group; #P<0.05 compared with C and H₁ group.

Table III. Comparison of T lymphocyte subgroups in peripheral blood.

Groups	CD4 ⁺ (%)	CD8 ⁺ (%)	CD4 ⁺ /CD8 ⁺
C	15.87±2.11 ^a	16.83±1.56	0.96±0.20 ^a
H ₁	28.62±4.21	16.99±1.29	1.68±0.18
H ₂	23.41±2.15 ^b	17.32±1.35	1.35±0.11 ^b

n=5, ^aP<0.05 compared with H₁ and H₂ groups; ^bP<0.05 compared with H₁ group.

increased serum IFN-γ levels, with H₁ group (62.33±3.01) pg/ml, H₂ group (72.78±2.88) pg/ml and C group (41.07±3.65) pg/ml (P<0.05). IFN-γ levels of H₂ group was significantly higher than H₁ group (P<0.05; Fig. 6).

Discussion

Progress in material science, temperature measurement technology (18) and magnetic field equipment allowed development of magnetic induction hyperthermia as a tumor treatment. Thermoseeds are a special alloy made from a non-magnetic substance and magnetic metal that restricts the maximum temperature by the Curie point. Thermoseeds with a narrow range of Curie points (generally several degrees Centigrade) produce better effects in tumor treatment. The gold-plated, nickel-copper alloy thermoseed could reach its Curie point temperature. Gold plating thermoseed have good biocompatibility, and the length (6 mm) allows accurate implantation with an implantation gun. Thermoseed treatment of malignant tumor of the biliary duct and advanced cancer of the esophagus are being pursued in clinical trials (19,20).

Mammary cancer is the most common malignant tumor in women in most developed countries. In China, the incidence

of mammary cancer increases at an annual rate of 3%, and is the most common malignant tumor in women in large cities (21). The therapeutic concept of mammary cancer has changed from 'maximum tolerated' to 'minimally effective' (22,23), and magnetic induction hyperthermia complies with this therapeutic mode. The mammary location allows thermoseeds to be implanted easily and located accurately. Thermoseeds are regarded as a new method to combined modality therapy. It is minimally invasive and displays a high safety profile. Thus, it is possible to prolong survival of patients and improve the quality of life.

We inoculated mammary cancer cells into the mammary gland to produce invasive tumors that induce mammary epithelial proliferation and nuclear atypia. Hyperthermia treatment caused tumor cells apoptosis and necrosis, with complete tumor tissues necrosis in some rats within 24 h, consistent with Tucker *et al* (3). Hyperthermia also decreased PCNA expression. PCNA is a cyclin critical for eukaryotic DNA synthesis that associates with DNA polymerase D and influences tumor metastasis (24). Hyperthermia can also improve immunological function as it induces tumor cells to produce antigenic HSPs (25). HSPs can produce a signal to activate dendritic cells and induce protective immunity (26). HSP70 can strengthen the reaction of CD4⁺ T cells to MHC II stimuli (27) by making T cells, macrophages and dendritic cells to infiltrate tumor tissue and stimulate production of the cytokines, IFN-γ, TNF-α and IL-12 (28). CD4⁺ T cells help generate an effective immune response with T helper 1 (Th1) and Th2 cells (29). CD4⁺ T cells perform immunological surveillance and regulation, and a consistent CD4/CD8 ratio maintains the balance of cell-mediated immunity. Therefore, the CD4/CD8 ratio is an important index of anti-tumoral immune function (30). Here, hyperthermia increased CD4⁺ T lymphocytes and the ratio of CD4/CD8 cells. Interleukin-2 (IL-2) is a potent stimulator of lymphocyte proliferation and cytotoxic T lymphocytes and used in cancer therapy to enhance cellular immunity and the cytotoxic activity of effector cells (31). IFN-γ can inhibit tumor growth, recurrence and metastasis by inducing apoptosis, inhibiting cell proliferation and differentiation, inhibiting tumor angiogenesis

and regulating immune responses (32,33). Hyperthermia increased levels of IL-2 and IFN- γ and Th1 cell activity (10,34). CD4⁺ T cells in H₁ group were higher than in H₂ group, but IFN- γ levels in H₂ group were higher than in H₁ group, potentially due to differences in treatment temperature. Specifics need further investigation. Three rats in H₁ group and 6 rats in H₂ group were cured, but other cases have developed local uncontrolled growth or recurrence. H₂ group seems to have advantage over H₁ group, which needs further research. Repeated hyperthermia may show longer-term inhibition of tumor growth and potentiation of immunological function.

Tumor temperatures reached 45°C in H₁ group and 55°C in H₂ group, temperature increased rapidly, but normal tissue did not exceed 40°C. This specificity and control of temperature is an advantage over other methods. Ultrasonic wave is not through gas-bearing cavity, it can be reflected and absorbed by bone. Radiofrequency show excess heating of subcutaneous fat, unexpected heating caused by edge effects and insufficient penetration (13). Thermoseed characteristics, such as self-controlled temperature, internal heating and repeatability, increase the accuracy and safety of tumor treatments by hyperthermia.

In conclusion, both thermoseed induction hyperthermia methods could inhibit tumor growth and improve immune function *in vivo*. Hyperthermia caused tumor tissues to undergo apoptosis and necrosis and reduced PCNA expression, indicating growth inhibition. Hyperthermia increased the population of CD4⁺ T lymphocytes, probably after up-regulation of HSP70, IL-2 and IFN- γ . Hyperthermia seemed well tolerated and reasonably safe, providing a basis for future clinical research on mammary cancer.

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