Original Contribution

Long Duration Mild Hyperthermia as Adjuvant Therapy
for Experimental Breast Tumors in Rats.

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Abstract : The feasibility and efficacy of long duration-low temperature whole body hyperthermia (LL-WBH, at
40°C for 6 h) as adjuvant therapy was investigated in rats bearing highly metastatic mammary adenocarcinoma
(MTLn 3). All animals in the non-surgery control group demonstrated a progression of axillary lymph node
metastases (ALM) on day 24 after inoculation. In a preoperative treatment study, 67% of the animals developed
ALM in both the treatment and control groups, however, the local recurrence rate was significantly suppressed to
0% in the treated rats compared to the control (67%) (p < 0.05). In a postoperative treatment study, 30% and 33%
developed ALM, in treatment and control groups, respectively (p < 0.01, compared to the non-surgery control),
however, the incidence of local recurrence was 40% and 11% for the treatment and control groups, respectively. No
significant differences were seen between any of the groups (20-55%) with respect to tumor cure. The authors thus
conclude that LL-WBH appears to be a valuable adjuvant therapy to surgery, however, an ideal therapeutic design
still needs to be developed for its clinical application. Therefore, additional studies of LL-WBH as an adjuvant
treatment to surgery are called for.

Key words : Mild hyperthermia, Adjuvant therapy, Local recurrence, Neoadjuvant therapy, Mammary
adenocarcinoma, Whole body hyperthermia.

Introduction

For the majority of primary solid malignancies, surgery is still the most reliable treatment modality. However,
local recurrence at the site of the primary tumor and regional lymph node metastasis after a curative resection remain
significant problems in the management of many human malignancies. In the case of human breast cancer, these
modalities of recurrence continue to cause substantial problems even after curative surgery.

Radiation and chemotherapy have been used as perioperative adjuvant therapy, and preoperative local
hyperthermia combined with radiation and chemotherapy has also been proven to be an effective treatment either to
prevent local recurrences and/or tumor cell spread during surgical procedures in the patients with rectal cancer \(^1\) or to improve the long term survival after operation in the patients with esophageal carcinoma \(^2\).

Long duration mild whole body hyperthermia (long duration-low temperature whole body hyperthermia; LL-WBH) at 40.0°C for 4-10 hours has been shown to have a significant antitumor activity in the experimental mammary adenocarcinoma in rats and has also been demonstrated to have few adverse effects in our experimental system \(^3\). We thus applied this long duration mild hyperthermia either before or after a curative resection of a primary breast tumor in the same rat experimental model, and thereby studied the feasibility and efficacy of this treatment modality as an adjuvant therapy for a surgical resection.

**Materials and Methods**

**Animals.**

Experiments were performed using Fischer 344 rats (Harlan Sprague-Dawley, Inc., Indianapolis, IN.) weighing about 100 g (6-7 weeks old) at the time of tumor cell inoculation. The rats were fed standard laboratory chow, were allowed free access to water, and were housed under controlled conditions with a 12 h light/dark cycle. All rats were allowed a 1-week environmental adaptation period before being used for the experiments.

**Tumor model.**

The rats were inoculated with \(5 \times 10^5\) MTLn3 cells (high passage; passage number 45) in 0.25 ml of alpha-modified minimum essential medium (AMEM, Life Technologies, Inc., Grand Island, NY.), subcutaneously under the left abdominal nipple in the region of the mammary fat pad, under light anesthesia with ether. Thereafter, solid tumors were palpable at the inoculated site in 100% of the rats by day 6 post-implantation. Subsequently, the rats were treated when the tumor size reached from 100-200 mm\(^3\) in size (about 10 days after inoculation). A microscopic examination of lymph nodes revealed no evidence of metastatic invasion at this time \(^4\).

The MTLn 3 tumor is one of the clones derived from 13762 mammary adenocarcinoma and possesses a high potential to metastasize spontaneously to either the regional lymph nodes or lung when inoculated in the mammary fat pad \(^5\). In this tumor model, axillary lymph adenopathy (metastasis) was detectable in 100% of the animals by 24 days following tumor inoculation.

Axillary lymph node metastasis was thus determined by palpation 24 days after inoculation, since axillary lymphadenopathy is easily accessible in 100% of the non-treatment controls \(^5\). Local recurrences were also easily assessed and usually appeared in the operative wounds.

**Whole Body Hyperthermia (WBH).**

WBH was induced by partially immersing the halothane anesthetized rats into a thermostatically controlled circulating water bath maintained at 40.0 ± 0.1°C for LL-WBH, by a Haake Model E 12 circulator/heater as described previously \(^6\). The rectal temperature was continuously measured in all rats treated and recorded every 5 min. An average of 15 min was required for the rectal temperature to reach 40.0°C. The rats were thereafter maintained 6 h at 40.0°C. General anesthesia of 1% halothane in pure oxygen as described previously \(^6\) was used and this anesthesia effected neither tumor growth \(^7\) nor normal tissue toxicity \(^6\). The anesthetized normothermia control
rats were maintained at 37.0°C for 6 h. Both the LL-WBH and/or 6 h anesthesia have been proved to have minimal toxicity in our experimental system. A thermistor probe was connected to a YSI model 4002 12 channel switch box and displayed on a YSI model 49 TA digital telethermometer (Yellow Springs Instrument Co., Yellow Springs, OH.). The probes were calibrated against a mercury thermometer (Ertco ASTM 64c) certified by the National Bureau of Standards.

Surgery.

The tumors that grew in the mammary fat pad were completely resected by a combined resection of the skin flap including the nipple under light anesthesia with ether on day 12 after inoculation. The skin defect was closed with the use of surgical staplers. These staples were removed 10 days after surgery. Neither any wound infection nor delayed healing was seen in this experiment. About half of the rats who underwent surgery on day 12 developed axillary lymph node metastases on day 24 after inoculation (unpublished data).

Experimental design.

The animals were randomly assigned to either a treatment or control group in each study on the treatment date. Preoperative treatment study: The animals in the treatment group first received LL-WBH on day 10 and then surgery on day 12. The rats in the control group received normothermic control treatment on day 10 and surgery on day 12. Postoperative treatment study: The animals in the treatment group first received surgery on day 12 and then LL-WBH on day 14. The rats in the control group received the same surgery on day 12 and normothermic control treatment on day 14. Non-surgery control: The animals in this control group received neither surgery nor treatment, and were only observed.

Statistics.

Significant differences in the incidence between groups was evaluated using Fisher's direct probability test.

Results

The data pertaining to axillary lymph node metastasis are shown in Table 1. All of the non-surgery controls developed axillary lymph node metastasis on day 24 after inoculation. Sixty-seven percent of the animals developed axillary lymph node metastasis on day 24 in the preoperative treatment study. In the postoperative treatment study, however, 3 of 10 (30%) and 3 of 9 (33%) developed axillary lymph

<table>
<thead>
<tr>
<th>Number of rats</th>
<th>Preoperative treatment study</th>
<th>Postoperative treatment study</th>
<th>Non-surgery control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Treatment</td>
<td>Control</td>
<td>Treatment</td>
</tr>
<tr>
<td>Number of rats</td>
<td>9 (67)</td>
<td>9 (67)</td>
<td>10</td>
</tr>
<tr>
<td>Axillary lymph node metastasis (day 24)</td>
<td>6 (67)</td>
<td>6 (67)</td>
<td>3 (30)</td>
</tr>
<tr>
<td>Detected date</td>
<td>20.0 ± 2.5</td>
<td>18.7 ± 2.4</td>
<td>20.0 ± 2.0</td>
</tr>
</tbody>
</table>

a, p < 0.01 compared to non-surgery control
node metastasis on day 24, for treatment and control groups, respectively (p < 0.01, compared to the non-surgery controls).

In the animals showing axillary lymph node metastasis, no differences were seen in the detected date between the groups.

The data pertaining to local recurrence and tumor cure are shown in Table 2. In the preoperative treatment study, local recurrence was seen in 6 of 9 (67%) of the control rats, whereas no rats developed local recurrence in the LL-WBH treatment group (p < 0.05). In the postoperative treatment study, however, the control rats showed a low incidence of local recurrences (1/9; 11%) while the treated rats showed a higher 4/10 (40%) local recurrence rate. In addition, no significant statistical difference was observed between the groups in the postoperative treatment study.

Regarding tumor cure, no significant differences were seen between the treatment groups (20-55%) in both studies.

Table 2. Local recurrence and tumor cure after each treatment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>Treatment</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of rats</td>
<td>9</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>Local recurrences</td>
<td>0 (0)</td>
<td>6 (67)</td>
<td>4 (40)</td>
</tr>
<tr>
<td>Tumor cure</td>
<td>3 (33)</td>
<td>2 (22)</td>
<td>3 (30)</td>
</tr>
<tr>
<td>Non-surgery control</td>
<td>0 (0)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Discussion

Nature’s systemic hyperthermia, fever, is one of the body’s defense mechanisms against infectious challenge. Mild hyperthermia at 40°C for several hours is just an induced fever and thus is expected to induce some antitumor action. Most early studies on hyperthermia aimed at higher temperatures (42-45°C) than the range of mild hyperthermia, based on the downward turn of the slope of the Arrhenius plot of thermotoxicity in the region of 43°C, which was originally shown by Westra and Dewey. Recently, some groups have shown that some human cell lines are still sensitive to mild heat for protracted long duration due to a lack of chronic thermotolerance. We thus showed the feasibility of administering mild hyperthermia for a long duration (up to 12 h) and thereby increased the antitumor activity of LL-WBH in the same animal model as that used in the current study. There are still few experimental investigations on long duration mild hyperthermia in vivo, due in part to its technical difficulties, and we conducted the present study to reveal the efficacy of LL-WBH on locoregional recurrence as an adjuvant therapy to surgical removal.

As a result, whether treatment group or control, lower axillary lymph node metastatic rates were seen in the postoperative treatment study compared to the preoperative treatment groups. In addition, the preoperative application of LL-WBH was significantly effective in preventing local recurrences compared to the controls but postoperative treatment was not. Osten et al. reported that WBH decreased the incidence of local recurrence in the animals treated with chemotherapy and an incomplete excision of the primary tumor but did not prevent regional nodal metastasis. Dalfen et al. also showed the effectiveness of radiofrequency regional hyperthermia as an adjuvant therapy following a surgical resection to prevent local recurrence but heat did not increase the tumor cure.
rate\(^{(13)}\). Their results are relatively compatible with our current data, however, in their study preoperative treatment did not seem to be profitable in the event of regional lymph node metastasis.

It has been reported that WBH at a relatively high temperature (41.5-42°C) is immunosuppressive in tumor bearing animals\(^{(14,15,16)}\), however, several other studies have shown that mild hyperthermia (38.5-40°C) in animals is accompanied by an increased immune response\(^{(17,18,19)}\). In our current study, the preoperative application of LL-WBH was effective in preventing subsequent local recurrences following surgery, however, it was not effective in preventing regional lymph node metastasis. It can thus be considered that the increased host defense mechanism induced by LL-WBH played a role in preventing subsequent local recurrences, whereas some factors associated with treatments and/or anesthesia may compromise this locoregional lymphatic system. Because of the discrepancy in the influence of WBH on the antitumor defense mechanisms, additional studies are thus called for to clarify these questions.

References
手術合併療法としての長時間軽温熱療法の意義
：ラット乳癌モデルにおける実験的検討

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要  旨：近年、温度を比較的下げた軽度の温熱療法（mild hyperthermia）が注目を集めている。また、全身温熱療法では高体温が生体に及ぼす副作用が大きな問題となるため、我々はこの軽度温熱療法を全身温熱療法に応用し動物実験で長時間（6時間）施行することにより抗腫瘍効果が得られることを報告した。今回、長時間軽温熱療法（LL-WBH, 40；6時間）の手術合併療法としての有用性をラットの高転移性乳癌モデル（MTLn 3）を用いて検討した。非手術対照群の動物は全例腫瘍接種後24目目に腋窩リンパ節転移をきたした。術前治療実験では腋窩リンパ節転移は温熱療法治療群、対照群ともに67％の動物で認められたが、局所再発は温熱療法群で0％対照群の67％に比し有意に抑制された（p < 0.05）。術後治療実験では腋窩リンパ節転移は療法群、対照群でそれぞれ30、33％と両群間に差はなかったが、非手術群に較べると有意に低値であった（p < 0.01）。また局所再発率は治療群、対照群でそれぞれ40、11％と温熱療法の効果は認められなかった。腫瘍の完全治癒は20～55％に認められたが各群間では有意差は認められなかった。以上、LL-WBH手術的治療における術前合併療法として有用と思われるが、臨床応用のためにはまだ治療計画などの工夫が必要である。今後さらにこの領域での研究が望まれるところである。