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# Lorus study report

# STUDY REPORT

## *Preclinical Efficacy Evaluation of Oncothermia (LAB-EHY Hyperthermia Device) in a Human Pancreatic Xenograft Tumor Model in Mice*

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1

# Preclinical Efficacy Evaluation of Oncothermia (LAB-EHY Hyperthermia Device) in a BxPC-3 Human Pancreatic Xenograft Tumor Model in Mice

## Purpose

To evaluate the therapeutic efficacy of Oncothermia (LAB-EHY) in the treatment of BxPC-3 human pancreatic adenocarcinoma xenograft in CD-1- nude mice.

## Materials and Methods

### *Cell line*

Human pancreatic adenocarcinoma cell line (BxPC-3) was grown as monolayer culture in minimum essential medium ($\alpha$-MEM) supplemented with 10% fetal bovine serum (FBS), 0.1 mM non-essential amino acid, 1.0 mM sodium pyruvate, 100 U/ml penicillin, 100 $\mu$g/ml streptomycin, 0.25 $\mu$g/ml amphotericin B and 2mM L-alanyl-l-glutamine at 37°C in an atmosphere of 5% $CO_2$ in air. The tumor cells were routinely subcultured twice weekly by trypsin-EDTA treatment. The cells were harvested from subconfluent logarithmically growing culture by treatment with trypsin-EDTA and counted for tumor inoculation.

### *Tumor Inoculation*

An acclimation period of at least 7 days was allowed between animal receipt and commencement of tumor inoculation. When the female CD-1 mice were 7 weeks of age (~25 g), each mouse was subcutaneously injected at the right flank with $5.5 \times 10^6$ BxPC-3 human pancreatic adenocarcinoma cells in 0.1 ml of PBS to induce tumor growth.

### *Treatments*

The following treatment (or control) conditions were evaluated for this experiment.

Group 1: Untreated Control (n=10)
Group 2: Oncothermia (n=10)

After the tumor size reached an approximate volume of 50 $mm^3$, treatments are initiated. Each group contained 10 tumor bearing mice.

## *Oncothermia Treatment*

Method of treatment and dosing schedule and duration were as described (see Results and Discussion). Test articles were provided from Oncotherm Kft., including 1) Lab-EHY device 2) IPITEK temperature monitoring device.

## *Anesthetic Regimen*

The mice were treated with Lab-EHY device under general anesthesia. A cocktail mix of Ketamine and Xylazine was used at a dosage of 100 mg/kg and 10 mg/kg, respectively. The drug mixture was formulated as follows: 1.0 ml Ketamine (100 mg/ml conc.) + 0.5 ml Xylazine (20 mg/ml conc.) + 3.5 ml sterile distilled water. The dose volume was administered (IP) intraperitoneally to each mouse before treatment. Anesthesia was induced and maintained for a good 30 minute period. No adverse reactions were observed during recovery from anesthesia.

## *Endpoints*

The major endpoint was to evaluate the anti-tumor effects of Oncothermia (EHY). Antitumor activity was estimated by the inhibition of tumor volume which was measured at intervals as described (Results and Discussion) in two dimensions using a caliper, and the volume was expressed in $mm^3$ using the formula: *$V = 0.5 (a \times b \times h)$, where a, b and h are the long, short diameters and height of the tumor,* respectively. Mean tumor volumes calculated from each measurement were then plotted in a standard graph to compare the anti-tumor efficacy of Oncothermia (EHY) treatments to that of untreated control. On Day 72, tumors were excised from the animals and tumor weights (TW) were measured. A standard bar graph is used to demonstrate the differences in tumor weights with each bar representing mean tumor weight calculated from 10 animals. Percent inhibition of tumor growth is determined using the formula: [(mean TW of controls – mean TW of treated group)/mean TW of controls) x100]. Body weights were measured at intervals as described (Results and Discussion) to assess toxicity.

## *Termination Procedure and Statistical Analysis*

The mice were sacrificed by neck dislocation at termination of the trial on Day 72. Body and tumor weights of each mouse were determined. Statistical differences in tumor volumes between control and treatment group were assessed as described (Results and Discussion). A p-value of $\leq 0.05$ is considered to be statistically significant. Error bars in the figures represent the standard error of the sampling distribution of the means.

## Results and Discussion

This study report describes an experiment intending to evaluate the efficacy of hyperthermia therapy using Lab-EHY (Onco-therm) device for the treatment of human pancreatic tumors in mice. Twenty female 7 wk old CD-1-nude mice were subcutaneously implanted with 5.5 x $10^6$ Bx-PC3 cells /mouse on Day 0. The mice were divided into Test and Control groups with 10 mice each. On Day 7 post-tumor cell inoculation, treatment with Lab-EHY device was started. The treatment conditions used were as follows: First Cycle: Power: 6 watts; time exposure: 30 min; and water cooling system in place during treatment. Second Cycle: Power: varies from 7.0 to 8.0 watts; time exposure: 30 min; and water cooling system optional. The treatment set-up was followed according to the instruction of Prof. Szasz. The first cycle was performed on Days 7, 9, 11, 14, 16, 18 and the second cycle was performed on Day 30, 35, 37, and 49. There was no adverse skin injury observed after the first cycle of treatment. In contrast, there were moderate to severe skin burns observed in three out of the ten mice treated due to higher power output being tested during the second cycle of treatment. The affected mice were given an analgesic, buprenorphine, at 0.1 mg/kg SC to relieve the pain and stress from the treatment. The body weight and tumor sizes were measured three times a week during the treatment period and continued on after the treatment cycles were completed. The experiment was terminated on Day 72 when endpoints were reached in accord with the Animals for Research Act and guideline of the Lorus Animal Care Committee. The results indicate that the tumors treated with the Lab-EHY device in the test group had shown a 73% and 66% tumor growth inhibitions based on mean tumor volume and excised tumor weight measurements, respectively, when compared to the untreated control group on Day 72 (Table 1 & Figure 1&2). A statistical analysis to compare the difference between the initial and final tumor volume measurements was performed using TTEST and showed a p-value = 0.046. Moreover, the total weight of excised tumors in the control group was considerably bigger than that of the treated tumors (Figure 2). There was no significant body weight change observed between the treated and control groups at the end of the study (Figure 3). Likewise post-mortem examination revealed no difference in the gross morphology and condition of internal organs between the two groups. These observations suggest that hyperthermia may be an effective mode of treatment of xenograft tumors in mice in this particular study, however, more studies should be done to optimize the treatment conditions and minimize the onset of severe skin burns.

**Table 1. Measurements of Tumor Volumes (mm3) on Day 72**

| Group | Length | Width | Height | Final Vol. | - Initial Vol. | Difference* |
|---|---|---|---|---|---|---|
| Treated Mice | | | | | | |
| 1 | 0.00 | 0.00 | 0.00 | 0 | 63 | -63 |
| 2 | 3.86 | 2.85 | 1.78 | 10 | 74 | -64 |
| 3 | 9.57 | 7.86 | 6.86 | 258 | 63 | 195 |
| 4 | 8.01 | 6.57 | 5.03 | 132 | 31 | 101 |
| 5 | 10.73 | 10.12 | 5.09 | 276 | 63 | 213 |
| 6 | 9.65 | 8.19 | 4.41 | 174 | 63 | 111 |
| 7 | 0.00 | 0.00 | 0.00 | 0 | 63 | -63 |
| 8 | 3.96 | 2.90 | 1.96 | 11 | 54 | -43 |
| 9 | 3.50 | 3.25 | 1.65 | 9 | 63 | -54 |
| 10 | 2.00 | 1.65 | 0.68 | 1 | 41 | -40 |
| | | | Mean | 87 | | |
| | | | SD | 113 | | |
| Control Mice | | | | | | |
| 1 | 5.50 | 5.34 | 3.34 | 49 | 74 | -25 |
| 2 | 12.57 | 10.40 | 8.73 | 571 | 41 | 530 |
| 3 | 4.31 | 3.79 | 2.45 | 20 | 45 | -25 |
| 4 | 8.66 | 7.93 | 7.81 | 268 | 63 | 205 |
| 5 | 13.92 | 12.58 | 11.45 | 1003 | 80 | 923 |
| 6 | 3.85 | 2.93 | 1.90 | 11 | 63 | -52 |
| 7 | 11.92 | 9.38 | 8.80 | 492 | 50 | 443 |
| 8 | 13.87 | 10.63 | 9.10 | 671 | 58 | 613 |
| 9 | 2.97 | 2.00 | 0.00 | 0 | 50 | -50 |
| 10 | 9.05 | 7.08 | 6.07 | 194 | 38 | 157 |
| | | | Mean | 328 | | |
| | | | SD | 343 | | |

* The difference between control and treated groups was statistically significant $p \leq 0.046$

Based on the results, it is apparent that the tumors in both the control and treated groups were not behaving uniformly with some tumors growing aggressively and others regressing in size over time. As a result, higher standard deviations were obtained than anticipated. For statistical analysis, the initial tumor volumes were subtracted from the final measurements and analyzed by TTEST using the Excel Program. Percent growth inhibition was obtained by subtracting the mean tumor volume of the treated (87) from the control (328) and dividing by the control mean and multiplying by 100.

**Figure 1. Tumor Growth Curves (Treated vs Control)**

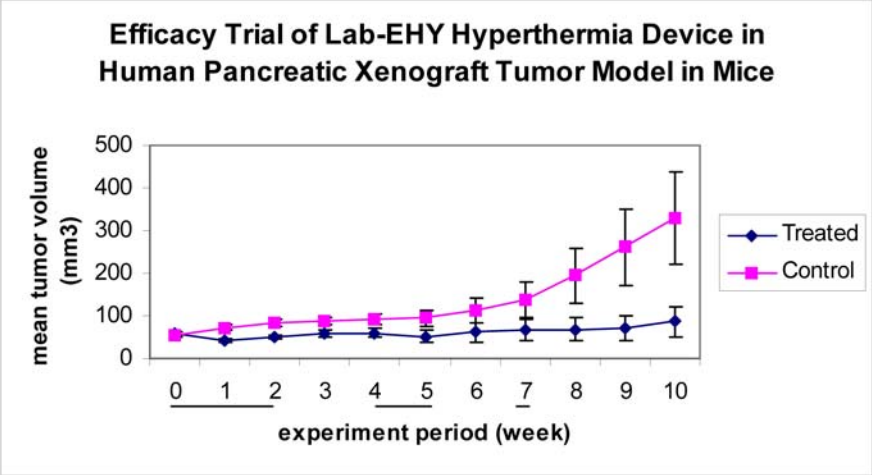
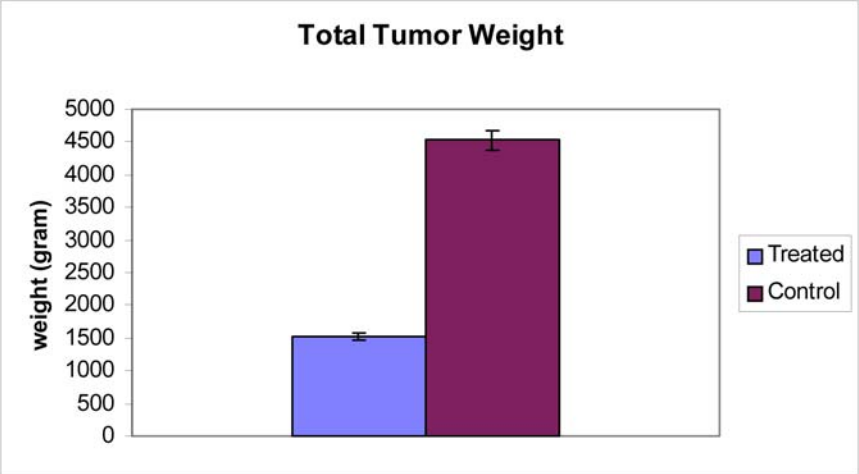
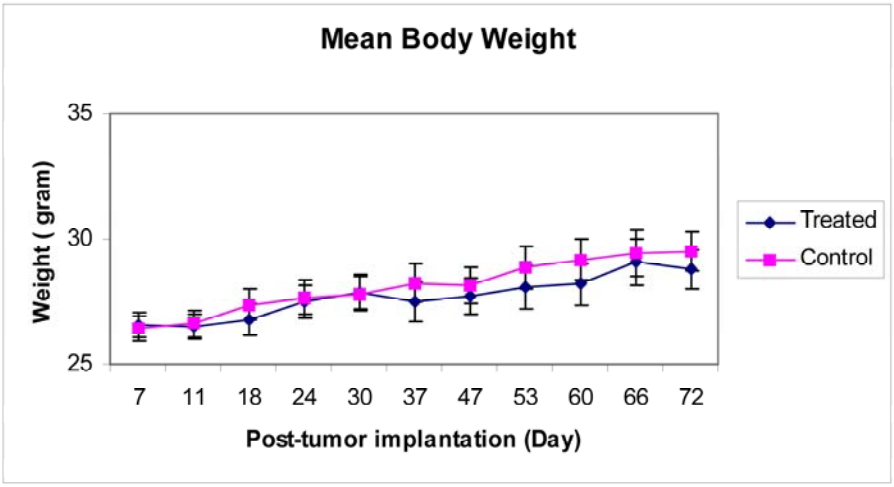



**Figure 2. Excised Tumors**




On Day 72, mice were sacrificed and their tumors excised and weighed. There was a big difference observed in the total weight of excised tumors between control and treated groups. A 66% tumor growth inhibition was observed based on excised tumor weights.

**Figure 3. Body Weight Change**




There was no significant mean body weight change observed between the control and treated mice during the entire duration of the study.

Table 2 describes the setting parameters (power output and time exposure) for treatment cycle 1 and treatment cycle 2 and indicates the mice which had registered the readings for Lab-EHY device and IPITEK temperature monitoring device, respectively. A small commentary was included describing the observations during the conduct of the experiment.

Table 3 indicates the highest temperature readings achieved within the 30 minute duration of the treatment. Two temperature sensors were used, one was placed in the adjacent skin area of the tumor and the other was placed in the rectum of a mouse being treated. The data showed a variation in the level of hyperthermia achieved in each mouse and between treatment sessions and on different days of treatment.

Figure 4 illustrates the treatment set-up for the trial which was demonstrated by Prof Szasz during his visit to the animal facility. The top picture shows the LAB-EHY device linked to a computer laptop during treatment cycle 1 when IPITEK temperature monitoring device was not available. The bottom picture shows the set-up with the IPITEK device linked to the computer during treatment cycle 2. The pictures also show actual mice undergoing hyperthermia treatment on closer view.

**Table 2: HYPERTHERMIA TREATMENT REGIMEN:**

| | Treatment Conditions w/ Lab-EHY : | | | | | | Comments: |
|---|---|---|---|---|---|---|---|
| **Cycle 1** | **Power: 6.0W Time: 30 min**<br>**Cooling system: On**<br>**IPITEK Thermoscope: none**<br>**Treatment Day:** | | | | | | Day 7 and 9, I had problem getting hyperterminal connection. We had to try a different R232 cable. When a connection was achieved, the hyperterminal reading of S11 during treatment was not consistent hence, only a small number of mice had readings saved on file (as shown with *). However, the LCD panel of Lab-EHY device was consistently displaying S11 reading for each treated mouse. There was no adverse skin reaction observed (e.g.burn,edema, swelling,etc.)after treatment. I later found out with the use of thermoscope that this conditions resulted in increase of adjacent skin temperature to a maximum of 36ºC. With the cooling system in place, the skin temperature went down to about 34ºC. |
| **CD-1 Nude Mice** | **7** | **9** | **11** | **14** | **16** | **18** | |
| **1** | | | | * | * | * | |
| **2** | | | * | * | * | * | |
| **3** | | | * | * | | | |
| **4** | | | | * | | | |
| **5** | | | | | * | | |
| **6** | | | | | | | |
| **7** | | | | * | | | |
| **8** | | | | | * | * | |
| **9** | | | | | | * | |
| **10** | | | | | | | |
| | * mouse which registered Lab-EHY-Hyperterminal readings of S11 | | | | | | |
| | | | | | | | |
| **Cycle 2** | **Power: 7.0-7.5-8.0 W Time: 30 min**<br>**Cooling system: Off**<br>**IPITEK Thermoscope: In-use**<br>**Treatment Day:** | | | | | | **Comments:** |
| | **30** | **35** | **37** | **49** | | | A consistent IPITEK-hyperterminal connection was achieved hence, temperature readings were saved on file for all mice except for one reading. Because the laptop computer can only handle one connection, I chose IPITEK so I can monitor the induction of hyperthermia.I also gradually increased the power output to try to reach an adjacent skin temperature of 42ºC. The use of cooling system was limited- only to prevent temperature reaching over 42ºC. With these conditions, severe adverse skin reactions (e.g. burn,edema,swelling,discoloration, etc.) were observed on two mice on Day 49 so treatment was discontinued. |
| **1** | | ✓ | ✓ | ✓ | | | |
| **2** | ✓ | ✓ | ✓ | ✓ | | | |
| **3** | ✓ | ✓ | ✓ | ✓ | | | |
| **4** | ✓ | ✓ | ✓ | ✓ | | | |
| **5** | ✓ | ✓ | ✓ | ✓ | | | |
| **6** | ✓ | ✓ | ✓ | ✓ | | | |
| **7** | ✓ | ✓ | ✓ | ✓ | | | |
| **8** | ✓ | ✓ | ✓ | ✓ | | | |
| **9** | ✓ | ✓ | ✓ | ✓ | | | |
| **10** | ✓ | ✓ | ✓ | ✓ | | | |
| | ✓mouse which registered IPITEK-Hyperterminal temperature readings | | | | | | |

**Table 3.**

| Highest Temperatures (ºC) Recorded During Second Cycle Treatment | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Day 30 | | Day 35 | | Day 37 | | Day 49 | |
| Lab-EHY-treated mice | Adjacent Skin area | Rectal | Adjacent Skin area | Rectal | Adjacent Skin area | Rectal | Adjacent Skin area | Rectal |
| # 1 | | | 36.7 | 37.7 | 38.0 | 37.3 | 37.5 | 36.6 |
| # 2 | 34.4 | 36.0 | 39.4 | 36.0 | 35.9 | 35.8 | 39.1 | 36.3 |
| # 3 | 36.5 | 37.2 | 39.0 | 36.5 | 41.9 | 37.5 | 42.1 | 38.0 |
| # 4 | 41.11 | 37.5 | 41.6 | 36.7 | 37.7 | 37.0 | 38.3 | 37.0 |
| # 5 | 42.16 | 39.2 | 40.3 | 36.7 | 37.4 | 35.8 | 42.4 | 38.7 |
| # 6 | 40.00 | 37.0 | 42.9 | 37.5 | 37.7 | 37.0 | 43.0 | 36.5 |
| # 7 | 33.15 | 32.0 | 40.8 | 38.0 | 38.7 | 37.1 | 42.8 | 38.7 |
| # 8 | 38.0 | 37.7 | 42.4 | 38.5 | 37.0 | 32.8 | 39.2 | 36.5 |
| # 9 | 42.4 | 38.0 | 40.0 | 37.14 | 40.3 | 38.0 | 42.4 | 37.9 |
| # 10 | 39.7 | 38.2 | 40.1 | 38.25 | 41.3 | 37.5 | 39.8 | 37.0 |
| | | | | | | | | |

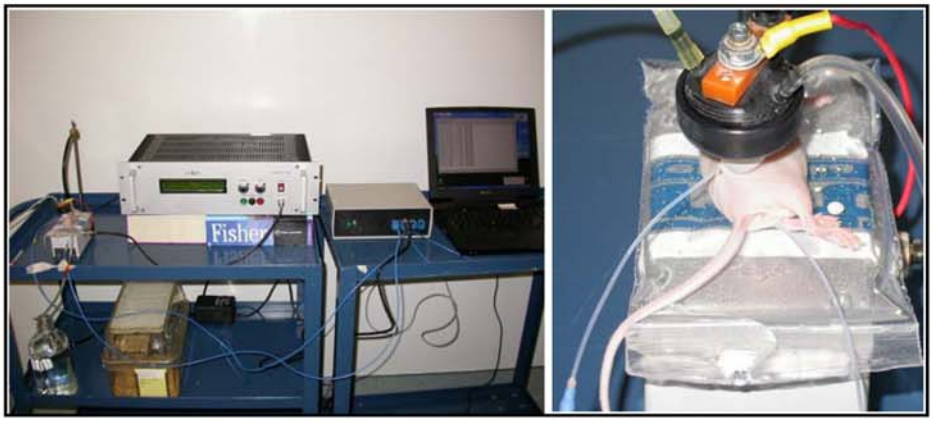
## Treatment Set-Up

On April 11, 2007, Prof. Szasz visited the Animal Facility at Lorus Therapeutics, Inc. to demonstrate the treatment set-up for the use of Lab-EHY device. Although a test run was not performed due to technical problems with the laptop computer and the IPITEK device at that time, sufficient instructions and information were provided to carry out the proper set-up and actual experiment on schedule. Figure 4 illustrates the set-up for the hyperthermia treatment with the Lab-EHY or IPITEK device linked to a laptop computer.

Figure 4.

Lab-EHY-Treatment Set-Up

Lab-EHY- Treatment and IPITEK Thermoscope Set-Up



10

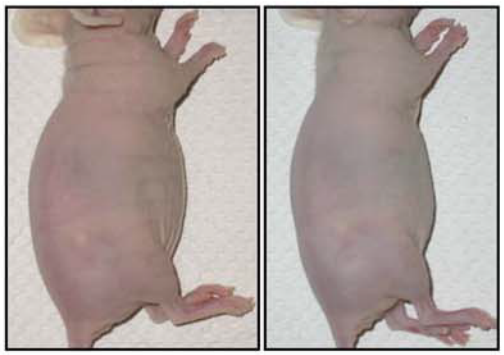
## Clinical Observations :

Each mouse was observed for adverse skin lesions after treatment. In these examples, the skin over the tumor area where hyperthermia was induced did not result in major skin burns during treatment cycle 1. Figure 5 shows 3 mice with no marked difference in the skin area over the tumor before and after treatment.

**Figure 5**

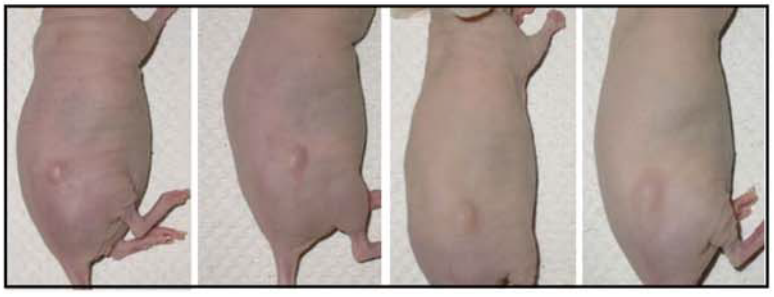
Lab-EHY Treatment: Power 6.0W , Time 30 min

Before After



Lab-EHY Treatment: Power 6.0W , Time 30 min

Before After Before After



11

However, during cycle 2, wherein higher power output was tested, moderate to severe skin burns were observed in three mice out of the ten mice treated. Figure 6 shows a mouse with moderate skin burn with skin discoloration, reddening of the adjacent area, and minor swelling.

**Figure 6.**

Lab-EHY Treatment: Power 7.0-7.5-8.0W , Time 30 min

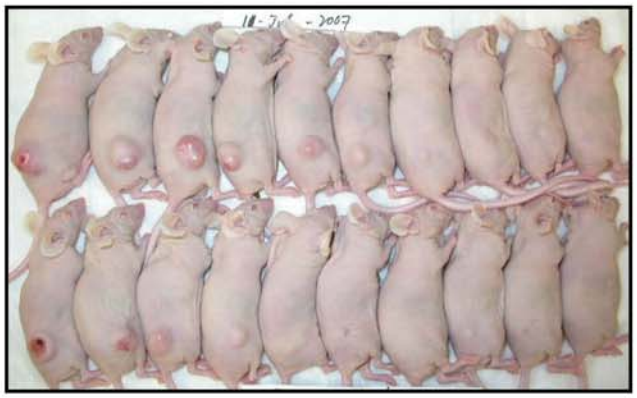
Moderate skin lesions: skin burn, hyperemia, mild edema,and swelling

In summary, there were a total of 10 treatment doses administered to each mouse (6 doses in cycle 1 and 4 doses in cycle 2) using the Lab-EHY device. The mice responded well to the treatment during cycle 1 and no adverse side effects were observed. Tumor growth inhibition was found to be significant when tumor volumes were compared between the control and treated groups on Day 22 when cycle 1 was concluded ($p \leq 0.0005$) and 40% growth inhibition was achieved (Data not shown). Because the tumors at this stage were not growing aggressively as expected, it was advised to extend the efficacy trial period and to administer a second cycle of treatment. Cycle 2 provided opportunity to explore the advantage of higher power output because the availability of the IPITEK temperature sensing device allowed a close monitoring of the induction of hyperthermia. In addition, it was observed that the power output used in cycle 1 (Power: 6.0W and 30 min) did not result in temperature elevation above 37°C in the adjacent skin area when monitored by the IPITEK device. Cycle 2 treatment doses induced hyperthermia in the adjacent skin area at power ranging from 7.0W to 8.0W during the 30 min dosing administration. However, three ouf of ten mice suffered moderate to severe skin burns as described previously. Further

studies should be done to optimize the tumor growth rate, treatment conditions, treatment schedule and regimen to carefully assess the therapeutic value of Lab-EHY device. However, despite the first-time experience with the device and the preliminary stage of the study, it was observed that the use of Lab-EHY device for treatment of human pancreatic tumors in mice in this study could bring about efficacious benefits. Figure 7 depicts the tumor size difference between the control and treated groups as seen visually in-situ at the conclusion of the study.

**Figure 7. Gross tumor sizes (Treated vs Control)**

In-Vivo Efficacy Evaluation of Oncothermia (Lab-EHY) in Human Pancreatic Tumor Model in Mice



Control

Treated

## Recommendations

1. The mice should be carefully monitored individually during treatment because each mouse responds differently inspite of similar treatment conditon (power level and time exposure) and may result to skin burns in more sensitive animals at higher power output.
2. Further studies should be done to optimize the treatment conditions such that hyperthermia could be induced (42ºC) without necessarily causing skin burns. Factors like the tumor size, the water balloon size (electrode), and type of contact between the skin and the electrode may play a role.
3. In an optimized experimental trial, at least two technicians should be present to monitor simultaneously the LAB-EHY device and the IPITEK temperature sensing device. Two laptop computers are preferable to record the readings (one for each device).
4. Lab-EHY device should be checked why it was not consistently registering readings with the hyperterminal link.

**Appendix** (To be sent on a separate electronic file)

I. Raw data 1) tumor volume measurements
2) body weight measurements
3) excised tumor weights
II. Lab-EHY readings
III. IPITEK temperature readings.

**This study report was prepared by:**

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**Approved by:**

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