Modulated electro hyperthermia inhibits tumor progression in a triple negative mouse breast cancer model

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1 Institute of Clinical Experimental Research, Semmelweis University, Budapest

Introduction
The effective therapy of triple-negative breast cancer (TNBC) has not yet been achieved. Modulated electro-hyperthermia (mEHT) is a novel adjuvant antitumor therapy, based on the highly selective heating of the tumor tissue by a 13.56 MHz radiofrequency current induced electric field.

Aims
Our aim was to investigate the effects of repeated mEHT treatment in a triple-negative mammary carcinoma bearing mouse model.

Methods
4T07 cells were inoculated orthotopically in female BALB/c mice. Tumor growth was monitored in vivo by digital caliper and ultrasound (Phillips Sonos 5500). The mEHT (n=8) or sham (n=9) treatments started 7 days after inoculation and were repeated 5 times, on every other day. Mice were euthanized 1 day after the fifth treatment and the tumors were dissected, weighed and processed for histology and molecular biology techniques. The ratio of the damaged area compared to the whole tumor area (Tissue Destruction Ratio, TDR) was evaluated on H&E and cleaved caspase-3 stained sections, while HSP70, a common damage-associated molecular signal, Ki67, a proliferation marker and p21, a tumor suppressor protein expression were analyzed on immunohistochemical staining with the HistoQuant module of the CaseViewer Software (3DHistech).

Results
There was a significant decrease in tumor growth (sham: 5.7x, mEHT: 2.4x relative to pre-treatment (day 6) size, p<0.0001) and weight (sham: 288.3±58.1 mg vs mEHT: 85.3±21.3 mg, p<0.05) in the mEHT treated group, compared to the sham group. The HSP70 stained area in the non-destructed tumor tissue was 5.2 fold higher in the mEHT treated group, compared to the sham group (p<0.05). Moreover, the Ki67 positive nucleus / mm2 count was significantly lower (sham: 2823.4±211.9 pcs/mm2 vs mEHT: 1736.7±315.3 pcs/mm2, p<0.05) and the p21 positive nucleus / mm2 count showed increasing tendency (sham: 127.0±25.3 pcs/mm2 vs mEHT: 242.2±78.2 pcs/mm2, p = 0.073) in the mEHT treated group, compared to the sham group.

Conclusion
Our findings suggest, that repeated mEHT could lower tumor cell proliferation by promoting cell cycle arrest in vivo. Thus, mEHT could be a possible alternative adjuvant therapeutic strategy for TNBC cancer patients. We plan next generation sequencing to elucidate the biological mechanism behind the effects of mEHT.

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36th Conference of the International Clinical Hyperthermia Society

NVKP-16-1-2016-0042 project

Modulated electrohyperthermia

Highly-selective heating of the tumor

Szász et al. (2011) Oncothermia – Principles and Practice

Oncothermia Journal, Volume 24, October 2018
TRIPLE-NEGATIVE BREAST CANCER (TNBC)

Molecular Subtypes of Breast Cancer
Berrocal et al. (2017) AJHO, 13(6):16-19

ER/PR – Estrogen/Progesterone receptor
Her2 – Human Epidermal growth factor Receptor

Isogenic clones of a spontaneous mouse triple-negative breast cancer
- Different metastatic potential
  67NR < 168FARN < 4T07 < 4T1

Dykxhoorn D, Lieberman J: Plos ONE, 2009
Korpal et al: Nat Med
Treatment settings:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heating pad temperature</td>
<td>37-38 °C</td>
</tr>
<tr>
<td>Skin temperature</td>
<td>40 °C</td>
</tr>
<tr>
<td>Rectal temperature</td>
<td>37-38 °C</td>
</tr>
<tr>
<td>Power</td>
<td>0.7±0.3 W</td>
</tr>
<tr>
<td>Time</td>
<td>35 min</td>
</tr>
</tbody>
</table>
ONE-TREATMENT PROTOCOL

Female Balb/C mice (N = 14)

1X modulated electrohyperthermia
LabEHY-200
40°C 30min

10⁶ 4T1-GFP-mCherry-Luciferase
TNBC cells / 100 µl PBS:Matrigel

<table>
<thead>
<tr>
<th>Day after inoculation</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
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<tbody>
<tr>
<td>4T1 cell inoculation</td>
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<td></td>
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<td>IVIS</td>
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<tr>
<td>Ultrasound</td>
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<td>x</td>
<td>x</td>
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<td>mEHT</td>
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<td>x</td>
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<tr>
<td>Harvest</td>
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<td></td>
<td></td>
<td>x</td>
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</tr>
</tbody>
</table>

RESULTS – one treatment

Total flux (p/s)

\[ \text{Total flux} = 2.0 \times 10^8 \]

\[ p = 0.1221 \]

Day

Caliper

Ultrasound

Tumor volume (mm³)

mEHT vs Sham+Ctr

Oncothermia Journal, Volume 24, October 2018  447
TWO-TREATMENT PROTOCOL

Female Balb/C mice (N = 12)

2X modulated electrophyperthermia
LabEHY-200
40°C 30min

10⁶ 4T1-GFP-mCherry-Luciferase
TNBC cells / 50 μl PBS:Matrigel

<table>
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<th>Day after inoculation</th>
<th>0</th>
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<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
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</thead>
<tbody>
<tr>
<td>4T1 cell inoculation</td>
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<tr>
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</tr>
</tbody>
</table>

RESULTS – two treatments

![Graph showing Total Flux (p/s) over Day]

- **Total Flux (p/s)**
  - mEHT
  - Sham

![Graph showing Ultrasound analysis]

- **Ultrasound**
  - Sham
  - mEHT

![Graph showing Digital caliper analysis]

- **Digital caliper**
  - Sham
  - mEHT

![Graph showing Tumor weight analysis]

- **Tumor weight**
  - Sham
  - mEHT
RESULTS – two treatments

Tissue destruction ratio

Cleaved Casp3

relative Masked Area (%)

SHAM

MEHT

HSP70 – damage associated molecular marker

SHAM

MEHT
## Immune profile of TNBC isografts

### Expression of checkpoint inhibitors

<table>
<thead>
<tr>
<th>CTLA4 expression</th>
<th>PD-1 expression</th>
<th>PD-L1 expression</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="Bar graph for CTLA4 expression" /></td>
<td><img src="image2" alt="Bar graph for PD-1 expression" /></td>
<td><img src="image3" alt="Bar graph for PD-L1 expression" /></td>
</tr>
</tbody>
</table>

**CTLA4**: Cytotoxic T-lymphocyte-associated protein 4  
**PD-L1**: Programmed cell death protein 1  
**PD-L1**: Programmed cell death ligand 1

### FIVE-TREATMENT PROTOCOL

**Female Balb/C mice (N = 18)**

1. **5X modulated electrohyperthermia**  
   LabEHT-200  
   40°C 30min

2. **10⁶ 4T07 TNBC cells / 50 μl PBS**

<table>
<thead>
<tr>
<th>Day after inoculation</th>
<th>-1</th>
<th>0</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
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<th>11</th>
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<th>13</th>
<th>14</th>
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<tbody>
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<tr>
<td>Tumor size (US, caliper)</td>
<td>X</td>
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<td>X</td>
<td>X</td>
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<td>mEHT</td>
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<td>X</td>
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</tbody>
</table>

**NVKP-16-1-2016-0042 project**
RESULTS – five treatments

Tumor volume (mm³)

Days

Sham
mEHT

RESULTS
five treatments

HSP70 – damage associated molecular marker

HSP70

Relative Mask Area (%)

Sham mEHT

p=0.0176

p=0.0206

Oncothermia Journal, Volume 24, October 2018
RESULTS

**Ki67** – proliferation marker

**HSP70** – damage associated molecular marker

*after two treatments*  
*after five treatments*

**Sham:** 4, 14, 2, 15, 12, 16, 10, 7, 9,

**mEHT:** 18, 8, 1, 13, 2, 17, 11, 9,

**Sham:** 4, 14, 2, 15, 12, 16, 10, 1, 9,

**mEHT:** 18, 8, 1, 13, 2, 17, 11, 9

**Ki67**

- **Sham:** 4000 nuclei/mm²
- **mEHT:** 2000 nuclei/mm²

$p = 0.0116$

**Sham:** 4000 nuclei/mm²

**mEHT:** 2000 nuclei/mm²
RESULTS
five treatments

p21 - common cyclin-dependent kinase inhibitor

Summary

Short-term effects

- tissue damage (TDR, cCasp3)
- tumor cell death (IVIS)
- no reduction in tumor size with traditional methods (US, caliper) but with IVIS and TDR (weight and volume)

Long-term effects

- heat-shock (Hsp70)
- decrease tumor cell proliferation (Ki67)
- Inhibit tumor growth (weight and volume)
Thank you for your kind attention!

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MD, PhD, postdoc

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MD, PhD student

Zita Zolcsák
MD, resident

Bettina Farkas
Pharm. student
TDK

Hentriett Mesterházi
Pharm. Student
TDK

NVKP-6-1-2016-0042 project