Strong Synergy of Heat and Modulated Electromagnetic Field in Tumor Cell Killing

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Background and Purpose: Hyperthermia is an emerging complementary method in radiooncology. Despite many positive studies and comprehensive reviews, the method is not widely accepted as a combination to radiotherapy. Modulated electrohyperthermia (mEHT; capacitive, electric field modulated, 13.56 MHz) has been used in clinical practice for almost 2 decades in Germany, Austria and Hungary. This in vivo study in nude mice xenograft tumors compares mEHT with "classic" radiative hyperthermia (radHT).
Material and Methods: Nude mice were xenografted with HT29 human colorectal carcinoma cells. 28 mice in four groups with seven animals each and two tumors per animal (totally 56 tumors) were included in the present study: group 1 as untreated control; group 2 treated with radHT at 42 °C; group 3 treated with mEHT at identical 42 °C; group 4 treated with mEHT at 38 °C (by intensively cooling down the tumor). 24 h after treatment, animals were sacrificed and the tumor cross sections studied by precise morphological methods for the respective relative amount of "dead" tumor cells.
Results: The effect of mEHT established a double effect as a synergy between the purely thermal (temperature-dependent) and nonthermal (not directly temperature-dependent) effects. The solely thermal enhancement ratio (TER) of cell killing was shown to be 2.9. The field enhancement ratio (FER) at a constant temperature of 42 °C was measured as 3.2. Their complex application significantly increased the therapeutic enhancement to 9.4.
Conclusion: mEHT had a remarkable cancer cell-killing effect in a nude mice xenograft model.

Key Words: Hyperthermia · Modulated electric field · Tumor treatment · Bioelectromagnetics

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Ausgeprägte Synergie zwischen Hyperthermie und moduliertem elektromagnetischem Feld bei der Abtötung von Tumorzellen

Hintergrund und Ziel: Die Hyperthermie ist eine aufstrebende ergänzende Therapie in der Radioonkologie. Trotz zahlreicher positiver Studien und umfassender Reviews ist diese Methode immer noch nicht als Kombination zur Radiotherapie anerkannt. Die modulierte Elektrohyperthermie (mEHT; kapazitiv mit moduliertem elektrischem Feld, 13,56 MHz) wird seit fast 2 Jahrzehnten in Deutschland, Österreich und Ungarn klinisch angewandt. Die vorliegende In-vivo-Studie vergleicht in einem Xenograft-Nacktmaus-Tumormodell die mEHT mit der „klassischen" radiativen Hyperthermie (radHT).
Material und Methodik: Nacktmäuse wurden mit humanen kolorektalen HT29-Tumorzellen xenotransplantiert. 28 Mäuse in vier Gruppen zu je sieben Tieren mit zwei Tumoren pro Tier (gesamt 56 Tumoren) wurden in diese Studie einbezogen: Gruppe 1 als unbehandelte Kontrollgruppe; Gruppe 2 behandelt mit radHT bei 42 °C; Gruppe 3 behandelt mit mEHT ebenfalls bei 42 °C; Gruppe 4 behandelt mit mEHT bei 38 °C (durch intensive Kühlung des Tumors). 24 h nach der Behandlung wurden die Tiere getötet und die Tumorquerschnitte morphologisch auf den jeweiligen Anteil „toter" Tumorzellen untersucht.
Ergebnisse: Die Behandlung mit mEHT zeigte eine doppelte Wirkung als Synergie zwischen dem ausschließlich thermalen (temperaturabhängigen) und dem nichtthermalen (nicht direkt temperaturabhängigen) Effekt. Folgende Faktoren wurden gemessen: die durch alleinige Hyperthermie bedingte Verstärkung der Zellzerstörung („thermal enhancement ratio" [TER]) mit dem Faktor 2,9; der alleinige Feldverstärkungseffekt („field enhancement ratio" [FER]) bei konstanter Temperatur von 42 °C mit dem Faktor 3,2; die Kombination beider Effekte mit einem signifikant erhöhten Faktor von 9,4.
Schlussfolgerung: Die durch ein moduliertes elektrisches Feld (13,56 MHz) erzeugte mEHT hatte in einem Nacktmaus-Xenograft-Tumormodell einen ausgeprägten tumorzellabtötenden Effekt.

Schlüsselwörter: Hyperthermie · Moduliertes elektrisches Feld · Tumorbehandlung · Bioelektromagnetismus

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Introduction

Despite the long, more than 2,000-year history of hyperthermia, it is not a widely acknowledged treatment yet. Numerous questions were formulated in the medical literature about its applications in oncology [25, 44]. The real challenges are, of course, the controversial results, which address a lot of further questions and raise the doubts.

The thermal (temperature-dependent) and nonthermal (non-temperature-derived) effects are simultaneous driving forces of the complex effect of hyperthermia. The temperature-determined thermal equilibrium characterizes the requested dynamism for the cellular distortions of the tumor. The nonthermal chemical and physiological machinery make more than only increase the temperature in the actual volume.

Hyperthermia is applied simultaneously with radiotherapy [43] or radiochemotherapy [24] or would well be suitable in innovative regimens [31, 32]. The goal of hyperthermia is to enhance radioefficacy. Preradiative hyperthermia in the hypoxic tumors could enhance blood perfusion (oxygenation) boosting the radioefficacy [11]. In good blood flow conditions radiotherapy has to be applied first and postradiative hyperthermia finishes the cell damages. In both cases, the control [10] and localization [15] of hyperthermia are crucial.

Cell distortion by hyperthermia needs selectivity to avoid considerable damage in healthy tissue. The selection is realized by the applied radiative or field-conducted energy-transfer techniques. Both the heat therapy (thermal) and the electric field applications (nonthermal) have a long history [33, 46]. The task of selection is definitely technical: to accurately deliver energy to deeply situated malignant tissues [37]. There are numerous indications that the electric properties of the malignant tissue differ from their healthy counterpart [30]. The bioimpedance is an investigated [14, 27] and applied diagnostic [6, 17, 28, 34] method to select the tissues. On this way the electric field could select the tissues and so could play an essential role in tumor regression [19, 20]. On the other hand, the electric fields could promote proliferation [13], can cause differentiation [2] as well as re- [1], and dedifferentiation [7], and could also control cellular division [8, 16].

Yet few studies discuss the biological mechanisms involved in electromagnetic field-induced hyperthermia [18, 32]. However, the bioeffect of electric field remains to be a hot topic in science [23, 35].

The objective of our study is to detect and evaluate the possible thermal and nonthermal effects and document the synergy of temperature and applied electric field in the cell-death rate in a xenograft model.

Material and Methods

Tumor Model

Experimental animals were female nude BALB/c (nu/nu) mice provided by the Division of Animal Experiments of the National Research Institute for Radiobiology, Budapest, Hungary. All animals were maintained in a sterile environment, sterilized food and water was provided ad libitum. All animals were kept on daily 12-h light/12-h dark cycle. The animals were 6–8 weeks old and weighed 22–25 g at the time of tumor induction. All studies were approved by the local animal experimentation committee and were carried out in compliance with national guidelines for the care and use of experimental animals.

The xenograft was an HT29 human colorectal carcinoma cell line. The cells were maintained in DMEM + GlutaMax, high-glucose (4.5 g/l) medium (GIBCO, Invitrogen, Carlsbad, USA), supplemented with 10% heat-inactivated fetal calf serum (FCS, GIBCO, Invitrogen) and gentamicin (10 µg/ml). Cells were grown in 75-cm^2 tissue-culture flasks (BD, Falcon, Franklin Lakes, USA) incubated at 37 °C with 5% CO_2 in humidified air.

Cells were harvested with 0.25% trypsin + EDTA (GIBCO, Invitrogen) of subconfluent monolayers, were washed once in DMEM serum-free medium and counted. Cells were resuspended in serum-free medium to achieve the desired cell concentration.

Each mouse was subcutaneously injected at the femoral region on both sides with 6×10^6 cells in 0.1 ml of medium to induce tumor growth on both sides, symmetrically. Animals were used for experiment 18 days after tumor inoculation, when the tumor volume had reached approximately 0.5–0.8 cm^3. For the experiment we only used animals, which developed their tumors symmetrically and at approximately the same size. No animals were paralyzed by the growing tumors.

Hyperthermia Treatment

The radiative hyperthermia (radHT) treatment was provided by infrared radiation (IR) emitter fitting to tumor sizes. It was composed of a 5 cm in diameter round-shaped IR-A reflector and eight tiny infrared-emitting bulbs. The radiated energy was controlled by the measured temperature, adjusted to keep the steady-state temperature plateau in the tumor during treatment. Temperature of the treatment pad was kept at 37 °C to avoid the mice get cooled down.

Electric Field Heating Treatment

The modulated electrohyperthermia heating (mEHT) was made by capacitive arrangement, using the fractal physiology achievements [41, 42]. The mice were placed in between the plan-parallel electrodes in asymmetric arrangement [3]. Electrode sizes were 72.0 cm^2 (lower) and 2.5 cm^2 (upper), respectively; the upper electrode was located precisely above the treated tumor with flexible arm. Permanent electric controls of the tumor were made by radiofrequency (RF) current in a resonant circuit. The electrodes were coated with water boluses to equalize the surface inequalities. The treatment pad was temperature-controlled like in the hyperthermia case. The upper electrode was cooled by precisely controlled Peltier units. The applied radiofrequency electromagnetic field (RF-EMF)

was provided on 13.56 MHz with modulation (Oncotherm®, Hot Oncotherm GmbH, Troisdorf, Germany). An ultrafast tuning system enabled the sudden automatic adjustments. Sampling frequency of the actual software was 60/min. The treatment setup is shown in Figure 1.

The actual temperature of mEHT treatment was adjusted by the absorbed RF power. In the case of low-temperature mEHT, the tumor was cooled down by the upper bolus. The intensive cooling kept the tumor near the physiological temperature (38 °C) while the mEHT field was identical with the previous heating conditions.

Temperature Measurement

Temperature measurements were done (a) intratumorally in both tumors, (b) systemically (rectal), and (c) on the outer surface over the treated tumor in each animal. The temperature was accurately measured in real time by a metal-free fiber-optic temperature measurement system (m3300, Luxtron Corp.. Santa Clara, USA; sensing accuracy ~ 0.1 °C, repetition accuracy ~ 0.4 °C). The precise equivalence of the actual temperature in all phases of the processes were managed by regulating the applied energy. The heating-up and cooling-down periods were also kept identical to control the dynamic physiological parameters, like the synthesis of heat-shock proteins.

Treatment Groups

Four experimental groups were formed: each group had seven animals with 14 tumors in pairs.

Group 1 was the untreated control group. The animals in this group did not receive any kind of treatment.

Group 2 was treated by radHT reaching and keeping 42 °C in the left-sided tumor, while the right-sided tumor was kept untreated.

Group 3 was devoted to the same 42 °C temperature on the left-sided tumor, but heated by mEHT. The identical temperature history was carefully managed within the experimental accuracy.

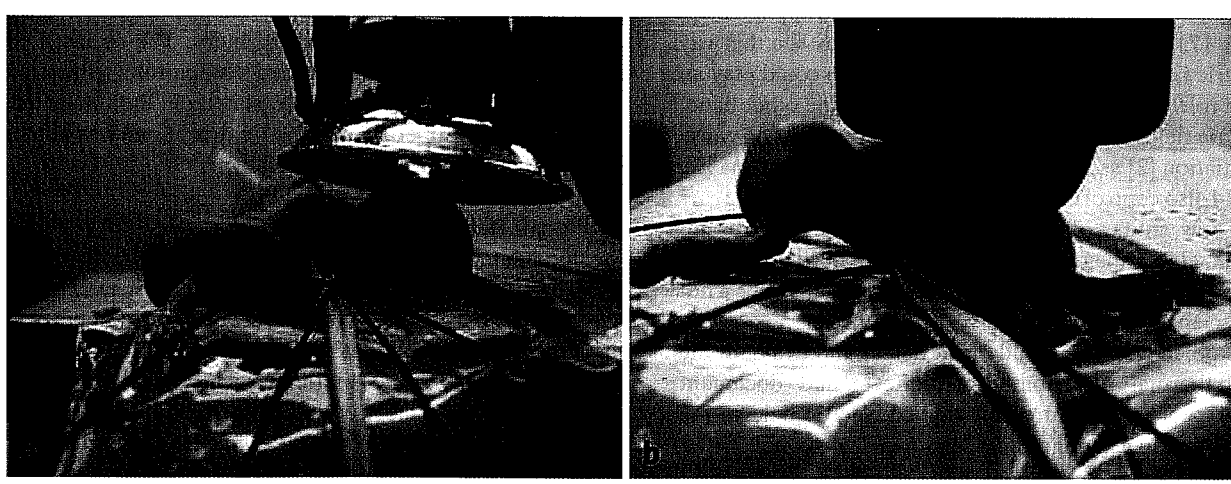
Group 4 was treated by mEHT, identically with group 3 animals, only the treated tumor was cooled down to 38 °C.

Treatments were systematically made only on the left tumor of the animals, while the right was kept for individual control. Only one single treatment was applied for clear comparison. All treatments were provided for 30 min with an average power of 4 W. The reason to have group 1 was to have an entirely untreated control group for validation of the method. The rectal temperature of group 1 was 37.5 °C, while that of the other groups was 38 °C due to the treatment of one tumor side. For this reason we had chosen the intratumoral temperature for group 4 as 38 °C.

Evaluation Method

Animals were sacrificed 24 h after treatment and both the control and treated tumors were removed and studied in pairs. The removed tumors were cut accurately at their centerline, and the tumor volume was calculated. After fixing the samples in 4% buffered formalin, all histological samples were stained with hemalum-eosin.

Morphological comparison of the pathologic differences was used to calculate the cell-killing rate of the treatments. Histological tumor samples were imaged using dedicated slide scanner (MiraxScan, 3DHISTECH Ltd., Budapest,



Figures 1a and 1b. Experimental setup: only the left xenotransplanted tumor was treated with radHT (infrared; a), or with mEHT (b). Temperature sensors inserted in both transplanted tumors, in the rectum, and on the surface of the treated tumor.

Abbildungen 1a und 1b. Versuchsanordnung: Jeweils nur der linke xenotransplantierte Tumor wurde mit radHT (Infrarot; a) oder mit mEHT (b) behandelt. Temperaturmesssonden in beiden Transplantattumoren, im Rektum und an der Oberfläche des behandelten Tumors.

Hungary). Microscopic analysis was used to distinguish the living and the dead tumor cells, based on the staining differences of the destroyed cells from their intact counterparts. No differentiation was done to distinguish apoptotic from necrotic cells [22]. The detection and marking of the vivid/dead cell areas were performed by morphology-analyzing software (MiraxViewer, 3DHISTECH Ltd., Budapest, Hungary). The whole area of the tumor cross section was defined as 100%, the differently stained dead cell areas were defined in respective percentage to compare the change of the dead part in the control and treated tumor originated from the same animal.

In addition to the standard descriptive statistics, data were evaluated by Wilcoxon's matched pairs signed-ranks test. The coincidence limit was chosen for 95%.

Table 1. Control tumors (without treatment) and their parameters of comparison: group 1 (n = 14) and groups 2–4 (n = 21); respective intratumoral temperature, respective dead cell areas as ratio of the total areas of the respective whole-tumor cross sections in percent (± 95% confidence interval [CI]) measured histomorphologically.

Tabelle 1. Kontrolltumoren ohne Behandlung und deren Vergleichsparameter: Gruppe 1 (n = 14) und Gruppen 2–4 (n = 21); jeweilige intratumorale Temperatur, kein moduliertes elektrisches Feld, jeweiliger Flächenanteil toter Zellen im Verhältnis zur Gesamtschnittfläche des jeweiligen Tumors in Prozent (± 95%-Konfidenzintervall [CI]).

Parameter	Group 1 n = 14 Left	Right	Both	Groups 2–4 n = 21 Only left
Intratumoral temperature (°C)	37.5	37.5	37.5	38.0
± 95% CI	0.3	0.3	0.3	0.4
Modulated electric field	No	No	No	No
Average of dead cell ratio (%)	6.1	5.2	5.7	6.1
± 95% CI	3.3	2.9	2.1	2.5

Results

Control Tumors

Sizes of the bilateral tumors were statistically equal, the p-value was > 0.57 in all groups. The percentage of the dead cell areas to the full tumor size areas was 5.7% ± 2.1% in all 14 tumors of group 1, and 6.1% ± 2.5% in the 21 untreated tumors in groups 2–4 (Table 1). All control tumors were considered identical ($p > 0.72$). The Pearson correlation of the individual differences between the bilateral tumors was 0.88 and the dead cell ratio was identical on both sides ($p > 0.93$; [$W^+ = 15$, $W^- = 13$]) in group 1, which validated the evaluation method. A histomorphological pattern of a typical control tumor is shown in Figure 2a.

Treated Tumors

To calculate the enhancement of the dead cell ratio in the different treatment groups 2–4, the percentage of the dead cell area was compared to their respective own control tumors: the dead cell area percentage of the nontreated (normothermia) reference tumor was deducted from the treated tumor in the same animal. This value was used to characterize the efficacy of the actual treatment. Calculation was done for all animals. The average percentage of the dead cell areas to the full tumor size areas in groups 2, 3, and 4 was 17.9%, 57.1%, and 45.9%, respectively. The differences between the treated and untreated tumors were statistically significant ($p < 0.02$). The histomorphological patterns of typical dead cell areas are shown in Figures 2b to 2d. Results and therapeutic gain are collected in Tables 2 and 3, as well as comparatively shown for groups 2–4 in Figure 3.

Discussion

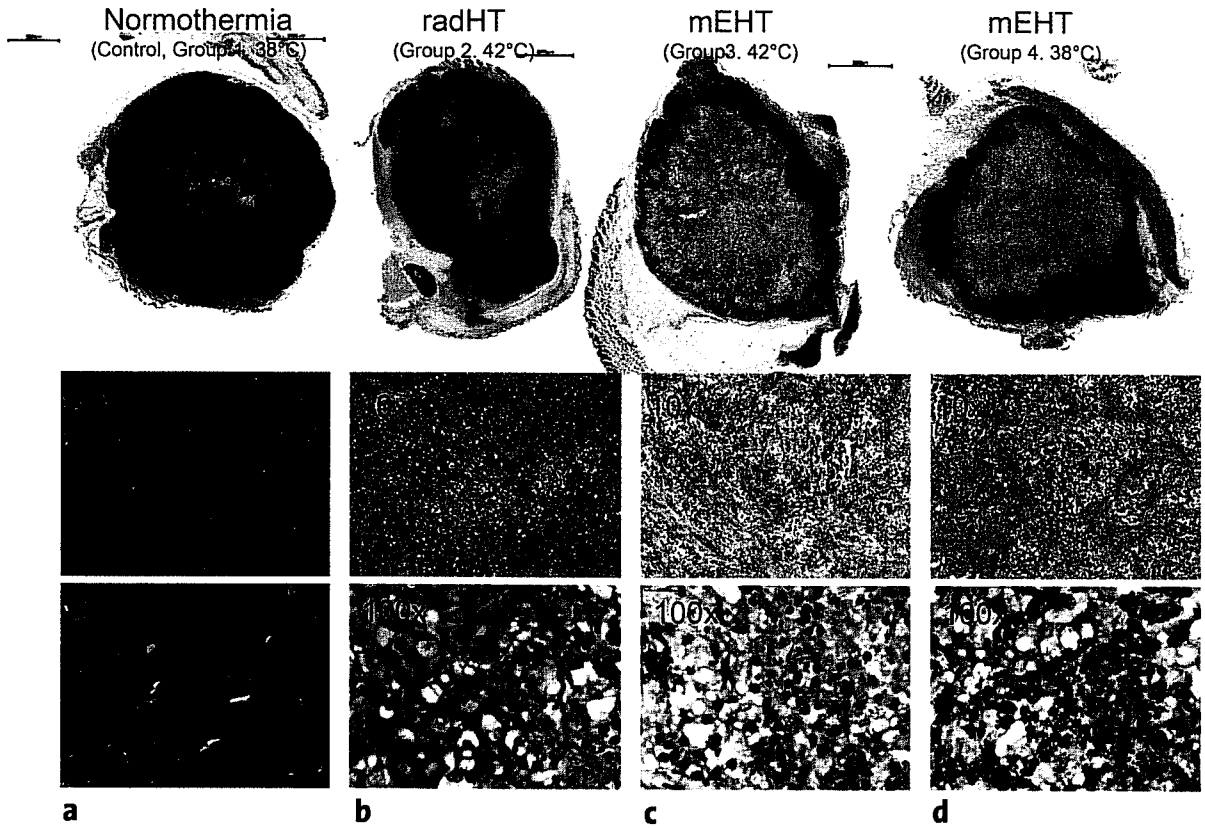
The above results show clear differences between the temperature-dependent and field-dependent cell-killing effects by hyperthermia in an HT29 xenograft tumor model in nude mice.

The thermal enhancement ratio (TER) of the tumor cell-killing process was introduced by Overgaard [26]. The TER in our experiments without field application is 2.9 at a temperature of 42 °C (Table 3).

In addition, we have to introduce a field enhancement ratio (FER), whose effect was theoretically predicted [40, 45]. The mEHT enhances the TER at a constant temperature of 42 °C by further 3.2 (Table 3). Consequently, the combination of heat and field at a temperature of 42 °C enhances the cell killing by 9.4. The efficacy could be controlled by absolute gain with comparison of the cooled mEHT experiment excluding the temperature effect (Tables 2 and 3 and Figure 3).

Applications of hyperthermia in oncology [9, 21] and in radiooncology [29] induce a reasonable part of discussions. The change of the temperature paradigm was urged on a theoretical basis [36, 39], supported by clinical observations as well [12, 38]. In the light of the present experimental results, the temperature effect alone misleads the discussions [39]. We argue that the controlling parameter of hyperthermia, in general, is not solely the temperature, but the electric field effect is probable initialized by it. The RF current makes a temperature gradient microscopically between the extra- and intracellular electrolytes and by this way damages the cell membrane, and could destroy the membrane by various parallel actions [40]. This is supported by zero-order noise structure [45].

Our present results indicate that mEHT extends the thermal treatment efficiency, probably by nonequilibrium heating [40]. The thermally induced mechanisms of cell killing could be different from the equilibrium-based thermal ones. The difference was observed in various experiments [4, 5], which are currently in progress.



Figures 2a to 2d. Histomorphological analysis 24 h after treatment (hemalum-eosin staining). Total cross section of the whole tumor (upper row), partial section at 10x and 100x magnification (lower rows). The areas of dead cells as lighter areas show a clearly distinctive staining. Control (a), radHT 42 °C (b), mEHT 42 °C (c), mEHT 38 °C (d).

Abbildungen 2a bis 2d. Histomorphologische Untersuchungen 24 h nach Behandlung (Hämalaun-Eosin-Färbung). Gesamtschnittfläche des ganzen Tumors (obere Reihe), Ausschnitt in 10- bzw. 100facher Vergrößerung (untere Reihen). Die Areale toter Zellen zeigen als helle Bezirke ein eindeutig unterschiedliches Färbeverhalten. Kontrolle (a), radHT 42 °C (b), mEHT 42 °C (c), mEHT 38 °C (d).

Table 2. Tumors with treatment and their parameters: groups 2–4 (n = 7 per group); treatment results measured histomorphologically: the respective dead cell areas are calculated as ratio of the total areas of the respective whole-tumor cross sections in percent (± 95% confidence interval [CI]), in dependence on the respective intratumoral temperature and the respective transtumoral modulated electric field. The therapeutic result of the three experimental groups is statistically highly significant ($p < 0.02$).

Tabelle 2. Tumoren mit Behandlung und deren Parameter: Gruppen 2–4 (n = 7 pro Gruppe); Therapieergebnis als jeweiliger Flächenanteil toter Zellen im Verhältnis zur Gesamtschnittfläche des jeweiligen Tumors in Prozent (± 95%-Konfidenzintervall [CI]) in Abhängigkeit von der intratumoralen Temperatur und dem transtumoralen modulierten elektrischen Feld. Das Therapieergebnis ist in allen drei Versuchsgruppen statistisch hochsignifikant ($p < 0{,}02$).

Parameter	Group 2 Control n = 7	radHT n = 7	Group 3 Control n = 7	mEHT n = 7	Group 4 Control n = 7	mEHT n = 7
Intratumoral temperature (°C)	38.0	42.0	38.0	42.0	38.0	38.0
± 95% CI	0.3	0.3	0.3	0.3	0.5	0.5
Modulated electric field (V/m)	No	No	No	32.0	No	32.0
± 95% CI				5.0		5.0
Average dead cell ratio (%)	9.2	17.9	4.8	57.1	5.2	45.9
± 95% CI	7.6	10.8	2.5	8.1	3.7	15.7
Statistic significance	$p < 0.02$		$p < 0.02$		$p < 0.02$	

Table 3. Therapeutic gain in dependence on the different therapeutic parameters: different intratumoral temperature (→) or different transtumoral modulated electric field (→) or constant intratumoral temperature (=) or constant transtumoral modulated electric field (=). Measured histomorphologically: the respective dead cell areas are calculated as ratio of the total areas of the respective whole-tumor cross sections in percent (see values in Figure 3) and the enhancement factors calculated out of these. FER: field enhancement ratio; mEHT: modulated electrohyperthermia; radHT: radiative hyperthermia; TER: thermal enhancement ratio.

Tabelle 3. Therapeutischer Gewinn in Abhängigkeit von den unterschiedlichen Therapieparametern mit unterschiedlicher intratumoraler Temperatur (→) bzw. unterschiedlichem transtumoralem moduliertem elektrischem Feld (→) bzw. mit konstanter intratumoraler Temperatur (=) und konstantem transtumoralem moduliertem elektrischem Feld (=). Gemessen histomorphologisch als relativer jeweiliger Flächenanteil toter Zellen im Verhältnis zur Gesamtschnittfläche des jeweiligen Tumors in Prozent (s. Werte in Abbildung 3) und daraus errechnete Verstärkungsfaktoren. FER: „field enhancement ratio"; mEHT: modulierte Elektrohyperthermie; radHT: radiative Hyperthermie; TER: „thermal enhancement ratio".

Process	Temperature (°C)			Field (V/m)			Gain (%)	TER	FER
Temperature changes	38 (control)	→	42 (radHT)	0 (control)	=	0 (radHT)	11.8	2.93	
At constant field	38 (mEHT)	→	42 (mEHT)	32 (mEHT)	=	32 (mEHT)	11.2	1.24	
Field changes	38 (control)	=	38 (mEHT)	0 (control)	→	32 (mEHT)	39.8		7.52
At constant temperature	42 (radHT)	=	42 (mEHT)	0 (radHT)	→	32 (mEHT)	39.2		3.19
Combined effect	38 (control)	→	42 (mEHT)	0 (control)	→	32 (mEHT)	51	9.36	

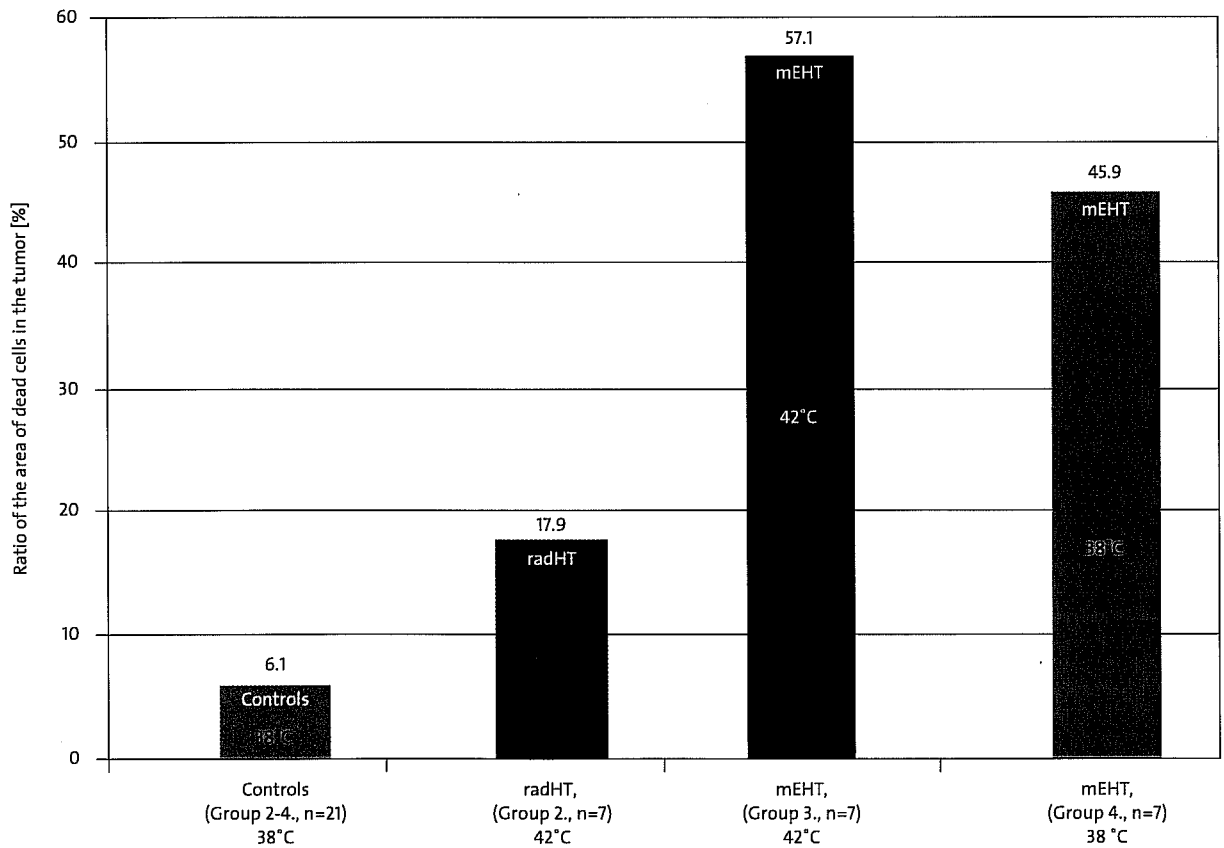


Figure 3. Therapeutic effects of intratumoral temperature and transtumoral modulated electric field as a survey. Experimental results of the histomorphological analysis of the respective dead cell areas as ratio of the total areas of the respective whole tumor cross sections in percent.

Abbildung 3. Therapieeffekte von intratumoraler Temperatur und transtumoralem moduliertem elektrischem Feld in der Übersicht. Ergebnisse der histomorphologischen Untersuchungen des jeweiligen Flächenanteils toter Zellen im Verhältnis zur Gesamtschnittfläche des jeweiligen Tumors in Prozent.

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