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Broadband microwave spiral applicator (105–125 MHz) for *in vitro* examinations of hyperthermia-induced tumor cell death forms – first analyses with human breast cancer cells

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ABSTRACT

Purpose: Local tumor heating with microwave applicators has been used in multimodal breast cancer therapies. This hyperthermia allows to target small regions while marginally affecting healthy tissue. However, most preclinical examinations only use simplified heating methods. Microwave applicators employed for deep heating to provide the greatest depth of penetration operate in the tens to hundreds frequency. Therefore, we aimed to adapt and test a clinically often used broadband spiral applicator (105–125 MHz) for hyperthermia with clinically wanted temperatures of 41 and 44 °C in *in vitro* settings with human breast cancer cell lines and with simulations.

Material and Methods: A clinically used spiral-microwave applicator (105–125 MHz) was the basis for the construction, simulation, and optimization of the *in vitro* HT set-up under stationary conditions. Microwave effects on tumor cell death of two human breast cancer cell lines (hormone-receptor positive MCF-7 and triple-negative MDA-MB-231) were compared with conventional heating in a contact-heating chamber. Cell death forms were analyzed by AnnexinV/Propidium iodide staining.

Results: An *in vitro* spiral applicator microwave-based heating system that is effective at applying heat directly to adherent breast cancer cells in cell culture flasks with medium was developed. Simulations with COMSOL proved appropriate heat delivery and an optimal energy coupling at a frequency of 111 ± 2.5 MHz. Apoptosis and necrosis induction and significantly higher cell death rates than conventional heating at both temperatures were observed, and MCF-7 showed higher death rates than MDA-MB-231 tumor cells.

Conclusions: Well-characterized *in vitro* heating systems are mandatory for a better understanding of the biological effects of hyperthermia in tumor therapies and to finally determine optimized clinical treatment schemes.

Abbreviations: AxV: AnnexinV; AxV-FITC: Annexin-V-fluorescein isothiocyanate; cos: Cosinus; CT: Chemotherapy; DMEM: Dulbeccós modified Eaglés medium; DNA: Deoxyribonucleic acid; FACS: Fluorescence-activated cell sorting; FBS: Fetal bovine serum; FSc: Forward scatter; HT: Hyperthermia; PBS: Phosphate buffered saline; PCR: Polymerase chain reaction; PI: Propidium-iodide; PS: Phosphatidylserine; RT: Radiotherapy; S.D.: Standard deviation; sin: Sinus; SSc: Side scatter

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Introduction

Cancer is the most common non-communicable disease in industrialized countries [1], with breast cancer being the most prevalent human malignancy concerning women [2]. The prognosis (e.g., overall survival, progression-free survival) for breast cancer heavily depends on classical prognostic factors, including cancer grade, lymph vascular status, stage of progression and age at diagnosis [3]. Different subtypes of breast cancer can be determined, and their unique molecular and histological structure can be used for more specific therapies including immune therapies [4]. Classical breast cancer therapies include surgery, chemotherapy (CT), and radiotherapy (RT), while more individualized multimodal breast cancer therapies with distinct targeted and immune therapies arose just recently [5].

Locally applied hyperthermia (HT), a process that heats the cancer cells and their microenvironment to supraphysiological temperatures from 39–44 °C [6], has been shown to induce immune alterations and to be effective in multimodal settings for breast cancer treatment [7]. One must emphasize that HT should always be used as an additional treatment in multimodal cancer therapies, mostly to increase the

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effectiveness of RT and CT [8]. Adding hyperthermia to RT and/or CT showed significant clinical success for different cancers in various clinical trials [9–12].

Nowadays, it is agreed that the main therapeutic effects of HT rely on synergistic effects with CT, RT, and the immune system [13–15]. However, divagating results regarding biological modes of action of HT have been published, mainly caused by methodological differences like the examination temperature or the applied heating method [14,16]. Preclinical model systems tend to make use of conventional heating methods with warm water baths or contact-heating plates, whereas clinical treatment mainly utilizes microwave heating techniques ($\leq$ 2.45 GHz) [17].

Recently, more experiments focused on the effects of microwave heating on the cell death of cancer cells and the expression of immune-checkpoint molecules have been carried out. These experiments were, e.g., performed with closed-loop devices operating with 915 MHz or 2.45 GHz heating systems. They concluded that microwave heating is superior to warm water heating in inducing cell death in cancer cells and that the operational frequency has a significant impact on the immune phenotype of breast cancer cells [15].

An important observation is that microwave applicators employed for deep heating provide the greatest depth of penetration when operating in the frequency region of tens to hundreds MHz [18]. Therefore, preclinical knowledge about the effects on tumor cells of HT delivered with microwave applicators operating at that frequency range is needed. Here, we present a first study focusing on the optimization and characterization of a 105–125 MHz spiral microwave heating system for *in vitro* treatment of adherent tumor cells.

Local and systemic effects of HT treatments rely highly on the temperature, timing, time interval and heating method [19–21]. Quality assurance guidelines for HT treatments suggest a single treatment duration of 30–60 min with a target tumor temperature between 40–44 °C and a short interval between HT treatment and RT (less than 4 h) [22–24]. We adapted a broadband microwave applicator system (Spiral applicator, S/N 980603-015; Pyrexar Medical, Salt Lake City, UT, USA, distributed by Dr. Sennewald Medizintechnik, Munich, Germany; 105–125 MHz) for *in vitro* tumor cell culture experiments. In advantage to other preclinical microwave heating systems, it does not perform or require a circulation of the cell medium (periodic heating). Therefore, constant heating of the entire cell medium and the adherent cells can be achieved. Spiral HT heating is a technique that involves the use of a spiral-shaped applicator to deliver microwave energy to the medium, i.e., cancer cells and their microenvironment. The microwave energy is absorbed by the tissue, which leads to an increase in temperature. It is more selective in terms of the tissue that is heated, compared to other heating methods [25]. This can be useful for minimizing side effects and maximizing the therapeutic benefits of the treatment. In addition, spiral HT heating can be used to heat larger volumes of tissue, which may be more practical and effective in certain situations. There are different types of spiral hyperthermia heating systems, and the specific design and characteristics of these systems can influence the effectiveness of the treatment [26]. For example, the frequency of the microwave energy, the shape and size of the applicator, and the duration of the treatment can all affect the thermal dose and distribution within the tissue. The physical basis of spiral applicator heating is tissue absorption of microwave energy. When microwave energy is applied to tissue, it is absorbed by the molecules within the tissue, which leads to an increase in temperature. This process is known as dielectric heating. For standardized results obtained by *in vitro* experiments, clear characteristics of the used spiral applicator are necessary.

Therefore, the key aim of this work was to adapt and test the 105–125 MHz broadband spiral applicator with clinically relevant temperatures of 41 and 44 °C in an *in vitro* setting with human breast cancer cell lines as first cell model system based on COMSOL simulations. Further, tumor cell death induction including analysis of the key tumor cell forms of this stationary microwave heating was compared to a conventional contact-heating method with a self-developed heating chamber.

Material and methods

Cell lines, cultivation, and hyperthermia treatments

The adherent MDA-MB-231 (ECACC catalog no. 92020424; Merck KGaA, Darmstadt, Germany) and the adherent MCF-7 (ECACC catalog no. 86012803; Merck KGaA, Darmstadt, Germany) human breast cancer cell lines were maintained in Dulbeccós modified Eaglés medium (DMEM; PAN-Biotech GmbH, Aidenbach, Germany) supplemented with 10% heat inactivated (60 °C, 30 min) fetal bovine serum (FBS; Biochrome AG, Berlin, Germany) and 1% Penicillin-Streptomycin (PenStrep; Gibco, Carlsbad, USA) under sterile conditions (37 °C, 5% CO_2, 90% humidity). They were tested for mycoplasma spp. by polymerase chain reaction (PCR) detection kit and confluency was regularly checked with light microscope. Splitting at 80–90% confluency and harvesting for experiments were performed with 1% trypsin solution (Thermo Fisher Scientific, Erlangen, Germany).

All experiments were performed in 75cm^2 or 25cm^2 cell culture flasks (Cellstar; Greiner BioOne, Nürtlingen, Germany). According to the size of the cell culture flask, the corresponding medium in the experiments was scaled (75cm^2: 15 ml medium; 25cm^2: 5 ml medium). The variation of the temperature during the treatment was less than 0.2 °C and one session of HT lasts for 60 min at the target temperature (41 or 44 °C).

For the conventional contact-heating experiments, 75cm^2 cell culture t-flasks were placed in a self-constructed heating device, consisting of a temperature control unit, heating wire, temperature sensor, and the connection box for the heating wire [19]. The heating chamber (see Figure 2(G,H)) is automatically self-controlled for target temperature, set to 41 or 44 °C *via* a thermosensor controller. The same HT chamber was already used and published by *Stoll* et al. and further characteristics can be found there [27].

The spiral applicator heating (see Figure 2(A–F)) was performed in 25cm^2 cell culture flasks, because of the smaller surface of the spiral applicator; cell count and volume of medium was correlated to the conventional heating methods, i.e., similar number of cells/volume and volume/area. The cell culture flasks were placed on the spiral applicator. Temperature was controlled with microwave-compatible temperature probes inside the cell culture flasks. All cell culture flasks contained the same medium, as described above.

Cell death form analysis by AnnexinV/PI staining

To determine the form of tumor cell death after the distinct HT treatment, multicolor flow cytometry after staining of the tumor cell suspensions with AnnexinV/propidium-iodide (PI) was used. The tumor cells were centrifuged (180xg, 5 min, 20 °C), resuspended with 1 ml FACS-buffer (98% PBS and 2% FBS) and counted with CASY®-Cell-Counter (Innovatis AG, Reutlingen, Germany); 1x10^5 cells were washed and centrifuged (400xg, 6 min, 20 °C) on a 96-well-plate, resuspended in Ringer's solution (B. Braun, Melsungen, Germany), and stained with 1 µg/ml of PI (Sigma Aldrich, Munich, Germany) and 0.5 µg/ml FITC-labelled AnnexinV (Geneart, Life Technologies, Regensburg, Germany). After incubation (30 min, 4 °C) in the dark, the stained cells were analyzed with a CytoFLEX S flow cytometer (CytExpert 2.3 Software; Beckman Coulter, Krefeld, Germany) on two time points d0 (direct after the treatment) and d1 (24 h after the treatment). Resulting data were analyzed using Kaluza analysis software 2.1 (Beckman Coulter, Krefeld, Germany) and GraphPad Prsim 8 (GraphPad Software Inc., La Jolla, USA).

Characteristic morphological changes of the dead cells were detected in the forward scatter (FSc) against side scatter (SSc) plot (Figure 1(A)). PI penetrates cells that have lost their membrane integrity and intercalates with DNA, marking primary and secondary necrotic cells, while AxV-FITC binds phosphatidylserine (PS) on the outer membrane of apoptotic cells or in addition on the inner membrane of necrotic cells [28]. Thereby, viable cells and apoptotic, primary or secondary necrotic cell death forms can be distinguished [29] (Figure 1(B)).

Simulations and statistical analysis

COMSOL simulation program was used (COMSOL Version 5.5; COMSOL Multiphysics GmbH, Goettingen, Germany) to evaluate the temperature profile, the electric field strength (V/m) and the local/global total heat balance (in W/m3 or W/kg) of the commercially available spiral applicator, operating at around 105 - 125 MHz. COMSOL software is used to model and analyze various physical phenomena, such as heat transfer, fluid flow, and electromagnetism. It is based on the finite element method, which is a mathematical technique that is used to solve partial differential equations that describe the physical phenomena being studied. To simulate the spiral applicator, we defined the geometry of the system, the material properties and boundary conditions.

Statistical analyses were performed using the Kruskal-Walli's test with uncorrected Dunn's multiple comparisons. All independent samples from the same distribution were compared relative to the corresponding untreated control conditions. Results were considered statistically significant for: * ($p < 0.05$), ** ($p < 0.01$), *** (< 0.001). All graphs and statistics were performed by Prism 8 software (Graph Pad, San Diego, CA, USA). Results were shown with individual p values and the mean rank difference.

Results

Set-up and operation of the microwave spiral applicator

First, we constructed a set-up with the spiral applicator and a cell culture t-flask attached. To measure the temperature inside the bottle, a microwave-compatible temperature

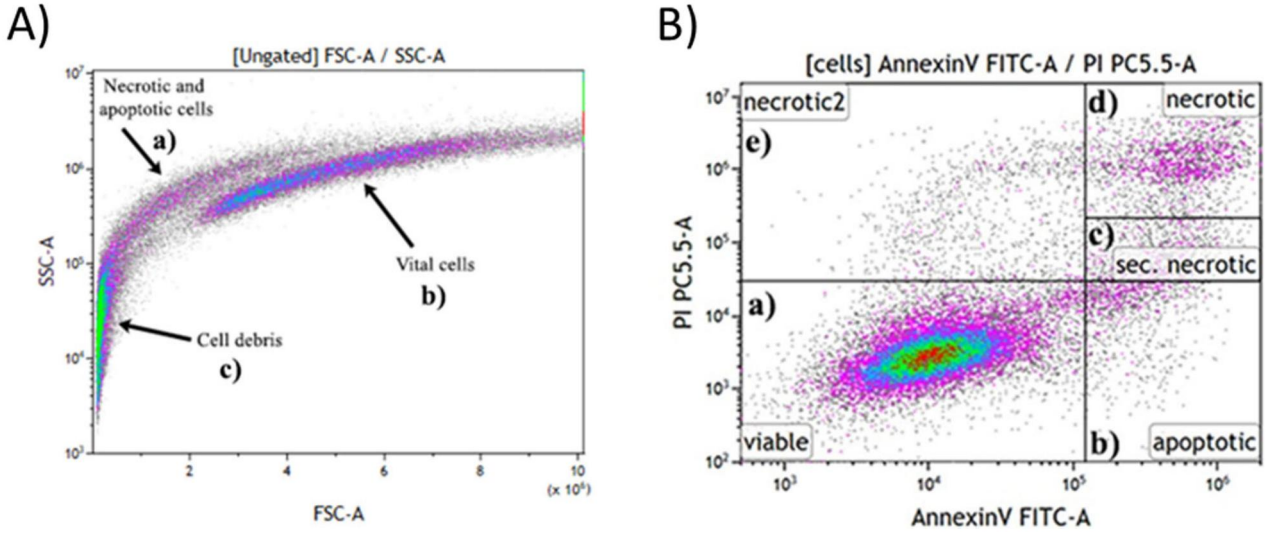

Figure 1. Differentiation of the tumor cell death forms by AnnexinV/PI staining and measurement with flow cytometry. A) Characteristic changes of the morphology of the cells. The apoptotic and necrotic cells (a) lose in granularity and size. B) Five different gates were set to differentiate viable cells (a; AxV-/PI-), apoptotic cells (b; AxV+/PI-), secondary necrotic cells (c; AxV+/PIlow) and primary necrotic cells in two gates (d, e; PI+) were chosen. Exemplarily data of MCF-7 breast cancer cells 24 h after treatment with 44 °C spiral applicator heating are displayed.

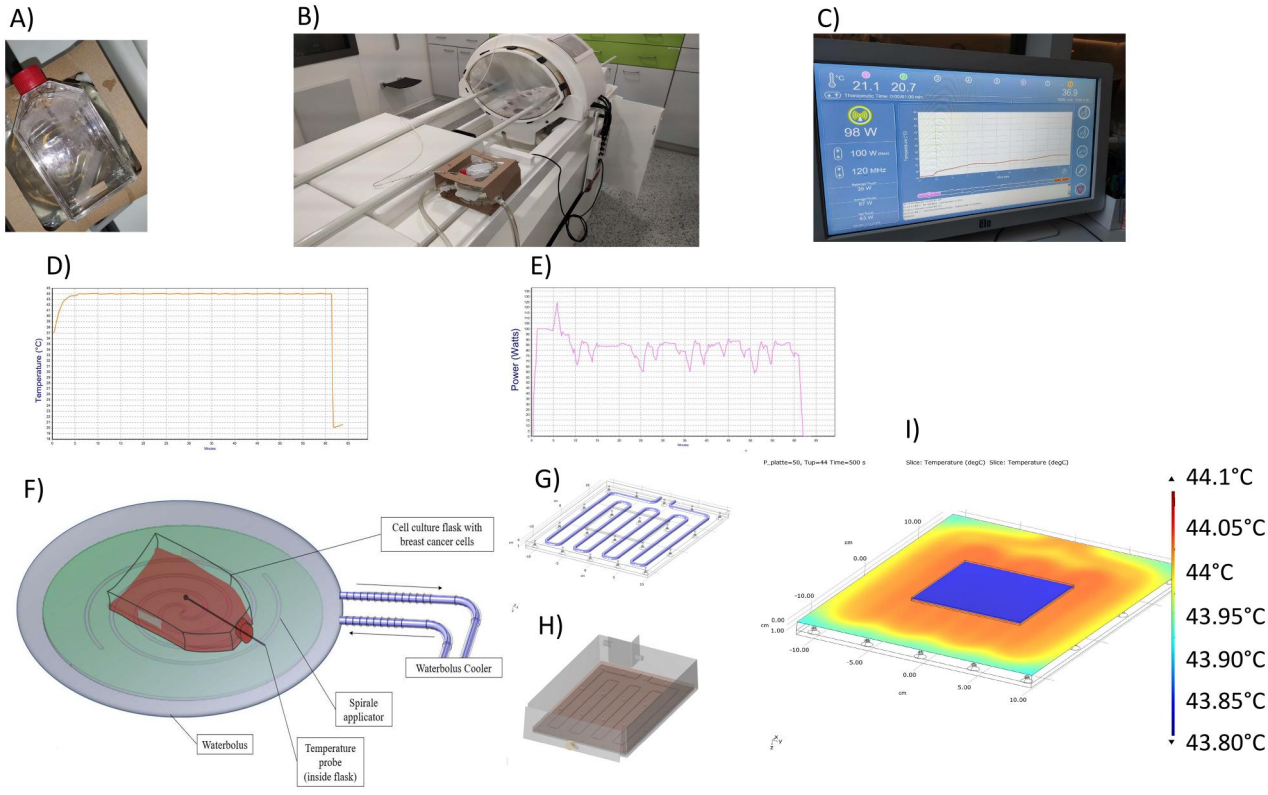

Figure 2. Set-up and operation of the spiral applicator-based microwave HT treatment of tumor cells in cell culture flasks and schematics/COMSOL simulation of the hyperthermia chamber. Mounting of a cell culture flask on the spiral applicator (a), and practical setup coupled to a MSD2000 machine, with the addition of the water cooling (water bolus) and the MSR unit (B,C). The latter is used for (automatic) adjustment of power and frequency as well as for recording the temperature. Exemplary temperature (D) and power (E) curve for treatment with the 105–125 MHz applicator at 44 °C over a period of 60 min. (F) Draft of the optimized set-up. Heat-wiring of the HT chamber (G), full schematics of the HT chamber with thermosensor controller (H) and COMSOL simulation of the HT chamber (I).

probe was glued into the bottle and its position could be varied longitudinal by control unit using a sleeve. In the consecutive biological experiments, four 25 cm^2 cell culture bottles were each placed on the applicator, of which one bottle was used as a temperature control dummy and three bottles were filled with a fixed number of tumor cells and 5 ml of cell culture medium.

Figure 2(A,B) shows the set-up of the spiral applicator coupled to a BSD2000 machine (Pyrexar Medical, Salt Lake City, UT, USA, distributed by Dr. Sennewald Medizintechnik, Munich, Germany), as well as a screenshot of the measurement, control and regulation unit of the deep hyperthermia system, which is also used for the spiral applicator in the clinics (Figure 2(C)). The operation of the spiral applicator *via* the SigmaVision® computer software was adapted to the requirements of the experimental setup for the experiments in agreement with *Dr. Sennewald Medizintechnik*. This includes the adjustment of the frequency range as well as the free parameter selection of the temperature hysteresis in fully automated operation, adapted to the 25 cm^2 cell culture flasks used in the biological experiments. Exemplary temperature and power curves for treatment with the 105–125 MHz applicator for a HT at 44 °C over a period of 60 min are shown in Figure 2(D,E), respectively. We identified a good energy coupling at a frequency of 111 ± 2.5 MHz (theoretical optimum at 112 MHz).

In addition to the actual construction of the 105–125 MHz broadband spiral applicator for heating cell culture flasks, a process characterization was carried out with regard to the optimum resonance frequency range in which good coupling of the microwave into the cell culture liquid takes place, as well as the determination of a power-temperature characteristic curve. For the consecutive biological experiments, the water bolus for cooling the spiral applicator had to be operated with 3 °C cooler water than the target temperature (41, 44 °C) and the power had to be re-adjusted automatically in the SigmaVision® (Dr. Sennewald Medizintechnik, Munich, Germany) program. As an example, Figure 2(D) shows the temperature curve for the target temperature 44 °C and Figure 2(E) the corresponding output power of the spiral applicator from 65 W to 85 W. After 60 min of effective heating, the treatment was terminated, recognizable by the steep drop in temperature and power. A draft of the optimized set-up is shown in Figure 2(F).

In comparison, the heat-wiring of the HT chamber, a full schematic of the HT chamber with thermosensor controller and COMSOL simulation of the HT chamber for conventional heating are displayed in Figure 2(G–I).

Microwave spiral applicator for heat treatments of cancer cells – simulations

As performed with previous hyperthermia systems and models examined by our group [14, 15], COMSOL simulation program was used to evaluate the temperature profile, the electric field strength (V/m) and the local/global total heat

balance (in W/m3 or W/kg) of the commercially available spiral applicator, operating at around 105 - 125 MHz. To simulate the spiral applicator, we defined the geometry of the system, the material properties and boundary conditions.

Thus, a perfect electric conductor (sphere) was placed around the calculation domain to calculate the electric field of the 105–125 MHz spiral applicator. The spiral applicator itself was constructed analogously to the real system with two spirals offset by 180° according to the values listed in Table 1 hereby the spirals are tuned to the frequency range around 111 MHz and thus correspond to the real system. The resonant frequency depends strongly on the steady inlet temperature of the cooling bolus (37 °C in our experiments) and therefore the cooling of the condensator.

In the simulation model (Figure 3), an isothermal water bolus (blue area in Figure 3(D)), as used in the clinical field for superficial cooling of the skin, a polystyrene plate (red area in Figure 3(E)), representing the bottom of the cell culture bottle, and four water-filled cuboids with a surface area of 25 cm^2 (yellow area in Figure 3(E)), representing the cell cultures, were added.

Analogous to the other models, the investigation of the target temperatures of 41 and 44 °C was also performed here. As an example, the logarithmized E-field strength profile [20·log10(normE)] at the frequency 111 MHz as well as the port power 72 W (microwave voltage 60 V and impedance 50 Ω; $P = U^2/Z$)) can be seen in Figure 3(F). The wave-like E-field with the global maximum in the center and other local maxima near the spiral bands is clearly visible. Figure 3(D) shows the temperature distribution in the cell culture flasks at 72 W. The target temperature corresponded to 44 °C. It can be seen that the temperature distribution in the

Table 1. Geometric parameters and equations for the design of the spiral of the 105–125 MHz microwave applicator.

	spiral 1	spiral 2
yw / mm	1,5·2s·1,5·cos(s)	(1,5 + 1,5·2s) ·1,5·cos(s)
yw / mm	1,5·2s·1,5·sin(s)	(1,5 + 1,5·2s) ·1,5·sin(s)
start (microwave port)	0	4·π
end	0	4·π
polygon (xw/yw) / mm	50 & 60 / 0 & 0	
rectangle / mm or °	wide: sqrt(22); height: 1,2; rotation: atan2(1,sqrt(14))/π·180	
microwave-port / mm or °	side length: 1,2; rotation: atan2(1,sqrt(14))/π·180	

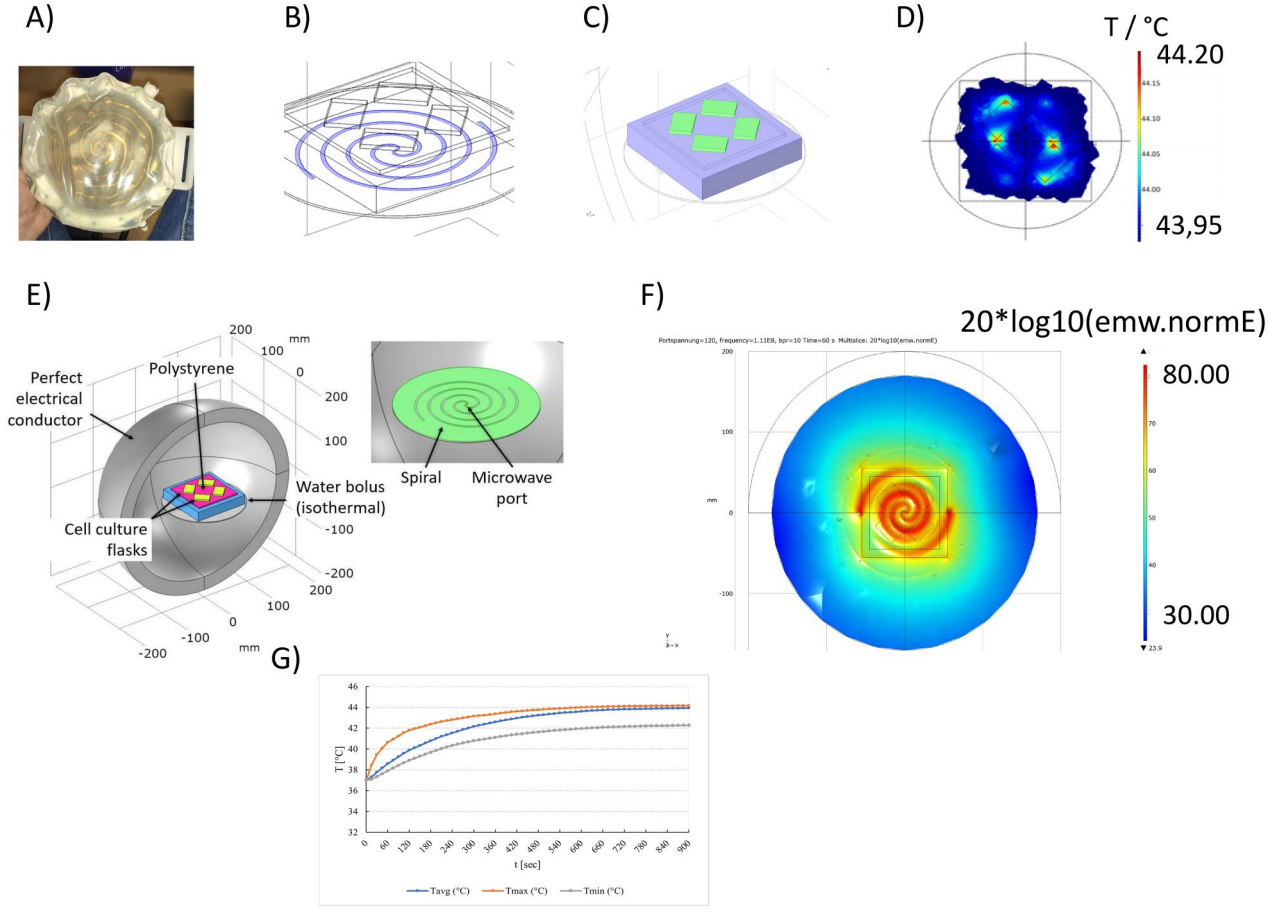

Figure 3. Simulation of heat application to cell culture medium with adherent cells in cell culture flasks with a microwave spiral applicator clinically used for the treatment of surface tumors. (A) Spiral applicator model in COMSOL (B) with spirals (blue), schematic cell culture flasks on the spiral applicator (C), exemplary temperature profile simulation with COMSOL (D), domain in COMSOL to calculate the microwave field (E) and exemplary E-field simulation with COMSOL with unit 20*log10(emw.normE) (F). The temperature profile from COMSOL simulations with 44 °C is shown in (G).

flasks is sufficiently homogeneous. Only slight excess temperatures (+ 0.2 °C) occur, preferably in the corners of the cell culture domains. This was also demonstrated in the COMSOL simulations, as shown in Figure 3(G) for volume integrated temperature profiles (Tmin, Tavg, Tmax) at 72 W, i.e., to practically achieve a Ttarget of 44 °C.

Viability and cell death forms of human breast cancer cells after spiral applicator heating in comparison to heat-chamber treatment

MCF-7 and MDA-MB-231 breast cancer cells were treated for 60 min with either the optimized and characterized 105–125 MHz spiral-microwave applicator developed here or a conventional heating chamber [19]. Directly and 24 h after the treatment, tumor cell death forms were analyzed.

The MCF-7 breast cancer cells, which are caspase-3 deficient [30], are significantly (d0: 41 °C $p = 0.028/\pm 11.00$, 44 °C $p = 0.032/\pm 10.75$; d1: 41 °C $p = 0.024/\pm 11.25$, 44 °C $p < 0.001/\pm 17.00$) affected by the microwave heating (Figure 4(A,C)), while the conventional heating resulted in no increase of the cell death rates (Figure 4(B,D)).

The MDA-MB-231 breast cancer cells were also significantly affected by microwave heating with exception of 41 °C at d0 (Figure 4(E,G)) (d0: 41 °C ns, 44 °C $p = 0.0004/\pm 17.75$; d1: 41 °C $p = 0.0044/\pm 14.25$, 44 °C p= <0.0404/±10.25). Again, the conventional heating had no significant impact on the tumor cell death rates (Figure 4(F,H)).

Both temperatures tested with the spiral-applicator-based microwave heating led to increased cell death rates in both cell lines (Figure 4(A,C,E,G)). Overall, the microwave heating induced significantly higher cell death rates compared to conventional heating.

The detailed analyses of cell death forms after conventional and microwave heating showed slight, but significant effects on the occurrence of apoptotic cells. In comparison to the non-treated controls, the MCF-7 breast cancer cells show significant increased rates of apoptotic cells after the microwave heating (d0: 41 °C $p = 0.0316/\pm 10.75$, 44 °C ns; d1: 41 °C $p = 0.0019/\pm 15.50$, 44 °C $p = 0.0069/\pm 13.50$; Figure 4(A,C)). Directly after the treatment with 41 °C and in the follow-up after 24 h, a significant increase of apoptotic cell death could be observed. On the other hand, the triple-negative MDA-MB-231 breast cancer cells showed only a significant increase directly after the microwave treatment at 41 °C (d0: 41 °C $p = 0.0278/\pm 11.00$). Overall, the conventional heating had no significant effects on apoptosis induction at both examined temperatures, time points and cell lines (Figure 4(B,D,F,H)). To conclude, apoptotic cell death was induced in dependence the heating method, and in contrast to the significant increase of apoptosis on d1 in the MCF-7 after microwave heating (Figure 4(A,C)), the microwave heating had no significant effects on the apoptotic cell death rates of the MDA-MB-231 on d1 (Figure 4(E,G)).

Regarding primary necrosis, after the microwave heating, highly significant changes were observed in both cell lines (Figure 4(A,C,E,G)). Directly after the microwave heating with both temperatures, the MCF-7 breast cancer cells showed a significant increases of primary necrotic cell death (d0: 41 °C $p = 0.0044/\pm 14.25$, 44 °C $p = 0.0037/\pm 14.50$; d1: 41 °C ns,

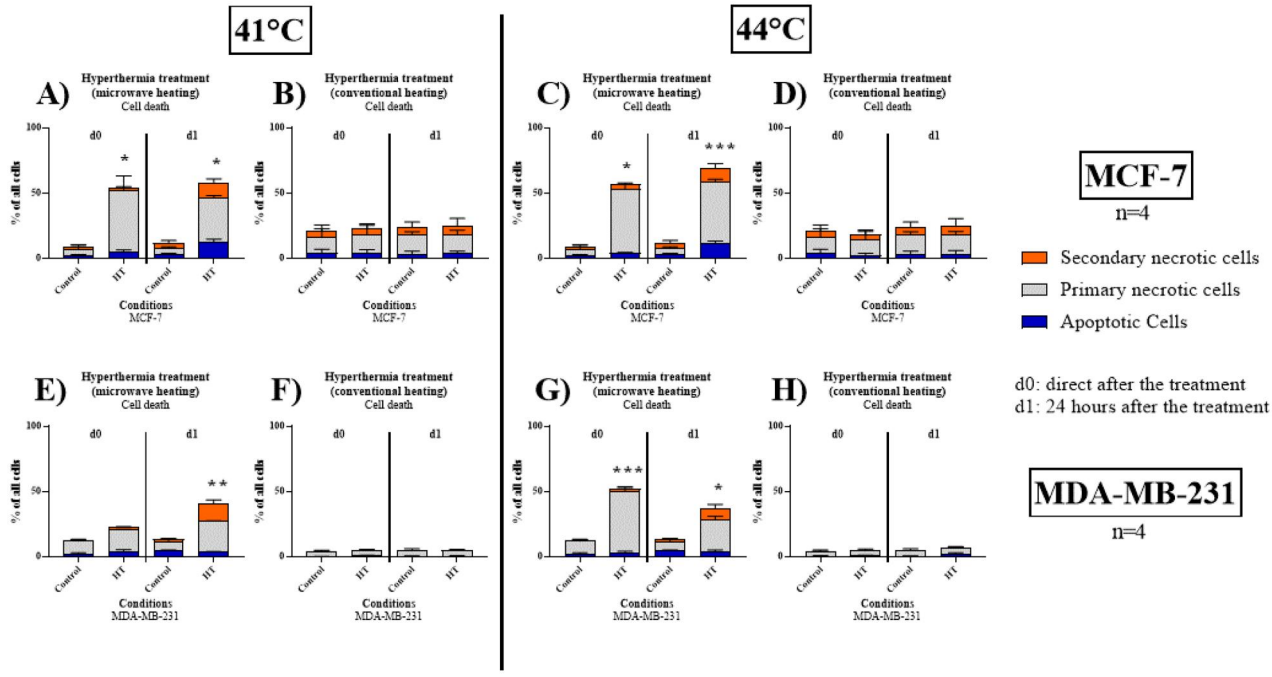

Figure 4. Comparison of cell death rates and forms of human breast cancer cells after spiral-applicator-based microwave heating compared to conventional heating. The cell death forms of MDA-MB-231 and MCF-7 were analyzed using multicolor flow cytometry and AnnexinV-FITC/propidiumiodid-staining (AxV/PI-staining) after spiral applicator-based and conventional and hyperthermia treatment with 41 and 44 °C, respectively. The viable cells, the apoptotic cells (blue), the primary necrotic cells (gray), and the secondary necrotic cells (orange) were differentiated with AxV/PI-staining on two different time points d0 (direct after the treatment) and d1 (24 h after the treatment). mean ± SD are presented from four independent experiments, each measured in duplicates. A test for significance was conducted using Kruskal-Walli's test with uncorrected dunn's multiple comparisons, by comparing the treatment-related total percentages of killed cells to the corresponding controls of untreated cells at the indicated time points (d0, d1); * ($p < 0.05$), ** ($p < 0.01$), *** (< 0.001).

44 °C $p = 0.0069/\pm 13.50$; Figure 4(A,C)). The effect lasts until the follow-up on d1 with the higher temperature of 44 °C. Higher temperatures induced by the microwave heating (44 °C) led also to higher rate of primary necrotic cell death in the triple-negative MDA-MB-231 breast cancer cell line, especially direct after the heating. In the follow-up after 24 h, both temperatures affected the primary necrotic cell death significantly (d0: 41 °C ns, 44 °C $p = 0.001/\pm 16.50$; d1: 41 °C $p = 0.0115/\pm 12.63$, 44 °C $p = 0.004/\pm 14.38$; Figure 4(E,G)). Overall, the conventional heating had no significant effects on primary necrotic cell death induction in both cell lines at both time points.

The analyses of the secondary necrotic cell death rates revealed that only the microwave heating increased this cell death form in the MDA-MB-231 cell line 24 h after the treatment significantly. Furthermore, this significant effect was observed independent of the temperature (d1: 41 °C $p = 0.0044/\pm 14.25$, 44 °C $p = 0.0244/\pm 11.25$; Figure 4(E,G)). All other examined conditions showed no significant effects on the secondary necrotic cell death rate.

In comparison, the MCF-7 showed higher rates than the MDA-MB-231, in both heating experiments at all temperatures, except the significant effects mentioned above. Especially directly after the heating, all rates of secondary necrotic cells remained on low base levels, independent of the temperature.

Discussion

While the use of microwave heating systems for hyperthermia treatments is common in clinical practice [25], the preclinical knowledge of the effects of around 105–125 MHz frequency microwave exposure on cancer cells is limited. The used clinically relevant spiral applicator operates with frequencies at around 105–125 MHz, analogous to our previous experiments with e.g., 434 or 915 MHz [15]. Our simulations with COMSOL showed that frequencies around 110 to 114 MHz can effectively heat cell culture flasks and the breast cancer cells to supraphysiological temperatures, e.g., 41 and 44 °C, with only a slight excess temperature (±0.2 °C) and within a short period of time, i.e., 7–8 min. From our experimental data, we found the optimum energy coupling into the cell culture flask at 111 MHz. This depends, among other things, on the dielectric constant of the heated material and the distance from the microwave source. All together, these findings suggest that microwave heating at around 105–125 MHz may be a promising approach for inducing cell death in cancer cells and enhancing the effectiveness of cancer therapies.

Kok and colleagues demonstrated that radiative heating with microwave achieves a more favorable specific absorption rate and temperature distributions for superficial tumors, compared with capacitive heating [31]. We demonstrate that when heat is delivered with the spiral applicator around 105–125 MHz, HT alone at 41 and 44 °C is sufficient to induce breast cancer cell death. In addition, microwave heating also affects healthy breast cells, they are heated in the same way as the tumor cells. Due to their generally lower sensitivity to heat (e.g., intact cell cycle, metabolism, DNA repair mechanisms) and the intact heat dissipation with normal vascularization of the healthy tissue, they can regenerate from any cell damage that occurs and the sum of the applied heat is not sufficient to damage healthy cells. This highlights that the frequency of the microwave determines cellular effects.

It has already been demonstrated that the frequency of microwave heating impacts on tumor cell death induction [14,15]. But also other heating systems such as radiofrequency waves, focused ultrasound and infrared heating might impact differently on tumor cell death. Generally, the temperature ant the heating method have to be considered when analyzing tumor cell death induction by HT [8]. Future work should additionally analyze how adding RT or using other heating systems to the here examined settings contributes to breast cancer cell death induction.

We developed and characterized for the first time an *in vitro* spiral applicator heating system that is effective at applying heat directly to the adherent breast cancer cells in medium in cell culture flasks. We validated the HT application with temperature probes inside the cell culture flasks. This easily allows monitoring of the temperature on the bottom of the cell culture flasks during the spiral heating process. The applied power (W) varied multiple times around ±25 W to stabilize the temperature inside the cell culture flask. Such *in vitro* settings reflect the clinical situation more precisely and should be used for preclinical examinations for effects of HT on tumor cells alone and in multimodal settings [32]. One has to note that the measuring range is correspondingly large (10–15%), since clear highs and lows can be detected around the spiral(s) of the applicator. This is always the case with microwaves. The main reason why a small temperature difference is present, although the electric field strength is heterogeneous, is the water bolus under the cell culture bottle where the cell suspension is located, which ensures heat dissipation or a constant temperature in the bottle without large hot spots.

Overall, the spiral heating static microwave system induced significantly cell death in human breast cancer cell lines. Compared to a conventional heating method, the cell death increased significantly after sole HT treatment. All investigated cell death forms could be increased by the spiral applicator treatment. Especially the primary necrotic and apoptotic cells were increased. A mixture of apoptosis and necrosis is discussed to be particular immunogenic [33] and future *in vivo* settings should analyze how anti-tumor immune responses are affected by microwave-based local HT treatment of tumors. Primary necrosis as a type of cell death that occurs due to external cell damages and is characterized by swelling of the cell and the organelles until the membrane integrity is lost and cell content is released into the extracellular space. This triggers an inflammatory immune response. In contrast, at any time point during the process of apoptosis, the cells might lose their membrane integrity, thereby becoming secondary necrotic ones [28]. The latter one release already degraded DNA bound to histones and are discussed to be particularly immunogenic [34]. However, one has to consider that secondary necrosis appears more

under *in vitro* conditions, as *in vivo*, apoptotic cells are often phagocytosed by macrophages before becoming secondary necrotic.

Our *in vitro* examinations further revealed that triple-negative MDA-MB-231 breast cancer cells need higher temperatures being applied with the 111 MHz broadband spiral applicator than caspase-3 deficient MCF-7 cells. MCF-7 cell death could be increased significantly by lower temperatures (41 °C) than MDA-MB-231 cells (44 °C). They also showed a direct response on the spiral applicator hyperthermia treatment on time point d0, while the MDA-MB-231 cells reacted mostly 24 h after the treatment. Compared to the conventional heating method, both cell lines showed higher cell death rates. This suggests for the first time that microwave heating with a broadband spiral applicator is capable of inducing caspase-3 independent cell [35].

Based on our findings, we conclude that the microwave spiral applicator heating method is more effective at inducing cell death in breast cancer cells and may additionally alter the immunogenicity of the tumor microenvironment compared to conventional heating methods. Complementary *in vivo* studies help to optimize clinical multimodal therapies with microwave hyperthermia [36]. The understanding of the effects of different heating methods on the immune phenotype of tumor cells could be critical for improving the effectiveness of cancer treatments, especially the new area of immune-checkpoint inhibitor-based therapies and radiation techniques [37,38]. We succeeded to adapt and to test a clinically often used 105–125 MHz spiral applicator for *in vitro* settings that can be used for manifold settings to test such multimodal treatments of tumor cells preclinically. To adapt such experiments better to physiological conditions, three-dimensional *in vitro* cell culture models with adjusted frequencies (according to the best energy coupling) and extended thermo-monitoring could be used, instead of adherent cell lines.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The data presented in this study are available on reasonable request from the corresponding author.

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