Irreversible Electroporation, Electrochemotherapy and Calcium Electroporation on Hepatocellular Carcinoma Cells in Vitro

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Pulse Parameters and Thresholds for (ir)Reversible Electroporation on Hepatocellular Carcinoma Cells *in Vitro*

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Abstract

Hepatocellular carcinoma is a leading cause of cancer-related death in many parts of the world. Traditional treatment options are not always effective. During the promising minimally invasive electroporation-based therapies, biological cell membranes are exposed to an external, sufficiently high, pulsed electric field which creates so-called nanopores into the lipid bilayer of the cell membrane. These pores can either be permanent (irreversible electroporation (IRE)), leading to apoptosis, or repairable (reversible electroporation (RE)), with continued cell function. In tumor therapy, RE is used to increase the diffusion of a chemotherapeutic drug during electrochemotherapy. For both IRE and RE, the success of the treatment is dependent on application of the appropriate electric field. Therefore, this study aims to define the pulse parameters and thresholds for IRE and RE on hepatocellular carcinoma (HepG2) cells *in-vitro*.

In a custom-made *in-vitro* setup, HepG2 cell viability (0, 5, 10, and 15 min), and the peak temperature were measured after electroporation with the different IRE and RE pulsing protocols, to determine the most successful settings for IRE and RE. A CAM/PI flow cytometric assay was performed to confirm cell permeabilization for the RE pulsing protocols with the highest cell viability.

The results indicated that an IRE pulsing protocol (70 pulses, 100 μs pulse length, and 100 ms interval) with an electric field strength of 4000 V/cm was needed as threshold for almost complete cell death of HepG2 cells. A RE pulsing protocol (8 pulses, 100 μs pulse length, and 1000 ms interval) with an electric field strength of 1000 V/cm was needed as threshold for viable and permeabilized HepG2 cells. The low peak temperatures (max 30.1°C for IRE, max 23.1°C for RE) within this study indicated that the reduction in HepG2 cell viability was caused by the applied electric field and was not a result of Joule heating.

Keywords

hepatic cancer, HepG2, IRE, RE, ECT, minimally invasive, electric field, viability, thermal effects, cell permeabilization

Abbreviations

CAM, calcein acetoxymethyl ester; ECT, electrochemotherapy; EP, electroporation; ESOPE, European Standard Operating Procedure on ECT; HCC, hepatocellular carcinoma; IRE, irreversible electroporation; MWA, microwave ablation; PEF, pulsed electric field; PI, propidium iodine; RE, reversible electroporation; RFA, radiofrequency ablation; SD, standard deviation

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Introduction

Hepatocellular carcinoma (>80% of primary liver cancers) is a leading cause of cancer-related death in many parts of the world.[1,2] For various tumor diseases, the liver is also a frequent location of metastases.[3,4] For cancer patients, traditional treatment options like surgery, radiation therapy, and systemic chemotherapy are not always efficient in eradicating the tumor.

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Although preferred as method, resection of the primary tumor and its metastasis turned out to be only suitable for 25% of the patients in our department.[5] This emphasizes the necessity of new treatment methods.

In this era, minimally invasive interventional cancer therapies are a popular area of research. These techniques limit the size of incisions needed resulting in less pain, decreased risk of infection, and fastened recovery of the patient.[6] The ablation method can be either thermal (such as radiofrequency ablation (RFA) and microwave ablation (MWA), most commonly used for liver metastases) or nonthermal (such as electroporation (EP)-based therapies). Nonthermal EP-based therapies seem the most promising, mainly due to their possibility to induce apoptosis instead of necrosis, which can lead to a superior immune effect, and their avoidance of thermal side effects.[6,7] The latter makes it possible to operate in proximity to vulnerable structures such as blood vessels, nerves, and ducts.[6,8,9] In EP-based therapies, biological cell membranes are exposed to an external, sufficiently high, pulsed electric field (PEF), which can lead to a rapid and large increase in electric conductivity and permeability, creating so-called nanopores into the lipid bilayer of the cell membrane.[10] When these PEF are applied to cells, two different phenomena are observed: irreversible electroporation (IRE) or reversible electroporation (RE).[10]

During the clinical treatment modality IRE, the cell membrane cannot repair the induced nanopores because of their size and amount, which causes the cell to undergo apoptosis, the natural cell death.[8–11] At present, there are not many IRE devices on the market, so the classic NanoKnife® System (AngioDynamics, New York, US), which used to be the only approved system for IRE treatment in Europe for years, remains widely used.[12] It can be used to treat a variety of soft tissue tumors. Several applicator designs have been developed, but the system combined with straight monopolar needles remains the most popular.[13] Hence, the need for further research and development, to improve this promising treatment.

During RE, the cells can repair their phospholipid bilayer and continue with their normal cell functions. In tumor therapy, those hydrophilic pores are used to increase the diffusion of a chemotherapeutic drug which is known as electrochemotherapy (ECT).[14,15] Most antitumor drugs are (nearly) nonpermeant, due to their physico-chemical properties and lack of membrane transport mechanisms, which limits their antitumor effectiveness.[16] ECT is an established therapy option in the clinic for the treatment of (sub)cutaneous tumors.[17,18] The treatment protocol was standardized in the framework of the European Standard Operating Procedure on ECT (ESOPE), which also introduced the widely used Cliniporator™ (IGEA, Carpi, Italy) to perform ECT in clinical practice.[15,20,21,22] More research needs to be done, to extend the application of ECT to deep-seated tumors like liver cancer.[10]

Working closely with radiologists, our department focusses on developing an improved electroporation probe, which enables the combination of IRE with ECT treatment for liver cancer.[5–23] In both IRE and ECT, the success of the treatment is dependent on application of the appropriate electric field. The electric field can be affected by the following parameters: tissue-specific electrical properties, geometry and positions of electrodes, and electric pulse parameters.[24,25]

Therefore, this study aims to define the pulse parameters and thresholds for IRE and RE on hepatocellular carcinoma (HepG2) cells *in-vitro*.

Methods

A translational study with several HepG2 (ATCC, Manassas, US) *in-vitro* experiments was performed to define the pulse parameters and thresholds for IRE and RE.

Cell Culture

The human Caucasian hepatocellular carcinoma cell line HepG2 was used for all *in-vitro* experiments. The cells were subcultured by trypsinization twice a week (1:5 ratio) in Eagle's minimum essential medium (EMEM) (ATCC, Manassas, US) supplemented with 10% fetal bovine serum (FBS) (100 μL/mL) and 1% penicillin/streptomycin (100 U/mL and 100 μg/mL). The cells were grown in a humidified incubator at 37°C with 5% CO_2.

Electroporation Setup in-Vitro

All electroporation experiments were performed *in-vitro*, using a custom-made setup designed in our department. The setup consisted of a photopolymer spacer (RGD720) with a thickness of 0.2 cm (serving as 0.7 mL cell suspension reservoir), placed between two block electrodes (1.4571 stainless steel) and PVC isolators, powered by the ECM 830 Square Wave Electroporation System (BTX, Holliston, US) (Figure 1). This setup has been described, and successfully used in several related projects over the past few years.[26] It was made sure that the custom set-up produced a homogeneous field, and the suspension was carefully added to avoid shear stress.

Settings for (ir)Reversible Electroporation on HepG2 Cells

First, the cell viability and heat production were measured after electroporation on HepG2 cells with different pulsing protocols, to determine the most successful pulse parameters and threshold for IRE and RE. In addition, cell permeabilization was examined to confirm pore formation for the selected RE pulsing protocols with the highest cell viability.

Viability. HepG2 cells were harvested, and a suspension of 1 million cells/mL was prepared in HEPES electroporation buffer (HEPES 10 mM, Sucrose 250 mM, and $MgCL_2$ 1 mM). 500 μL cell suspension was added to the spacer in the *in-vitro* electroporation setup.

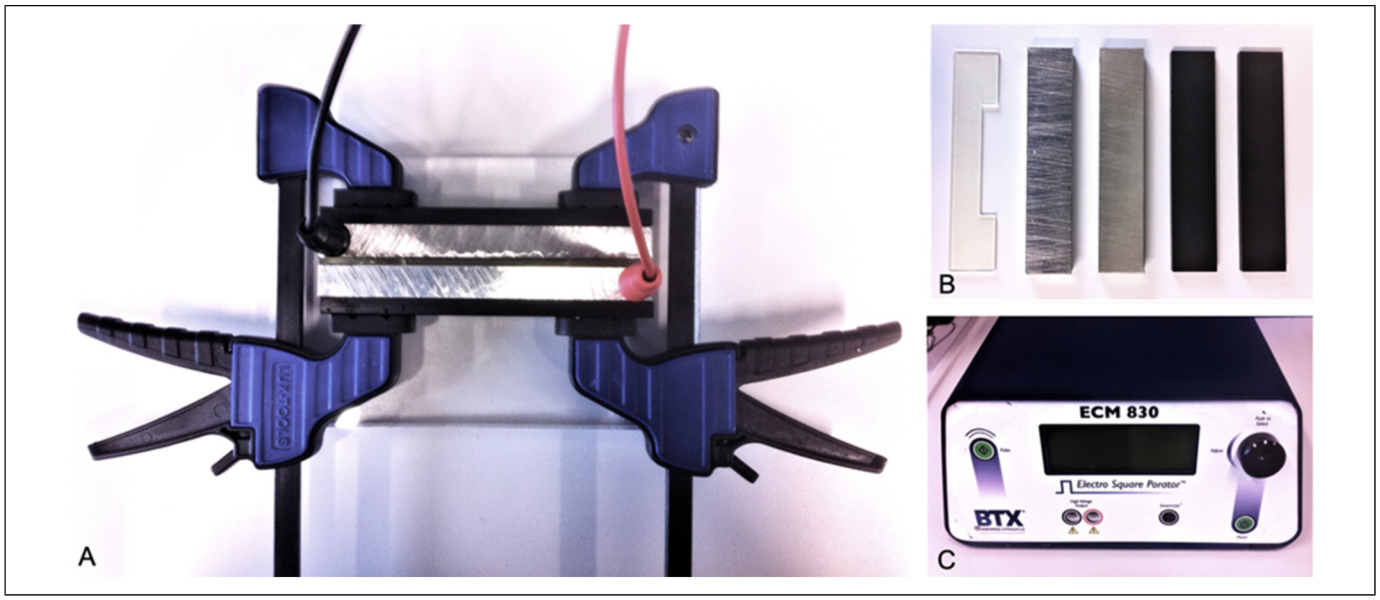

Figure 1. (A) Overview *in-vitro* electroporation setup. (B) Photopolymer spacer 0.2 cm (RGD720), two block electrodes (1.4571 stainless steel) and two insulators (PVC). (C) ECM 830 square wave electroporation system.

For the IRE experiments, the following fixed parameters were used: 70 pulses, 100 μs pulse length, 100 ms interval. The electric field strength was varied between 0 (control), 1000, 2000, 3000, and 4000 V/cm.

For the RE experiments, the following fixed parameters were used: 8 pulses, 100 μs pulse length, 1000 ms interval. The electric field strength was varied between 0 (control), 500, 1000, 2000, and 4000 V/cm.

The number of dead and living cells, as well as the viability of the HepG2 was measured at 0, 5, 10, and 15 min after application of the different pulsing protocols. This was done using a 0.4% Trypan Blue (Gibco, Waltham, US) dye exclusion assay (1:1 ratio) on a LUNA-FL Dual Fluorescence Cell Counter (BioCat GmbH, Heidelberg, DE).

The collected data were exported to a spreadsheet for further analysis. Control groups were set to 100% viability. Accordingly, the cell viability at 0, 5, 10, and 15 min after application of the different pulsing protocols was normalized to the corresponding control group and visualized using GraphPad Prism 5 (GraphPad Software, San Diego, US). Results were displayed in graphs as mean $\pm$ standard deviation (SD). A two-way ANOVA with Bonferroni correction was performed and a two-tailed p-value of <0.05 was set for statistical significance (*). For both IRE and RE, the experimental test series consisted of nine independent experiments, measured in duplicates ($n = 9$). The sample size was based on preliminary data.

Heat Production. In accordance with the viability experiments, 500 μL cell suspension (1 million HepG2 cells/mL in HEPES buffer) was added to the spacer in the *in-vitro* electroporation setup. For both the IRE and RE experiments, the same corresponding fixed and variable electroporation parameters were applied during the viability measurements described above.

While applying these different pulsing protocols to the cells, the temperature in °C was closely monitored using the Testo 882 Thermal Imaging Camera (Testo, Lenzkirch, DE). To ensure reproducibility, the thermal camera was used in combination with the Velbon Vel-flo 9 PH-368 panhead and Velbon DV-7000 tripod (Velbon Corporation, Tokyo, JP), at a 30-degree angle with a ϵ of 0.96. For each pulsing protocol, the peak temperature was determined, the data was exported to a spreadsheet for further analysis and visualized using GraphPad Prism 5. Results were displayed in graphs as mean $\pm$ SD. A 1-way ANOVA with Bonferroni correction was performed and a two-tailed p-value of $<.05$ was set for statistical significance (*). For both IRE and RE, the experimental test series consisted of six independent experiments ($n = 6$).

Cell Permeabilization. Generally, a Calcein acetoxymethyl ester (CAM)/Propidium Iodine (PI) staining is used as a life/death staining, but the combined staining of cells with these two dyes enables quantification of viability and permeability. For this flow cytometric assay, 500 μL cell suspension (1 million HepG2 cells/mL in HEPES buffer), with 40 μM PI (Sigma-Aldrich, St. Louis, US) was added to the spacer in the *in-vitro* electroporation setup. To confirm cell permeabilization for two selected reversible electroporation pulsing protocols, the same fixed electroporation parameters were applied as during the viability measurements described above. The electric field strength was varied between 0 (control), 500 and 1000 V/cm.

After electroporation, the cells were incubated for 5 min at room temperature and washed with FACS-buffer (1x PBS, 5% FBS, 2 mM EDTA). The cells were suspended in FACS-buffer containing 0.2 μM (CAM) (Sigma-Aldrich, St. Louis, US) and incubated for 10 min at RT. A BD

LRSFortessa™ Cell Analyzer (BD, Franklin Lakes, US) flow cytometry device was used to process the samples. CAM signal was measured in the FITC channel, and PI signal was measured in the Texas RED channel. For each sample, 15,000 events were acquired, and the raw fluorescence data were then quantitatively analyzed and visualized with the FlowJo™ software (Ashland, US). The cells were characterized as viable and nonpermeabilized (CAM + /PI−; Q3), viable and permeabilized (CAM + /PI + ; Q2), dead (CAM−/PI + ; Q1), and unstained debris (CAM−/PI−; Q4). The test series consisted of three independent experiments, measured in duplicates ($n = 3$).

Results

Viability

The HepG2 cell viability was measured at 0, 5, 10, and 15 min after electroporation with the different IRE and RE pulsing protocols, as previously described.

For the IRE experiments, the following results were found. Original cell viability of the control group was approximately 91%. Within the same pulsing protocol, no significant difference was found in cell viability between the different measuring time points. When comparing the cell viability at the same time point between different pulsing protocols a significant difference was found. The increase of electric field strength from 0 to 1000, 1000 to 2000, 2000 to 3000, and 3000 to 4000 V/cm resulted in a significant reduction of HepG2 cell viability ($P < .001$). Application of 4000 V/cm resulted in less than 1% cell viability, indicating almost complete cell death (Figure 2A).

For the RE experiments, the following results were found. Original cell viability of the control group was approximately 90%. Within the same pulsing protocol, a significant difference was found in cell viability between the different measuring time points. Values laid within 1% and standard deviations were less than 0.6%. When comparing the cell viability at the same time point between different pulsing protocols a significant difference was found. The increase of electric field strength from 0 to 500, 500 to 1000, 1000 to 2000, and 2000 to 4000 V/cm resulted in a significant reduction of HepG2 cell viability ($P < .001$). Application of 4000 V/cm caused, with approximately 78%, the lowest percentage of cell viability (Figure 2B).

Heat Production

The heat production was measured after electroporation with the different IRE and RE pulsing protocols, as previously described.

For the IRE experiments, the following results were found. The increase of electric field strength from 0 (23.2°C base temperature) to 1000 V/cm (23.5°C), did not significantly increase the temperature. The increase from 1000 to 2000 (24.7°C), 2000 to 3000 (26.4°C), and 3000 to 4000 V/cm (30.1°C) did significantly increase the temperature ($P < .05$, $P < .001$, and $P < .001$, respectively). The total temperature increase was 6.9°C (Figure 3A).

For the RE experiments, the following results were found. The increase of electric field strength from 0 (22.4°C base temperature) to 500 (22.6°C), 500 to 1000 (22.7°C), and 1000 to 2000 V/cm (23.1°C) did not significantly increase the temperature. The increase from 2000 to 4000 V/cm (23.7°C) did significantly increase the temperature ($P < .01$). The total temperature increase was 1.3°C (Figure 3B).

Cell Permeabilization

A CAM/PI flow cytometric assay was performed to confirm cell permeabilization for the RE pulsing protocols with the highest cell viability (Figure 4).

After exposure to an electric field strength of 0 V/cm (control) and 500 V/cm, respectively, 82.2% and 82.8% of the cells were characterized as viable and nonpermeabilized (CAM + /PI−; Q3), whereas 17.8% and 17.2% were characterized as viable and permeabilized (CAM + /PI + ; Q2). After exposure to an electric field strength of 1000 V/cm the majority of cells, 94.3%, were characterized as viable and permeabilized, whereas only 5.7% remained viable and nonpermeabilized. The number of dead cells (CAM−/PI + ; Q1), and unstained debris (CAM−/PI−; Q4) was neglectable in all experimental groups.

Discussion

The human Caucasian hepatocellular carcinoma cell line HepG2 was chosen because it is a good representation of the patient population that is treated in our department and literature.[27–29] Several *in vitro* model systems for IRE have been used in literature, including artificial membrane systems, isolated single cells, and cell suspensions.[30] The latter is the most widely used, as it allows observation of a group of cells under external electrical stimulation, reflecting the average behavior within a cell population.[30] In contrast to classic EP cuvettes, a custom-made cell suspension setup, designed in our department, was used. This setup had been successfully used in previous studies, and, in order to compare the results, the conditions were kept similar.[26] Moreover, classic plastic cuvettes do not have a heat sink effect due to their low heat conductivity, whereas our setup with stainless-steel electrodes does. This is closer to reality, as the liver also has heat sink characteristics due to its vascular structure and high blood flow rate.[31] To maximize the area of a homogeneous field and to minimize effects on the borders and corners as well as boundary effects of the electric field, it was made sure that the surface of the electroporated zone was large compared to the marginal zone. Also, the suspension reservoir was not completely filled, so the suspension was not placed on the edge of the electrodes.

It is demonstrated that electroporation buffer composition can influence cell viability.[32] Therefore, pH changes due to pulsing were minimized by using a buffered, low-conductivity solution and stainless-steel electrodes.[33] Although used widely

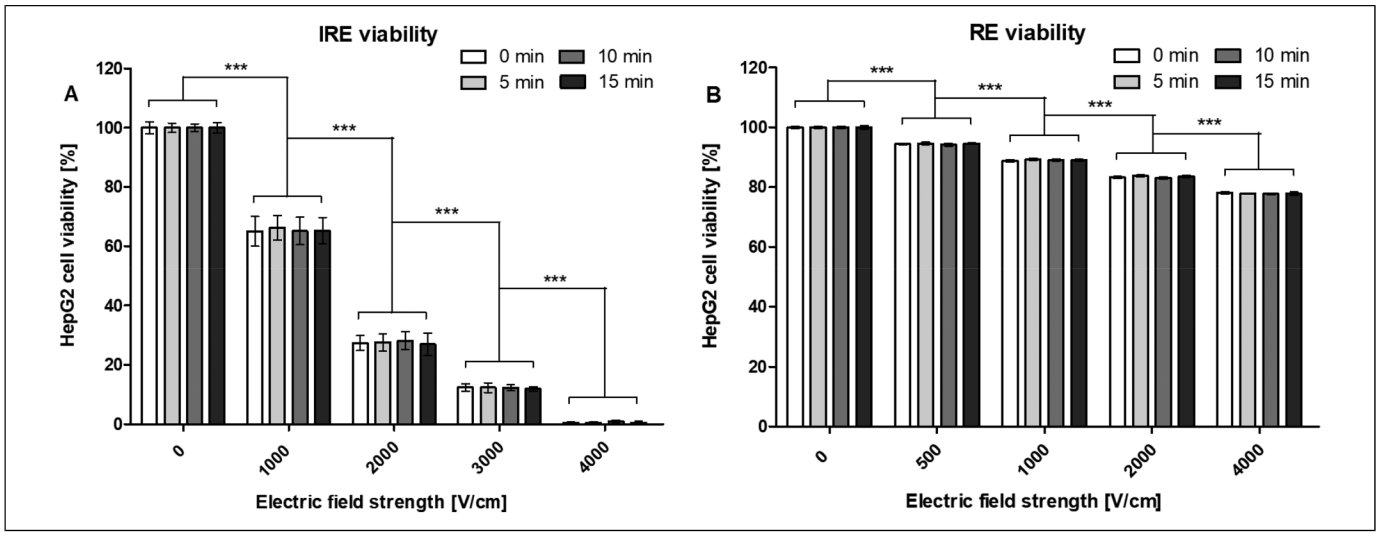

Figure 2. Viability (%) of HepG2 cells at 0, 5, 10, and 15 min after electroporation with the different (A) IRE and (B) RE pulsing protocols. Results are displayed as mean ± SD. A two-way ANOVA with Bonferroni correction was performed (*$P < .05$, **$P < .01$, ***$P < .001$). $n = 9$, in duplicates.

in EP studies, an increase in metal ions present in stainless steel electrodes (Fe, Ni, Cr, and Mn) has recently shown to decrease cell viability in RE studies.[34] So, ion metal release from the electrodes during pulsing should be kept minimal.

Standard IRE and RE pulsing protocols were used to compare the results with the literature and increase the translatability to the clinic. Previous studies from our department also indicated that these protocols were the most effective.[26]

The Trypan Blue dye exclusion assay was used to measure the cell viability at 0, 5, 10, and 15 min after electroporation with the different IRE and RE pulsing protocols. Incubation times were kept short, to avoid any toxicity caused by Trypan Blue. Based on the biological principle, conducting viability measurements with trypan blue after permeabilization of cells may not be optimal. However, pore closure time is completed within tens to hundreds of nanoseconds, so should not interfere with the viability measurements afterwards.[35] This accounts for all samples, as pore closure time is practically independent of the field by which the pore was induced.[35]

Both the control groups for the IRE and RE electroporation experiments had a high and steady starting viability of 91% and 90%, respectively, which indicates a healthy cell culture needed for reliable results.

For the IRE experiments, no significant difference was found in cell viability between the different measuring time points. Within the RE experiments a significant difference was found, but given the close values, small standard deviation, and error of the technique, the difference can be disregarded. This proved that the reduction in cell viability was caused by the applied electric field and could not be attributed to external factors.

Regardless of the measuring timepoint, for both the IRE and RE experiments, the stepwise increase of electric field strength from 0 to 4000 V/cm resulted in a significant reduction of HepG2 cell viability. These results were expected, as increasing the electric field strength leads to the creation of more and bigger nanopores, which eventually lead to cell death.[18] Although the pore formation of electric stimulated membranes is not yet completely understood, it is assumed that the electric field causes lipid molecule rearrangement on the bilayer that supports pore formation.[10,36]

For the IRE experiment, application of 4000 V/cm resulted in less than 0.5% cell viability, indicating almost complete cell death. When comparing this threshold to the data from previous studies performed in our lab with the same setup and different human pancreatic and cholangiocellular cancer cell lines, the HepG2 cells have a similar threshold to BxPc3 cells, Panc1 cells, CCLP1 cells, and SNU1079 cells, but a higher threshold than MiaPaCa2 cells (2000 V/cm).[26] The variation in threshold for different cell lines, is in line with literature, and can be explained by differences in their size and membrane composition, as lipid differences in the outer leaflet of the cell membrane can potentially influence the level of permeabilisation.[37,38] The variation in threshold among different types of cancer cells might be an important factor when considering the treatment planning for IRE in the clinic. Nevertheless, 637 ± 43 V/cm, a value determined by Davalos et al solely on rat liver tissue, is widely accepted as threshold for IRE in most cell types.[39] In addition, it would be ideal if electroporation settings in vitro were directly translatable to human. But based on literature (1000–2000 V/cm) and our results (2000–4000 V/cm), the IRE thresholds for cells in suspension are higher than those measured in tissue.[30,38] This could be explained by the compactness and spherical shape of cells in suspension, compared to their native morphology in tissue. Currently, a similar IRE pulsing protocol with a maximum of 1400 V/cm electric field strength (between the two needle electrodes) is used with the NanoKnife device for the treatment of liver cancer in the clinic.

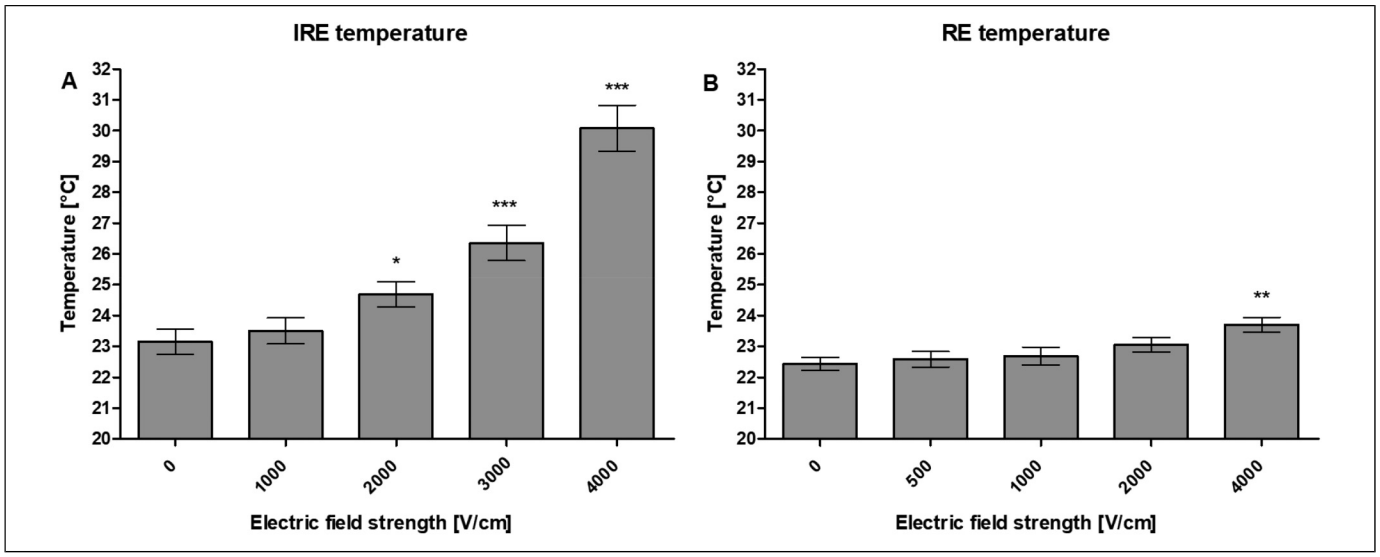

Figure 3. Temperature (°C) of HepG2 suspension after electroporation with the different (A) IRE and (B) RE pulsing protocols. Results are displayed as mean ± SD. A one-way ANOVA with Bonferroni correction was performed (*$P < .05$, **$P < .01$, ***$P < .001$). $n = 6$.

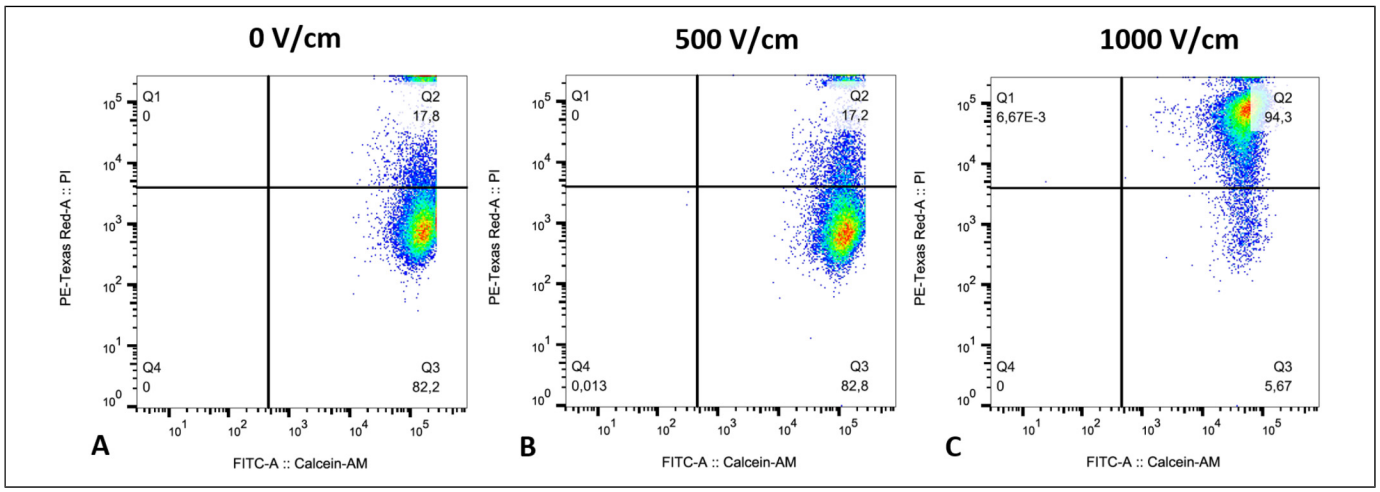

Figure 4. Representative dot-plot graph from CAM/pi flow cytometric assay on HepG2 cells. RE pulse protocol with an electric field strength of (A) 0 V/cm, (B) 500 V/cm, and (C) 1000 V/cm. Cells were characterized as viable and nonpermeabilized (CAM + /PI−; Q3), viable and permeabilized (CAM + /PI + ; Q2), dead (CAM−/PI + ; Q1), and unstained debris (CAM−/PI−; Q4). $n = 3$, in duplicates.

Although IRE is classified as a nonthermal ablation method, reports have shown that Joule heating can lead to thermal damage, especially near the needles.[40] Therefore, the peak temperature was measured during the IRE and RE experiments, with a total temperature increase of 6.9°C and 1.3°C, respectively, starting at ~23°C base temperature. Temperature induces cell death differently; apoptosis occurs around 43°C to 50°C, whereas necrosis occurs at 50°C or higher.[41] This means that the low-temperature changes within this study will not induce cell-death; thus, this data indicates that the reduction in HepG2 cell viability was caused by the applied electric field and was not a result of Joule heating.

For RE, induction of cell death due to the applied electric field is unwanted. The goal is to use a field that creates a majority of viable and permeabilized cells. Therefore, the RE pulsing protocols with the highest cell viability were chosen for further analysis on their pore formation. Application of a RE pulsing protocol with an electrical field strength of 0 V/cm (control) and 500 V/cm resulted in, respectively, 82.2% and 82.8% viable and nonpermeabilized cells, whereas an electric field strength of 1000 V/cm resulted in 94.3%, viable and permeabilized cells. This means that an electric field strength of 500 V/cm was not high enough to induce pore formation in the HepG2 cells, but 1000 V/cm was. This finding is also in line with the standardized treatment protocol for (sub)cutaneous tumors in the framework of ESOPE (8 rectangular pulses, 1000 V/cm, 100 µs), currently used in the clinic.[20–22]

To conclude, an IRE pulsing protocol (70 pulses, 100 μs pulse length, 100 ms interval) with an electric field strength

of 4000 V/cm was needed as threshold for almost complete cell death of HepG2 cells. A RE pulsing protocol (8 pulses, 100 μs pulse length, and 1000 ms interval) with an electric field strength of 1000 V/cm was needed as threshold for viable and permeabilized HepG2 cells. The low peak temperatures within this study indicated that the reduction in HepG2 cell viability was caused by the applied electric field and was not a result of Joule heating.

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Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical Statement

The ethics committee decided no ethical board approval was needed, since the experiments were performed on a commercially available cell line and not on human tissue/samples.

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LABORATORY INVESTIGATION INTERVENTIONAL ONCOLOGY

Electrochemotherapy and Calcium Electroporation on Hepatocellular Carcinoma Cells: An In-Vitro Investigation

K. H. K. Lindelauf[1,2] · M. Baragona[2] · T. Lemainque[1] · R. T. H. Maessen[2] · A. Ritter[1]

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Abstract

Purpose Electrochemotherapy, clinically established for treating (sub)cutaneous tumors, has been standardized in the framework of the European Standard Operating Procedure on Electrochemotherapy (ESOPE). Due to common side effects of chemotherapeutic drugs, recent advances focus on non-cytotoxic agents, like calcium, to induce cell death (calcium electroporation). Therefore, this study aims to determine the efficacy of electrochemotherapy with bleomycin or cisplatin, or calcium electroporation on human hepatocellular carcinoma cells (HepG2) in vitro using the ESOPE protocol.

Methods HepG2 cell viability was measured with a MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay after electrochemotherapy with the chemotherapeutic drugs bleomycin or cisplatin (0–20 μM), or after calcium electroporation (0–20 mM), to determine its efficacy on HepG2 cells in vitro using the ESOPE protocol (8 rectangular pulses, 1000 V/cm, 100 μs) compared to non-electroporated drug treatment.

Results Cell viability was significantly lower in electroporated samples, compared to their non-electroporated controls (27–75% difference). Electrochemotherapy with bleomycin and calcium electroporation, reached (almost) complete cell death ($-1 \pm 3\%$ and $2.5 \pm 2\%$), in the lowest concentration of 2.5 μM and 2.5 mM, respectively. Electrochemotherapy with 2.5 μM cisplatin, significantly decreased cell viability to only 68% ($\pm$ 7%).

Conclusion Electrochemotherapy with bleomycin or cisplatin, or calcium electroporation were more effective in reducing the HepG2 cell viability in vitro using the ESOPE protocol compared to the non-electroporated drug treatments alone. When comparing electrochemotherapy, HepG2 cells are more sensitive to bleomycin than cisplatin, in similar concentrations. Calcium electroporation has the same effectiveness as electrochemotherapy with bleomycin, but calcium potentially has a better safety profile and several treatment advantages.

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Graphical Abstract

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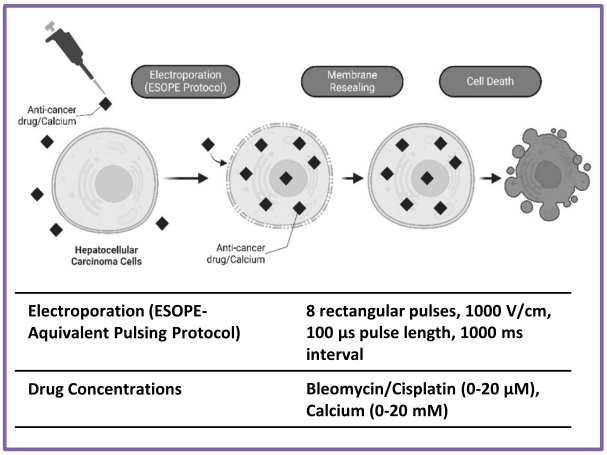
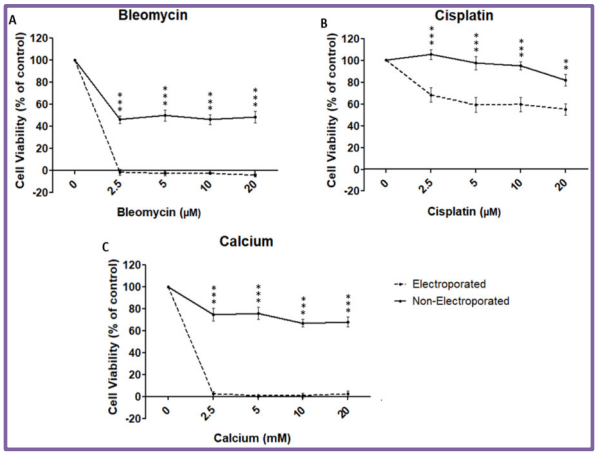
Electrochemotherapy and Calcium Electroporation on Hepatocellular Carcinoma Cells – an In-Vitro Investigation



Electroporation (ESOPE-Aquivalent Pulsing Protocol)	8 rectangular pulses, 1000 V/cm, 100 µs pulse length, 1000 ms interval
Drug Concentrations	Bleomycin/Cisplatin (0-20 µM), Calcium (0-20 mM)



Electrochemotherapy with bleomycin/cisplatin, or calcium electroporation was more effective compared to the non-electroporated drug treatments alone. HepG2 cells are more sensitive to bleomycin than cisplatin. Calcium electroporation has the same effectiveness as electrochemotherapy with bleomycin, but calcium potentially has a better safety profile and several treatment advantages.

Keywords Electrochemotherapy · Calcium electroporation · ESOPE · Hepatic cancer · HepG2 · In vitro

Abbreviations

ANOVA	Analysis of variance
ATP	Adenosine triphosphate
CaEP	Calcium electroporation
CAM	Calcein acetoxymethyl ester
ECT	Electrochemotherapy
EMEM	Eagle's minimum essential medium
EP	Electroporation;
ESOPE	European Standard Operating Procedure on Electrochemotherapy
FBS	Fetal bovine serum
HCC	Hepatocellular carcinoma
IRE	Irreversible electroporation
PEF	Pulsed electric field
PI	Propidium iodine
RE	Reversible electroporation
SEM	Standard error of the mean

Introduction

Cancer is the second leading cause of death worldwide, and the number of patients is still rising rapidly in aging populations [1]. Hepatocellular carcinoma (HCC), accounting for $> 80\%$ of primary liver cancers, has a heavy disease burden and is a leading cause of cancer-related death worldwide [2, 3]. The liver is also a frequent location of metastases for various tumor diseases [4, 5]. Traditional treatment options are not always effective. As with any cancer, the treatment and prognosis of HCC varies depending on the specifics of tumor histology, size, metastatic status, and the overall health of the patient. [6]

Over the past years, minimally invasive cancer therapies have been an important area of research, with nonthermal electroporation (EP)-based therapies among the most promising approaches, as it provides the possibility to operate in proximity to vulnerable structures [7]. In EP-based therapies, a sufficiently high pulsed electric field (PEF) is applied to induce permeabilization of the cell membrane, creating so-called nanopores. [7, 8]

During irreversible electroporation (IRE), these nanopores cannot be repaired because of their size and amount which leads to cell death [7]. During reversible electroporation (RE), the cell can repair its membrane and continue normal cell function. In clinical practice, those transient hydrophilic pores are used to promote the

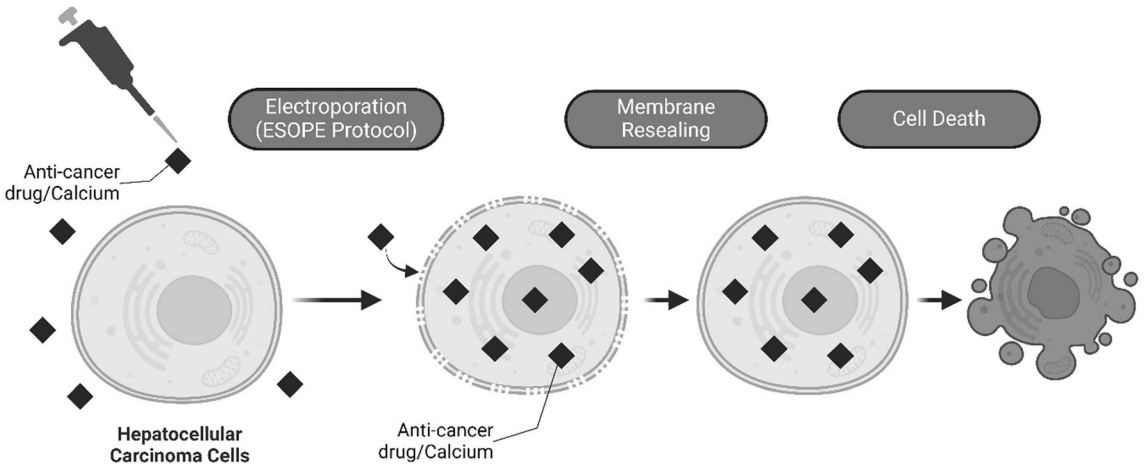


Fig. 1 Principles of electrochemotherapy (ECT) and calcium electroporation (CaEP)

diffusion of hydrophilic chemotherapeutic drugs, most commonly bleomycin or cisplatin. This therapeutic approach is known as Electrochemotherapy (ECT) (Fig. 1) [8, 9]. Currently, ECT is an established and efficient option in the clinic for the treatment of (sub)cutaneous tumors [10]. Its treatment protocol has been standardized in the framework of the European Standard Operating Procedure on ECT (ESOPE). [11, 12] Recent advances focus on the development of ECT for deep-seated tumors, such as liver cancer. Although the first clinical studies showed promising results, the intraoperative procedure during open surgery was considered a limitation [13]. This limitation has recently been overcome with the development of a new pulse generator Cliniporator®VITAE (IGEA SpA, Carpi, Italy) in combination with long needle electrodes, which can generate sufficient power to treat deep-seated tumors percutaneously. The first attempts on HCC patients have shown this method to be safe, feasible and efficient. [14, 15]

Due to common side effects of chemotherapeutic drugs, recent advances focus on the investigation of non-cytotoxic agents, such as calcium (as compound often administered as the salt calcium chloride), which can also be internalized into the cells in high concentration by electroporation to induce cell death (Fig. 1) [16, 17]. Calcium is a tightly regulated ubiquitous intracellular second messenger involved in many cellular processes, including cell death [18]. Calcium electroporation (CaEP) has shown to efficiently induce cell death in vitro, in vivo, and in clinical trials, through adenosine triphosphate (ATP) depletion, using electroporation parameters similar to the ESOPE protocol for ECT [19–22]. In a previous study, we have verified the ESOPE protocol for RE on human HCC cells (HepG2) in vitro, but without performing ECT or CaEP yet, i.e., without the application of chemotherapeutic drugs or calcium. [23]

Based on these promising results, more basic research needs to be done to transfer CaEP to clinical practice, and to extend the application of ECT to deep-seated tumors like liver cancer.

Therefore, this follow-up study aims to determine the efficacy of ECT with bleomycin or cisplatin, or CaEP on HepG2 cells in vitro using the ESOPE protocol, compared to non-electroporated drug treatment.

Methods

In this translational study, the HepG2 cell viability was measured after ECT with the chemotherapeutic drugs bleomycin or cisplatin, or after CaEP, to determine its efficacy on HepG2 cells in vitro using the ESOPE protocol, compared to non-electroporated drug treatment.

Cell Culture

The human HCC cell line HepG2 (ATCC, Manassas, US), derived from a Caucasian male, was used for all in vitro experiments. As described previously, the cells were routinely subcultured twice a week (1:5 ratio) by trypsinization. Eagle's Minimum Essential Medium (EMEM) (ATCC, Manassas, US) supplemented with 1% penicillin/streptomycin (100 U/mL and 100 µg/mL) and 10% fetal bovine serum (FBS) (100 µL/mL) was used as culture medium. The cells were grown in a humidified incubator (37 °C with 5% CO2). [23]

Electroporation Parameters

The RE electroporation parameters for HepG2 cells were determined and verified in a previously conducted study,

where a detailed description of the materials and methods can be found. [23]

In an in vitro setup, HepG2 cell viability was measured with a Trypan Blue dye exclusion assay at 0, 5, 10 and 15 min after electroporation with a RE pulsing protocol (8 rectangular pulses, 100 µs pulse length, 1000 ms interval resp. 1 Hz) combined with variable electric field strengths (0–4000 V/cm), to determine the most successful settings for RE (n = 9, in duplicate). To confirm cell permeabilization for two selected RE pulsing protocols (500 and 1000 V/cm), a Calcein acetoxymethyl ester (CAM)/Propidium Iodine (PI) flow cytometric assay was performed (n = 3, in duplicate).

Electrochemotherapy and Calcium Electroporation

A suspension of 1 million HepG2 cells/mL was prepared in a HEPES-based low-conductivity electroporation buffer (HEPES 10 mM, Sucrose 250 mM, $MgCL_2$ 1 mM). A variety of cell-drug suspensions was prepared. The final drug concentration was varied between 0 (control), 2.5, 5, 10 or 20 µM bleomycin (BLEO-cell, STADAPHARM GmbH, Bad Vilbel, DE) or cisplatin (Cisplatin Teva, TEVA GmbH, Ulm, DE), and 0 (control), 2.5, 5, 10 or 20 mM calcium (calcium chloride dihydrate, $CaCl_2{\cdot}2H_2O$, Sigma-Aldrich, St. Louis, USA). 400 µL of the above-mentioned cell-drug suspensions was added to a BTX Electroporation Cuvette Plus (2 mm, 400 µL) (BTX, Holliston, US), powered by a Gemini Twin Wave Electroporator (BTX, Holliston, US). The samples were either electroporated following the ESOPE protocol (8 rectangular pulses, 1000 V/cm, 100 µs pulse length, 1000 ms interval resp. 1 Hz), previously confirmed for this cell type, or sham-exposed and non-electroporated [23]. The sample size was based on preliminary data, and consisted of 9 independent experiments, measured in duplicates (n = 9). [23]

The samples were centrifuged (5000 rpm, 5 min) and the supernatant was discarded. The samples were resuspended in culturing medium and seeded (10.000 cells/well, in duplicates) in a 96 well plate (VWR, Radnor, US). A blank well with only culturing medium served as background control.

After the plates were incubated for 72 h at 37 °C, a MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide) assay (Thermo Fisher Scientific, Waltham, US) was performed to measure the HepG2 cell viability. The medium was replaced with 100 µL of fresh medium, and 10 µl of 12 mM MTT solution was added to each well. The plates were incubated for 4 h at 37 °C. After the incubation, 25 µl medium was kept in the wells and 50 µl DMSO (AppliChem GmbH, Darmstadt, DE) was added and mixed thoroughly. After incubation for 10 min at 37 °C the samples were mixed again, and the absorbance was read at 540 nm using a BioTek Synergy HT Microplate Reader and Gen5 microplate reader software (BioTek Instruments, Winooski, US).

Statistics

The collected data was analyzed. For all samples, the duplicates were averaged, the background absorbance was subtracted, and the cell viability was calculated. Control groups without drug were set to 100% viability. Accordingly, the cell viability of the experimental samples was normalized to the corresponding control group and visualized using GraphPad Prism 5 (GraphPad Software, San Diego, US). Obvious outliers have been removed from the data set. Results were displayed in graphs as mean ± standard error of the mean (SEM). A two-way analysis of variance (ANOVA), with Bonferroni-correction as pairwise post-hoc comparison, was performed between corresponding electroporated and non-electroporated samples (visually indicated in Fig. 3), and between the different drug concentrations (not visually indicated in Fig. 3, to avoid confusion). two-tailed p-values of $p < 0.05$ were interpreted as statistically significant (*).

Results

Electroporation parameters

A RE pulsing protocol (8 rectangular pulses, 100 µs pulse length, 1000 ms interval resp. 1 Hz) with an electric field strength of 1000 V/cm was needed as threshold for viable and permeabilized HepG2 cells (Fig. 2). These parameters correspond to the ESOPE protocol. [23]

Electrochemotherapy and Calcium Electroporation

The HepG2 cell viability was measured and compared after ECT with increasing concentrations of the chemotherapeutic drugs bleomycin or cisplatin, or after CaEP following the ESOPE pulsing protocol.

For all administered concentrations of bleomycin, cisplatin, or calcium, the HepG2 cell viability was significantly lower in the electroporated sample, compared to its non-electroporated control ($p < 0.001$) (Fig. 3).

After ECT with 2.5 µM bleomycin, the cell viability significantly decreased to -1% (± 3%), indicating complete cell death ($p < 0.001$). After administration of 2.5 µM bleomycin alone, without electroporation, the cell viability significantly decreased, but only to 46% (± 4%) ($p < 0.001$). For both ECT and the non-electroporated treatment, the further increase of bleomycin concentration

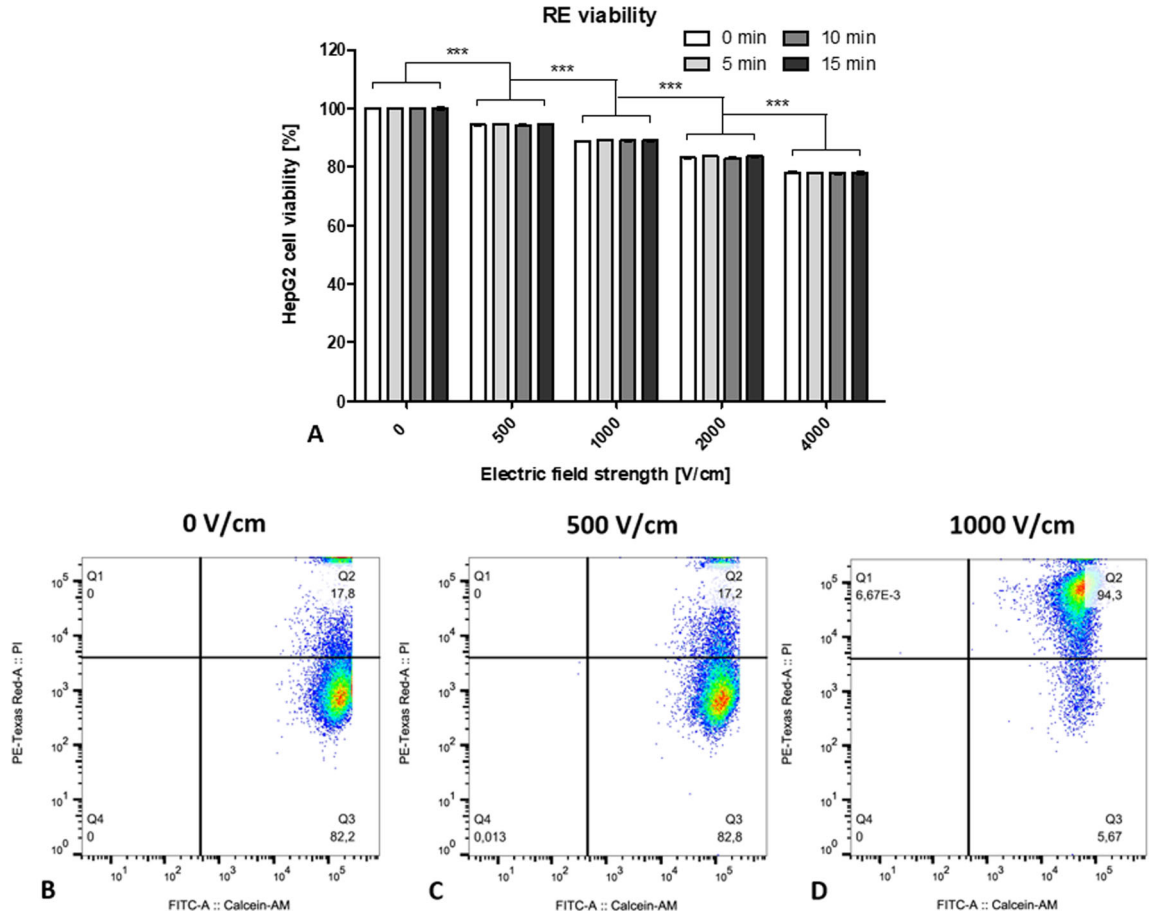


Fig. 2 **A** Cell viability (% of control) of HepG2 cells at 0, 5, 10 and 15 min after electroporation with a RE pulsing protocol (8 pulses, 100 µs pulse length) combined with variable electric field strengths (0–4000 V/cm), measured with a Trypan Blue dye exclusion assay. The results are displayed as mean ± SD. A two-way ANOVA with Bonferroni correction was performed between the different field strengths (results of pair-wise post hoc comparisons indicated above the data points, $*p < 0.05$, $**p < 0$ .01, $***p < 0.001$). n = 9, in duplicates. **B** Representative dot-plot graph from CAM/PI flow cytometric assay on HepG2 cells. RE pulsing protocol with an electric field strength of 0 V/cm (**B**), 500 V/cm (**C**), and 1000 V/cm (**D**). The number of cells is represented by the color intensity. Cells were characterized as viable and nonpermeabilized (Q3; CAM+/PI−), viable and permeabilized (Q2; CAM+/PI+), dead (Q1; CAM−/PI+), and unstained debris (Q4; CAM−/PI−). n = 3, in duplicates.[23]

to 5, 10, and 20 µM, did not result in a significant change in HepG2 cell viability ($p > 0.05$) (Fig. 3A).

After ECT with 2.5 µM cisplatin, the cell viability significantly decreased to 68% (± 7%), whereas 20 µM Cisplatin was needed to significantly decrease the cell viability to 82% (± 5%) in the non-electroporated sample ($p < 0.001$). Within the ECT treatment, the further increase of cisplatin concentration to 5, 10, and 20 µM, did not result in a significant change in HepG2 cell viability ($p > 0.05$) (Fig. 3B).

After CaEP with 2.5 mM calcium, the cell viability significantly decreased to 2.5% (± 2%), indicating almost complete cell death ($p < 0.001$). After administration of 2.5 mM calcium alone, without electroporation, the cell viability significantly decreased, but only to 75% (± 6%) ($p < 0.001$). For both CaEP and the non-electroporated treatment, the further increase of calcium concentration to 5, 10, and 20 mM, did not result in a significant change in HepG2 cell viability ($p > 0.05$) (Fig. 3C).

Discussion

In this in vitro study, the HepG2 cell viability was measured after ECT with increasing concentrations of the chemotherapeutic drugs bleomycin or cisplatin, or after CaEP, to determine its efficacy on HepG2 cells in vitro using the ESOPE protocol, compared to non-electroporated drug treatment.

The colorimetric MTT assay, which assesses cell metabolic activity, was used as a representation of HepG2 cell viability [19]. One might argue that relatively high concentrations of bleomycin are used in this in vitro study. However, the concentration range for the drug bleomycin,

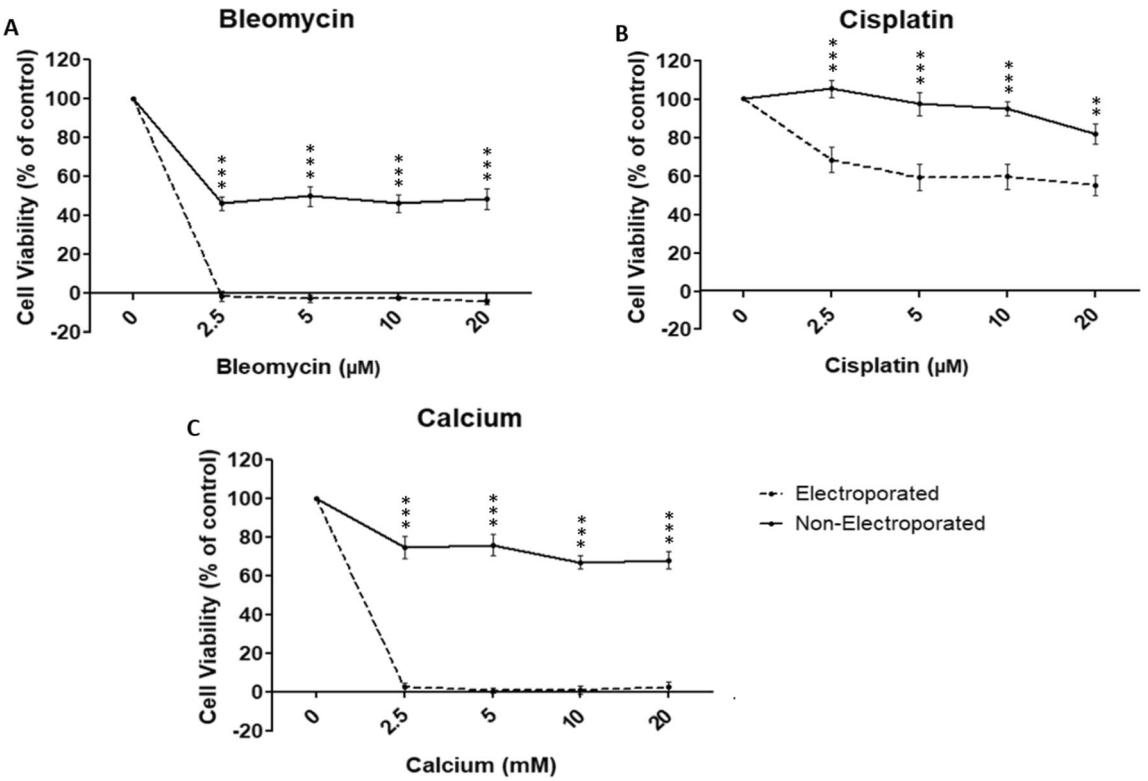


Fig. 3 Cell Viability (% of control) of HepG2 cells after ECT with the chemotherapeutic drugs bleomycin (**A**) or cisplatin (**B**), or after calcium-electroporation (**C**) with the ESOPE pulsing protocol (8 pulses, 1000 V/cm, 100 μs), measured with an MTT assay. Sham-exposed non-electroporated samples served as control. Results are displayed as mean ± SEM. A two-way ANOVA with Bonferroni correction was performed between corresponding electroporated and non-electroporated samples (results of pair-wise post hoc comparisons indicated above the data points, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$), and between the different drug concentrations (post hoc comparison results not visually indicated). n = 9, in duplicates

as well as cisplatin and calcium was chosen to include the dosage used in literature for relevant and comparable in vitro studies, with proven effectiveness [12, 19, 21, 24–26]. These studies selected 10 μM bleomycin as optimal in vitro concentration from a test range of 0.1–50 μM bleomycin [21, 24]. In addition, the goal of this paper was not to find the optimal dosage for the drugs, but to confirm the effectiveness of electroporation combined with classic chemotherapeutic drugs, and CaEP as a novel addition, on HepG2 cells. By using similar literature, and adding a few higher and lower concentrations, the test range of the dosage needed for an effective treatment could be limited.

For all administered concentrations of bleomycin, cisplatin, or calcium, the combination with electroporation was more effective (minimum 27% and maximum 75% difference) in reducing the HepG2 cell viability compared to the non-electroporated controls, emphasizing the overall potential for ECT and CaEP. In general, to treat tumors with the same efficiency, ECT and CaEP use less drugs than conventional chemotherapies. This is in line with literature, as electro-permeabilization studies with classic chemotherapeutics have reported an enhanced cytotoxicity up to several thousand-fold for bleomycin, and up to 80-fold for cisplatin due to an increased cellular uptake and accumulation of the drug [27, 28]. Moreover, our results also show that, within the same concentration range, ECT with bleomycin is more effective than with cisplatin to reduce HepG2 cell viability. Thus, supporting the role of bleomycin as the chemotherapeutic drug of choice for traditional ECT.

ECT with bleomycin and CaEP had a similar effect on HepG2 cell viability, reaching (almost) complete cell death in the lowest concentration of 2.5 μM and 2.5 mM, respectively. Further concentration increase of the drugs did not cause any significant changes. However, it has been reported that 10 μM bleomycin and 5 mM calcium are considered to be the optimal concentrations in vitro as they cause 80% cell death [19, 21, 24, 25]. This suggests that the HepG2 cells within our study were more sensitive to both drugs in combination with electroporation. This underlines that more research is needed to optimize the drug concentrations for different cell types. It has been reported that several physical and biological cell properties can affect electroporation efficiency. [12, 29]

Despite their equal effectiveness combined with electroporation, bleomycin and calcium display different cytotoxic profiles when used as drug alone without electroporation. Calcium alone caused a reduction of HepG2 cell viability to 75%, compared to 46% for bleomycin, this suggests that CaEP is an equally effective but safer option. Similar studies, for different cell lines, have even reported that calcium alone had no significant impact on cell viability in comparable concentrations up to 20 mM. [12, 19, 30] Initial clinical studies have also found high doses of calcium (220–225 mM) to be well tolerated, contrary to classic chemotherapeutics. [22, 31]

Besides calcium's potentially good safety profile, especially for patients, but also for the staff handling the drug, it is efficient, easily available, inexpensive, heat-stable and has a long shelf life [32]. This shows that CaEP, compared to traditional ECT, has the potential to be an applicable treatment for high-, middle, and low-income countries.

In this study, the HepG2 cell line was again chosen for experiments because of its good representation of the patient population with liver cancer treated in our department, where the majority classifies as Caucasian male, and in literature [23]. However, the previously used cell suspension setup, designed in our department, was replaced by classic EP cuvettes. Together with the ESOPE-protocol, of which the RE effectiveness had been previously proven for the HepG2 cell line (Fig. 2), it allowed for better comparison with relevant literature. An effect of pH and temperature changes due to pulsing on cell viability can be excluded in this study. As before, pH changes were minimized by using stainless-steel electrodes and a buffered, low-conductivity solution. In addition, the ESOPE protocol has shown not to significantly change the temperature of the samples. [23]

However, there are some limitations to the used methods. During (almost) complete cell death, some HepG2 cell viability values (ECT with bleomycin) measured by the MTT assay, appeared to be negative. However, there was no significant difference between the positive and negative values, resulting in an overall 0% cell viability. Some values exceeded 100% viability (2.5 μM cisplatin non-electroporated). This specific treatment might have stressed the cells, causing an increase in their metabolism as a response, detected by the MTT assay [33]. In addition, to get an initial idea about the suitable IRE and RE pulse parameters, and the efficacy of ECT and CaEP, the initial study as well as this follow-up study have been performed in vitro using HepG2 cells. As IRE and ECT work in cell suspension as well as in deep seated tumors, in vitro studies for CaEP with hepatic cell lines are an important indicator and basic prerequisite for animal experiments. As limitation, these results are not directly translatable to the clinic, as the in vivo environment is more complex.

To conclude the current study, ECT with bleomycin or cisplatin, or CaEP were more effective in reducing the HepG2 cell viability in vitro using the ESOPE protocol compared to the non-electroporated drug treatments alone. When comparing ECT, HepG2 cells are more sensitive to bleomycin than cisplatin, in similar concentrations. CaEP has the same effectiveness as ECT with bleomycin, but calcium potentially has a better safety profile and several treatment advantages. Despite these promising results, more research is needed to transfer CaEP as novel cancer treatment to the clinic, and to extend the application of traditional ECT to deep-seated tumors like liver cancer.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors. The ethics committee decided no ethical board approval was needed, since the experiments were performed on a commercially available cell line.

Consent for Publication For this type of study consent for publication is not required.

Informed Consent For this type of study informed consent is not required.

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Affidavit according to § 5 (1) for Data Retention

Hiermit erkläre ich, dass die dieser Dissertation zu Grunde liegenden Originaldaten bei meinem Betreuer, Dr.-Ing. Andreas Ritter, Klinik für Diagnostische und Interventionelle Radiologie, des Universitätsklinikums Aachen, hinterlegt sind.

Affidavit according to § 5 (1) and (2), and § 11 (3) 12 of the doctoral studies regulations

I, Kim Hella Karla Lindelauf, hereby declare on oath, that I have contributed a significant part, and thus majority, of the publication:
Lindelauf, K. H. K., Baragona, M., Baumann, M., Maessen, R. T. H., and Ritter, A.: Pulse Parameters and Thresholds for (ir)Reversible Electroporation on Hepatocellular Carcinoma Cells in Vitro; Technology in Cancer Research & Treatment. 2023;22:1-8.
The contributions to the publication were as follows:

Names	Candidate (K.H.K. Lindelauf)	Practical supervisor (M. Baragona)	Collaborative partner (R.T.H. Maessen)	Collaborative partner (M. Baumann)	Doctoral supervisor (A. Ritter)	Sum (%)
Study supervision		40			60	100
Study design/conception	60	10	10		20	100
Collection of data	100					100
Data analysis	100					100
Performing viability experiments	100					100
Performing temperature experiments	100					100
Performing pore formation experiments	100					100
Statistical evaluation	100					100
Delivery of materials			25	25	50	100
Interpretation of data evaluation	80	10			10	100
Writing/draft of the manuscript	100					100
Correction of the manuscript		40		20	40	100

The position as the first author obviously arises from this significant contribution.

_____
Signature of the doctoral candidate

As supervisor and / or corresponding author I confirm the statements of Kim Hella Karla Lindelauf and as a representative of the collaborative partners, M. Baragona and R.T.H Maessen

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Signature of the doctoral supervisor Signature of the corresp. author (if different)

As co-author I endorse the statement of Univ.-Prof. Dr. med. Philipp Bruners

M. Baumann: A. Ritter:
Names and signatures of all German speaking co-authors

Affidavit according to § 5 (1) and (2), and § 11 (3) 12 of the doctoral studies regulations

I, Kim Hella Karla Lindelauf, hereby declare on oath, that I have contributed a significant part, and thus majority, of the publication:
Lindelauf, K. H. K., Baragona, M., Lemainque T., Maessen, R. T. H., and Ritter, A.: Electrochemotherapy and Calcium Electroporation on Hepatocellular Carcinoma Cells: An In-Vitro Investigation; CardioVascular and Interventional Radiology. 2024;47(10):1384-1391.
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Study supervision		40			60	100
Study design/conception	60	10		10	20	100
Collection of data	100					100
Data analysis	100					100
Performing viability experiments	100					100
Statistical evaluation	90		10			100
Delivery of materials				50	50	100
Interpretation of data evaluation	80	10			10	100
Writing/draft of the manuscript	100					100
Correction of the manuscript		40	20		40	100

The position as the first author obviously arises from this significant contribution.

Signature of the doctoral candidate

As supervisor and / or corresponding author I confirm the statements of Kim Hella Karla Lindelauf and as a representative of the collaborative partners M. Baragona and R.T.H. Maessen

Signature of the doctoral supervisor

Signature of the corresp. author (if different)

As co-author I endorse the statement of Univ.-Prof. Dr. med. Philipp Bruners

T. Lemainque: A. Ritter:

Names and signatures of all German speaking co-authors