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ORIGINAL PAPER

Treatment of cervical cancer by electrochemotherapy with bleomycin, cisplatin, and calcium: an in vitro experimental study

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Abstract

Low-dose chemotherapy in advanced stages of cancer does not give a positive response in treatment. The use of high-dose antineoplastic drugs creates significant side effects. The limiting situation in treatment creates a need for new generation drugs with less side effects and new treatment methods that will enable low-dose drug use. Electroporation (EP), a phenomenon, is a technique in which the membrane permeability is increased by the establishment of hydrophilic pores in the cell membrane with short and high-voltage electrical pulses. In the present investigation study, we aimed to inspect the effects of EP plus bleomycin, cisplatin, and calcium administration (CaEP) on cell viability, apoptotic activity, gene expression p53, Bax/Bcl-2 rate mitochondrial membrane potential ($\Delta\Psi$m), and cell cycle in HeLa cervical cancer cell line. The permeabilization of the membrane was evaluated in flow cytometry with the PI method, and cell viability was measured in an ELISA reader with the WST-8 method. For bleomycin and cisplatin doses applied to HeLa cells, the concentration values (IC_{50}) that inhibited 50% of the cells were found to be 214.11 ± 4.7 µM and 35.16 ± 3.3 µM, respectively. The IC_{50} values of the groups administered together with EP were calculated as 0.44 ± 0.3 µM for bleomycin and 20.55 ± 4.3 µM for cisplatin. There was no change in cell viability in calcium alone application, but a statistically notable reduction in cell vitality was observed in CaEP application. An increase in $\Delta\Psi$m was found in bleomycin and CaEP exposure with EP. It was determined that EP exposure caused G0/G1 arrest in the cell cycle at all electric field intensities. It was determined that EP application in HeLa cells increased bleomycin cytotoxicity 487 times and cisplatin cytotoxicity 1.71 times, and CaEP could be an alternative treatment method.

Keywords Electroporation · Electrochemotherapy · Cervical cancer treatment · Cell viability · Cell cycle

Introduction

Cancer is an major public health issue with the highest mortality in the world after cardiovascular diseases. Cervical cancer at world is the fourth eventual widespread sort of cancer in women [1]. Treatment for cervical cancer varies depending on the stage of the cancer. The most commonly used methods in the treatment of cervical cancer; surgery, chemotherapy, and radiotherapy. Bleomycin, cisplatin, carboplatin, bevacizumab, and gemcitabine are accepted chemotherapeutic drugs in cervical cancer. These drugs cause serious side effects when administered at higher doses and significantly affect the quality lifetime patients [2, 3]. Electroporation (EP) is a biophysical event membrane permeability are rised by creating nanometer (nm) hydrophilic pores in the membrane with the application of pulsed electric field to cells and tissues [4–9]. The duration of electroporation pulses ranges from milliseconds (ms) to microseconds (µs), and the intensity of the pulses ranges from hundreds to thousands of volts per centimeter (V/cm) [10]. EP may agent reversible or irreversible pore creation in the membrane contingent on the E pulse parameters (shape-number-duration and intensity of the pulse) applied. Reversible electroporation is the temporary high conductivity state of the membrane. It is used in medicine and biotechnology, which is excluded by the membrane under usual circumstances where the cells maintain their vitality, to ensure the entry of substances changing from until small molecules (Drugs,

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fluorescent dye, etc.) to large molecules (DNA, antibodies, etc.) into the cell [11–13]. Electrochemotherapy (ECT) is a tumor therapy that improves drug diffusion by combining the administration of a poorly penetrating cytotoxic agent with reversible electroporation. This method includes a locally applied and non-thermal treatment. Reversible EP applied at the high extracellular drug concentration provides an effective anti-tumor activity by facilitating the uptake of chemotherapeutic drugs into the cell. [14–16]. ECT therapy considerably increases the efficacy of chemotherapeutic agents over a standard chemotherapy. In this case, the dose of chemotherapeutic agents is reduced and their side effects can be reduced. Various studies have demonstrated the efficacy of ECT using different chemotherapeutic agents. Intracellular and extracellular Ca^{2+} are responsible for many cellular activities, neural transmission, and muscle contraction-relaxation. Ca^{2+} concentration is tightly regulated within cells. The intracellular concentration of Ca^{2+} is about 10^{-7} M, which is much lower than the extracellular calcium level 10^{-3} M. [17–20]. Disturbances or changes in Ca^{2+} homeostasis can agent destroyed life of cells via apoptosis or necrosis. With the application of EP to cells in the attendance of Ca^{2+}, the concentration of intracellular Ca^{2+} greatly rising. With the application of calcium electroporation (CaEP), the increase in the concentration of intracellular Ca^{2+} ions can lead to cell death (apoptosis and necrosis) by several different mechanisms. These mechanisms are; It can be explained as ATP depletion and production of reactive oxygen species due to the formation of pores in the mitochondrial membrane and the loss of electrochemical gradient leading to ATP production, and increased activity of Ca^{2+}-ATPase and other ATPases [21–26].

The research has actual four primary purposes: (1) research the efficacy of chemotherapeutic drugs + EP (Cisplatin and bleomycin) in the therapy of in vitro cervical cancer cells, (2) research whether underdose of cisplatin and bleomycin treatment with EP was as effective high doses of therapy in extinction of HeLa cervical cancer cells, (3) examine the cytotoxic effect of CaEP in HeLa cells and whether it is as effective as chemotherapeutic drugs, and (4) search of the mechanism of action of ECT and CaEP applications in human cervical cancer cell line HeLa.

Materials and methods

Cell culture and bleomycin, cisplatin, and calcium treatment

In this study, we used the HeLa cell line. The cell line was acquired from the Health Sciences University Gülhane Stem Cell Research Laboratory. HeLa cells were cultured in 75-cm^2 culture flasks in Dulbecco's modified Eagle's medium (DMEM Capricorn, DMEM-HA) enhance with 10% fetal bovine serum (FBS Invitrogen, Capricorn, FBS-11A) and 1% penicillin/streptomycin antibiotic (PSA, HyClone). HeLa cells were treated with different concentrations (10–800 µM) of bleomycin (Bleomycn Sulfate, Sigma-Aldrich), (10–100 µM) of cisplatin (Sigma-Aldrich), and (1–5 mM) calcium chloride ($CaCl_2$, Sigma-Aldrich).

Electroporation protocol

100 µL of cell suspension at an intensity of 1×10^6 cells/mL was placed in EP cuvettes with 4 mm gaps and 10 µL of propidium iodide (PI, ABP Biosciences) was added. Cuvettes were placed in the electroporation chamber in the BTX square wave pulse generator (BTX ECM 830). With the BTX electroporator system, increasing electric field intensities between 0.4 and 3.2 kV/cm for 100 µs were applied to each EP cuvette as 8 square wave pulses at a repetition frequency of 1 Hz. Control cells in the electroporation cuvette were placed in the electroporation chamber, but no electrical pulse was applied. Experiments were repeated three times.

Determination of electropermeabilization

In this study, a non-membrane-penetrating fluorescent dye, PI, was used to determine the membrane permeability of electroporated cells. If a pore is formed after electroporation, the membrane becomes permeable and PI enters the cell. In this case, PI stains both DNA and RNA and emits red fluorescence. 10 µL of propidium iodide (PI) was added to 100 µL of cell suspension at a intensity of 1×10^6 cells/mL just prior to EP application. EP was then applied and cells were incubated for 10 min at room temperature. Mean fluorescence intensity of permeabilized cells and of percentage were obtained by using fluorescence microscopy (Leica) and flow cytometry (BD Accuri C6 Plus). The percentage of permeabilized cells was obtained compared to non-electroporated control cells. Each sample was measured with at least 10,000 cells.

Cell viability assay

The viability of cells after 24 h the application of electroporation (30 min and 24 h), bleomycin, cisplatin, $CaCl_2$, bleomycin + electroporation, cisplatin + electroporation. and CaEP was measured by the WST-8 assay kit. 100 µL of cell suspension was placed in 96-well plates. It was incubated at 37 °C in 5% CO_2, 95% humidity. After incubation, 10 µL of cell proliferation reagent WST-8 (ABP Biosciences, A014-1, A014-2) was added to each well. After 3 h, absorbance values at 450 nm wavelength were measured with an ELISA reader spectrophotometer (Molecular Devices Filter

Max F5). Results were presented as percent of cell viability (% of control cells).

Mitochondria membrane potential assay

The mitochondria membrane potential of cells 24 h after the treatments of bleomycin, cisplatin, $CaCl_2$, bleomycin + electroporation, cisplatin + electroporation, and CaEP was measured by the JC-1 (ABP Biosciences, A048) assay. Briefly, cell suspensions were seeded into 96 wells. After 24 h of incubation, the cell culture medium was aspirated and 200 µL of PBS was added. 2 µL of JC-1 stock solution was added to each well and incubated for 30 min at 37 °C, 5% CO_2. After incubation, cells were washed twice with 200 µL of PBS and 200 µL of PBS was added to each well again. Red fluorescence and green fluorescence (respectively, values excitation and emission of 550 nm, 600 nm for red fluorescence and 485 nm, 535 nm for green fluorescence) were measured using a spectrofluorometric plate reader. The red/green fluorescence ratio was identified. Results were presented as percent of change in mitochondria membrane potential ($\Delta\Psi m$) (% of control cells).

Cell cycle assay

100 µL of HeLa cell suspension at a density of 1×10^6 cells/mL was placed in electroporation cuvettes with 4 mm gaps. With the BTX electroporator system, 8 square wave pulses were applied to each electroporation cuvette at a repetition frequency of 1 Hz with an intensity of 0.4–3.2 kV/cm for 100 µs. Bleomycin (10 and 100 µM), cisplatin (10 and 25 µM), and $CaCl_2$ (1 and 5 mM) were applied to HeLa cells alone and with electroporation. For cell cycle analysis after the treatments, cells were placed in 6-well cell plates in the form of 200 µL suspension and incubated at 37 °C in 5% CO_2, 95% humidity. After incubation, cells were trypsinized and centrifuged and supernatants were discarded. The cells were fixed for 15 min with pure ethanol, which was kept at − 20 °C for a day, and the ethanol was removed by centrifugation again. Fixed cells were washed 2 times with PBS (Capricorn PBS-1A). Finally, 0.5 mL of PI/RNAse solution (ABP Biosciences, A056) was added and cell cycle analysis was performed by flow cytometry.

Determination of bax, Bcl-2 and P53 by polymerase chain reaction (PCR) method

In order to determine Bax, Bcl-2, and p53 gene expressions by PCR method, the applications of the experimental groups determined in HeLa cells were carried out. Cells were incubated for 24 h at 37 °C and 5% CO_2. Cells were removed from the flask surface by trypsinization after incubation. Trypsin was removed, cells were washed with fresh medium, and experimental groups were delivered to the unit where PCR analysis would be performed under appropriate conditions. RNA isolation and Cdna synthesis with appropriate primers and real-time (RT) PCR analysis were performed by the Molecular Research and Application Unit of the University of Health Sciences as service procurement. QIAGEN brand Cat.No.74104 RNeasy mini kit was used for mRNA isolation. In order to perform gene expression study from the mRNA molecule, first of all, the single-stranded RNA molecule must be double-stranded with the reverse transcriptase enzyme. ELK Biotechnology EntiLink 1st Strand cDNA Synthesis Kit was used for this procedure. Total RNA was calculated as 2 µg/µL for each sample. Quantitative RT-PCR method following cDNA synthesis is the most preferred method in gene expression analysis. SYBR Green dye was used for fluorescent glows. TOMBO cyber green kit and ABI 7500 PCR device were used for this process. The "GAPDH" gene was used as a reference gene in the analysis.

Data analysis

Statistical analyses were made using the GraphPad Prism 9 program. All experiments were performed in triplicate and data are stated as mean ± standard deviation (mean ± SD). While the distribution of the data was determined by the Shapiro–Wilk test, the analysis of variance was determined by the Levene test. Comparisons to control were made using the one-way ANOVA test. Tukey test was used as post hoc while analysis of variance (two-way ANOVA) was performed to judge the distinctness between multiple groups. Significance level was considered statistically significant by taking $p < 0.0001$.

Results

Electropermeabilization and viability of HeLa cell

Permeabilization in cells due to electric field change was determined by PI method. The change in permeabilization with electric field (0–3.2 kV/cm) is shown in Fig. 1. As seen in the figure, the percentage of permeabilized cells increased due to the electric field change for 100 µs at a repetition frequency of 1 Hz. No dramatic increase in permeability of HeLa cells was seen at low pulse intensities. The permeability of the cells was very low. (Fig. 2). A strikingly increased alteration in the quantity of permeabilized cells was observed at 1.6 kV/cm. At this electric field strength, approximately 57.65% of the cells were permeable. We measured cell viability in HeLa cells after electroporation with the WST-8 assay after both 30 min and 24 h of incubation. (Fig. 3,4). The viability of the cells after short-term incubation did not change at the low amplitude of the

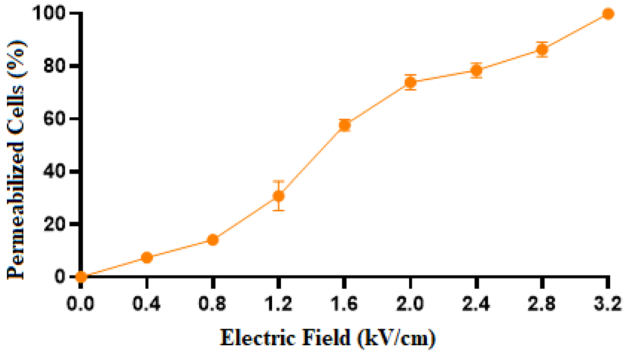


Fig. 1 Percentage of electropermeabilizated HeLa cells. The data were obtained from 3 separate experiments. Results are given as mean and ± SD

electrical pulses (Fig. 3). However, cell vitality started to decrease after 0.8 kV/cm. The viability of cells was 83.54, 67.58, 43.9, and 42.4% for 1.2, 1.6, 2.4, and 3.2 kV/cm, respectively, 30 min after electroporation application. After long-term incubation, cell viability decreased depending on the electric field strength. Viability of HeLa cells 91.47% at 0.4 kV/cm, 85.11% at 0.8 kV/cm, 65.3% at 1.6 kV/cm, 42.6% at 2.4 kV/cm and 32.4% at 3.2 kV/cm was founded (Fig. 4). In this study, we chose electric field value of 1,6 kV/cm (at this value, cells have 67,58% viability and 57,65% permeabilization) for ECT and CaEP treatments at HeLa cells.

Effect of bleomycin and cisplatin on the viability of HeLa cells with and without electroporation

The effect of using the determined bleomycin and cisplatin with and without electroporation was investigated (Figs. 5, 6). The effect of bleomycin + EP on cell viability was given with the application of bleomycin alone. In bleomycin alone application, cell viability is 97.57% at 10 μM concentration, 63.73% at 100 μM, 52.98% at 200 μM, and 42.34% at 400 μM, while at 10 μM + EP when applied together with EP, cell viability was determined as 24.11%, 21.30% at 100 μM + EP, 20.29% at 200 μM + EP, and 21.9% at 400 μM + EP. When bleomycin + EP application was compared with bleomycin application alone, there was a statistically important reduction in cell viability with EP at 10, 100, 200, and 400 μM bleomycin concentrations ($**p < 0.01$, $****p < 0.0001$) (Fig. 5). The half-maximum inhibitory concentration (IC_{50}) value was calculated as 214.11 ± 4.7 μM in lonely bleomycin treatment. IC_{50} value after bleomycin + EP application was calculated 0,44 ± 0,3 μM. These results showed that bleomycin + EP treatment can be an effective treatment method in cervical cancer at lower doses of bleomycin. In Fig. 6, the cell viability of the cisplatin and cisplatin + EP groups as measured by the WST-8 assay is presented as a percentage of viability. There were striking dissimilarity in cell viability between the low-dose cisplatin treatment lonely and the cisplatin + EP groups. It caused only 9.83% decline in cell viability at a dose of 10 mm cisplatin. On the other hand, 67.4% dramatically decrease in cell viability was observed in the treatment of the same dose of cisplatin with EP. Cisplatin 10 μM treatment alone did not cause a significant decrease in the control group ($p > 0.01$). On the other hand, it was observed that other concentrations of cisplatin caused a statistically important reduction in cell viability opposed to the control group ($****p < 0.0001$). When cisplatin + EP treatment was compared with the control, it was observed that there was a statistically significant decrease in all drug concentrations ($****p < 0.0001$). When 10 μM cisplatin alone and 10 μM + EP treatment were compared, it was determined that there was a statistically significant decrease in cell viability ($****p < 0.0001$). The cisplatin treatment alone resulted in IC_{50} value of 35,16 ± 3,3 μM. On the contrary, IC_{50} value after cisplatin + EP treatment of HeLa cells was observed at 20,55 ± 4,3 μM. These results indicated that use to be low dose of cisplatin with EP can be an effective treatment method in cervical cancer (Fig. 8).

Contrasting of the consequence of calcium alone and calcium electroporation on cell viability

The effect of $CaCl_2$ alone and CaEP treatment on cell viability is given comparatively in Fig. 7. No statistically significant change was viewed in cell viability in comparison to the control group at all concentrations in $CaCl_2$ treatment alone ($p > 0.01$). In opposition to this situation, a dramatic reduction in cell viability was observed in CaEP treatment. Cell viability was 51.13% at 1 mM $CaCl_2$, 63.19% at 2 mM, 46.77% at 3 mM, 37.55% at 4 mM, and 26.82% at 5 mM, respectively. A statistically significant change was observed with CaEP treatment at all $CaCl_2$ doses in comparison with the control group ($****p < 0.0001$) (Figs. 7, 8). These results exhibit something that the combined treatment of $CaCl_2$ with EP could be an effective treatment method in cervical cancer.

Effect of ECT and CaEP application on mitochondrial membrane potential (ΔΨm)

The effects of doses of bleomycin, cisplatin, and calcium alone and of combined treatments with EP on ΔΨm were found by calculating the red/green fluorescence ratio using JC-1 fluorescent staining. The depolarization or hyperpolarization that occurs in ΔΨm is an important indicator of apoptosis. The red/green fluorescence ratio of cells treated with various concentration of bleomycin showed a slight rise in comparison with control cells, but this change was not statistically significant ($p > 0.01$) (Fig. 9a). In the bleomycin + EP

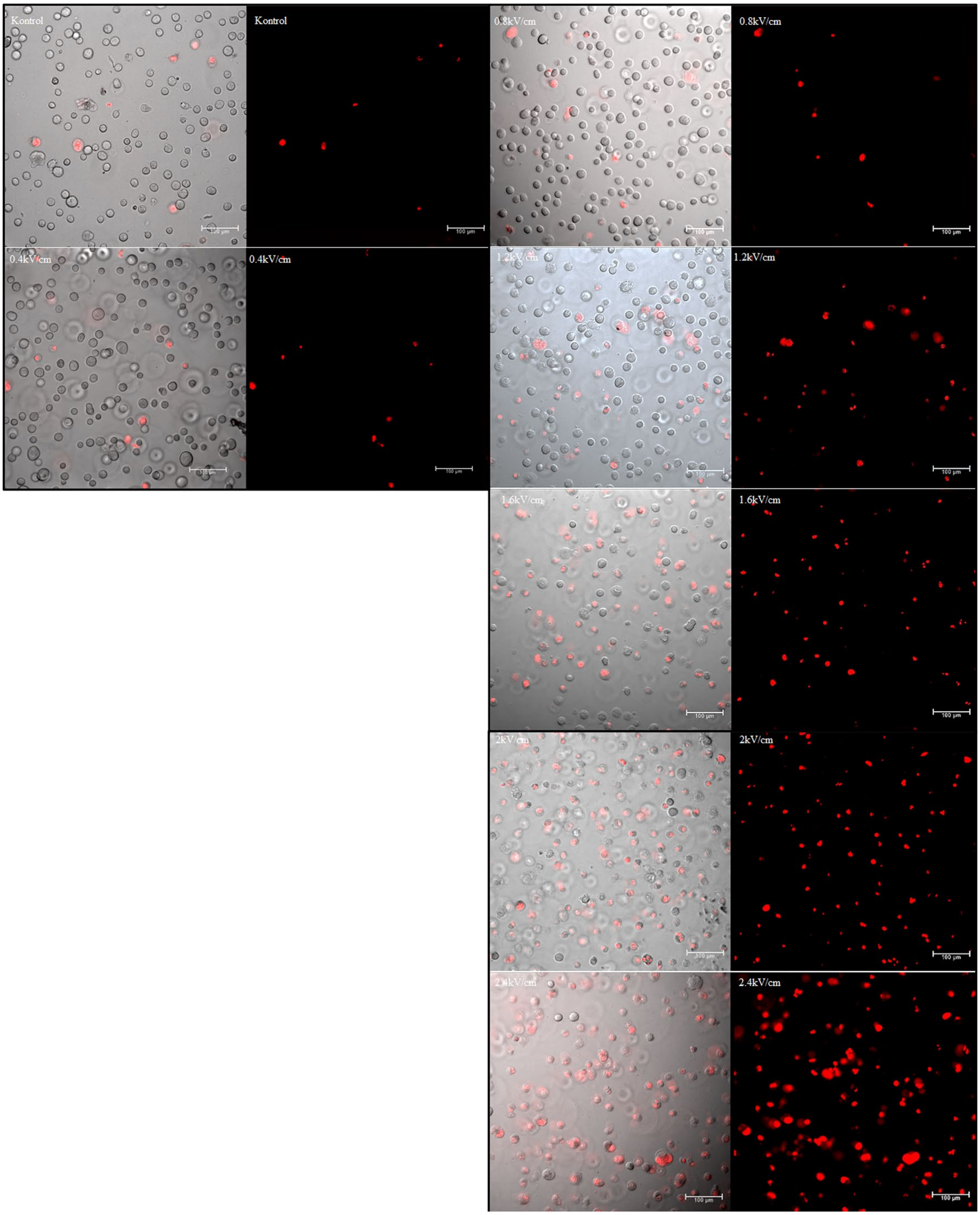


Fig. 2 İmmunofluorescence microscope images and PI-stained phase contrast of HeLa cells at varying electric field intensities. (×20 Objective)

treatment, a statistically significant change was observed in the red/green fluorescence ratio of the cells compared to the control cells at all bleomycin doses (**$p < 0.01$) (Fig. 9b). The results show that the increased intracellular cytotoxicity with bleomycin + EP treatment causes an increase in $\Delta\Psi$m, resulting in hyperpolarization of the mitochondrial membrane. $\Delta\Psi$m results after cisplatin + EP treatment with cisplatin alone are given in Fig. 10a, b. A 32.8% reduction in $\Delta\Psi$m was found in 10 µM cisplatin treatment alone. This change was found to be significant compared to the control group (***$p < 0.001$)(Fig. 10a). This result shows that exposure to 10 µM cisplatin induces depolarization of the mitochondrial membrane. Contrary to this situation, there was no statistically significant difference in other ciplatin

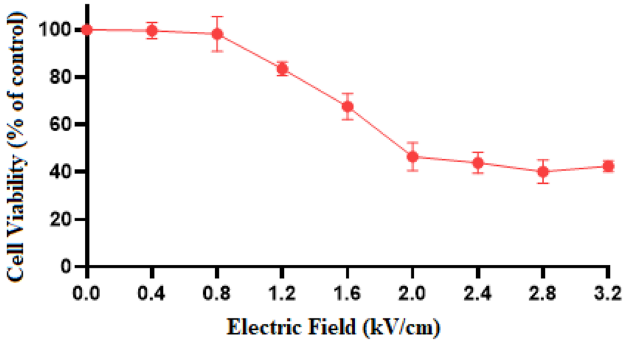


Fig. 3 Cell viability in HeLa cells after short-term incubation due to 0–3.2 kV/cm E field strength increase in 100 μs at 1 Hz repetition frequency

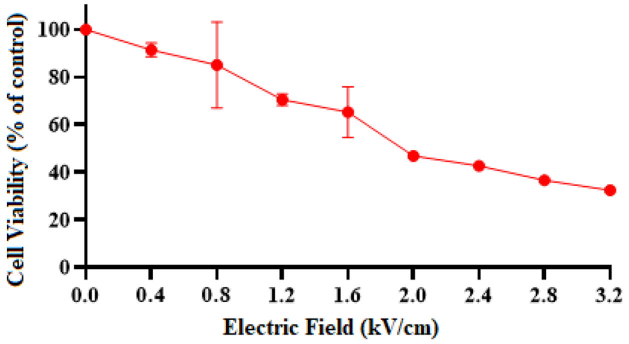


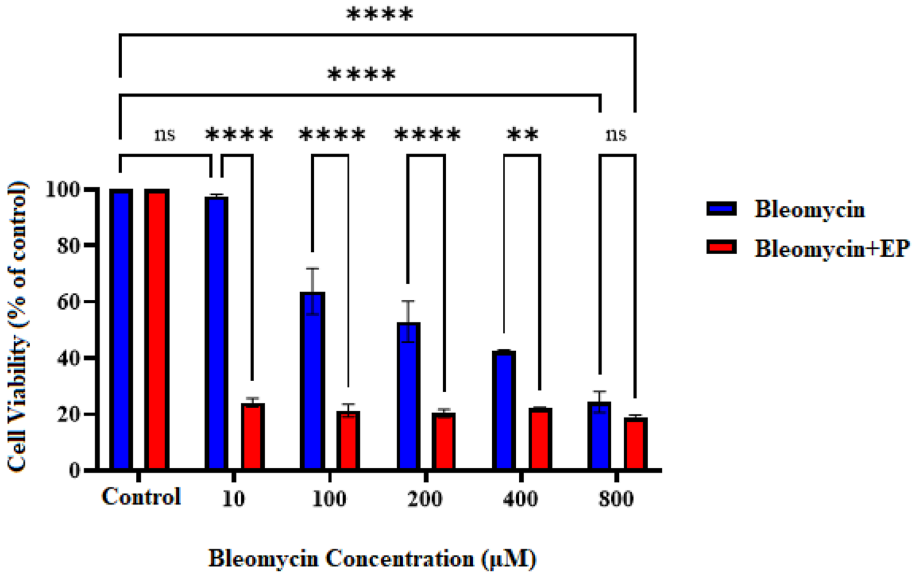
Fig. 4 Cell viability in HeLa cells after long-term incubation due to 0–3.2 kV/cm E field strength increase in 100 μs at 1 Hz repetition frequency

doses ($p > 0.01$). A partial increase in $\Delta\Psi m$ of 10 μM was observed in the cisplatin + EP treatment. However, at other doses, 10% decrease at 25 μM, 26.2% at 50 μM, and 22.7% at 75 μM, and 8.3% at 100 μM were determined, respectively. However, this change in cisplatin + EP treatment was not statistically significant when compared to the control group ($p > 0.01$)(Fig. 10b). It was observed that in $CaCl_2$ application, a decrease of 11.2% occurred in the red/green fluorescence ratio and at 2 mM concentration. Only the change in 4 mM was found to be statistically significant ($^{**}p < 0.01$). It was determined that 2 mM $CaCl_2$ caused a decrease in the red/green fluorescence ratio and depolarization in the mitochondrial membrane, but this change was not significant compared to the control group ($p > 0.01$) (Fig. 11a). The increase in $\Delta\Psi m$ at 1, 4, and 5 mM $CaCl_2$ concentrations after CaEP was also found to be statistically significant compared to the control group ($^{*}p < 0.05$, $^{**}p < 0.01$). It is seen that CaEP application at these concentrations causes hyperpolarization in the mitochondrial membrane (Fig. 11b).

Effects of electroporation, electrochemotherapy, and calcium electroporation on cell cycle

The distribution in the phases of the cell cycle (G0/G1, S and G2/M) in HeLa cells with 8 square pulses and 0.4–3.2 kV/cm electroporation parameters during 100 μs pulse at 1 Hz repetition frequency is given in Fig. 12. The distribution of HeLa cells in the control group in the G0/G, S and G2/M phases of the cell cycle was 87.5%, 2.5%, and 9.8%, respectively. When the cells were exposed to 0.4 kV/cm electric field intensity, the distribution percentages in the phases of the cell cycle were 95.5% for G0/G1, 2.5% for S, and 1.5% for G2/M. The distribution of phases in the 1.2 kV/cm electric field intensity group was 95.2% for G0/G1, 1.2% for S, and 3.6% for G2/M. In the electroporation application, where the electric field strength was 1.6 kV/cm, the G0/G1

Fig. 5 Cell viability of HeLa cervical cancer cells as a result of bleomycin and electrochemotheraphy (Bleomycin + EP) treatment. The data were obtained from 3 separate experiments. Results are given as percentage of control and mean ± SD. Statistical data ns: not significant, $^{**}p < 0.01$ and $^{****}p < 0.0001$



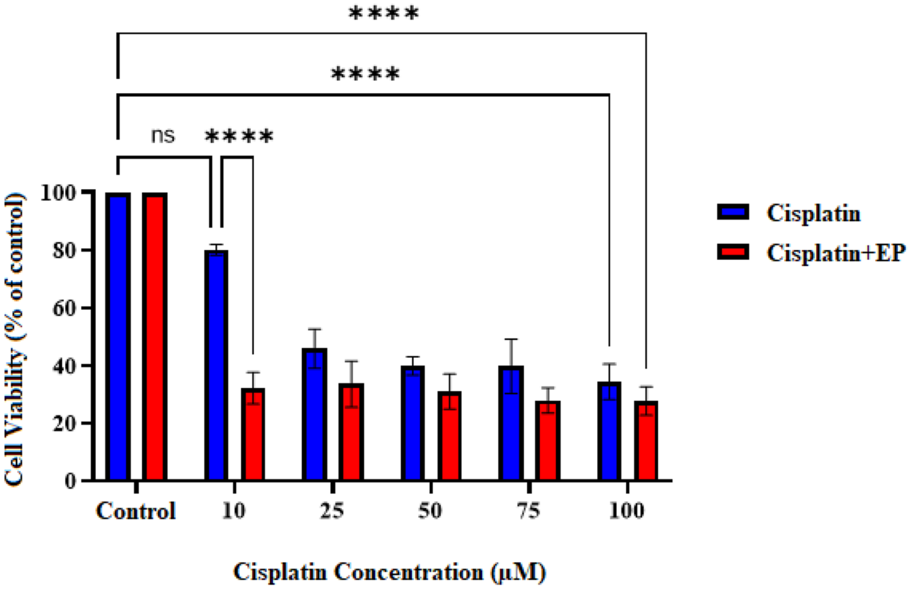


Fig. 6 Cell viability of HeLa cervical cancer cells by cause of ciplatin and electrochemotheraphy (Cisplatin + EP) treatment. The data were obtained from 3 separate experiments. Results are given as percentage of control and mean ± SD. Statistical data ns: not significant and ****$p < 0.0001$

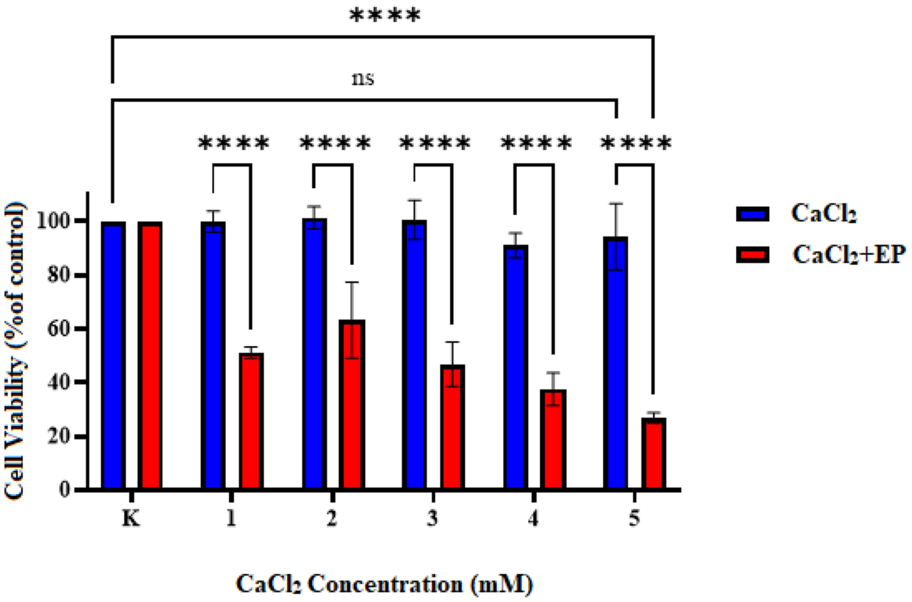


Fig. 7 Cell viability of HeLa cervical cancer cells due to calcium and CaEP treatment. The data were obtained from 3 separate experiments. Results are given as percentage of control and mean ± SD. Statistical data ns: not significant and ****$p < 0.0001$

phase was determined as 95.3%, the S phase as 1.8%, and the G2/M phase as 2.7%. If the selected highest E field intensity was 3.2 kV/cm exposure, G0/G1 phase was determined as 98.5%, S phase as 0.7%, and G2/M phase as 0.6%. It was observed that EP exposures at all electric field intensities applied in HeLa cells caused G0/G1 arrest in the cell cycle compared to the control group.

The effects of only 10 and 100 µM bleomycin exposure to HeLa cells and ECT at the same concentrations on the cell cycle are presented in Fig. 13a. It was observed that 87.5% of the cells in the control group were in G0/G1 phase, 2.5% were in S phase, and 9.8% were in G2/M phase. The distribution in the cell cycle at 10 and 100 µM bleomycin exposures was 59%–53.7% in G0/G1 phase, 2.2%–2.7% in S phase, and 38%–42.8% in G2/M phase, respectively. Compared to the control group, it was observed that there was accumulation in the cells in the G2/M phase in bleomycin exposure and the accumulation increased with the increase of the dose. In the presence of 10 and 100 µM bleomycin with EP, the distribution in the cycle is 88.2%—99% in the G0/G1 phase, 3.6%—0.9% in the S phase, and 8.2%–0.1% in the G2/M phase, respectively, determined. It was observed that bleomycin + EP exposure caused a higher increase in G0/G1 phase cells compared to the control group. It was determined that EP + bleomycin application caused a significant decrease in the cells in the G2/M phase compared to the

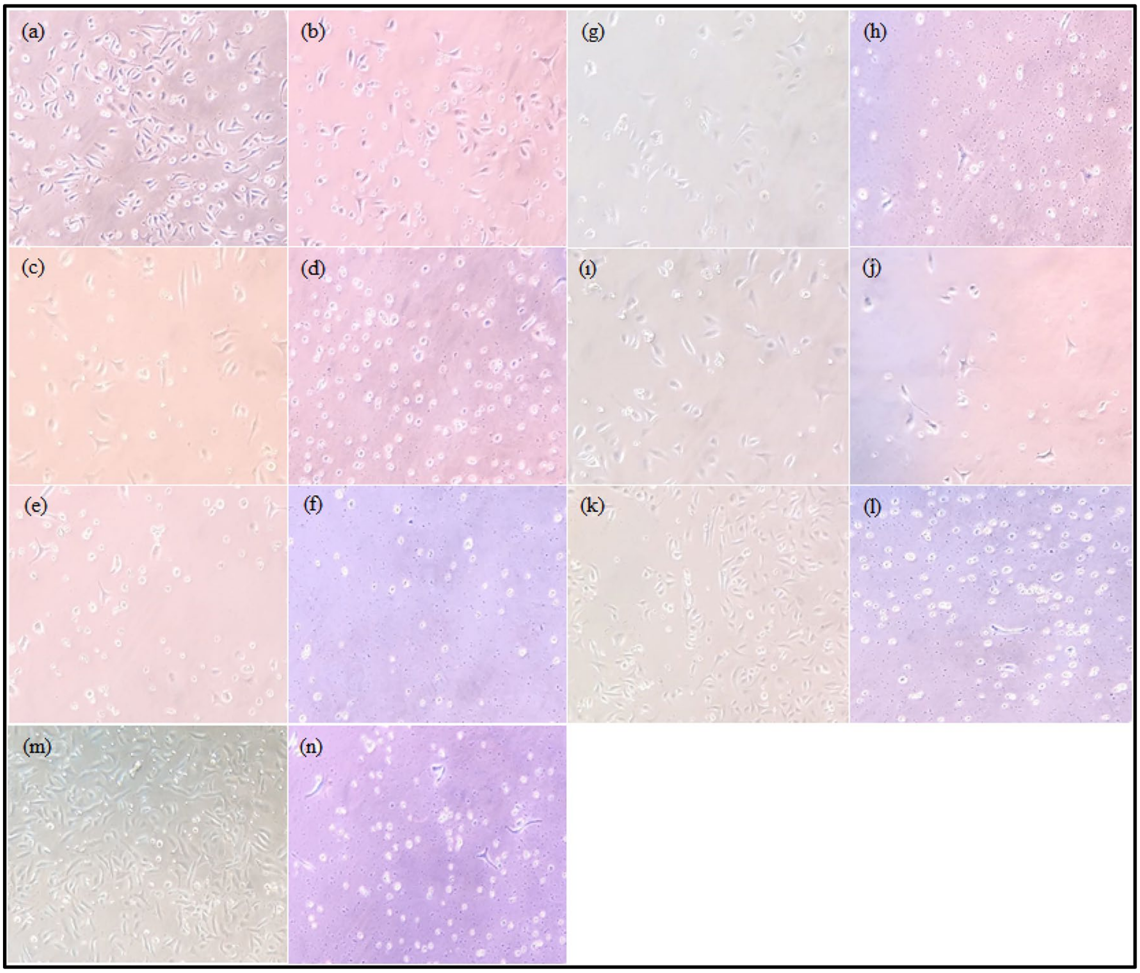


Fig. 8 Invert microscope images at ×100 magnification after 24 h of EP applications with Bleomycin, Cisplatin, $CaCl_2$ in HeLa cell line, **a** Control, **b** 1.6 kV/cm EP, **c** Bleomycin 10 µM, **d** Bleomycin + EP10 µM, **e** Bleomycin 100 µM, **f** Bleomycin 100 µM + EP, **g** Cisplatin 10 µM, **h** Cisplatin + 10 µM + EP, **i** Cisplatin 25 µM, **j** Cisplatin 25 µM + EP, **k** $CaCl_2$ 1 mM, **l** $CaCl_2$ 1 mM + EP, **m** $CaCl_2$ 5 mM, **n** $CaCl_2$ 5 mM + EP

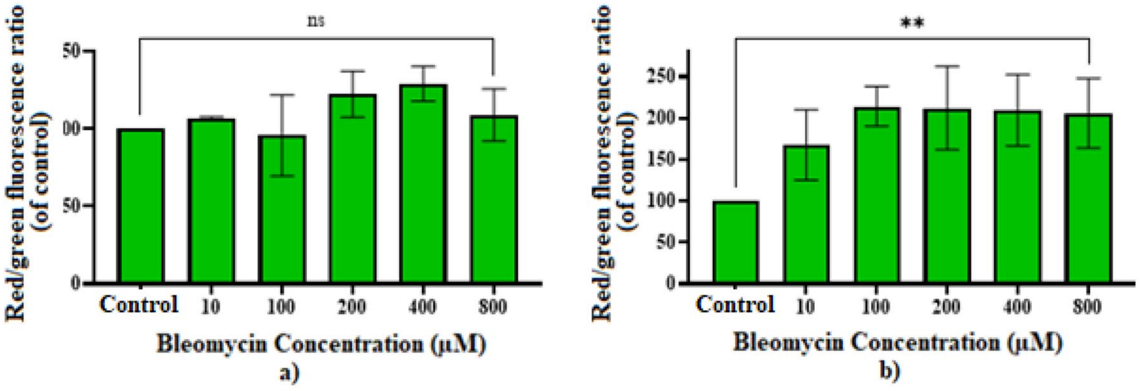


Fig. 9 Change in mitochondrial membrane potential (ΔΨm) of HeLa cervical cancer cells due to bleomycin (**a**) and bleomycin + EP treatment (**b**). The data were obtained from 3 separate experiments. Results are given as percentage of control and mean ± SD. Statistical data ns: not significant and $^{**}p < 0.01$

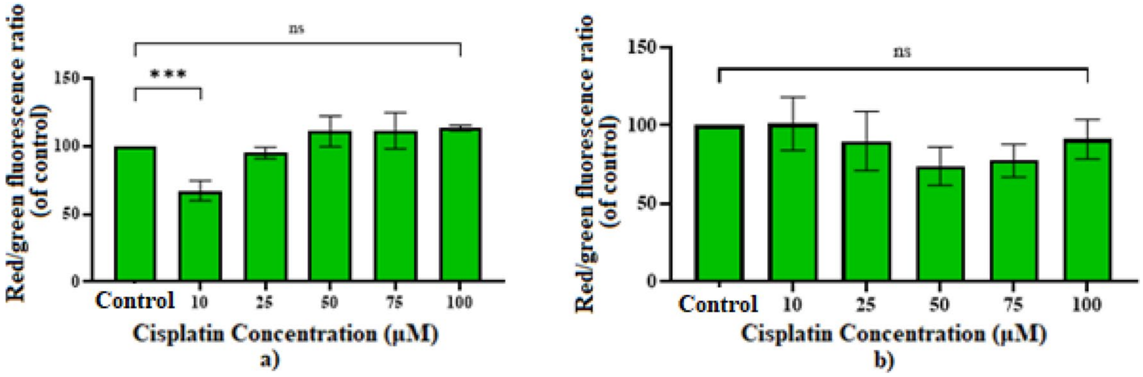


Fig. 10 Change in mitochondrial membrane potential (ΔΨm) of HeLa cervical cancer cells due to cisplatin (**a**) and cisplatin + EP treatment (**b**). The data were obtained from 3 separate experiments. Results are given as percentage of control and mean ± SD. Statistical data ns: not significant and $***p < 0.001$

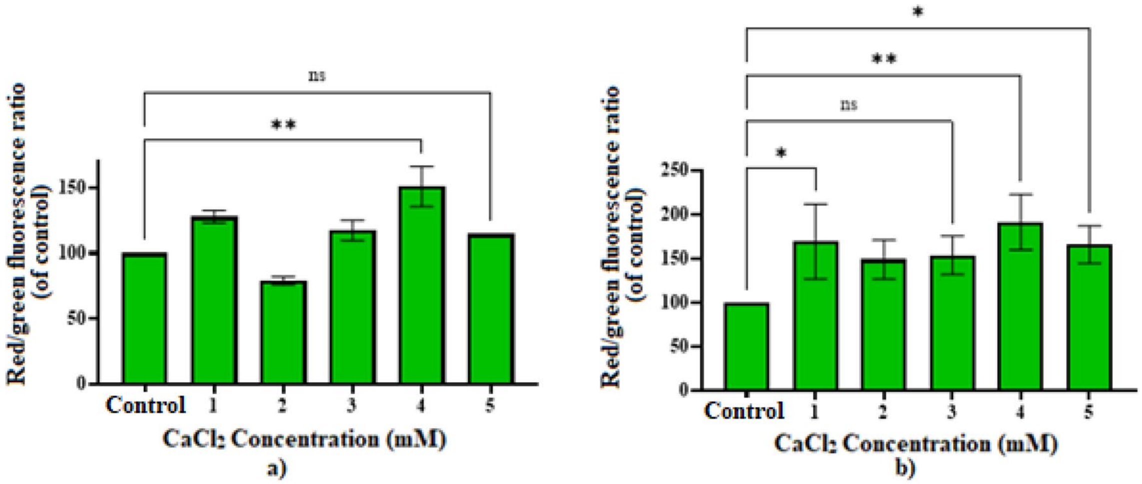


Fig. 11 Change in mitochondrial membrane potential (ΔΨm) of HeLa cervical cancer cells due to $CaCl_2$ (**a**) and CaEP treatment (**b**). The data were obtained from 3 separate experiments. Results are given as percentage of control and mean ± SD. Statistical data ns: not significant, $*p < 0.05$ and $**p < 0.01$

distributions in the presence of bleomycin alone, causing an increase in the G0/G1 phase and preventing the cells from transitioning to the S phase.

The effects of 10 and 25 µM cisplatin exposure and the same concentrations of ECT on the cell cycle of HeLa cells are given in Fig. 13b. A distribution of 87.5% in the G0/G1 phase, 2.5% in the S phase, and 9.8% in the G2/M phase was determined in the cells in the control group. At 10 and 25 µM cisplatin exposure, the distribution in the cell cycle was determined as 88.8%–75% in G0/G1 phase, 2%–1.9% in S phase, and 9.3%–22% in G2/M phase, respectively. Compared to the control group, 10 µM cisplatin exposure caused an increase in G0/G1 phase, but 25 µM cisplatin exposure caused a decrease. It was determined that the presence of 25 µM cisplatin caused an increase in the percentage of cells in the G2/M phase compared to the control group. In the presence of 10 and 25 µM cisplatin with EP, the distribution in the cycle is 96.4%–90.6% in the G0/G1 phase, 1.5%–1.8% in the S phase, and 2.2%–7% in the G2/M phase, respectively. It was observed that cisplatin + EP exposure caused an increase in cells in G0/G1 phase compared to the control group. It was observed that cisplatin + EP administration caused an increase in G0/G1 phase compared to the distributions in the presence of cisplatin alone, prevented the cells from transitioning to S phase, and caused a significant decrease in cells in G2/M phase.

The effects of only 1 and 5 mM $CaCl_2$ exposure to HeLa cells and the application of CaEP at the same concentrations on the cell cycle are given in Fig. 13c. The cyclic distribution of cells in the control group was 87.5% in G0/G1 phase, 2.5% in S phase, and 9.8% in G2/M phase. In the presence of 1 mM $CaCl_2$, a distribution of 46.9 in G0/G1 phase, 1.8% in

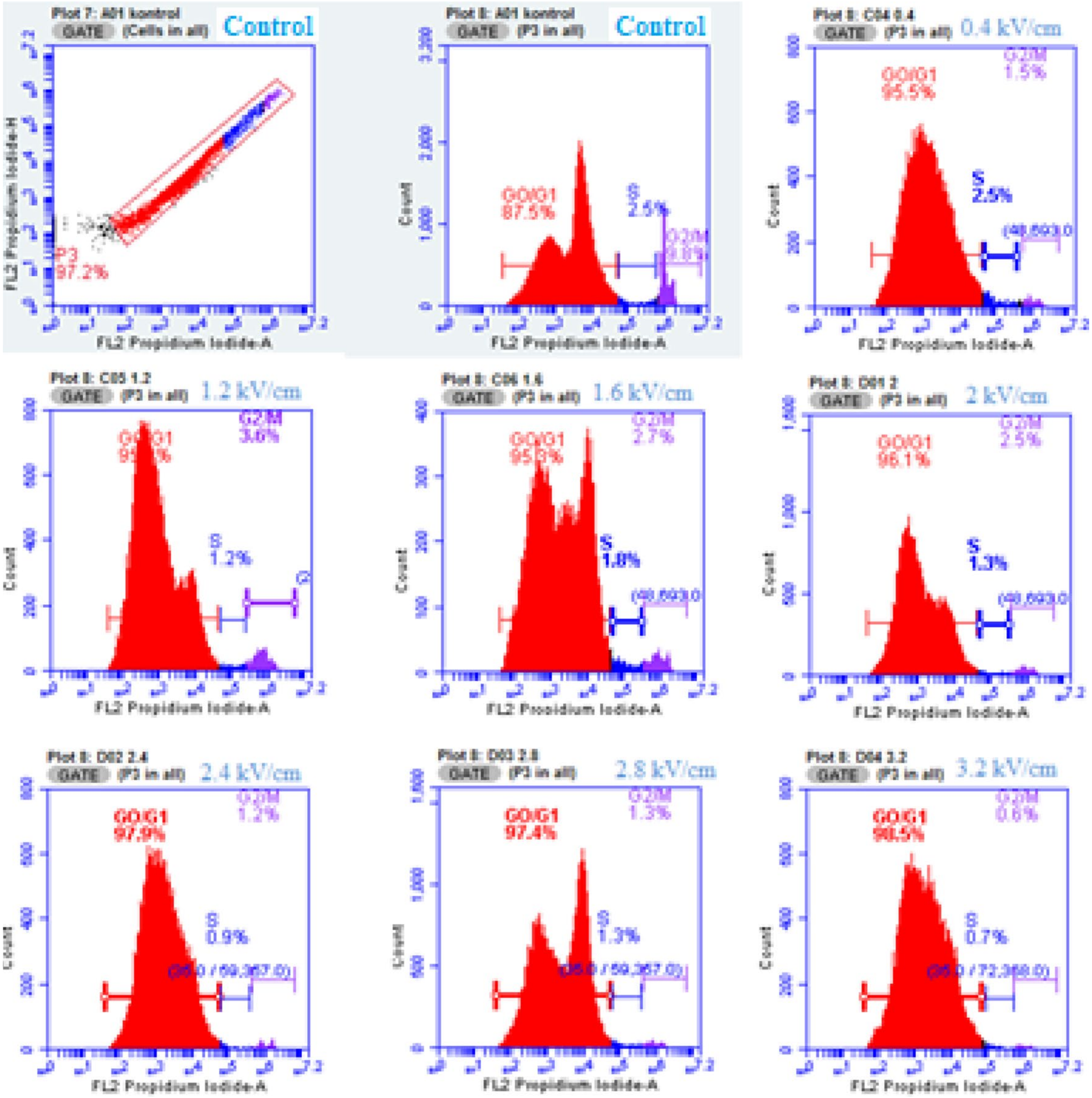


Fig. 12 Results of electroporation on cell cycle of HeLa cells

S phase, and 51% in M phase was determined in cells. In the presence of 5 mM $CaCl_2$, the cyclic distribution was 36.4% in the G0/G1 phase, 2.2% in the S phase, and 60.7% in the G2/M phase. Compared to the control group, it was observed that only the presence of 1 and 5 mM $CaCl_2$ caused accumulation in the cells in the G2/M phase. Cell cycle distributions of CaEP application in the presence of 1 and 5 mM $CaCl_2$ are 99%–97.4% in G0/G1 phase, 0.7%–1.7% in S phase, and 0.3% and 0.9% in G2/M phase, respectively, determined. It was observed that CaEP application significantly reduced cell distribution in G2/M phase and caused G0/G1 arrest within two dose values compared to only 1 and 5 mM $CaCl_2$ applications.

PCR analysis: results of bax/Bcl-2 and p53 gene expression

Bax/Bcl-2 gene expression ratios and p53 gene expression fold increase values of 10 µM bleomycin, 10 µM cisplatin, and 1 mM $CaCl_2$ applied to HeLa cells in combination with EP at the same doses are given in Fig. 14c, b. Changes in Bax/Bcl-2 ratio were determined as a result of the lowest dose values of chemotherapeutic drugs and $CaCl_2$ to HeLa cells and the application of these doses together with EP (Fig. 14a). In the applications of 10 M bleomycin and 10 µM cisplatin, an increase in Bax/Bcl-2 gene expression rate was observed by 1.2 and 1.4 times, respectively, while the increase was found to be 1.4 and 2.3 times, respectively, in combination with EP. However, it was observed that

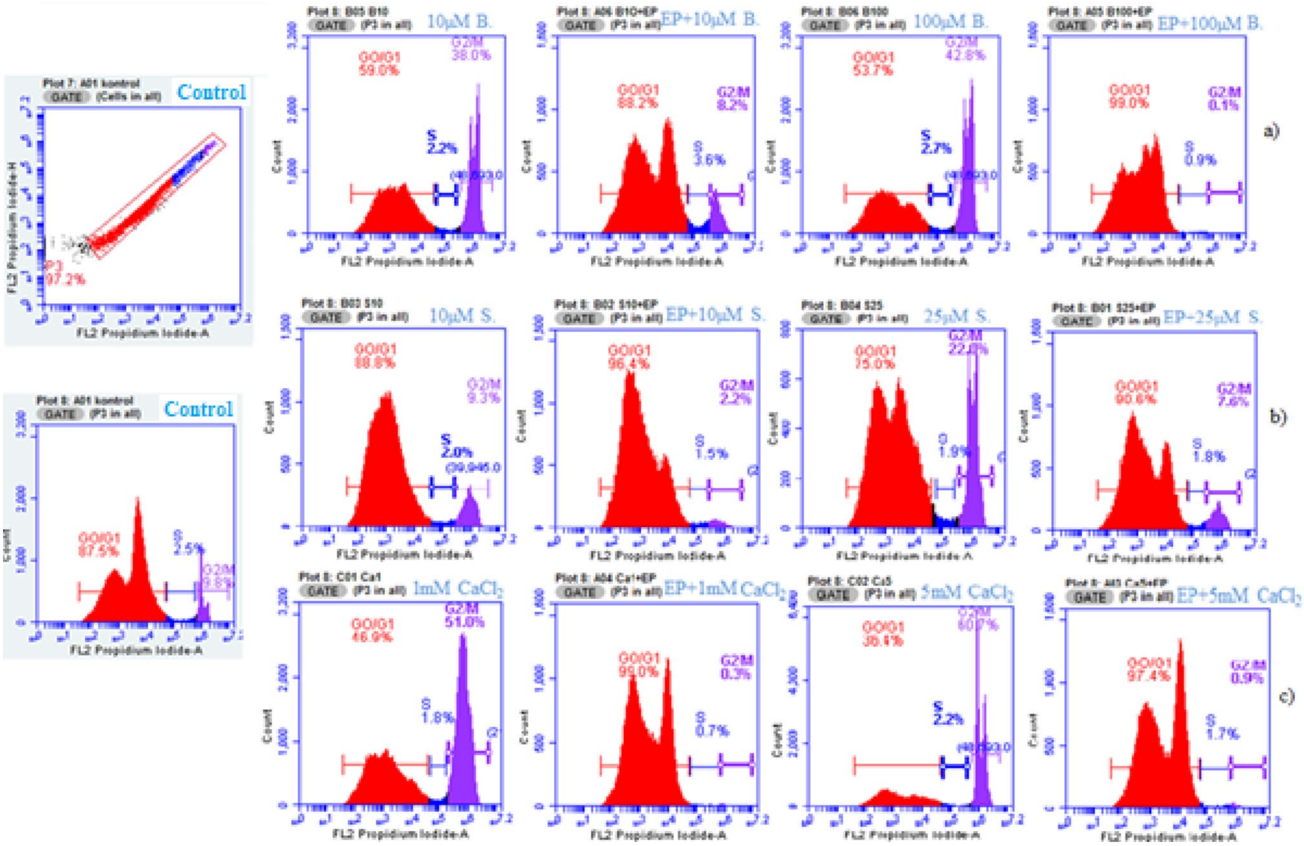
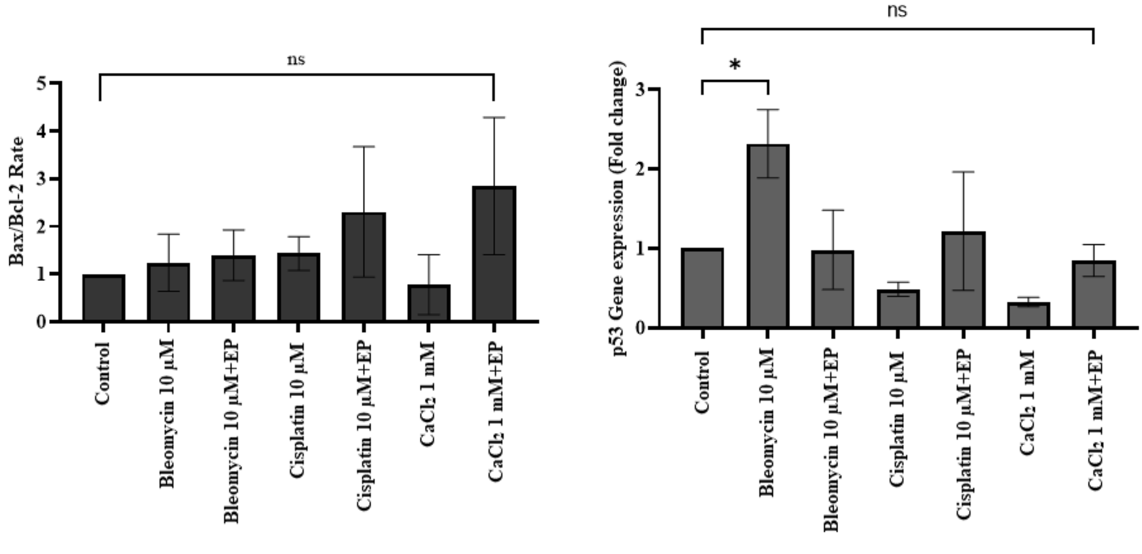


Fig. 13 HeLa cell cycle analysis. **a** The effects of bleomycin alone and bleomycin + EP on the cell cycle of HeLa cells. **b** The effects of cisplatin alone and cisplatin + EP on the cell cycle of HeLa cells. **c** The effects of $CaCl_2$ alone and CaEP on the cell cycle of HeLa cells



a) b)

Fig. 14 **a** Effect of ECT and CaEP Applications on Bax/Bcl-2 ratio and **b** p53 gene expression level. The data were obtained from 3 separate experiments. Results are given as percentage of control and mean ± SD. Statistical data. Statistical data ns: not significant and $*p < 0.05$.

the determined increases were not statistically significant ($p > 0.01$). While a decrease was observed in the Bax/Bcl-2 gene expression rate compared to the control group at a dose of 1 mM $CaCl_2$ applied to the cells, it was determined that it increased 2.8 times with CaEP application. However, the determined increase was not statistically significant ($p > 0.01$). Changes in p53 gene expression were determined as a result of the lowest dose values of chemotherapeutic drugs and $CaCl_2$ to HeLa cells and the application of these doses together with EP (Fig. 14b). A statistically significant increase was found in p53 expression compared to the control group in 10 µM bleomycin application ($*p < 0.05$). There was a slight decrease in EP + 10 µM bleomycin exposure, but this change was not statistically significant ($p > 0.01$). In the application of 10 µM cisplatin, a partial decrease in p53 expression was determined compared to the control group, while a 1.2-fold increase was observed in the combined application with EP, but these changes were not statistically significant ($p > 0.01$). In the presence of 1 mM $CaCl_2$, a decrease in p53 expression was found compared to the control group. In CaEP application, the p53 expression value increased by 2.5 times compared to the 1 mM $CaCl_2$ application alone, while there was no significant change compared to the control group ($p > 0.01$).

Discussion

Cancer is an important public health problem with the highest mortality in the world after cardiovascular diseases. The increase in cancer morbidity and mortality increases the interest of researchers in studies on cancer treatment. It is cervical cancer after breast cancer in women worldwide. In the treatment of cervical cancer, chemotherapy is widely used alone or in combination with other treatment options. Bleomycin and cisplatin, an antineoplastic drug, are among the most usually used drugs in the treatment of the disease. Due to the phospholipid structure of the cell membrane, the effectiveness of these chemotherapeutic agents, which cannot easily pass through the membrane, significantly decreases. In order to rise the influence, the dose of the drug must be increased, but this also brings with it important side effects. This restrictive situation in chemotherapy treatment reveals the necessity of increasing and developing researches on finding new methods and drugs in treatment. EP is a physical method that creates hydrophilic pores in the cell membrane, allowing large molecules to pass through the membrane. In ECT application, more cytotoxic effect can be achieved with less drug dose by allowing the passage of chemotherapeutic agents through the membrane together with EP. Thus, while the doses of drugs used in chemotherapy can be reduced, the side effects can be significantly reduced. Ca^{2+} plays an important role in many cellular activities, including cell death. The Ca^{+2} concentration in the intracellular medium is much lower than in the extracellular medium, and this balance is tightly regulated in the cell. The supraphysiological increase in the amount of intracellular Ca^{+2} provides the formation of pores in the mitochondria and enables the activation of some cell death mechanisms. With the increase in the amount of intracellular Ca^{+2} with the CaEP method, these intracellular pathways are stimulated, resulting in the death of cancerous cells.

In our study, the effective E field value to be applied to HeLa cells for ECT and CaEP application was found to be 1.6 kV/cm by using cell viability and permeabilization values. In the literature, there are many studies on the effective E field value determined for different cell lines. The effective E field values determined for ECT in the in vitro study by Jaroszeski et al. were 1100 V/cm for human ovarian cancer (LN1) cell line, 1250 V/cm for rat hepatocellular carcinoma (N1-S1) cells, and human endometrial adenocarcinoma (Ishikawa) cells and 1200 V/cm for mouse melanoma cells (B16) [15]. In another ECT study with human dermal microvascular endothelial cells (HMEC-1), the electropermeability of HMEC-1 cells was 30% at 1000 V/cm E field, and only 85% of cells at 1800 V/cm E field exposure. It has been reported that 1400 V/cm E field parameter was chosen for electrochemotherapy experiments, in which only 60% of the cells were permeabilized [27]. Esmekaya et al. reported the optimal EP parameters as 1 Hz, 100 µs pulse duration and 1750 V/cm E field intensity in their in vitro study to determine the effects of tamoxifen on human breast carcinoma (MCF-7) cells with ECT. In another study with human neuroblastoma cells (SH-SY5Y), effective EP parameters for cisplatin electroporation were determined as 1 Hz, 100 µs pulse duration, and 1500 V/cm E field strength [28, 29]. Studies in the literature have shown that there are different effective E field values for different cell lines. Studies show a broad spectrum of effective E field value according to different cell types.

In our study, the IC_{50} value for HeLa cells was found to be 214.11 ± 4.7 µM in the presence of bleomycin alone, and 0.44 ± 0.3 µM after ECT with bleomycin. It was determined that effective cell death occurred even at low doses of bleomycin in ECT application. As a result of the obtained data, it was observed that ECT application increased bleomycin cytotoxicity 487 times in HeLa cells. In the presence of only cisplatin chemotherapeutic drug, the IC_{50} value for HeLa cells was found to be 35.16 ± 3.3 µM, while the IC_{50} value was calculated as 20.55 ± 4.3 µM after ECT application with cisplatin. It was determined that ECT application increased cisplatin cytotoxicity 1.71 times in HeLa cells.

In an in vitro study by Jaroszeski et al., it was shown that cytotoxic effects were increased in all seven different cell lines treated with bleomycin with ECT, and when bleomycin was combined with a pulsed E field, there was a 100 to 5000-fold increase compared to drug administration alone [15]. In another study by Eşmekaya et al. with human neuroblastoma cells (SH-SY5Y), the IC_{50} value of the cisplatin-administered group was determined as 5.03 µg/mL, while the IC_{50} value of cisplatin after ECT was reported as 0.51 µg/mL [28]. In the study performed by Saczko et al., human ovarian clear cell carcinoma (OvBH-1) and epithelial ovarian carcinoma cells (SKOV-3), 24 h of cisplatin alone administration to OvBH-1 cells increased the IC_{50} to 34.97 µM and to SKOV-3 cells. It has been reported as 62.59 µM. In bleomycin exposure with 1.5 kV/cm E field, the IC_{50} value was reported as 4.74 µM in OvBH-1 cells and 84.25 µM in SKOV-3 cells. In the application with 2 kV/cm E field, the IC_{50} value was given as 0.12 µM for OvBH-1 cells and 3.09 µM for SKOV-3 cells. In the study, it has been reported that EP is safe up to 2 kV/cm in 24-h incubation applications [30]. Gehl et al., in their in vitro study with Chinese hamster lung fibroblast (DC-3F) cells with 1 Hz, 99 µs pulse duration and 1200 V/cm EP parameters, showed that the cytotoxic effect of bleomycin increased 300 times and cisplatin increased 2.3 times with EP application [31]. In an in vitro study conducted by Jaroszeski et al. on seven different cell lines and 44 different chemotherapeutic drugs, it was found that cisplatin and carboplatin with EP were 3–13 times more effective on six different cell lines, and bleomycin administration with EP increased the IC_{50} value compared to bleomycin alone. It has been found that vincristine, another chemotherapeutic agent, has a 1.3–3.4-fold increase in cytotoxicity in various cell lines [15]. In the study conducted by Orlowski et al. in Chinese hamster lung fibroblast (DC-3F) cells under in vitro conditions, it was reported that the cytotoxicity for netropsin and bleomycin increased by 200 and 700 times, respectively, with EP, while the increase in cytotoxicity was 3 times with the use of actinomycin D, which is a lipophilic drug [32]. In the in vitro study of Scuderi et al. on mouse melanoma (B16-F1) cells, 1 Hz repetition frequency, 8 × 100 µs pulse duration and severe monopolar pulse parameters with 1.2 kV/cm E, and IC_{50} value of 85 µM, 1 Hz repetition frequency in cisplatin exposure, 8 pulses of 1 µs and bipolar pulses of 3 kV/cm (HF-EP), and cisplatin exposure have reported an IC_{50} of 45 µM [33]. Todorovic et al.'s study on murine colorectal carcinoma cells (CMT-93) used 1 Hz repetition frequency, 100 µs pulse duration and 1300 V/cm EP parameters. It has been reported that with EP, cells are 500 times more sensitive to bleomycin 2.8 times to cisplatin as determined by the IC_{50} value [34]. In an in vitro study by Ramachandran et al. on human cervical cancer (ME180), acute myeloid leukemia (KG1), and promyelocytic leukemia (HL60) cell lines, in the presence of EP (1200 V/cm, 100 µs) and 10 µM bleomycin, a mortality rate of 39.2% for HL60 cells, 35.8% for KG1, and 35.5% for ME180 has been reported [35]. When we examine the results of many studies in the literature, it is seen that ECT application for different cell types increases the cytotoxicity of bleomycin and cisplatin at different rates. It is seen that these differences in the rate of increase vary according to the cell type and the EP parameters used (E field strength, pulse duration). However, there is one thing that is clearly stated that ECT application significantly reduces the IC_{50} value of bleomycin and cisplatin chemotherapeutic drugs and decreases the amount of dose needed for treatment. In our study, it is seen that the IC_{50} dose change rate in cisplatin and bleomycin applications, when cisplatin is applied together with EP, is lower compared to cisplatin alone. This may be because cisplatin is more toxic to HeLa cells at lower doses when administered alone.

In our study, $CaCl_2$ alone or combined with EP, which is thought to be an alternative to chemotherapeutic agents in cancer treatment, was performed. It was observed that the administration of $CaCl_2$ doses alone did not have a significant effect on the viability of HeLa cells. However, with the CaEP method, it was found that $CaCl_2$ had a significant cytotoxic effect on HeLa cells. In the application of CaEP with 1 mM and 5 mM $CaCl_2$, mortality rates of 48.87% and 73.18% were found, respectively. In addition, it was determined that the combined applications of 10 µM bleomycin and ciplatin concentrations and $CaCl_2$ at all doses did not have a synergistic effect on cell viability. In the first preclinical study showing the effects of in vitro and in vivo CaEP on cancer cells, increased cellular ATP usage with CaEP application causes mitochondrial changes and decreases ATP production. It has also been reported to cause acute ATP depletion due to loss of ATP through the permeability-enhanced cell membrane [21]. Human leukemia cell (K-562), murine lung carcinoma cell (Lewis Lung Carcinoma) and Chinese hamster lung fibroblast cells (DC-3F) were treated with CaEP in the presence of increased calcium concentration. It has been reported that there is a dose-dependent decrease in cell viability after 1 and 2 days of incubation after treatment, ECT application with CaEP and bleomycin has similar effects on cell viability, and the combined application of bleomycin and calcium has no synergistic effect. In the same study, the researchers reported that the effect of CaEP is independent of the calcium compound (calcium chloride and calcium glubionate) when compared at the molar level, and therefore, both clinically available calcium compounds are applicable in CaEP [26]. In a study performed on small-cell lung cancer (H69), bladder cancer (SW780), and leukemia cells (U937) to determine the effects of CaEP, they found a decrease in the amount of intracellular ATP and low viability in H69 and SW780 cells depending on the calcium concentration and E field strength [36]. Zielichowska et al. used 1000 V/cm, 1200 V/cm, and 1500 V/cm E field intensities in a CaEP study, they conducted on normal rat skeletal muscle and cancer muscle cells (L6 and Wehi-164-Fibrosarcoma). In the study conducted with 0.5 mM and 5 mM $CaCl_2$

concentration, CaEP was found to be cytotoxic in Wehi-164 cells; besides, it was safe for normal muscle cells, and EP containing 0.5 mM Ca^{+2} increased the proliferation of normal muscle cells L6 [37].

In our study, cell cycle analyses were performed to determine the mechanism of action of EP, ECT, and CaEP in HeLa cells. It was observed that all E field intensities applied to HeLa cells between 0.4 and 3.2 kV/cm caused arrest in G0/G1 in the cell cycle. When 10 and 100 µM doses of bleomycin were applied to HeLa cells compared to the control group, it was determined that the cell cycle percentages increased in the G2/M phase. It was observed that the cell cycle was stopped in G2 depending on the increase in the applied bleomycin dose. While a decrease was observed in the percentage of cells in the G2/M phase with ECT application together with bleomycin, it was observed that the increase shifted toward the G0/G1 phase. A similar situation was valid for 10 and 25 µM cisplatin applications. Depending on the dose increase, an increase in the percentage of cells in the G2/M phase was detected in drug administration alone. It was determined that cisplatin exposure, proportional to the increasing concentration, stopped the cell cycle in the G2 phase in HeLa cells. With cisplatin + EP application, the percentage of cells in the G2/M phase decreased and the percentage of cells in the G0/G1 phase increased. It was determined that cisplatin + EP administration caused G0/G1 arrest in the cell cycle. It was determined that only 1 and 5 mM $CaCl_2$ application caused an accumulation of 51% and 60.7%, respectively, in the cell percentage in the G2/M phase of the cell cycle. It was observed that this situation changed with CaEP application and the cell percentage was concentrated in the G0/G1 phase. In the study of Chen et al., on immortalized keratinocyte (HaCaT) cells, it was reported that there was a distribution of 21.2% in the G1 phase, 24.4% in the S phase, and 54.4% in the G2/M phase of the cell cycle at 0.5 µM bleomycin exposure. In the exposure of 2 µM bleomycin to human cervical cancer (HeLa) cells, the distribution in cell cycle phases was reported as 38.3% in G1 phase, 21.1% in S phase, and 40.6% in G2/M phase [38]. In the study of Asgar et al. in two cholangiocarcinoma cells (KKU-100, poorly differentiated and KKU-M214, moderately differentiated), 10 µM cisplatin exposure resulted in growth arrest at the G1 checkpoint in KKU-100 cells, which resulted in a higher cell percentage in G0/G1. (57.9% ± 3.3%) reported that it caused. It has been reported that the cell cycle profile of KKU-M214 cells with the same dose of cisplatin did not show growth arrest at a certain stage when compared to control [39].

In most studies in the literature, it has been reported that $\Delta\Psi m$ reduction (depolarization of mitochondria) induces apoptotic cell death. However, some recent studies have shown that apoptosis can also be caused by increased $\Delta\Psi m$. Mitochondrial hyperpolarization has been demonstrated in some cancer cells in the literatüre [40, 41]. F_0F_1-ATPase uses extruded protons to synthesize ATP. Protons re-enter the inner membrane to prevent hyperpolarization. For this reason, it has been reported that dysfunction of F_0F_1-ATPase may lead to hyperpolarization of mitochondria. [42]. Eşmekaya et al., in their study on MCF-7 cells reported that exposure to 2.1 GHz MW radiation caused hyperpolarization in mitochondria, which in turn induced apoptosis in MCF-7 cells [43]. In our study, it was determined that bleomycin chemotherapeutic drug did not cause a significant change in $\Delta\Psi m$ in the applications made at the specified doses, while it was observed that bleomycin with EP caused hyperpolarization in $\Delta\Psi m$ in all dose applications. The situation was slightly different for the chemotherapeutic drug cisplatin. While depolarization was determined in $\Delta\Psi m$ in the presence of 10 µM cisplatin, no significant change was detected in $\Delta\Psi m$ at other cisplatin doses. There was no significant change in $\Delta\Psi m$ in all cisplatin doses together with EP. In $CaCl_2$ alone applications, hyperpolarization was determined in $\Delta\Psi m$ in the presence of only 4 mM $CaCl_2$, while a significant change was detected in other doses, while in CaEP application, hyperpolarization was determined in $\Delta\Psi m$ at 1, 3, 4, and 5 mM $CaCl_2$ concentrations.

In the study of Jan et al., DNA-damaging chemotherapeutic agents (cisplatin, bleomycin, etc.), such as DRs in the extrinsic pathway and various agents proposed to stimulate the apoptotic function of ligands and target Fas (DR2) expression [44]. Mungunsukh et al. reported bleomycin in pulmonary endothelial cells induces early activation of the extrinsic apoptotic pathway by proteolytic activation of caspase-8, not the intrinsic apoptotic pathway [45]. In the study of Sharifi et al. in paclitaxel-resistant MCF-7 cells, an increase in Bcl-2/Bax ratio was observed, while a decrease in caspase-9 levels and an increase in caspase-8 were reported [46]. In our study, a partial increase in Bax/Bcl-2 gene expression rate was observed in the application of 10 µM bleomycin and 10 µM cisplatin to HeLa cells, while the increase was higher when combined with EP. While a decrease was observed in the Bax/Bcl-2 gene expression ratio at a dose of 1 mM $CaCl_2$ compared to the control group, it was determined that this ratio increased with CaEP application. However, it was observed that the determined changes were not statistically significant ($p > 0.01$). There was a statistically significant increase in p53 gene expression in the control group in the presence of 10 µM bleomycin ($p < 0.05$). In the presence of 10 µM cisplatin and 1 mM $CaCl_2$, a decrease in p53 gene expression was determined compared to the control group. In the combined application with EP, a partial increase was observed, but these changes were not statistically significant ($p > 0.01$).

Conclusions

In this study we conducted to evaluate the effectiveness of ECT and CaEP applications in cervical cancer, we have shown that ECT application is an effective method in cancer treatment by significantly increasing the cytotoxicity of chemotherapeutic agents in cervical cancer cells. The results of CaEP obtained as a result of in vitro experiments on HeLa cells have shown in our study that calcium can be an alternative new treatment option that can be used instead of chemotherapeutic agents used in the treatment of cervical cancer with electroporation. In addition, we think that the application of $CaCl_2$ together with EP instead of chemotherapeutic agents can be used as an effective method in terms of reducing the treatment costs of cervical cancer and not causing side effects on other organs. These results should be supported by in vivo and preclinical studies. In addition, it shows that the results we have obtained regarding intracellular death mechanisms should be supported by different studies and further cell death pathways should be investigated. However, in vivo studies should be supported by in vitro studies and the differences between them should be revealed. Thus, the missing aspects of intracellular molecular mechanisms in the physiopathology of cervical cancer will be completed.

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Declarations

Conflicts of interest All authors declare that they have no conflict of interest.

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