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Rozprawa doktorska

„Wykorzystanie elektroporacji z jonami wapnia oraz estradiolem w leczeniu raka jajnika na modelu *in vitro*”

Doctoral thesis:

“Utilization of electroporation with calcium ions and estradiol in the treatment of ovarian cancer in an *in vitro* model”

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1. Streszczenie

Rak jajnika (ang. *ovarian cancer;* OC) jest ósmym najczęściej diagnozowanym nowotworem u kobiet. Choroba często rozwija się bezobjawowo, a pierwsze symptomy pojawiają się dopiero w zaawansowanych stadiach, co skutkuje późnym rozpoznaniem. Leczenie opiera się na terapii skojarzonej, łączącej rozwiązania chirurgiczne i chemioterapię. Niestety, dużą część pacjentek dotyka wznowa procesu nowotworowego, po której wyleczalność jest znikoma. Istnieje więc konieczność opracowywania nowych, bardziej skutecznych terapii pierwszego rzutu, które pozwolą opóźnić nawrót choroby, wydłużą wskaźniki przeżycia lub podniosą komfort życia pacjentek w czasie trwania leczenia.

Praca doktorska oparta jest o cykl 2 publikacji oryginalnych, jednej pracy przeglądowej oraz rezultatów badań własnych, do tej pory nieopublikowanych. Temat przewodni cyklu obejmuje analizę efektów biologicznych wywołanych elektroporacją z jonami wapnia (Ca^{2+}) oraz estradiolem w modelu *in vitro* raka jajnika. Elektroporacja wapniowa (ang. *calcium electroporation,* CaEP) jest nowatorską modyfikacją elektrochemioterapii (ECT). Obie techniki opierają się na zjawisku elektroporacji (EP), gdzie pod wpływem impulsywnego pola elektrycznego dochodzi do reorganizacji błony komórkowej. Skutkiem jest przejściowe formowanie porów, umożliwiając zwiększony transport molekuł (m.in. leków cytotoksycznych lub jonów) do wnętrza komórki. Dotychczasowe badania wskazują, że CaEP jest mniej toksyczny dla komórek prawidłowych niż klasyczna chemioterapia. Literatura naukowa przedstawia także potencjał terapeutyczny estrogenów (m.in. 17β-estradiolu) oraz ich stymulujący wpływ na wewnątrzkomórkowe stężenie Ca^{2+}. Biorąc pod uwagę powyższe, w pracy przeanalizowano jego potencjalnie wzmacniający efekt wobec terapii CaEP.

Wyniki wykazały obiecujący efekt terapeutyczny CaEP na ludzkie komórki raka jajnika. EP zwiększa cytotoksyczność cisplatyny oraz chlorku wapnia, zarówno przy zastosowaniu impulsów nanosekundowych, jak i mikrosekundowych. Efektywność zależy od długości czasu trwania impulsu, polarności oraz symetryczności zastosowanych protokołów. Pozwala to na kontrolowanie tego procesu i skuteczne dostarczenie leku. Nowe rozwiązanie przejawia większy potencjał toksyczny wobec komórek nowotworowych w porównaniu do chemioterapii oraz ECT opartych na cisplatynie. Wskazano, iż CaEP w mniejszym stopniu oddziałuje negatywnie na komórki prawidłowe w porównaniu do standardowej chemioterapii oraz ECT. Odnotowano również wzmacniający wpływ 17β-estradiolu na skuteczność analizowanej terapii.

2. Abstract

Ovarian cancer (OC) is the eighth most diagnosed cancer in women, accounting for 3.7% of cases and 4.7% of cancer-related deaths worldwide in 2020. The disease often develops asymptomatically, with the first symptoms typically appearing only at advanced stages, leading to late diagnosis. Treatment combines surgery and chemotherapy. Unfortunately, a significant number of patients experience recurrence of the cancer, after which the chances of cure are minimal. Therefore, there is a urgent need to develop new, more effective first-line therapies that can delay recurrence, extend survival rates, or improve the quality of life for patients during treatment.

Presented doctoral dissertation is based on a series of publications and non-published data focusing on the analysis of biological effects induced by electroporation with calcium ions (Ca^{2+}) and estradiol ovarian cancer model. Calcium electroporation (CaEP) is a novel technique that modifies standard electrochemotherapy (ECT). Both techniques are based on the phenomenon of electroporation (EP), where an impulsive electric field induces rearrangement of the cell membrane. This results in the transient formation of pores allowing increased transport of molecules (incl. cytotoxic drugs or ions) into the cancer cell. Moreover, previous studies indicate that CaEP has a less toxic impact on normal cells compared to conventional chemotherapy. Additionally, considering the therapeutic potential of high concentrations of estrogens, particularly 17β-estradiol, and its stimulatory effect on intracellular Ca^{2+} concentration, its potential stimulating effect on CaEP therapy was analyzed.

The results obtained demonstrated a promising therapeutic effect of CaEP on ovarian cancer cells. Electroporation enhances the cytotoxicity of cisplatin and calcium chloride with both nanosecond and microsecond pulses. The effectiveness depends on the pulse duration, polarity, and symmetry of the applied protocols, allowing for precise control of the process and effective drug delivery. This novel approach shows a greater toxic potential against cancer cells compared to chemotherapy and cisplatin-based ECT. It was also noted that CaEP has a lesser negative impact on normal cells compared to standard chemotherapy and ECT. Additionally, the potentiating effect of 17β-estradiol on the efficacy of the analyzed therapy was observed.

3. Wykaz publikacji stanowiących podstawę rozprawy doktorskiej

Artykuły zostały uszeregowane zgodnie z realizowanym w nich celem naukowym.

1) **Łapińska Zofia**, Dębiński Michał, Szewczyk Anna, Choromańska Anna, Kulbacka Julita, Saczko Jolanta: *Electrochemotherapy with calcium chloride and 17β-estradiol modulated viability and apoptosis pathway in human ovarian cancer*, Pharmaceutics, 2021, vol. 13, nr 1, art.19 [17 s.], DOI:10.3390/pharmaceutics13010019

   (IF: 6,525; MNiSW: 100 pkt.)

2) **Łapińska Zofia,** Novickij Vitalij, Rembiałkowska Nina, Szewczyk Anna, Dubińska-Magiera Magdalena, Kulbacka Julita, Saczko Jolanta Ewa: *The influence of asymmetrical bipolar pulses and interphase intervals on the bipolar cancellation phenomenon in the ovarian cancer cell line*, Bioelectrochemistry, 2023, vol. 153, art.108483 [10 s.], DOI: 10.1016/j.bioelechem.2023.108483

   (IF: 4,8; MNiSW: 100 pkt.)

3) **Łapińska Zofia,** Szwedowicz Urszula, Choromańska Anna, Saczko Jolanta: *Electroporation and electrochemotherapy in gynecological and breast cancer treatment*, Molecules, 2022, vol. 27, nr 8, art.2476 [32 s.], DOI:10.3390/molecules27082476

   (IF: 4,6; MNiSW: 140 pkt.)

Łączna punktacja cyklu publikacji: IF: 15,925; **MNiSW:** 340 pkt.

4. Wstęp

Podstawę rozprawy doktorskiej stanowi cykl trzech pełnotekstowych artykułów naukowych opublikowanych w latach 2021-2023. Artykuły zostały uszeregowane zgodnie z realizowanym w nich celem naukowym. Komentarz do cyklu publikacyjnego stanowi podsumowanie najważniejszych naukowych osiągnięć własnych, będących podstawą przedstawionej rozprawy doktorskiej. Sumaryczny współczynnik wpływu (Impact Factor; IF) czasopism, w których opublikowany został cykl publikacji wynosi **15,925** a łączna liczba punktów Ministerstwa Nauki i Szkolnictwa Wyższego (MNiSW) wynosi **340**. Praca została ponadto wzbogacona o dane dotychczas nieopublikowane.

Rak jajnika (ang. *ovarian cancer*; OC) stanowi ósmy pod względem częstości występowania nowotwór diagnozowany wśród kobiet, odpowiadając szacunkowo za 3,7% przypadków i 4,7% zgonów z powodu nowotworów odnotowanych w 2020 roku na świecie [1]. Do początku XXI wieku wskaźnik zapadalności standaryzowanej na wiek był najwyższy w Europie Północnej oraz Ameryce Północnej. Ta tendencja uległa jednak zmianie. Obecnie zapadalność na tę chorobę wzrasta w niektórych częściach Europy Wschodniej i Azji. Według danych Krajowego Rejestru Nowotworów w 2021 roku w Polsce zostało odnotowanych 3624 nowych przypadków raka jajnika oraz 2639 zgonów kobiet spowodowanych tą chorobą [2]. Tym samym, rak jajnika stanowi drugą najczęstszą przyczynę zachorowań na nowotwory złośliwe narządów płciowych wśród polskich pacjentek. Poza guzami o wydzielaniu dokrewnym, nowotwory jajnika, często we wczesnym etapie, rozwijają się w sposób bezobjawowy (z czym wiąże się popularne w literaturze naukowej określenie tej choroby jako „*cichego zabójcy”*). Co więcej, w przypadku raka jajnika próżno jest szukać tzw. objawów patognomonicznych. Gdy guz osiągnie już znaczny stopień zaawansowania, pojawiające się symptomy często są mylnie interpretowane przez pacjentki jako dolegliwości ze strony układu pokarmowego, co nie skłania do wizyt u lekarza ginekologa. Z tego powodu wczesna diagnoza choroby ma najczęściej charakter incydentalny, a blisko 70% pacjentek rozpoczyna leczenie w III lub IV stopniu zaawansowania klinicznego wg. klasyfikacji Międzynarodowej Federacji Ginekologii i Położnictwa (FIGO, fr. *Fédération internationale de gynécologie et d'obstétrique*), w którym wskaźnik 5-letniego przeżycia obniża się odpowiednio do 41 i 20% [3]. Leczenie choroby opiera się na terapii skojarzonej łączącej cytoredukcję oraz chemioterapię opartą na związkach platyny oraz taksanów [4,5]. Pomimo, iż duża część pacjentek obiecująco reaguje na chemioterapię, wg statystyk ~70% kobiet z zaawansowanym rakiem jajnika doświadcza wznowy procesu nowotworowego w przeciągu

3 lat, a 10-letni wskaźnik przeżycia wynosi wówczas 17% [6,7]. Nawrotowy zaawansowany rak jajnika jest najczęściej nieuleczalny, co podkreśla potrzebę rozwoju nowych strategii terapeutycznych. Na niekorzystne rokowania składa się m.in. duża heterogenność, jaką charakteryzuje się schorzenie [8,9]. Znikoma część przypadków rozwija się z komórek germinalnych, czy komórek zrębu jajnika. Zdecydowana ich większość (~90% odnotowanych nowotworów) to nowotwory nabłonkowe (z ang. *epithelial cancers*). Tę ostatnią grupę można dalej podzielić na pięć głównych histotypów, tj. nowotwory surowicze o wysokim i niskim stopniu złośliwości (ang. *high-grade and low-grade serous cancers*) oraz nowotwory endometrioidalne (ang. *endometrioid*), jasnokomórkowe (ang. *clear cell*) i śluzowe (ang. *mucinous*). Każdy z przytoczonych podtypów różni się między sobą podłożem genetycznym, etiologicznym, a także odpowiedzią na zastosowane leczenie. Biorąc po uwagę powyższe, istnieje nagląca potrzeba poszukiwania nowych rozwiązań terapeutycznych oraz poznawania podłoży molekularnych choroby, w celu zwiększania szans przeżycia pacjentek oraz podnoszenia jakości ich życia w trakcie trwającej terapii.

Rak jajnika klasyfikowany jest jako nowotwór zależny od estrogenów, ponieważ hormony te oraz inne czynniki związane z rozrodczością, mają istotny wpływ na jego rozwój i postęp choroby [10]. Dominującym wewnątrzkomórkowym estrogenem jest 17β-estradiol (E_2), który jest syntetyzowany z cholesterolu, a głównym źródłem jego sekrecji są jajniki. Główną funkcję E_2 stanowi rozwój układu rozrodczego oraz gruczołów sutkowych. Jego aktywność biologiczna opiera się na interakcji z określonymi receptorami, mianowicie receptorem estrogenowym α (ang. *estrogen receptor α*; ERα), receptorem estrogenowym β (ang. *estrogen receptor β*; ERβ) oraz receptorem estrogenowym sprzężonym z białkiem G (ang. *G protein-coupled estrogen receptor*; GPER-1) [11]. Mechanizm działania estradiolu można podzielić na genomowy i niegenomowy [12]. Pierwszy z nich, określany jako klasyczny mechanizm działania estrogenu, polega na przenikaniu E_2 przez błonę komórkową i oddziaływaniu z receptorami estrogenowymi znajdującymi się w cytoplazmie (zarówno ERα, ERβ, jak i obydwoma jednocześnie). Związanie liganda umożliwia dimeryzację receptora, a następnie translokację do jądra komórkowego. Kompleks ER-E_2 wiąże się z określonymi sekwencjami DNA, znanymi jako elementy odpowiedzi na estrogen (ang. *estrogen response elements*; ERE), obecnymi w promotorach genów docelowych, co umożliwia regulację ich ekspresji. Alternatywnym szlakiem działania estradiolu jest szlak niegenomowy, w którym to osiągnięcie efektu nie wymaga bezpośredniego wpływu na ekspresję genów [12]. Estradiol wiąże się w tym przypadku z receptorami estrogenowymi zlokalizowanymi na błonie komórkowej, takimi jak receptor

estrogenowy typu alfa (mERα) lub wspomniany wyżej receptor GPER-1. Po związaniu z receptorem pobudzone zostają kaskady sygnalizacyjne, które obejmują aktywację kinaz białkowych, takich jak kinaza białkowa C (ang. *protein kinase C;* PKC), kinaza białkowa aktywowana mitogenami (ang. *mitogen-activated protein kinase*; MAPK), czy -kinaza fosfatydyloinozytolu (ang. *phosphoinositide 3-kinases*; PI3K). Skutkuje to fosforylacją białek docelowych, a co za tym idzie zmianami w regulacji kanałów jonowych, potencjału błonowego, mobilizacją jonów wapnia, czy modulacją aktywności enzymów. Te szybkie odpowiedzi mogą wpływać na funkcje komórkowe, takie jak proliferacja, migracja, różnicowanie czy odpowiedzi komórkowe na stres. W przeciwieństwie do genomowego szlaku działania estradiolu, niegenomowy funkcjonuje na poziomie cytoplazmatycznym lub błonowym, a efekty działania następują w przeciągu od kilku sekund do kilku minut.

Od dekad estradiol rozpatrywany jest jako jeden z czynników odpowiadających za rozwój szeregu nowotworów. Estrogen może indukować produkcję oksydacyjnych metabolitów, co prowadzi do powstania adduktów DNA oraz podwójnych pęknięć nici DNA (ang. *DNA double-strand breaks*; DSBs) [13,14]. Ponadto, hiperaktywacja kompleksu ER:E_2 skutkuje wzmożoną proliferacją komórek, co sprzyja akumulacji uszkodzeń DNA [15]. Stan ten doprowadza ostatecznie do niestabilności genomu, która jest jedną z głównych przyczyn rozwoju nowotworu. Zaskakujący jest jednak fakt, iż w zależności od zastosowanego stężenia (>1 nM), E_2 może wykazywać hamujący wpływ na proliferację komórek nowotworowych, jednocześnie napędzając w nich proces apoptozy [16].

Do lat 70-tych XX wieku tzw. wysokie dawki estrogenów (ang. *high dose estrogens;* HDEs) były popularne w leczeniu nowotworów piersi pojawiających się po długotrwałych okresach niedoboru estrogenów [17]. Z czasem jednak, rozwiązanie to zostało wyparte przez hormonalne preparaty antyestrogenowe, po porównaniu ilości skutków ubocznych występujących po obu terapiach. Jednakże, HDEs mogą być brane pod uwagę jako alternatywne leczenie pierwszego rzutu pacjentek będących min. 5 lat po menopauzie. Co ciekawe, z punktu widzenia niniejszej pracy, w literaturze naukowej omawiano również stymulujący wpływ estrogenów na kanały jonowe, m.in. kanały wapniowe [18]. W przeprowadzonych badaniach *in vitro* wykazano, iż 17β-estradiol w zależności od stężenia (≥10 μM) skutkuje zwiększonym poziomem białka Cav1.3, które to jest izoformą kanału wapniowego typu L (ang. *L-type calcium channel*; LTCC) w komórkach MCF-7, będących modelem badawczym nowotworu piersi [19]. LTCC należy do większej grupy białek, mianowicie kanałów wapniowych bramkowanych napięciem (ang. *voltage-gated calcium channels*; VGCC). Inkubacja szczurzych komórek siatkówki oka

z 10 μM E_2 skutkowała przejściowo zwiększonym wewnątrzkomórkowym stężeniem jonów wapnia za pośrednictwem LTCC oraz kinazy fosfoinozytolu 3' (ang. *phosphoinositide 3-kinases*; PI3K) [20]. Rezultatem tego była ochrona komórek przed procesem apoptozy indukowanym 100 μM nadtlenkiem wodoru (ang. *hydrogen peroxide*; H_2O_2). Biorąc po uwagę powyższe, w niniejszej pracy postanowiono przeanalizować ewentualną rolę 17β-estradiolu jako czynnika stymulującego dodatkowo efektywność elektroporacji z jonami wapnia.

Stosunkowo nową metodą leczenia nowotworów jest elektrochemioterapia (ang. *electrochemotherapy*; ECT). Zjawisko to zostało opisane po raz pierwszy przez L.M. Mir i wsp. w 1991 roku i stanowi połączenie konwencjonalnej chemioterapii z elektroporacją (ang. *electroporation*; EP) [21]. Jako *„elektroporację”* rozpatruje się zastosowanie impulsowanego pola elektrycznego zwiększającego krótkotrwale przepuszczalność błony komórkowej, poprzez tworzenie hydrofilowych porów [22–24]. W momencie ekspozycji komórki na zewnętrzne pole elektryczne o odpowiedniej sile dochodzi do powstania tzw. indukowanego napięcia transmembranowego (ang. *induced transmembrane voltage;* iTMV), które istnieje tak długo, jak obecne jest to zewnętrzne źródło pola elektrycznego [25]. W chwili, gdy różnica potencjałów po obu stronach błony wyniesie ok. 200-250 mV, dochodzi to rearanżacji/lub reorganizacji dwuwarstwy lipidowej, w wyniku której powstają wspomniane wcześniej pory, a co za tym idzie, zwiększa się przepuszczalność błony komórkowej [26]. W zależności od zastosowanego protokołu elektroporacji (tj. natężenia pola elektrycznego, kształtu, długości i czasu trwania impulsów), a także charakterystyki celowanej komórki (jej rodzaju, kształtu, wielkości, składu oraz krzywizny dwuwarstwy lipidowej), możemy wyróżnić dwie główne odmiany elektroporacji: nieodwracalną i odwracalną. Pierwsza (ang. *irreversible electroporation*; IRE) z nich prowadzi do bezpowrotnego rozszczelnienia błony komórkowej, co znacząco zaburza homeostazę komórki, skutkując ostatecznie jej wkroczeniem na drogę śmierci komórkowej [27,28]. Zastosowanie elektroporacji typu odwracalnego (ang. *reversible electroporation*; RE) pozwala komórkom na zrekonstruowanie ciągłości błony komórkowej oraz przywrócenie homeostazy [29]. Przejściowy wzrost przepuszczalności dwuwarstwy lipidowej pozwala na zastosowanie RE w celu dokomórkowego, bardziej precyzyjnego, transportu materiału genetycznego, białek, czy leków cytotoksycznych [21,30]. Dodatkowy podział elektroporacji zależy od długości aplikowanych impulsów tj. wyróżniamy elektoporację (nsEP) nano-, (μsEP) mikro- oraz (msEP) milisekundową. Na efektywność EP może wpływać również kształt zastosowanych impulsów. Zostało wskazane, iż zastosowanie

impulsów bipolarnych (ang. *bipolar pulses*; BP) wpływa na permeabilizację błony komórkowej bardziej intensywnie niż impulsy unipolarne (ang. *unipolar pulses*; UP), przy osiągnięciu tych samych lub lepszych wskaźników przeżycia [31,32]. Pakhomov i wsp. opisali zjawisko, w którym działanie impulsu elektrycznego o dodatniej polarności (tzw. impuls katodowy; oznaczany symbolem: ↑) może zostać zniesione (z ang. *cancelled*) poprzez zastosowanie impulsu o odwrotnej polaryzacji (tzw. impuls anodowy; oznaczany symbolem: ↓) [33]. Jest to unikalna reakcja fizjologiczna komórki, określana w literaturze naukowej jako *„bipolar pulse cancellation"* oraz uważana dotychczas za wyłączną dla protokołów nanosekundowych (nsEP). Zjawisko to może być również kontrolowane poprzez symetrię impulsów oraz ewentualne przerwy wprowadzane pomiędzy aplikację każdego z nich. Chociaż terapie oparte na zjawisku elektroporacji znajdują coraz szersze zastosowanie, należy zaznaczyć (jak zostało wspomniane powyżej), iż efekt końcowy takiej terapii zależny jest od wielu parametrów, dlatego też powinno się analizować skuteczność tego rozwiązania w kontekście każdej tkanki indywidualnie.

Procedury kliniczne z zastosowaniem elektrochemioterapii opierają się w głównej mierze na europejskich wytycznych dotyczących standardowej procedury operacyjnej ECT (ang. *European Standard Operative Procedure ECT*; ESOPE) opublikowanych w 2006 r. [34,35]. Obejmują one aplikację 8 impulsów o długości czasu trwania 100 μs i częstotliwości równej 1 Hz lub 5 kHz (w zależności od typu zastosowanej elektrody). Skutkuje to odwracalną elektroporacją wspomagającą transport leku cytotoksycznego, który podawany jest dożylnie lub bezpośrednio do guza [29]. Głównymi chemioterapeutykami stosowanymi podczas ECT są cisplatyna (CPP) i bleomycyna (BLM) [21,36–39]. Połączenie impulsów elektrycznych oraz wymienionych wyżej chemioterapeutyków pozwala na wzrost ich cytotoksyczności odpowiednio 80- i 1000-krotnie [40–42]. Rośnie, jednakże liczba badań analizujących efektywność przeciwnowotworową innych niż standardowe leki cytotoksyczne substancji, jak np. jony wapnia (Ca^{2+}). Ta modyfikacja standardowej ECT znana jest w literaturze naukowej jako „*elektroporacja wapniowa"* (ang. *calcium electroporation*; CaEP) i została opisana po raz pierwszy w 2012 r. przez Frandsen i wsp. [43]. Stężenia jonów wapnia po obu stronach błony komórkowej są ściśle kontrolowane [44]. Ich masywny napływ do wnętrza komórki ułatwiony, powstającymi w wyniku EP porami hydrofilowymi, skutkuje zaburzeniem tej homeostazy [45]. W celu pozbycia się nadmiaru Ca^{2+} komórka uruchamia zależne od adenozyno-5′-trifosforanu (ATP) wymienniki oraz pompy. Aktywność tych mechanizmów połączona z zaburzonym gradientem elektrochemicznym, który jest niezbędny do produkcji ATP, ostatecznie

doprowadza do wyczerpania zapasów tej molekuły, aktywując w komórce mechanizmy prowadzące do jej śmierci [46]. Podobnie jak w przypadku standardowej ECT, CaEP skutkuje większym efektem cytotoksycznym wobec komórek nowotworowych w porównaniu do konwencjonalnej chemioterapii [47]. Co bardziej znaczące, w zestawieniu z pozostałymi wymienianymi wcześniej terapiami przeciwnowotworowymi, CaEP w mniejszym stopniu wpływa negatywnie na prawidłowe komórki organizmu [48–50].

5. Cele rozprawy doktorskiej

Celem niniejszej pracy była analiza efektywności połączenia elektroporacji z jonami wapnia (Ca^{2+}) w kontekście eliminacji komórek raka jajnika na zróżnicowanych modelach *in vitro*. W pracy zweryfikowano dwie główne hipotezy:

1.) Komórki raka jajnika wykazują wysoką wrażliwość na działanie impulsowego pola elektrycznego o wysokim natężeniu w połączeniu z cisplatyną lub chlorkiem wapnia ($CaCl_2$).
2.) 17β-estradiol (E_2) pełni rolę czynnika uwrażliwiającego komórki raka jajnika na terapie oparte na zjawisku elektroporacji, takie jak elektrochemioterapia z wykorzystaniem cisplatyny i chlorku wapnia ($CaCl_2$).

Dodatkowo, w pracy wyróżniłam hipotezy podrzędne:

1.) Prawidłowe komórki nabłonkowe jajnika wykazują zmniejszoną wrażliwość na impulsy elektryczne o wysokim natężeniu oraz ich kombinację z cisplatyną lub chlorkiem wapnia ($CaCl_2$) w porównaniu do komórek nowotworowych.
2.) Symetryczne impulsy bipolarne skutkują obecnością efektu *bipolar cancellation*, tj. redukcją permeabilizacji błony komórkowej, przy jednoczesnym wzroście przeżywalności komórek. Zastosowanie protokołów asymetrycznych powoduje ograniczeniem tego zjawiska.

Sformułowane przeze mnie hipotezy zostały zrealizowane i przedstawione w formie cyklu dwóch oryginalnych prac naukowych oraz danych dotychczas nieopublikowanych. Dodatkowo, cykl publikacji został wzbogacony o pracę przeglądową, która opisuje aktualny stan wiedzy oraz potencjalne kierunki rozwoju terapii przeciwnowotworowych opartych na zjawisku elektroporacji, ukierunkowanych na leczenia nowotworów ginekologicznych i raka piersi.

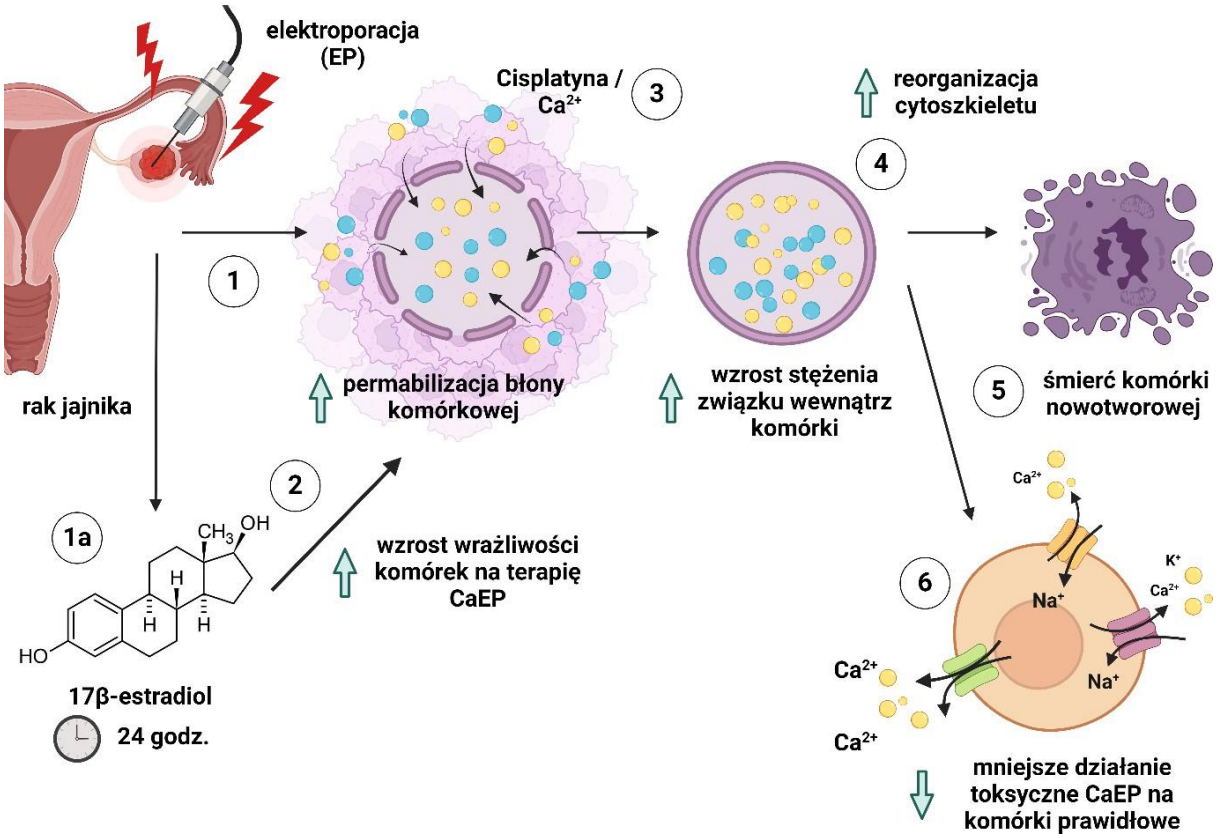

Figura 1. Graficzna prezentacja koncepcji pracy doktorskiej pt. „Wykorzystanie elektroporacji z jonami wapnia oraz estradiolem w leczeniu raka jajnika na modelu in vitro". Zastosowanie impulsywnego pola elektrycznego **(1)** skutkuje wzrostem stopnia permeabilizacji błony komórkowej **(3)**. Zjawisko to nosi nazwę elektroporacji (EP). Pozwala na podniesienie ilości związków chemioterapeutycznych lub jonów wapnia (Ca^{2+}), jakie są dostarczane do wnętrza komórki **(4)**. Ostatecznym skutkiem jest wzrost cytotoksyczności aplikowanych leków, przy jednoczesnym (w przypadku elektroporacji z jonami wapnia tj. elektroporacji wapniowej, CaEP) zmniejszeniu działań niepożądanych wobec komórek prawidłowych **(6).** Dodatkowo, ekspozycja komórek nowotworowych na działanie 17β-estradiolu **(1a)** pozwala uwrażliwić je na działanie stosowanych terapii **(2)**.

6. Materiały i metody

6.1 Linie komórkowe

Eksperymenty badawcze zostały przeprowadzone w laboratorium Katedry i Zakładu Biologii Molekularnej i Komórkowej Uniwersytetu Medycznego we Wrocławiu. W pracach doświadczalnych wykorzystano modele *in vitro* raka jajnika w postaci linii komórkowych pochodzenia ludzkiego: MDAH-2774 **[1]** oraz OvBH-1 **[*wyniki nieopublikowane*]**. Dodatkowo, w celu porównania wpływu analizowanych rozwiązań terapeutycznych na komórki prawidłowe (lub nienowotworowe) zastosowano prawidłowe komórki nabłonkowe jajnika chomika chińskiego: CHO-K1 **[*wyniki nieopublikowane*]**. Komórki są równocześnie modelem do badań nad elektroporacją, w związku z obniżoną ekspresją kanałów jonowych. Linie MDAH-2774 i CHO-K1 zostały nabyte w banku komórek ATCC® (American Type Culture Collection) i znajdują się na wyposażeniu Katedry i Zakładu Biologii Molekularnej i Komórkowej. Linia komórkowa OvBH-1 została udostępniona przez prof. J. Bar (Zakład Immunopatologii i Biologii Molekularnej, Katedra Podstaw Nauk Medycznych i Immunologii, Uniwersytet Medyczny im. Piastów Śląskich we Wrocławiu) i wyprowadzona z komórek 54-letniej kobiety zdiagnozowanej z jasnokomórkowym rakiem jajnika. Morfologiczne i fenotypowe cechy, jak również zależna od temperatury wrażliwość OvBH-1, zostały wcześniej opisane przez prof. J. Bar i współpracowników, wskazując OvBH-1 jako unikalny i interesujący model badawczy (patent polski nr: PL189880) [51,52]. Linię komórkową MDAH-2774 wyizolowano z guza endometrioidalnego raka jajnika (ang. *endometrioid ovarian carcinoma; EOC)* u 47-letniej kobiety. Po raz pierwszy opisane i zidentyfikowane zostały w 1979 roku. Wszystkie komórki były hodowane zgodnie z zaleceniami producentów w warunkach 37°C, 5% CO_2, 95% wilgotności powietrza. Linie komórkowej hodowano przy użyciu komercyjnych mediów hodowlanych tj. DMEM (OvBH-1), RPMI-1640 (MDAH-2774) oraz Ham's F-12 (CHO-K1). Pożywki wzbogacone zostały 100 U/ml penicyliną, 100 µg/ml streptomycyną, 10% płodową surowicą bydlęcą (z ang. *fetal bovine serum*; FBS) oraz 1% L-glutaminą.

6.2 Elektroporacja komórek oraz analiza efektywności

W doświadczeniach wykorzystano komórki adherentne, które przed ekspozycją na impulsowe pole elektryczne, odklejano od podłoża naczynia hodowlanego za pomocą enzymatycznego roztworu TrypLE™ Express Enzyme (Life Sciences— Thermo Fisher Scientific Inc., USA) i zawieszano w dwóch różnych buforach do elektroporacji (HEPES

wzbogacony o 250 mM sacharozę i 1 mM $MgCl_2$ dla linii OvBH-1 oraz CHO-K1 lub SMEM dla linii MDAH-2774) zawierającymi (lub nie) leki przeciwnowotworowe: cisplatynę (10 μM) lub chlorek wapnia (2,5 mM). Ekspozycję materiału na impulsy elektryczne poprzedzała ich 24-godzinna preinkubacja z roztworem 10 μM 17β-estradiolu (E_2) w medium hodowlanym. Zastosowane stężenie E_2 zostało zoptymalizowane dla każdej linii komórkowej raka jajnika **(Pub. 1; Fig. 1 oraz Fig. 2;** *dane nieopublikowane***)** poprzez analizę przeżywalności komórek na skutek 24- oraz 72-godzinnej inkubacji we wzrastających stężeniach związku. Na podstawie otrzymanych wyników wybrano najwyższe nietoksyczne stężenie E_2. Elektroporacji dokonywano w 1 lub 4-mm kuwetach z elektrodami (BioRad®, USA) przy użyciu kilku generatorów impulsów elektrycznych: 1.) ECM830 BTX (Harvard Apparatus, USA) – do wytworzenia impulsów mikrosekundowych; 2.) PPG-20 generator (FID Technology, Niemcy) – dla ultrakrótkich impulsów elektrycznych o długości czasu trwania równej 10 ns; 3.) High-Frequency Bipolar Electroporator (VGTU, Litwa) – do aplikacji krótszych (600 ns – 10 μs) impulsów elektrycznych uni- oraz bipolarnych o zmiennej symetrii. Każdy z zastosowanych protokołów został dokładnie opisany w pracach oryginalnych składających się na niniejszą rozprawę. W przypadku niepublikowanych wcześniej wyników, komórki OvBH-1 oraz CHO-K1 zostały wyeksponowane na działanie impulsów elektrycznych, które były dostarczane w postaci 8 impulsów elektrycznych o natężeniu pola w zakresie 0,8-2,5 kV/cm i czasie pojedynczego impulsu 100 μs z częstotliwością powtórzeń 1 Hz, zgodnie z klinicznie stosowanymi Europejskimi Standardowymi Procedurami Operacyjnymi ECT (ESOPE) [53]. Po ekspozycji na zewnętrze impulsy elektryczne komórki inkubowano przez 15 min. w temperaturze 37°C, a następnie zawieszono ponownie w medium hodowlanym i poddano dalszej analizie.

Skuteczność protokołów elektroporacji oceniano poprzez pomiar ilości barwnika fluorescencyjnego jodku Yo-Pro™-1 (zwanego również żółcią oksazolową; Thermo Scientific™, USA) zaabsorbowanego przez komórkę. Analizę przeprowadzono przy użyciu cytometru przepływowego CyFlow CUBE-6 (Sysmex, Polska). Komórki po trypsynizacji zawieszono w odpowiednim buforze do EP z dodatkiem 1 μl/ml barwnika Yo-Pro-1™. Po ekspozycji na zewnętrzne impulsy elektryczne, komórki odwirowano (2 min. × 100 RCF), a następnie zawieszono w 300 μl buforowanego fosforanem roztworu soli fizjologicznej (z ang. *Dulbecco's Phosphate Buffered Saline*; dPBS) lub 0,9% roztworu chlorku sodu (NaCl). Tak przygotowane próbki poddawano analizie w cytometrze przepływowym CyFlow Cube 6 flow cytometer (Sysmex, Poland). Każdorazowo analizie poddawano min. 10 000 komórek

w próbie. Kontrolę negatywną stanowiły komórki inkubowane z Yo-Pro-1^{TM}, które nie poddano elektroporacji. Procent permeabilizacji wyrażano jako liczbę komórek emitujących fluorescencję o długości fali 506 nm w stosunku do całkowitej liczby komórek, użytych do analizy. Eksperyment powtarzano 3 razy. Próbki kontrolne bez leczenia zostały użyte jako negatywna kontrola w celu określenia grupy komórek żywych przy użyciu detektorów FSC (z ang. *Forward Scatter Channel*) oraz SSC (z ang. *Side Scatter Channel*). Wyniki zebrano oraz analizowano za pomocą oprogramowania CyView software (Sysmex, Poland).

Żywotność komórek MDAH-2774, OvBH-1 oraz CHO-K1 po ich ekspozycji na zaaplikowane leczenie została oceniona przy użyciu testu MTT, który identyfikuje aktywność mitochondriów. Krótko po elektroporacji, komórki zostały wysiane na 96-dołkową płytkę i inkubowane przez 24-72 godz. Po tym czasie, medium hodowlane zostało usunięte i inkubowano z 0,5 mg/ml roztworem bromku 3-(4,5-dimetyltiazol-2-yl)-2,5-dofenylotatrazolowego (MTT; Sigma-Aldrich, USA) przez 2 godz. w 37^{o}C, 5% CO_2. Uformowane kryształy formazanu rozpuszczono przez dodanie 100 µl zakwaszonego izopropanolu (38% HCl w 99,7% izopropanolu). Do pomiaru absorbancji przy długości fali 570 nm wykorzystano czytnik mikropłytek GloMax® Discover (Promega, USA). Wyniki wyrażono jako procent żywych komórek w stosunku do nieleczonych komórek kontrolnych.

6.3 Wyznaczenie optymalnych stężeń analizowanych związków chemicznych

Metoda MTT została również wykorzystana w celu obserwacji przeżywalności komórek MDAH-227, OvBH-1 oraz CHO-K1 na działanie cisplatyny, chlorku wapnia ($CaCl_2$) oraz 17β-estradiolu (E_2). W pierwszej kolejności przeanalizowano toksyczność 17β-estradiolu, inkubując komórki ze związkami przez czas 24 i 72 godz. **(Pub. 1; Fig. 1 oraz Fig. 2;** ***dane nieopublikowane*****)**.

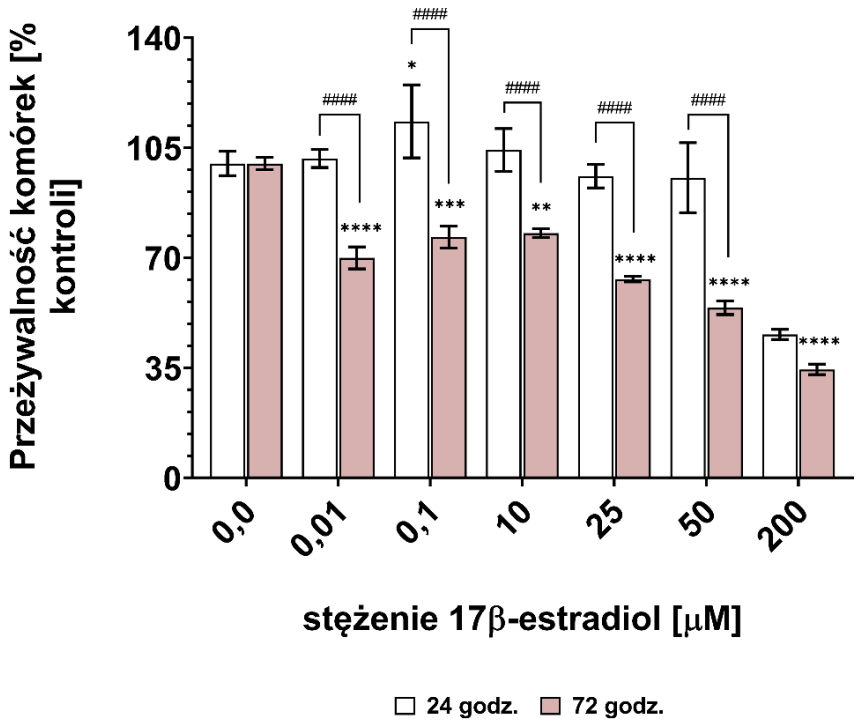

Figura 2. Optymalizacja stężenia 17β-estradiolu dla komórek OvBH-1. Komórki inkubowano we wzrastających stężeniach 17β-estradiolu przez 24 oraz 72 godziny; Uwagi: (średnia ± SD) N = 3, **p< 0.05, ** p< 0.01, *** p< 0.001, **** p< 0.0001* w przyrównaniu do kontroli (CTRL, 0 μM); *# p< 0.05, ## p< 0.01, ### p< 0.001, #### p< 0.0001* w przyrównaniu wyników dla poszczególnych stężeń pomiędzy różnymi czasami inkubacji; ***dane nieopublikowane.***

Na podstawie otrzymanych wyników wybrano optymalne stężenie E_2 stosowane na dalszych etapach prac badawczych (tj. 10 μM w przypadku obu analizowanych linii raka jajnika). Następnie, komórki (po uprzedniej preinkubacji z E_2) wystawiono na 24- i 72-godzinne działanie cisplatyny i $CaCl_2$, po czym dokonano inkubacji z odczynnikiem MTT. W tym miejscu, również na podstawie otrzymanych wartości, wyznaczono stężenia obydwu związków, które były wykorzystane w kolejnych eksperymentach.

6.4 Ocena morfologii komórek, zmian cytoszkieletu oraz struktury błony komórkowej

Zmiany morfologii komórek MDAH-2774 oraz OvBH-1 uwidoczniono stosując obrazowanie holotomograficzne przy użyciu holograficznego mikroskopu tomograficznego 3D Cell Explorerm (Nanolive Ecublens, Szwajcaria). Komórk preinkubowane z 17β-estradiolem zostały wystawione na działanie cisplatyny lub chlorku wapnia ($CaCl_2$) w połączeniu (lub bez) z impulsami elektrycznymi. Dokładny opis postępowania w przypadku linii MDAH-2774 został zawarty w rozdziale *Materiały i metody* w pracy oryginalnej nr **[1]**. W przypadku danych nieopublikowanych komórki linii OvBH-1 zostały odklejone od podłoża naczynia za pomocą TrypLE^{TM} Express Enzyme (Life Sciences— Thermo Fisher Scientific

Inc., USA), następnie zawieszone w buforze do EP z dodatkiem związków leczniczych (lub bez) i wystawione na działanie zewnętrznych impulsów elektrycznych. Następnie, komórki odwirowano (2 min. × 100 RCF) i zawieszono w medium hodowlanym, nasadzając je na naczynia do obrazowania Ibidi µ-Dish35mm Quad, a następnie inkubując w temp. 37°C, 5% CO_2 przez 24 godz. Porównanie dla leczenia wspieranego przez dodatek EP stanowiły komórki uprzednio nasadzone na naczynia firmy Ibidi, a następnie inkubowane z cisplatyną oraz $CaCl_2$ zawieszonymi w medium hodowlanym przez 24 godz. Po tym czasie wykonano zdjęcia przedstawione na **Fig. 10 (*dane nieopublikowane*)**. Część komórek została wcześniej wybarwiona przez 20 minut w temperaturze 37°C, 5% CO_2 barwnikiem Hoechst 33342 (Life Sciences— Thermo Fisher Scientific Inc., USA) w celu wizualizacji jąder komórkowych. Uzyskane obrazy poddano obróbce za pomocą oprogramowania Software for Tomographic Exploration of Living Cells w wersji 1.6.3496 (STEVE, Nanolive).

Przy użyciu mikroskopii konfokalnej oceniono zmiany w strukturze cytoszkieletu, wybarwiając materiał biologiczny przeciwciałami I-rzędowymi skierowanymi przeciwko F-aktynie (MDAH-2774 **[1]** i OvBH-1 ***dane nieopublikowane***; 1:300, nr kat. #A12381 Alexa Fluor™546 Phalloidin, nr kat. # A2228, Life Technologies-Thermo Fisher Scientifi, USA) oraz β-tubulinie (OvBH-1 **[2]**; 1:300, nr kat. #ab108342, Abcam, UK). Komórki, uprzednio wystawione na działanie impulsów elektrycznych z dodatkiem cisplatyny lub chlorku wapnia ($CaCl_2$), poprzedzone 24-godzinną preinkubacją z 17β-estradiolem, nasadzono na szkiełka mikroskopowe, utrwalono przy użyciu 4% paraformaldehydu w PBS, blokowano przy użyciu 5% roztworu płodowej surowicy bydlęcej (z ang. *fetal bovine serum*; FBS) w PBS i wybarwiono wymienionymi wyżej przeciwciałami przygotowanymi w 5% FBS w PBS. Dodatkowo, w celu analizy zmian zachodzących w błonie komórkowej, komórki OvBH-1 inkubowano z barwnikiem CellMask™ (Invitrogen™, USA) **[2]**. Oceny struktur dokonano przy pomocy skaningowego mikroskopu konfokalnego Olympus FluoView FV1000 (Olympus, Japonia).

6.5 Ocena poziomu wybranych białek (kaspazy-1/ -3/ -8 oraz -12)

Stosując metodę immunocytochemiczną określono poziom kaspazy-12 w komórkach MDAH-2774 inkubowanych przez 24 godz. z cisplatyną i chlorkiem wapnia ($CaCl_2$) **[1]**. Komórki były uprzednio (bądź nie) poddane 24-godz. preinkubacji z 17β-estradiolem. Obrazowania dokonano przy użyciu odwróconego mikroskopu świetlnego Olympus BCX43 (Olympus, Japonia).

Wykrycie obecności oraz poziomu białek kaspazy-1/-3/-8 oraz -12 zostało wykonane z udziałem techniki Western Blot. Komórki OvBH-1, wystawione wcześniej na działanie elektroporacji z jonami wapnia, poddano lizie przy użyciu buforu litycznego (50 mM Tris-HCl, pH 8,0, 150 mM NaCl, 1% NP-40, 0,1% SDS, 0,5% deoksycholanu sodu) uzupełnionego koktajlem inhibitorów proteaz (Promega, USA) oraz 0,2 mM fluorkiem fenylometylosulfonylu (PMSF, Sigma-Aldrich, USA) w celu pozyskania całkowitej frakcji komórkowej białek **(*dane nieopublikowane*)**. Stężenie białka zostało określone przy użyciu zestawu Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific Inc., USA). Następnie 50 µg białka/próbkę zostało zawieszonych w buforze Laemmli (Bio-Rad, Polska) i poddanych elektroforezie w warunkach denaturujących (SDS-PAGE) przy użyciu 10% żeli poliakrylamidowych. Rozdzielone białka zostały przeniesione na membranę nitrocelulozową przy użyciu systemu Transblot Bio-Rad i zablokowane w 5% roztworze mleka w buforze TBS-T (0,2 M Tris; 1,5 M NaCl; 0,1% Tween-20). Membrany były następnie inkubowane przez noc w 4°C z I-rzędowymi przeciwciałami króliczymi skierowanymi przeciwko kaspazie-1 (1:2000, nr kat. 22915-1-AP, Proteintech, Niemcy) i kaspazie-12 (1:1000, nr kat. PA5-19963, Thermo Fisher Scientific Inc., USA) oraz przeciwciałami mysimi przeciwko kaspazie-8 (1:200, nr kat. sc-56070, Santa Cruz Biotechnology, Inc., USA) i β-aktynie (kontrola wewnętrzna, 1:200, nr kat. sc-47778, Santa Cruz Biotechnology, Inc., USA). Po przemyciu membran 3x roztworem TBS-T, membrana inkubowano przez 1 godz. w 37°C z II-rzędowymi przeciwciałami sprzężonymi z peroksydazą chrzanową (HRP), tj. przeciwciałami przeciwko IgG króliczemu (1:5000, nr kat. A6154-1ML, Sigma-Aldrich, USA) lub przeciwciałami przeciwko IgGκ BP-HRP mysiemu (1:500, nr kat. sc-516102, Santa Cruz Biotechnology, Inc., USA). Wszystkie przeciwciała były rozcieńczane w buforze TBS-T. β-aktyna była stosowana w celu normalizacji ilości białka w każdej z prób. Sygnał chemiluminescencyjny został wykryty przy użyciu systemu obrazowania chemiluminescencyjnego i dokumentacji żelu G-Box Chemi XRQ (Syngen, Polska). Wyniki blottingu oceniano dalej poprzez analizę densytometryczną przy użyciu oprogramowania ImageJ w wersji 2.15.0 (https://imagej.net/).

6.6 Analiza śmierci komórkowej metodą kometową oraz cytofluorymetryczną

Śmierć komórkową oceniono pośrednio poprzez pomiar poziomu białek związanych ze szklakami apoptotycznymi. W tym celu wykorzystano metodę kometową, cytometrii przepływowej oraz **(*dane nieopublikowane*)**.

Analiza cytometryczna komórek OvBH-1 polegała na dodaniu do komórek barwników SYTOX oraz APC połączonego z Aneksyną V po upływie 6 oraz 24 godz. od ich uprzedniej ekspozycji na elektroporację z jonami wapnia (*leczenie wykonano wg. protokołu opisanego w podrozdziale 6.2*). Komórki oderwano od naczynia hodowlanego przy użyciu odczynnika TrypLE™ (Thermo Fisher Scientific Inc., USA), odwirowano (3 min., 1500 RPM), a następnie zawieszono w 0,5 ml roztworu PBS zawierającego SYTOX™ Green Nucleic Acid Stain (Thermo Fisher Scientific, USA) oraz Anneksynę V znakowaną allofikocyjaniną (APC Annexin V Apoptosis Detection Kit, BioLegend, USA), postępując zgodnie z zaleceniami producentów. Eksperyment został przeprowadzony przy użyciu cytometru BD FACSCanto™ Clinical Flow Cytometry System. Fluorescencja SYTOX™ była wzbudzana za pomocą lasera o długości fali 488 nm i mierzona detektorem 530/30, natomiast fluorescencja APC była wzbudzana za pomocą lasera o długości fali 640 nm i mierzona detektorem 670/30. Badanie przeprowadzono na dwóch grupach komórek OvBH-1 (niepreinkubowanych oraz preinkubowanych przez 24 godz. przed leczeniem, 37°C, 5% CO_2 z 10 μM E_2). Ponadto, kontrolami (CTRL) były próbki niebarwione i barwione, z użyciem tylko jednego z dwóch barwników. Komórki apoptotyczne wykrywano na podstawie ekspresji fosfatydyloseryny na zewnętrznej warstwie błony cytoplazmatycznej, którą oznaczano poprzez wiązanie aneksyny V. Komórki nekrotyczne barwiono odczynnikiem SYTOX, który przenika przez uszkodzoną błonę komórkową i tworzy kompleksy z kwasami nukleinowymi.

Wykonano również pomiar aktywności kaspazy-3 oraz -7, wykorzystując test Caspase-Glo® 3/7 Assay System (Promega, Niemcy). Poddane leczeniu komórki OvBH-1 nasadzono na 96-dołkową płytkę i inkubowano przez czas 4, 8, 12 i 24 godz., po czym do odpowiednich dołków dodano 100 μl Caspase-Glo® 3/7 Reagent i inkubowano zgodnie z zaleceniami producenta przez 1 godz., w temp. pokojowej. Odczyt sygnału luminescencyjnego umożliwił czytnik mikropłytek GloMax® Discover (Promega, USA). Wyniki zostały wyrażone jako procent aktywności kaspazy-3/7 w komórkach w porównaniu do komórek kontrolnych (CTRL) niepoddanych leczeniu.

6.7 Analiza statystyczna

Eksperymenty przeprowadzono w trzech powtórzeniach. Analizę statystyczną wykonano przy użyciu oprogramowania GraphPad Prism 8 (GraphPad Software Inc, USA). Dane przedstawiono jako średnia ± SD (odchylenie standardowe) i poddano analizie za pomocą dwuczynnikowej analizy wariancji (ANOVA). Gdy analiza ANOVA wykazała istotny

statystycznie wynik, zastosowano test wielokrotnych porównań Sidaka w celu oceny różnicy. Za istotne statystycznie uznano wartości $p < 0,05$.

7. Podsumowanie oraz wnioski

Cykl publikacji otwierają badania własne, gdzie porównano wrażliwość ludzkich komórek endometrioidalnego raka jajnika MDAH-2774 na klasyczną chemioterapię oraz elektrochemioterapię z cisplatyną lub chlorkiem wapnia ($CaCl_2$), który stanowił źródło jonów wapniowych **[1]**. Na wstępie należy zaznaczyć, iż linia MDAH-2774 wykazuje umiarkowaną oporność na konwencjonalną chemioterapię, tj. opartą na cisplatynie. W przypadku obu analizowanych związków, zwiększenie ich transportu poprzez zaaplikowanie zewnętrznego pola elektrycznego pozwoliło obniżyć przeżywalność komórek raka jajnika w porównaniu do cisplatyny oraz $CaCl_2$ podawanych samodzielnie. Najbardziej efektywny okazał się protokół oparty na impulsach mikrosekundowych (µsEP). Co więcej, preinkubacja komórek z 17β-estradiolem, jaka poprzedzała etap leczniczy uwrażliwiła linię MDAH-2774 na działanie cisplatyny oraz $CaCl_2$ **(Pub. 1; Fig. 3)**. Efekt utrzymywał się również w przypadku 72-godz. inkubacji ze związkami. Preinkubacja skutkowała również zwiększeniem poziomu cytotoksyczności klasycznej elektrochemioterapii oraz jej modyfikacji z jonami wapnia (Ca^{2+}). W celu określenia, czy obniżona przeżywalność komórek MDAH-2774 preinkubowanych z 17β-estradiolem jest związana ze zwiększoną syntezą reaktywnych form tlenu (ROS), przeanalizowano ekspresję kaspazy-12 **(Pub. 1; Fig. 4)**. Kaspaza-12 uczestniczy w apoptozie wywołanej stresem siateczki śródplazmatycznej i jest aktywowana w odpowiedzi na czynniki stresowe, takie jak reaktywne formy tlenu oraz wysokie stężenia jonów wapnia [54]. Barwienie immunocytochemiczne wykazało wzrost ekspresji kaspazy-12 w komórkach preinkubowanych z estradiolem, przy czym efekt ten był zróżnicowany w zależności od zastosowanego związku chemicznego. W komórkach traktowanych cisplatyną wzrost ten wynosił ok. 10%, niezależnie od stężenia cytostatyku. W przypadku zastosowania $CaCl_2$ w stężeniu 1 mM liczba komórek z ekspresją kaspazy-12 wzrosła o 45%. Należy zauważyć, że w przypadku zastosowania stężenia jonów wapnia 2,5 mM liczba komórek z obecnością kaspazy-12 nie zmieniała się na skutek preinkubacji z estradiolem, z uwagi na wysoką ekspresję tego białka niemal u 95% komórek poddanych terapii. Fluorescencyjne barwienie filamentów aktynowych ujawniło wyraźne zmiany morfologiczne w komórkach poddanych elektroporacji mikrosekundowej i nanosekundowej (przy natężeniu pola elektrycznego 50 kV/cm) **(Pub. 1; Fig. 5 i 6)**. W tych próbach zaobserwowano znaczące obkurczenie komórek oraz uszkodzenia cytoszkieletu. Zmiany morfologiczne zostały potwierdzone przy użyciu mikroskopii holotomograficznej **(Pub. 1; Fig. 8)**. W celu przeanalizowania czy wzrost toksyczności badanych rozwiązań terapeutycznych po preinkubacji komórek z estradiolem jest

związany z jego wpływem na strukturę błony komórkowej, komórki były elektroporowane (po uprzedniej preinkubacji) z barwnikiem fluorescencyjnym (Yo-Pro-1^{TM}), którego absorpcja była następnie mierzona za pomocą cytometru przepływowego **(Pub. 1; Fig. 7)**. Komórki preinkubowane z estradiolem wykazywały umiarkowanie niższą absorpcję barwnika. Uzyskane wyniki testu cytotoksyczności pozwalają wnioskować, iż mechanizm odpowiadający za spadek aktywności mitochondrialnej w tych komórkach nie wynika ze zmian w przepuszczalności dwuwarstwy lipidowej.

Jak już wcześniej wspomniano, linia komórkowa MDAH-2774 stanowiąca materiał badawczy pierwszej z prac oryginalnych zawartych w niniejszym cyklu **[1]** charakteryzuje się nieznaczną opornością wobec standardowej chemioterapii opartej na cisplatynie. Inaczej jest w przypadku linii OvBH-1, dla której efekt cytotoksyczny leku jest już obserwowany przy stężeniu 10 μM po 24-godzinnej inkubacji **(Fig. 3;** *dane nieopublikowane***)**.

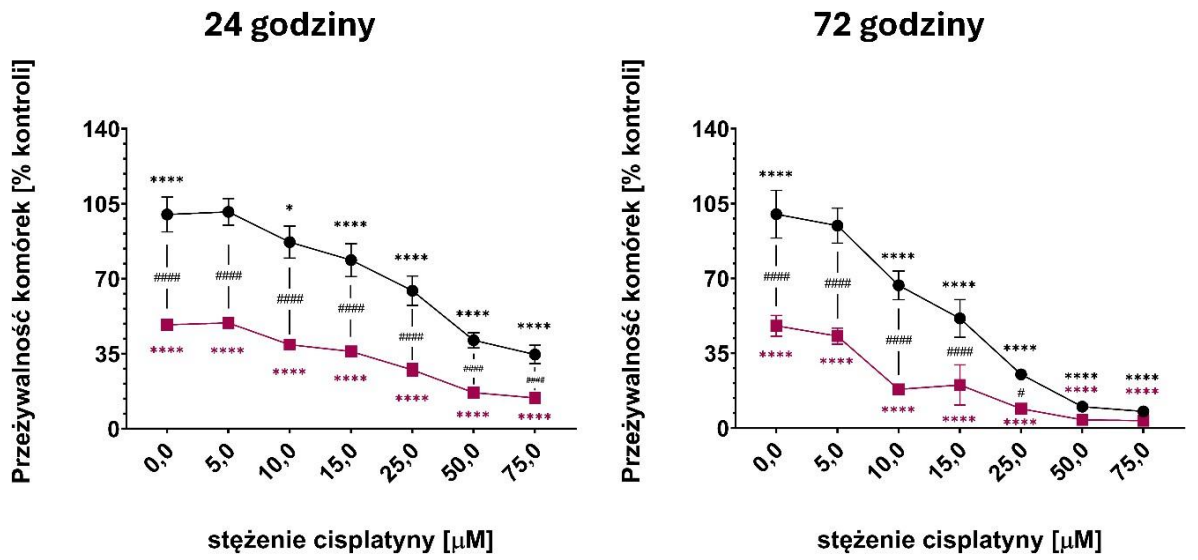

Figura 3. Przeżywalność linii komórkowej OvBH-1 podczas 24- oraz 72-godzinnej inkubacji na wzrastające stężenia cisplatyny. Eksperyment został przeprowadzony na dwóch grupach komórek, tj. nie- i preinkubowanych z 17β-estradiolem 24 godz. przed chemioterapią; Uwagi: (średnia ± SD) N = 3, *$p< 0.05$, ** $p< 0.01$, *** $p< 0.001$, **** $p< 0.0001$ w przyrównaniu do kontroli (CTRL, 0 μM); # $p< 0.05$, ## $p< 0.01$, ### $p< 0.001$, #### $p< 0.0001$ w przyrównaniu wyników pomiędzy grupami komórek; ***dane nieopublikowane.***

Może to być spowodowane różnicami w ich patofizjologii oraz patogenezie. MDAH-2774 to linia komórek endometrioidalnego raka jajnika (EOC) [55], natomiast linia OvBH-1 to komórki wyprowadzone od pacjentki ze zdiagnozowanym jasnokomórkowym rakiem jajnika *(szerzej opisane w sekcji „Materiały i metody")*. Oba typy histologiczne raka jajnika są powszechne wśród kobiet ze zdiagnozowaną endometriozą [56]. Jednakże posiadają odrębne cechy kliniczno-patologiczne i fenotypy molekularne. Charakterystyczne dla endometrioidalnego raka jajnika

są mutacje w genach *CTNNB1* (30-50%), *ARID1A* (~30%), *PIK3CA* (15-40%), *KRAS* (12-33%), *PTEN* (~20%) oraz *TP53* (6-24%) [57,58]. W porównaniu do innych typów raka jajnika, w endometrioidalnym raku jajnika rzadziej występują mutacje w genach *BRCA1/2*. EOC są przeważnie dodatnie wobec ekspresji receptorów estrogenowych, podczas gdy jasnokomórkowy rak jajnika zazwyczaj wykazuje niższy poziom ich ekspresji. EOC charakteryzuje się zazwyczaj lepszymi rokowaniami niż inne podtypy raka jajnika, szczególnie jeśli jest zdiagnozowany we wczesnym stadium.

Jasnokomórkowy rak jajnika (ang. *clear cel ovarian carcinoma; CCOC*) charakteryzuje się mniejszą częstością występowania. Jest za to bardziej agresywny i wiąże się ze zmniejszoną częstością odpowiedzi na chemioterapię opartą na platynie i gorszym rokowaniem, zwłaszcza w zaawansowanym stadium [57,59]. Co zaskakujące, dodatkowo wykonane eksperymenty wskazują na stosunkowo wysoką wrażliwość komórek OvBH-1 na chemioterapię **(Fig. 3;** *dane nieopublikowane***)**. Wśród najczęściej identyfikowanych mutacji w CCOC znajdują się mutacje genów *ARID1A* (47-57%), *PIK3CA* (30-60%), *PPP2R1A* (7-16%), *CTNNB1* (31-53.3%) [57,58]. Z uwagi na swoją agresywność i skłonność do szybkiego rozwoju, jasnokomórkowy rak jajnika często jest diagnozowany w bardziej zaawansowanych stadiach i wiąże się z gorszym rokowaniem. Biorąc to pod uwagę, powyższy model komórek również włączono do cyklu prowadzonych badań. Otrzymane wyniki nie zostały dotychczas opublikowane, jednakże włączono je do niniejszej pracy.

W kolejnym etapie prac wykonywanych w ramach niniejszej rozprawy doktorskiej podjęto próbę odpowiedzi na pytanie, czy dodatek zewnętrznych impulsów elektrycznych zwiększy efektywność cisplatyny oraz czy elektroporacja z jonami wapnia okaże się być bardziej efektywna w porównaniu do standardowej chemioterapii i elektrochemioterapii również w linii komórkowej jasnokomórkowego raka jajnika (model OvBH-1). Co więcej, podobnie jak w eksperymentach z udziałem linii MDAH-2274, podjęto próbę oceny roli 17β-estradiolu jako czynnika wspierającego badane terapie. Dodatkowo, przeanalizowano skutki biologiczne, jakie wywołają proponowane rozwiązania terapeutyczne na komórki prawidłowe nabłonka jajnika (tj. chomicza linia komórkowa CHO-K1).

Podobnie jak to miało miejsce w przypadku linii endometroidalnego raka jajnika, również tutaj obserwowano ewentualną wspierającą rolę preinkubacji z estradiolem leczenia cisplatyną **(Fig. 3 i 4;** *dane nieopublikowane***)**. Połączenie standardowego chemioterapeutyka z zewnętrznym polem elektrycznym w dużej mierze zwiększyło jego toksyczność wobec komórek OvBH-1 **(Fig. 4;** *dane nieopublikowane***)**.

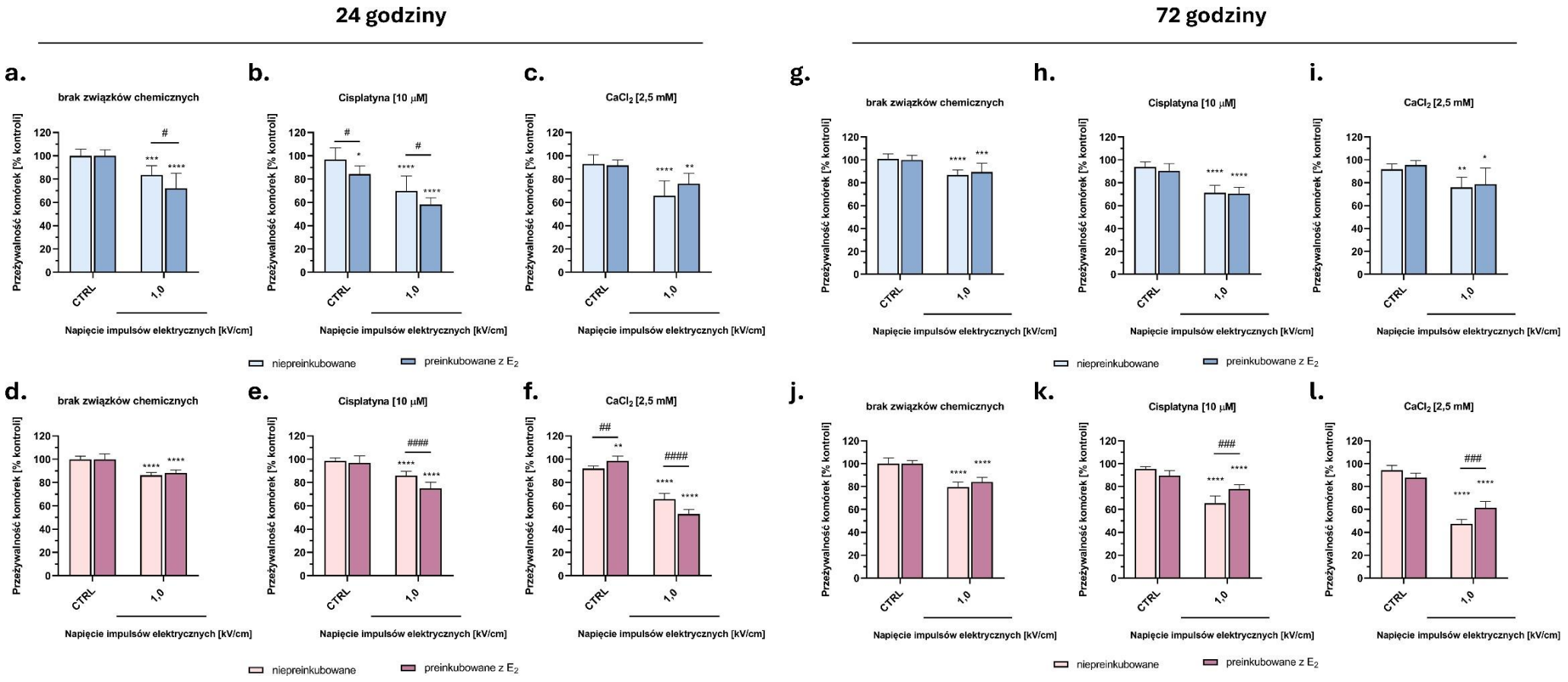

Figura 4. Analiza przeżywalności komórek CHO-K1 **(a-c** i **g-i)** oraz OvBH-1 **(d-f** i **j-l)** przeprowadzona za pomocą testu MTT **(a-f)** 24 godziny oraz **(g-l)** 72 godziny po ekspozycji na różne protokoły elektroporacji. Eksperyment przeprowadzono na dwóch grupach komórek (nieinkubowanych i inkubowanych przez 24 godziny z 10 μM E_2) z każdej linii komórkowej; Uwagi: (średnia ± SD) N = 3, *$p < 0,05$, ** $p < 0,01$, *** $p < 0,001$, **** $p < 0,0001$ w przyrównaniu do kontroli (CTRL); #$p < 0,05$, ## $p < 0,01$, ### $p < 0,001$, #### $p < 0,0001$ w przyrównaniu między grupami; ***dane nieopublikowane.***

Warto zaznaczyć, że zastosowanie samych impulsów elektrycznym zaburzyło żywotności komórek **(Fig. 4;** *dane nieopublikowane*). W przypadku komórek linii OvBH-1 odsetek przeżywalności po ekspozycji na impulsy elektryczne wynosił odpowiednio 86,27% po 24 godzinach oraz 79,5% po 72 godzinach od zakończenia terapii. W grupie komórek poddanych wcześniejszej preinkubacji z E_2 wartości te wynosiły odpowiednio 88% i 84%. Natomiast analiza za pomocą testu MTT wykazała żywotność komórek CHO-K1 na poziomie 83,5% po 24 godzinach oraz 86,67% po 72 godzinach. W przypadku preinkubacji z E_2 wartości te wynosiły 72,16% po 24 godzinach oraz 89,5% po 72 godzinach.

Elektroporacja z jonami wapnia okazała się być bardziej toksyczna wobec komórek nowotworowych, w porównaniu do standardowej chemioterapii oraz elektrochemioterapii z cisplatyną. Preinkubacja materiału badawczego z estradiolem uwrażliwiła komórki na działanie chlorku wapnia i cisplatyny podawanych pojedynczo oraz w połączeniu z elektroporacją **(Fig. 3 i 4;** *danie nieopublikowane***)**. Ciekawym jest fakt, że efekt ten został zniesiony po 72 godz. od leczenia, co niezaprzeczalnie wymaga dalszych badań i głębszej analizy. Co ważne, w przypadku obu linii komórkowych, podobnie jak we wcześniejszych eksperymentach z udziałem komórek MDAH-2774, poziom wychwytu barwnika Yo-Pro-1™ potwierdził, iż działanie estrogenu nie wpływa na stopień przepuszczalności błony komórkowej **(Fig. 5;** *danie nieopublikowane***)**.

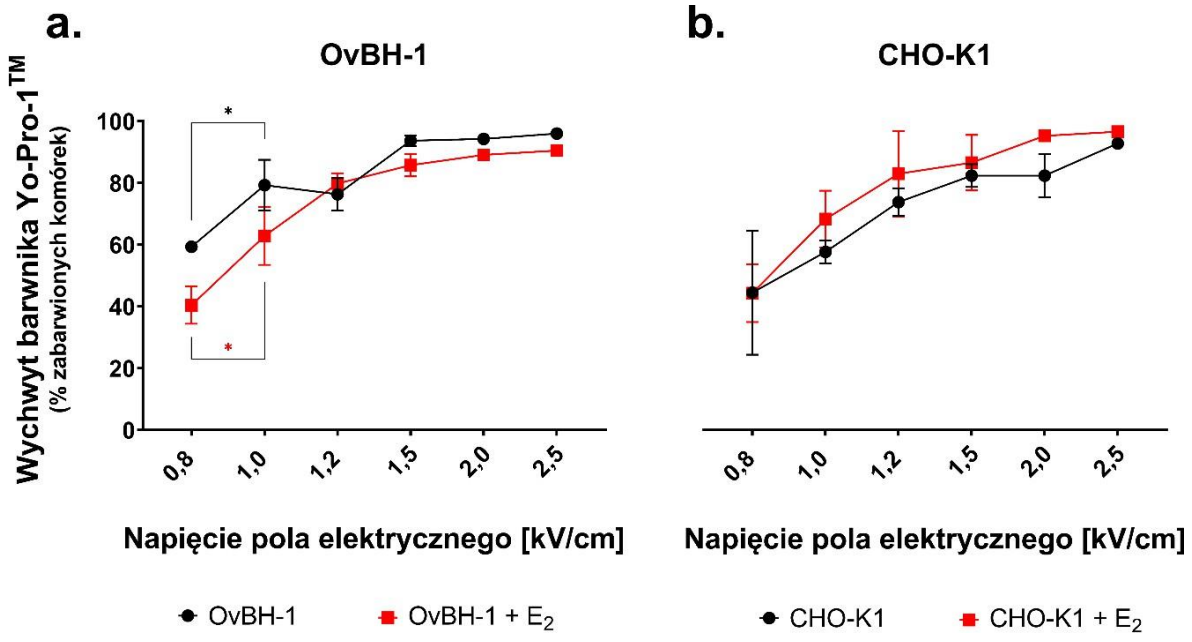

Figura 5. Wychwyt fluorescencyjnego barwnika Yo-Pro-1™, reprezentujące poziom permeabilizacji błony komórkowej dla linii komórkowych **(a)** OvBH-1 i **(b)** CHO-K1, zmierzone za pomocą cytometru przepływowego; Uwagi: (średnia ± SD) N = 3, **p < 0,05* w porównaniu między intensywnościami pola elektrycznego; ***dane nieopublikowane.***

Dzięki zastosowaniu mikroskopii konfokalnej odnotowano liczne zmiany w strukturze F-aktyny po zastosowanych terapiach **(Fig. 6;** ***dane nieopublikowane*****)**. Mianowicie, komórki eksponowane na CaEP wykazały zaburzenie lamellipodiów i włókien stresowych, połączone z akumulacją cytoszkieletu w centralnej części komórki. Zniszczenia były nasilone w grupie komórek poddanych wcześniej preinkubacji z E_2. Porównując obrazy uzyskane w 6 i 72 godz. po leczeniu można odnotować delikatną tendencję do rekonstrukcji cytoszkieletu, jednak jego ukształtowanie w trzeciej dobie po terapii nadal pozostaje w znacznym stopniu zaburzone.

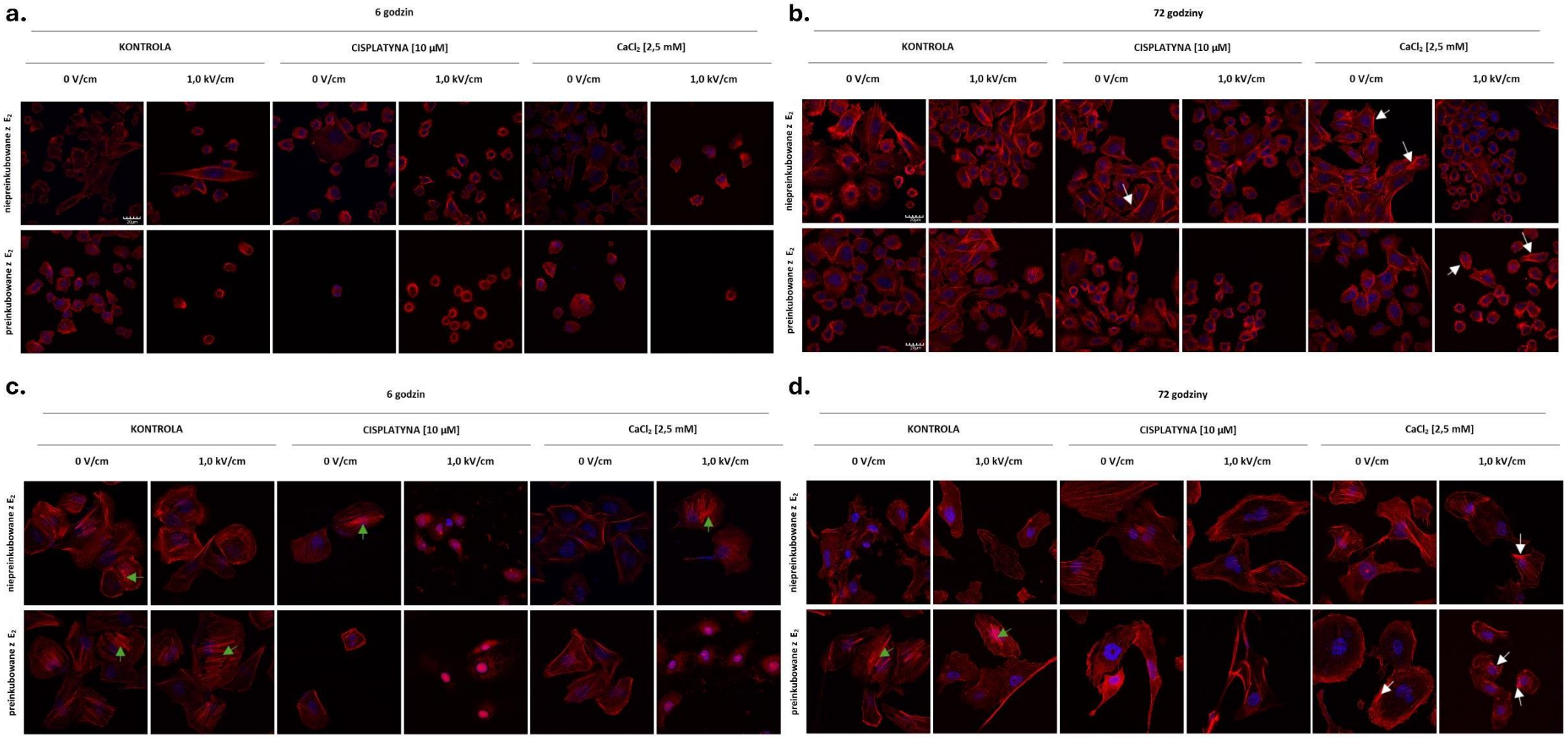

Figura 6. Organizacja F-aktyny w komórkach CHO-K1 **(a, b)** i OvBH-1 **(c, d)**. Komórki po ekspozycji na terapie zostały nasadzone na szkiełka nakrywkowe, a po 6 **(a, b)** oraz 72 **(c, d)** godzinach po ekspozycji na różne lecznicze utrwalone za pomocą 4% formaldehydu. Komórki zostały oznakowane w celu wizualizacji jąder **(niebieski)** oraz F-aktyny **(czerwony)**. Strzałki wskazują: **zielone** – lamellipodia, **białe** – włókna stresowe. Skala – 20 µm; ***dane nieopublikowane.***

W przypadku komórek prawidłowych, przeprowadzone testy przeżywalności wykazały podobny stopień toksyczności CaEP w porównaniu do elektrochemioterapii z cisplatyną **(Fig. 4;** *dane nieopublikowane***)**. Jednakże, zestawiając wyniki uzyskane po 24 i 72 godz. można zauważyć, iż komórki są w stanie przetrwać chwilowe zaburzenie homeostazy, powrócić do stanu pierwotnego i nadal się dzielić, co obrazuje wzrost przeżywalności pomiędzy tymi czasami. Co więcej, obrazy uzyskane dzięki mikroskopii konfokalnej, gdzie dokonano barwienia F-aktyny, ukazują rekonstrukcję lamellipodiów w komórkach CHO-K1 w czasie 72 godz. po CaEP **(Fig. 6;** *dane nieopublikowane***)**. Analizując potencjalne działanie wspierające toksyczność leczenia przez 17-βestradiol, zaobserwowano, iż komórki prawidłowe inkubowane wcześniej z estrogenem były mniej podatne na działanie chlorku wapnia ($CaCl_2$) i cisplatyny podawanych osobno. Badając terapie połączone z zewnętrznym polem elektrycznym nie odnotowano efektu wspierającego toksyczność leczenia przez estrogen, co popiera wcześniejsze obserwacje.

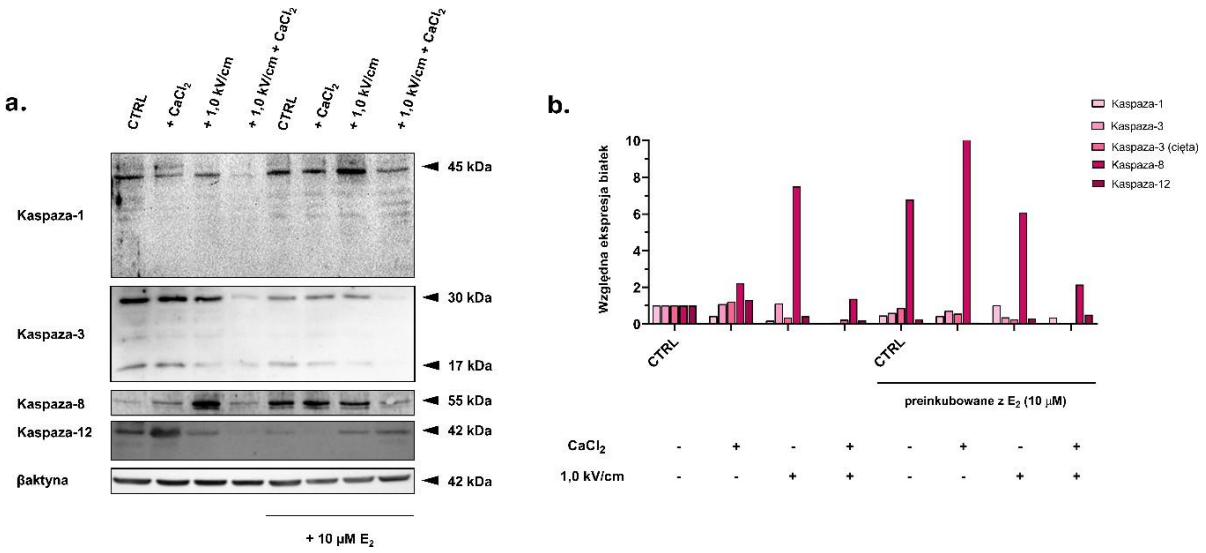

Figura 7. (a) Detekcja metodą Western-Blot kaspazy-1/ -3/ -8/ -12 w komórkach OvBH-1, 24 godziny po leczeniu CaEP; **(b)** względna ekspresja białek; β-aktyna została użyta jako kontrola załadunku; ***dane nieopublikowane.***

Biorąc pod uwagę wcześniejsze wyniki, ujawniające wzrost ekspresji kaspazy-12 po ekspozycji komórek MDAH-2774 na 2,5 mM $CaCl_2$, oraz brak w dostępnej literaturze informacji na temat alternatywnych szlaków śmierci komórkowej jako następstw CaEP, na tym etapie prac badawczych, wzięto pod uwagę również ten aspekt badawczy. Rzeczywiście, inkubacja komórek OvBH-1 z $CaCl_2$ również skutkowała wzrostem poziomu kaspazy-12, jednakże efekt ten został zniwelowany zarówno po dołączeniu impulsów elektrycznych, jak również w przypadku grupy komórek poddanych wcześniej preinkubacji z estradiolem **(Fig. 7;**

*dane nieopublikowane***)**. Wzrost poziomu kaspazy-8 został odnotowany w grupie komórek wcześniej preinkubowanych z 10 μM E_2, jednakże wśród tej grupy komórek jego najniższa ekspresja została zaobserwowana po leczeniu CaEP. W komórkach wcześniej niepreinkubowanych z estradiolem, wzrost poziomu tego białka zaobserwowano po aplikacji impulsów elektrycznych 1,0 kV/cm. W przypadku kaspazy-1 oraz -3, biorąc pod uwagę analizę densytometryczną otrzymanych wyników, nie odnotowano wzrostu względnego poziomu ich ekspresji ponad poziom komórek kontrolnych (tj. nieleczonych; CTRL).

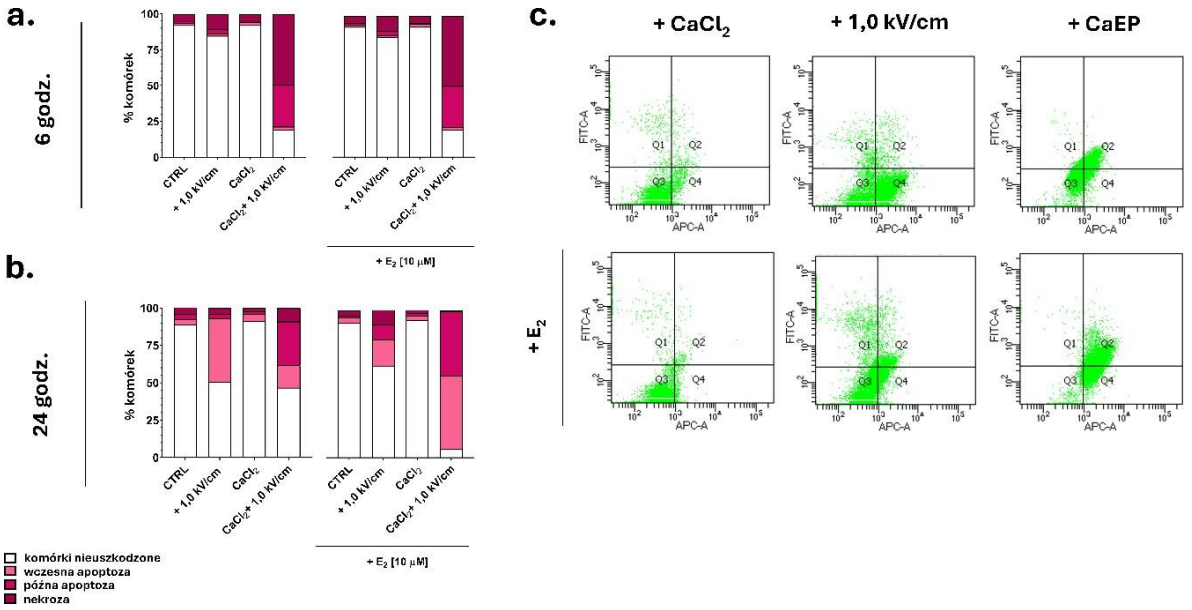

Figura 8. Procentowy rozkład apoptozy i nekrozy w komórkach OvBH-1 analizowana za pomocą pomiaru fluorescencji barwników SYTOX™ Green i APC-Annexin V. Barwienie przeprowadzono 6 i 24 godziny po dostarczeniu impulsów elektrycznych (PEFs). **(a, b)** reprezentatywne histogramy ukazujące rozkład zdrowych, wczesnych apoptotycznych, późnych apoptotycznych oraz nekrotycznych komórek po leczeniu; fluorescencja SYTOX™ Green — wysokość rozproszenia w przód; fluorescencja APC—Annexin V — wysokość rozproszenia w bok; **(c)** reprezentatywne histogramy z analizy cytometrii przepływowej 24 godziny po leczeniu; ***dane nieopublikowane.***

Analizując wyniki otrzymane z użyciem techniki Western Blot **(Fig. 7;** *dane nieopublikowane***),** cytometrii przepływowej **(Fig. 8;** *dane nieopublikowane***)**, oraz testu luminescencyjnego **(Fig. 9;** *dane nieopublikowane***)**, wysunięto wniosek, iż nekroza jest głównym szlakiem śmierci obieranym przez komórki w następstwie ich ekspozycji na elektroporację z jonami wapnia. Pomiar metodą cytometrii przepływowej wykazał znaczący wzrost sygnału emitowanego przez barwnik SYTOX™-Green, 6 godz. po leczeniu.

Dodatkowo, analiza luminescencyjna aktywności kaspazy-3 oraz -7 powiązanych z procesem apoptozy wykazała spadek ich aktywności 4 godz. po zastosowaniu terapii **(Fig. 9;** *dane nieopublikowane***)**, co można tłumaczyć gwałtowną redukcją ilości komórek w wyniku

nekrozy. Efekt utrzymywał się w czasie. Odnotowano również spadek poziomu kaspazy-1, która łączona jest z procesem pyroptozy [60].

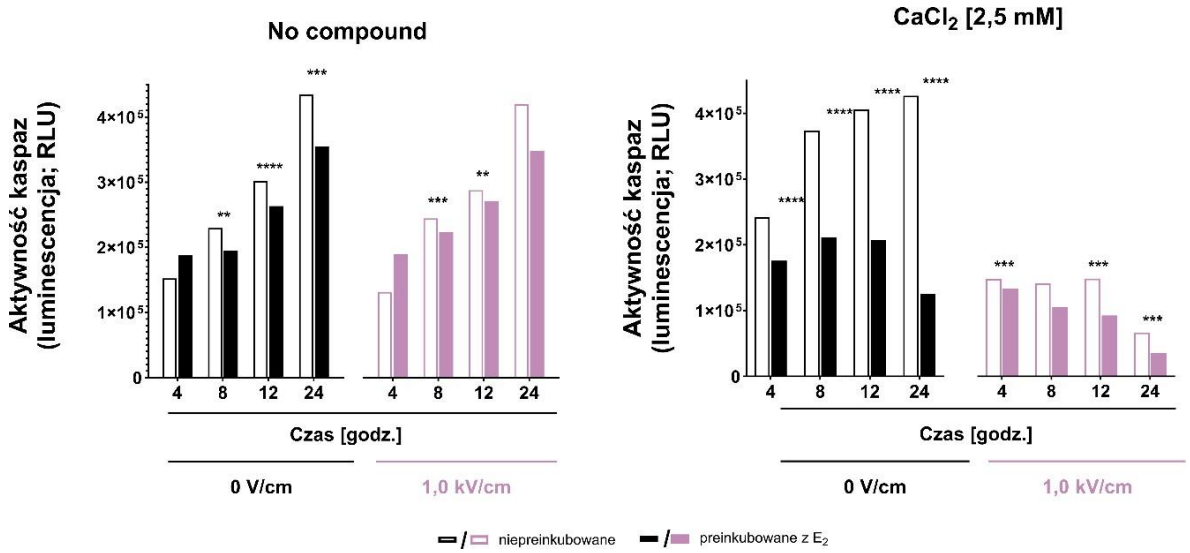

Figura 9. Pomiar aktywacji kaspazy 3/7 po ekspozycji na EP i CaEP. Uwagi: (średnia ± SD) N = 2, *$p < 0,05$, ** $p < 0,01$, *** $p < 0,001$, **** $p < 0,0001$ w porównaniu do kontroli (CTRL); ***dane nieopublikowane.***

Co ciekawe, interpretacja zdjęć otrzymanych dzięki mikroskopii holotomograficznej ujawniła istnienie pęcherzykowatych wypukłości na powierzchni błony komórkowej 24 godz. po elektroporacji z jonami wapnia, tzw. blebbing **(Fig. 10;** *dane nieopublikowane***)**. Fakt ten może wskazywać na obecność alternatywnych ścieżek śmierci komórkowej, jednak dostępna literatura odnotowuje również taką morfologię komórek w trakcie procesu nekrozy. Biorąc to pod uwagę, trudno jednoznacznie wskazać dominujący rodzaj śmierci komórkowej, powstałej w wyniku zastosowania CaEP, a problem wymaga dalszych badań i dokładniejszej analizy z uwzględnieniem krótszych punktów czasowych.

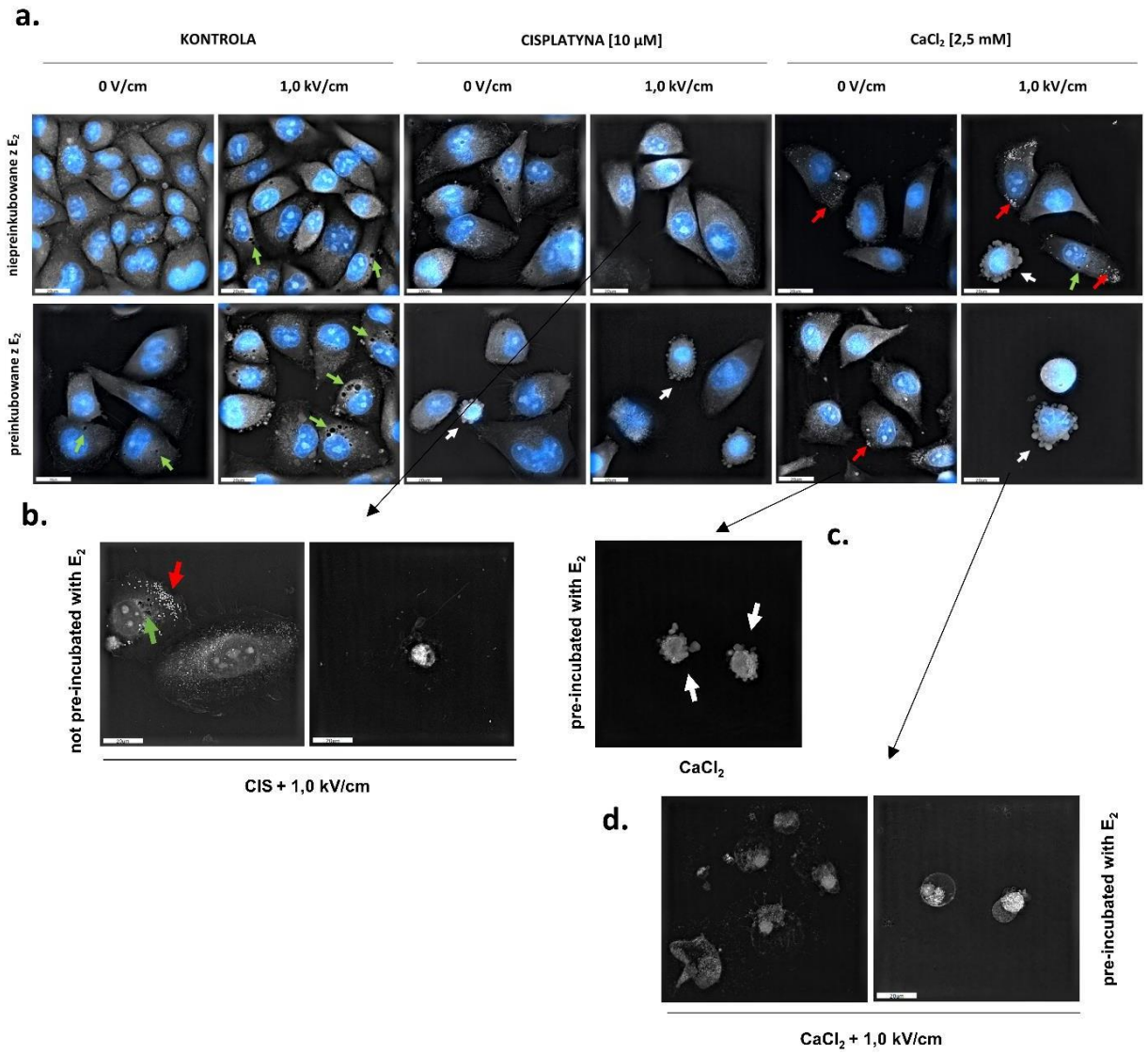

Figura 10. Wizualizacja morfologii komórek OvBH-1 za pomocą mikroskopii holotomograficznej (HTM) **(a)** 24 godziny po leczeniu, jądra zostały wybarwione fluorescencyjnym barwnikiem Hoechst 33342; **(b, c, d)** inne przykłady komórek obrazowanych 24 godziny po terapiach; strzałki wskazują: **zielone** – wakuole, **czerwone** – krople lipidowe, **białe** – blebbing. Skala – 20 µm; ***dane nieopublikowane.***

Druga z prac oryginalnych wchodzących w skład niniejszego cyklu publikacji obejmuje analizę wpływu symetryczności oraz obecności interwałów między impulsami w protokołach elektroporacji bipolarnej (BP EP) na obecność efektu *bipolar cancellation* **[2]**. Impulsy asymetryczne otwierają zupełnie nowy rozdział w zakresie elektrochemioterapii. Do tej pory jest niewiele badań dotyczących przeciwnowotworowego działania takich protokołów. Jako model badawczy wykorzystano ludzką linię jasnokomórkowego raka jajnika (OvBH-1). Przeprowadzone analizy przeżywalności komórek oraz absorpcji barwnika Yo-Pro-1^{TM} pozwoliły ustalić, iż w przypadku protokołów elektroporacji opartych na impulsach nanosekundowych uszkodzenie błony komórkowej wywołane zastosowaniem ↑600

nsEP może zostać zredukowane przez kolejny impuls o tej samej amplitudzie i czasie trwania, ale o przeciwnej polaryzacji, tj. ↑600 ns + ↓600 ns **(Pub. 2; Fig. 2 i 4)**. Jednocześnie, zastosowanie nanosekundowych symetrycznych protokołów bipolarnych skutkowało wyraźnym spadkiem wychwytu barwnika Yo-Pro-1^{TM} przez komórki OvBH-1 w porównaniu do wartości uzyskanych dla protokołu unipolarnego (↑600 nsEP). Co więcej, mikroskopia konfokalna ujawniła, iż oba rozwiązania skutkują zmianami w morfologii komórek raka jajnika, jednak są one mniejsze przy zastosowaniu protokołu ↑600 ns + ↓600 ns **(Pub. 2; Fig. 5 i 6)**. Wyniki te wskazują na obecność efektu *bipolar cancellation* w tym przypadku oraz potwierdzają jego unikalność dla protokołów angażujących impulsy nanosekundowe. Stwierdzenie to poparły również analizy zawarte w niniejszej pracy z udziałem uni- oraz bipolarnych protokołów symetrycznych, jednakże składających się z impulsów mikrosekundowych.

Rozpatrując wpływ symetryczności protokołu oraz wprowadzenia interwałów pomiędzy impulsami na obecność i intensywność efektu *bipolar cancellation*, dotychczasowe doniesienia podkreślały, iż aplikacja negatywnego impulsu o dłuższym czasie trwania skutkuje redukcją, bądź całkowitą eliminacją zjawiska [61,62]. Jednakże prace te obejmowały analizę jedynie impulsów nanosekundowych. Biorąc pod uwagę powyższe, w przytaczanej pracy stworzono protokoły bipolarne składające się z nanosekundowego impulsu dodatniego oraz następującego po nim negatywnego impulsu o długości czasu trwania 10 µs (↑600 ns + ↓10 µs) **(Pub. 2; Fig. 7)**. Otrzymane wyniki doprowadziły do konkluzji, iż efekt *bipolar cancellation* jest obecny w przypadku takiego protokołu, jednakże jest on znacząco słabszy niż przy zastosowaniu symetrycznego protokołu nanosekundowego (↑600 ns + ↓600 ns) **(Pub. 2; Fig. 2 i 4)**. Ponadto, wprowadzenie interwałów 1 lub 10 µs pomiędzy impulsami o przeciwnej polaryzacji nie wpłynęło znacząco na to zjawisko.

W aspekcie efektywności elektroporacji komórek OvBH-1 z jonami wapnia, zastosowanie impulsów nanosekundowych (uni- oraz bipolarnych) skutkowało mniejszą toksycznością, w porównaniu do protokołów z wykorzystaniem impulsów mikrosekundowych, co pokrywa się z wynikami osiągniętymi w publikacji **[1]**, stanowiącej część niniejszego cyklu **(Pub. 2; Fig. 4)**. Co więcej, asymetryczność protokołu pozwoliła osiągnąć podobne wartości przeżywalności komórek OvBH-1 jak protokół wzorowany na wytycznych ESOPE (tj. 100 µs; 1,2 kV/cm). Wprowadzenie dodatkowych interwałów pomiędzy impulsami nie miało istotnego wpływu na te wartości. Najsilniejszy efekt toksyczny wobec komórek raka

jajnika odnotowano dla symetrycznego protokołu bipolarnego opartego o impulsy mikrosekundowe.

Przeprowadzone badania dostarczają **nowych danych** na temat interakcji między impulsami o różnych polaryzacjach i czasach trwania, co może być wykorzystane do przyszłej optymalizacji protokołów elektrochemioterapii, szczególnie w kontekście leczenia nowotworów złośliwych.

Podsumowując, w wyniku przeprowadzonych badań wysunięto następujące wnioski:

- **Elektrochemioterapia:** Zastosowanie zewnętrznego impulsowego pola elektrycznego w połączeniu z konwencjonalną chemioterapią zwiększa lokalne stężenie leku w komórkach raka jajnika, co prowadzi do 20-70% spadku przeżywalności komórek w porównaniu do samej chemioterapii. Elektrochemioterapia, zarówno z cisplatyną, jak i z jonami wapnia, wykazuje antynowotworową aktywność wobec komórek raka jajnika, zarówno tych opornych, jak i podatnych na tradycyjną chemioterapię

- **Elektroporacja z jonami wapnia:** Otrzymane w ramach rozprawy wyniki wskazują, że jest to najbardziej obiecująca metoda leczenia, łącząca wysoką skuteczność w zwalczaniu komórek raka jajnika z relatywnie niską toksycznością wobec komórek prawidłowych, co czyni ją atrakcyjną opcją terapeutyczną.

- **Impulsy bipolarne:** W badaniach wykazano, że jeden impuls unipolarny może zostać „anulowany” przez drugi impuls o odwrotnej polaryzacji, co określono jako zjawisko bipolarnego anulowania (BPC). Badania wykazały, że opóźnienia między-impulsowe (1 i 10 μs) wpływają na zjawisko BPC, co sugeruje możliwość kontrolowania tego efektu poprzez modyfikację parametrów impulsów. Zjawisko BPC wpływa na skuteczność elektrochemioterapii z jonami wapnia, redukując permeabilizację błony komórkowej oraz przeżywalność komórek po terapii.

- **Rola 17β-estradiolu:** Może pełnić kluczową rolę jako czynnik uwrażliwiający komórki nowotworowe na terapie oparte na elektroporacji, co otwiera nowe możliwości w projektowaniu terapii skojarzonych.

W ramach cyklu publikacji dokonano również przeglądu aktualnego stanu wiedzy dot. efektywności terapii opartych na zjawisku elektroporacji (tj. ECT, CaEP, IRE itp.) w przypadku nowotworów ginekologicznych i nowotworu piersi **[3]**. W pracy przedstawiono krótkie charakterystyki każdej z omawianych metod leczniczych, ich korzyści oraz skutki uboczne. Zwrócono uwagę na znikomą ilość badań dot. stosowania elektrochemioterapii i jej pochodnych jako potencjalnej terapii raka jajnika. Biorąc pod uwagę stale rosnącą liczbę diagnozowanych przypadków oraz słabe prognozy związane z tych schorzeniem, poszukiwanie coraz to nowych rozwiązań terapeutycznych stanowi naglącą potrzebę. Autorzy publikacji dostrzegli także fakt, iż standardowa chemioterapia stymuluje pobudliwe tkanki i nerwy, powodując ból i skurcze mięśni [63] oraz nadzieję w ograniczeniu tych efektów ubocznych poprzez zastosowanie protokołów opartych na impulsach nanosekundowych.

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9. Spis załączonych w pracy figur

Figura 1. Graficzna prezentacja koncepcji pracy doktorskiej pt. „Wykorzystanie elektroporacji z jonami wapnia oraz estradiolem w leczeniu raka jajnika na modelu in vitro". Zastosowanie impulsywnego pola elektrycznego **(1)** skutkuje wzrostem stopnia permeabilizacji błony komórkowej **(3)**. Zjawisko to nosi nazwę elektroporacji (EP). Pozwala na podniesienie ilości związków chemioterapeutycznych lub jonów wapnia (Ca^{2+}), jakie są dostarczane do wnętrza komórki **(4)**. Ostatecznym skutkiem jest wzrost cytotoksyczności aplikowanych leków, przy jednoczesnym (w przypadku elektroporacji z jonami wapnia tj. elektroporacji wapniowej, CaEP) zmniejszeniu działań niepożądanych wobec komórek prawidłowych **(6)**. Dodatkowo, ekspozycja komórek nowotworowych na działanie 17β-estradiolu **(1a)** pozwala uwrażliwić je na działanie stosowanych terapii **(2).....** 15

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różne protokoły elektroporacji. Eksperyment przeprowadzono na dwóch grupach komórek (nieinkubowanych i inkubowanych przez 24 godziny z 10 μM E_2) z każdej linii komórkowej; Uwagi: (średnia ± SD) N = 3, *p < 0,05, ** p < 0,01, *** p < 0,001, **** p < 0,0001 w przyrównaniu do kontroli (CTRL); #p < 0,05, ## p < 0,01, ### p < 0,001, #### p < 0,0001 w przyrównaniu między grupami; **dane nieopublikowane.** 27

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Figura 9. Pomiar aktywacji kaspazy 3/7 po ekspozycji na EP i CaEP. Uwagi: (średnia ± SD) N = 2, *p < 0,05, ** p < 0,01, *** p < 0,001, **** p < 0,0001 w porównaniu do kontroli (CTRL); **dane nieopublikowane.** 33

Figura 10. Wizualizacja morfologii komórek OvBH-1 za pomocą mikroskopii holotomograficznej (HTM) **(a)** 24 godziny po leczeniu, jądra zostały wybarwione fluorescencyjnym barwnikiem Hoechst 33342; **(b, c, d)** inne przykłady komórek obrazowanych 24 godziny po terapiach; strzałki wskazują: **zielone** – wakuole, **czerwone** – krople lipidowe, **białe** – blebbing. Skala – 20 μm; **dane nieopublikowane.** 34

10. Artykuły z cyklu publikacji w formie załączników

Praca 1

Tytuł

"Electrochemotherapy with calcium chloride and 17β-estradiol modulated viability and apoptosis pathway in human ovarian cancer"

Autorzy

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Electrochemotherapy with Calcium Chloride and 17β-Estradiol Modulated Viability and Apoptosis Pathway in Human Ovarian Cancer

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Abstract: Estrogens (Es) play a significant role in the carcinogenesis and progression of ovarian malignancies. Depending on the concentration, Es may have a protective or toxic effect on cells. Moreover, they can directly or indirectly affect the activity of membrane ion channels. In the presented study, we investigated in vitro the effectiveness of the ovarian cancer cells (MDAH-2774) pre-incubation with 17β-estradiol (E_2; 10 μM) in the conventional chemotherapy (CT) and electrochemotherapy (ECT) with cisplatin or calcium chloride. We used three different protocols of electroporation including microseconds (μsEP) and nanoseconds (nsEP) range. The cytotoxic effect of the applied treatment was examined by the MTT assay. We used fluorescent staining and holotomographic imaging to observe morphological changes. The immunocytochemical staining evaluated the expression of the caspase-12. The electroporation process's effectiveness was analyzed by a flow cytometer using the Yo-Pro™-1 dye absorption assay. We found that pre-incubation of ovarian cancer cells with 17β-estradiol may effectively enhance the chemo- and electrochemotherapy with cisplatin and calcium chloride. At the same time, estradiol reduced the effectiveness of electroporation, which may indicate that the mechanism of increasing the effectiveness of ECT by E_2 is not related to the change of cell membrane permeability.

Keywords: 17β-estradiol; chemotherapy; electrochemotherapy; calcium electroporation; ovarian cancer; MDAH-2774

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1. Introduction

Ovarian cancer (OC) is the most common gynecological malignancies associated with the highest number of deaths [1]. The International Agency for Research on Cancer (IARC) prophesies that the number of women diagnosed with OC will increase by 47% before 2040 [2]. The poor prognosis and 5-year survival rates of <30% are mainly caused by the fact that nearly 3/4 of women are diagnosed when the disease is stage III or IV FIGO (Féderation Internationale de Gynécologie et d'Obstétrique) [3]. It is attributable to several factors, including vague symptoms, not effective preventative measures, and a lack of definitive screening tools [4,5]. The disease mostly affects women between 50 and 70 years of age, and is rare in young women under 30. The first-line treatment includes primary tumor debulking surgery and six cycles of subsequent chemotherapy (CT) with cisplatin or its derivatives [4]. Around 70% of patients relapse and then do not respond to the standard treatment method [6]. The reason is, cancer cells frequently acquire secondary drug resistance as a result of repeated chemotherapy [7]. The primary drug resistance of

cancer cells also continues to be a problem. Furthermore, because of the many side effects of conventional CT, there is an urgent need to search for new, more effective, and less toxic therapies.

OC belongs to estrogen-dependent malignancies because of hormones and reproductive factors' distinct influence on the disease's pathogenesis and progression [8]. Unfortunately, the exact molecular mechanism of this relationship remains unclear. Mungenast et al. describe that estrogens metabolism disorders may cause the formation of DNA adducts, which, together with free radicals from the metabolic activation to reactive catechol estrogens, are responsible for DNA damage [5].

The predominant intracellular estrogen is 17β-estradiol (E_2), of which increased production is noted in many OC patients [9]. The biological activity of E_2 is based on binding and interaction with specific receptors, namely, estrogen receptor α (ERα, also known as ER1 or Esr1), estrogen receptor β (ERβ, also known as ER2 or Esr2), and G protein-coupled estrogen receptor (GPER1, formerly known as GPR30) [10]. Most of the patients show increased expression of the ERα receptor, in contrast to ERβ, decreasing during tumor development [11]. Moreover, compounds that are antagonists of this protein exhibit a strong inhibitory effect on ovarian cells' growth, both healthy and neoplastic cells.

The mechanism of the action of estradiol is divided into genomic and non-genomic. By a non-genomic mechanism, estrogen, through interaction with membrane ERs (mERs), may influence the activation of mitogen-activated protein kinases (MAPKs), tyrosine kinases, or G proteins, or may modulate the function of ion channels [12,13]. One of the well-known non-genomic pathways of the estradiol activity is the activation of the G protein-coupled estrogen receptor [14]. Estradiol has the highest affinity for this receptor among all estrogens. After the E_2 binding to the receptor, the G protein is activated. That results in a signaling cascade, leading to the mobilization of calcium ions (Ca^{2+}) from the endoplasmic reticulum and the generation of secondary messengers: Inositol triphosphate (IP_3) and diacylglycerol (DAG) [12]. Activation of GPER-1 receptor could also, through the increase in the metalloproteinase production, lead to the Heparin-binding EGF-like growth factor (HB-EGF) release. HB-EGF binds to the epidermal growth factor receptor (EGFR, also known as ErbB-1 or HER1 in humans). This event results in the activation of nuclear receptors responsible for cell proliferation regulation [13–15]. A significant increase in the GPER-1 expression level was noted in ovarian cancer cells [12]. Unfortunately, its role has not been fully understood to date. Researchers point to increased proliferation and invasiveness of GPER-1 positive cells [14].

On the other hand, recent studies show the anti-proliferative and pro-apoptotic effect of GPER-1 stimulation, resulting from the arrest in the G2/M-phase of the cell cycle. Estrogens can also directly or indirectly influence membrane ion channels' activity by integrating into the cell membrane [13]. This action may include increasing the frequency and length of time these channels will be open, or vice versa, completely disabling their function. Estrogens can regulate intracellular calcium ion concentration in endothelial and smooth muscle cells by inhibiting L-type calcium channels [16]. They also affect K^+ ion concentration by opening Ca^{2+} channels and voltage-activated K^+ channels through cGMP-dependent phosphorylation.

Estrogens (Es), depending on their concentration, can also be a useful therapeutic agent for some carcinomas. For example, its high doses were used for breast cancer treatment, while it is known that estrogens are responsible for stimulating the growth of breast cancer cells [17,18]. This situation has been named an 'estrogen paradox'.

Electroporation (EP) is a technique in which short, high-voltage pulses induce cell membrane permeabilization by creating hydrophilic pores and reorganizing membrane lipids [19]. Depending on the electric field's applied parameters, this process may be reversible in time or irreversible (IRE) [20,21]. Another range of pulses used in the electroporation technique are nanoseconds, where we use terms of a nanosecond pulsed electric field (nsPEF) or nanosecond electroporation (nsEP) [22]. It is based on the use of pulses with a duration below 1 μs with very high intensity (even up to 100 kV/cm). It was observed that

nanosecond pulses can permeabilize and disrupt not only the outer cell membrane, but also inner membranes [23]. One of the essential features of nsEP is the fact that the death of cells electroporated in this way occurs through apoptosis [24].

The combination of electroporation with standard chemotherapy called electrochemotherapy (ECT) enables the effective delivery of low permeability chemotherapeutic agents to cells [25]. Electroporation facilitates their transmembrane transport and, as a result, increases cytotoxicity [21]. Research was carried out on many chemotherapeutic agents (i.e., paclitaxel, cisplatin, bleomycin, and carboplatin), but only two of these drugs have been identified as potential agents for use with electrochemotherapy and are currently used in clinical practice: Cisplatin and bleomycin [26–29]. The main advantage of ECT is that the doses of drugs needed to achieve a local cytotoxic effect are significantly reduced [30]. The cisplatin toxicity increases almost 80 times in this method, and in the case of bleomycin, it rises to 1000 times. ECT introduces rare side effects; it is easy to perform (the procedure takes about 30 min) and relatively cheap [20,21]. Its development is currently focused on adapting it to the treatment of broader and deeper tumors (e.g., liver, prostate) [20,31]. Unfortunately, to date, little is known about its application in ovarian cancer. In our experiments, we used settings optimized for reversible EP of tumor cells following the European Standard Operating Procedures of ECT (ESOPE) protocol [32].

Calcium electroporation (CaEP) is a new, experimental modification of electrochemotherapy, in which the chemotherapeutic agent is replaced by calcium ions (Ca^{2+}) [33,34]. Calcium is a second-order messenger involved in transcription regulation, metabolism, proliferation, and cell death [35]. The concentration of free calcium in the eukaryotic cell is tightly controlled and extremely low (10^{-7} mol/L), in contrast to its concentration in the plasma (10^{-3} mol/L). Even small changes in the permeability of the membrane can significantly increase the concentration of intracellular calcium. To balance the calcium concentration, the cell shows increased Ca^{2+}-ATPase activity. High intracellular Ca^{2+} concentration also causes disturbances in the permeability of mitochondrial membranes, loss of electrochemical gradient, and Na^{+}/K^{+}-ATPase activation. Intensive use of ATP combined with its synthesis inhibition results in a rapid depletion of its reserves and ultimately introduced the cell to the necrosis pathway. Moreover, overloading the cell with Ca^{2+} may induce the formation of reactive oxygen species (ROS) and the activation of lipases and proteases.

Apoptosis, also called programmed cell death, is a process by which a cell undergoes genetically regulated suicide death [36]. This process affects damaged or unnecessary cells, and its primary goal is to maintain homeostasis. It can be initiated by several extra- and intracellular factors. Depending on the type of factor, its amount and duration of action, as well as the type of cell, the process of apoptotic or necrotic cell death can be initiated [37]. At low concentrations, cytotoxic compounds may induce apoptosis, while at high concentrations, they will lead to necrosis. Several apoptosis pathways can be distinguished depending on the inducer, including the endoplasmic reticulum (ER) stress-induced pathway [38]. Inducing factors include disruption of ER homeostasis resulting from an accumulation of misfolded proteins, disturbances in calcium ion (Ca^{2+}) balance, or the activity of reactive oxygen species (ROS). The process is associated with the caspase-12 and c-Jun N-terminal kinases (JNK1) activity. Procaspase-12, located in the ER membrane, is transformed to caspase-12 in response to stress factors. Then, caspase-3 (activated by caspase-12) initiates the execution phase of apoptosis [37,39].

The available information allows hypothesizing that electroporation may be an effective method against ovarian cancer. Additionally, the controlled concentration of 17β-estradiol may have a protective or toxic effect on the MDAH-2774 ovarian cancer cell line, which expresses estrogen receptors (ERs) [40,41]. The presented research aimed to evaluate the modulating effect of 17β-estradiol on the effectiveness of chemotherapy and electrochemotherapy with the use of cisplatin and calcium chloride in human ovarian cancer.

2. Materials and Methods

2.1. Cell Culture

The research was carried out on human ovarian endometrioid adenocarcinoma cell line MDAH-2774, purchased from ATCC® (Manassas, VA, USA) originating from a patient who did not receive chemotherapy or radiation prior to the collection of cancer cells [42]. Cell culture was maintained at 37 °C, 5% CO_2, 95% air humidity in RPMI-1640 medium (GlutaMAX™ GIBCO; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO, USA). Cell passages were carried out 2–3 times a week when confluency was about 80–90%. Cells were then removed from the flasks by trypsinization (trypsin 0.25% and EDTA 0.02%; Sigma-Aldrich, St. Louis, MO, USA) and washed with DPBS buffer (Sigma-Aldrich, USA).

2.2. Examined Substances

Stock solutions of 17β-estradiol (10 mM) in 96% ethanol and calcium chloride ($CaCl_2$; 100 mM) in distilled water were performed. The stock solutions were stored at 2–8 °C temperature. The stock solution of cisplatin (1 mM) in distilled water was freshly prepared each time before the experiment due to the low stability of the solution. Subsequently, the proper amount of stock was mixed with RPMI-1640 to achieve the required concentration.

2.3. Cell Viability Assay

The MTT assay determines the viability of the cells following treatment. It evaluates the mitochondrial activity, which functions as a marker of cell viability. Briefly, MDAH-2774 cells were seeded into 96-well microculture plates at 1×10^4 cells/well and incubated with 17β-estradiol, cisplatin, and $CaCl_2$ at indicated concentrations and periods. Then, 100 µL/well of the MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] reagent (Sigma-Aldrich, USA) was added, and cells were incubated 2 h at 37 °C. Formazan crystals were dissolved by adding 100 µL of acidic isopropanol (38% HCl in 99.7% isopropanol). The absorbance value was measured at 570 nm using GloMax® Discover multimode microplate reader (Promega, Madison, WI, USA). The experiments were performed in three replicates. The results were expressed as the percentage of viable cells relative to untreated control cells.

2.4. Pulsed Electric Fields (PEF) Treatment

For electroporation, cells were trypsinized, counted, and distributed at 5×10^5 cells/falcon tube (15 mL), then centrifuged (5 min, 1000 rpm, Centrifuge 5430 R, Eppendorf AG, Hamburg, Germany) and resuspended in calcium-depleted medium—SMEM (Spinner's minimum essential medium, Sigma Merck M8167, a highly conductive medium where conductivity = 1.53 S/m) with the addition of selected concentrations of cisplatin (25 µM) and $CaCl_2$ (2.5 mM). The experiment was performed in sterile polycarbonate cuvettes with a 4 mm gap between electrodes (BioRad®, Hercules, CA, USA) by using three pulsing protocols: (1) 1.3 kV/cm × 100 µs × 100 Hz × 8 pulses (ESOPE); (2) 37.5 kV/cm × 10 ns × 1 Hz × 200 pulses; and (3) 50 kV/cm × 10 ns × 1 Hz × 200 pulses. BTX ECM 820 (Harvard Apparatus, Holliston, MA, USA) electric pulse generator delivered microsecond pulses, and PPG-20 generator (FID Technology, Burbach, Germany) 10 ns pulses with time rise of 2 ns. After the pulse delivery, cells were incubated 10 min at 37 °C and then centrifuged (5 min, 1000 rpm). The pellet of cells was suspended in the culture medium. Then, the MTT assay, microscopic, and cytometric analysis were performed.

2.5. Flow Cytometry Study

The efficiency of the electroporation protocols was by assessing the amount of the Yo-Pro™-1 Iodide fluorescent dye absorbed by the cell. Initially, cells were trypsinized and suspended in calcium-depleted electroporation buffer—SMEM (5×10^4 cells/400 µL), and Yo-Pro™-1 Iodide (YP-1, $\lambda_{exc}491/\lambda_{em}509$, Thermo Scientific™, Warszawa, Poland) was added. Cell suspensions were then transferred to cuvettes and electroporated. Then cells

were washed by DPBS to remove the content of the free dye, gently centrifuged 2 min × 100× *g*, and suspended in 500 µL of DPBS for measurements in polystyrene FACS tubes, 10^4 events/sample were measured in triplicate. Flow cytometric analysis was performed by using the CyFlow CUBE-6 flow cytometer (Sysmex, Warszawa, Poland). Data was analyzed using CyView Sofware (Sysmex, Warszawa, Polska).

2.6. Immunocytochemical (ICC) Staining

Immunocytochemical staining semi-quantitatively assessed the expression of caspase-12. Caspase-12 evaluates the induction of apoptosis. Cells were trypsinized and suspended in a culture medium with the addition of 10 µM 17β-estradiol so that the number of cells in suspension is equal to 5 × 10^4 cells/mL RPMI-1640. Cultures were harvested on 12-well PTFE microscopic diagnostic slides (Thermo Scientific™, Portsmouth, NH, USA) and incubated for 24 h, then exposed to cisplatin and $CaCl_2$ for 24 h at 37 °C and fixed using 4% paraformaldehyde for 10 min. EXPOSE Mouse and Rabbit Specific HRP/DAB Detection IHC kit (Abcam, Cambridge, MA, USA) was used to execute immunocytochemical staining. After rinsing in PBS (Bishop) (2 × 10 min), any remnant peroxidase activity was removed by incubation with Hydrogen Peroxide Block for 10 min. Samples were permeabilized by incubation with 1% Triton X-100 (Sigma-Aldrich, USA) in PBS (Bishop). The overnight exposure to a primary antibody: Rabbit polyclonal IgG (dilution rate: 1:100; SC-5627; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4 °C visualized the expression of caspase-12. Then the secondary antibody conjugated with horseradish peroxidase (HRP) was added to samples. The next step was incubation with diaminobenzidine–H_2O_2 mixture to visualize the HRP label and hematoxylin (Alchem, Toruń, Poland) for 1 min to stain cell nuclei. Slides were dehydrated using ethanol (Chempur, Piekary Śląskie, Poland) gradient (6 × 5 min) and xylene (Chempur, Poland) (3 × 5 min) and covered using DPX gel (Aqua-Med Zpam-Kolasa, Łódź, Poland). The upright microscope (Olympus BCX43, Warszawa, Poland) was used to examine the immunocytochemical reaction. The intensity of immunohistochemical staining was evaluated as (−) negative, (+) weak, (++) moderate, or (+++) strong.

2.7. Confocal Laser Scanning Microscopy (CLSM) Study

Cytoskeleton confocal microscope evaluated modifications in the structure of MDAH-2774 cells. Cells were suspended (5 × 10^4 cells/mL) in a culture medium, seeded into coverslips placed on a 6-well plate, and incubated for 24 h, 37 °C. Next, selected concentrations of cisplatin and $CaCl_2$ were added and incubated for 24 h, 37 °C. Then, cells were fixed in 4% paraformaldehyde for 10 min, raised in PBS (3 × 5 min), blocked with 1% Bovine Serum Albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA) in PBS for 1 h, and permeabilized using 1% Triton X-100 in PBS (3 × 5 min). After 1 h incubation with a solution of the Alexa Fluor™ 546 phalloidin (Life Technologies-Thermo Fisher Scientifi, Waltham, MA, USA) in PBS (dilution rate: 1:300), cells were raised in PBS (3 × 5 min), then were mounted in a fluorescence mounting medium (Fluoromount™ Aqueous Mounting Medium, Sigma-Aldrich, USA). The assessment of changes in the cytoskeleton structure was made based on images taken with Olympus FluoView FV1000 confocal laser scanning microscope (Olympus, Tokyo, Japan).

2.8. Living Cell Tomographic Microscopy

The experiment aimed to evaluate the morphological changes in MDAH-2774 cells after 24 h incubation with cisplatin and $CaCl_2$, depending on whether or not cells have been incubated with 10 µM 17β-estradiol. Firstly, cells were trypsinized and suspended in a culture medium (5 × 10^4 cells/mL). Suspensions of cells were then seeded into ibidi µ-Dish^{35mm} Quad imaging dishes and incubated 24 h, 37 °C. The next day, cisplatin and $CaCl_2$ were added, and the 24 h incubation was repeated. Visualization of the 3D live cell morphology of MDAH-2774 cells was performed using a live cell tomographic holographic 3D microscope Nanolive (3D Cell Explorer, Lausanne, Switzerland).

2.9. Statistical Analysis

The experiments were performed in 3 replicates. The statistical analysis was performed using the GraphPad Prism 8 (GraphPad Software Inc, San Diego, CA, USA). Data are expressed as mean ± SD (standard deviation) of the mean and were analyzed by two-way ANOVA (analysis of variance), with $p < 0.05$ being considered statistically significant.

3. Results

3.1. Cytotoxicity of the Examined Substances and IC_{50} Determination

MTT assay assessed the cytotoxicity of the 17β-estradiol (E_2), cisplatin, and calcium chloride ($CaCl_2$). Figure 1 shows the response of MDAH-2774 cells to the 24 h and 72 h incubation with E_2. After 24 h incubation, E_2 intensified cytotoxic activity in concentration 25–200 μM. However, extended incubation time (72 h) further decreased cells' viability. Estradiol in concentrations 0.01–0.1 μM did not significantly change the cells' mitochondrial activity compared to the untreated control. Based on the results presented above, the low-toxic concentration of 10 μM was selected for further experiments.

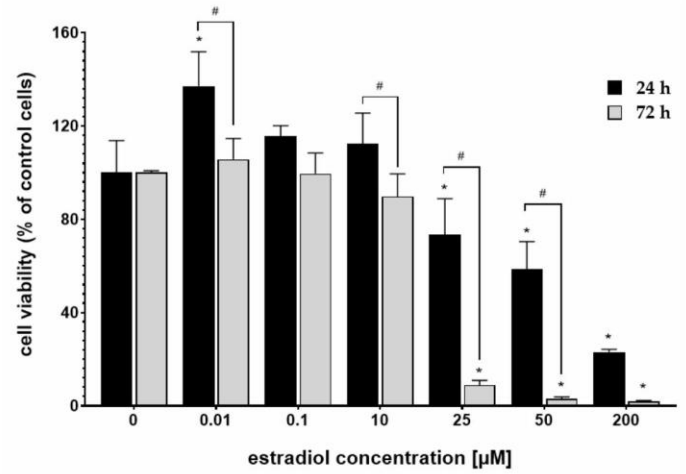

Figure 1. MDAH-2774 cells viability measured by the MTT assay after 24 h and 72 h incubation in different 17β-estradiol concentrations. Notes: (mean ± SD) $N = 3$, * $p < 0.05$ compared to control, # $p < 0.05$ compared between different incubation times.

Figure 2 shows the viability of MDAH-2774 cells after 24 h and 72 h exposure to increasing concentrations of cisplatin and $CaCl_2$. The experiment was carried out on two groups of cells. One of them was prior pre-incubated for 24 h with 10 μM 17β-estradiol (E_2). Control represents the viability of untreated cells.

After 24 h exposure, MDAH-2774 cells were resistant to the cytotoxic effects of cisplatin in the full range of concentrations (Figure 2a). Cells pre-incubated with 17β-estradiol had an increased sensitivity to both compounds. However, the decrease in the mitochondrial activity of pre-incubated cells at the two highest levels of cisplatin (50 μM and 75 μM) administered was a maximum of ~26%. In contrast, the viability of pre-incubated cells after the exposure to 10 mM and 25 mM of $CaCl_2$ was ≤20%. Extending the incubation time resulted in a more substantial cytotoxic effect of cisplatin (Figure 2b). In concentrations of 10–25 μM, it had a stronger than $CaCl_2$ cytotoxic effect on both groups of cells. Table 1 presents the IC_{50} values calculated for cisplatin and $CaCl_2$. The IC_{50} values calculated using Quest Graph™ IC_{50} Calculator (AAT Bioquest, Inc, Sunnyvale, CA, USA). IC_{50} was determined with a non-linear model.

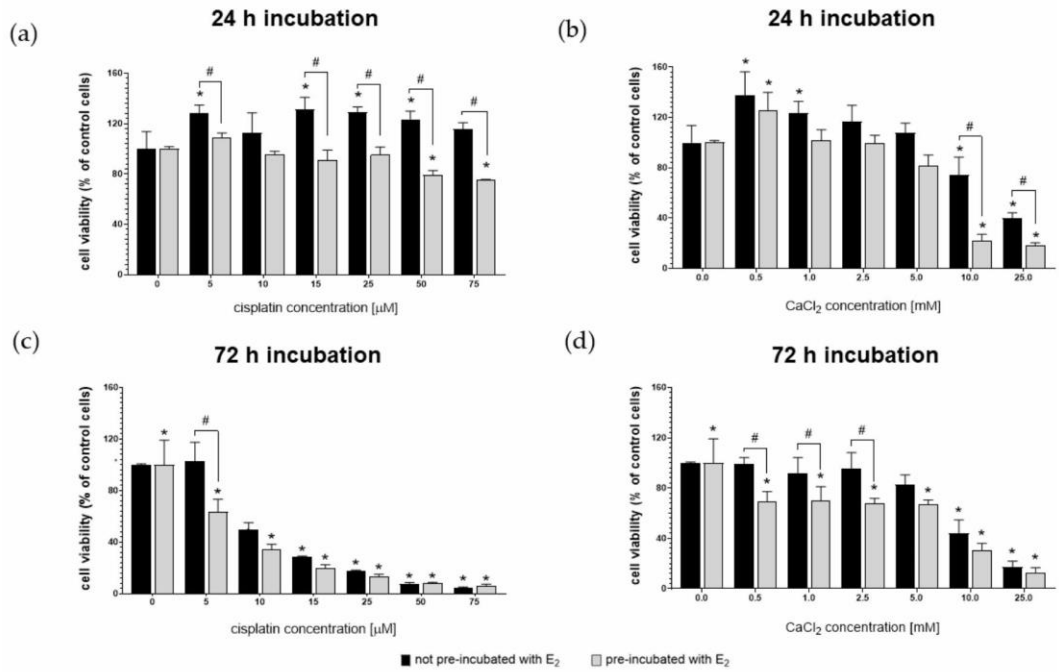

Figure 2. MDAH-2774 cells viability measured by the MTT assay after 24 h (**a**,**b**) and 72 h (**c**,**d**) incubation in increasing cisplatin (**a**,**c**) and $CaCl_2$ (**b**,**d**) concentrations. Notes: (mean ± SD) $N = 3$, * $p < 0.05$ compared to control, # $p < 0.05$ compared between different pre-incubation conditions.

Table 1. IC_{50} values for cisplatin and calcium chloride ($CaCl_2$).

	Cisplatin [μM]	$CaCl_2$ [mM]
IC_{50} for 24 h		
Not pre-incubated with E_2	727.47	20.03
Pre-incubated with E_2	129.53	13.29
IC_{50} for 72 h		
Not pre-incubated with E_2	9.567	16.637
Pre-incubated with E_2	6.072	8.503

Based on the obtained results, the highest non-toxic concentrations of cisplatin and $CaCl_2$ were selected for subsequent experiments, amounting to 25 μM and 2.5 mM, respectively.

3.2. Effect of Pulsed Electric Fields (PEF) on Cancer Viability

The viability of ovarian cancer cells after μEP and nsEP was evaluated for 25 μM cisplatin and 2.5 mM calcium concentrations. The experiment was performed on the two groups of cells (pre-incubated and not pre-incubated with 10 μM of 17β-estradiol). Figure 3 shows the viability of MDAH-2774 cells after exposure to μEP and nsEP with calcium ions or cisplatin relative to control. Control included non-electroporated cells. For both compounds, pre-incubation with E_2 improved the cytotoxic effect of electroporation in all electric field strengths. The highest values of mitochondrial activity for both compounds occurred by electroporated cells according to the nanosecond protocol with an electric field strength of 37.5 kV/cm. The lowest viability was achieved for the microsecond electroporation protocol. The use of 2.5 mM calcium ions instead of a cytostatic gave a more significant cytotoxic effect (Figure 3c).

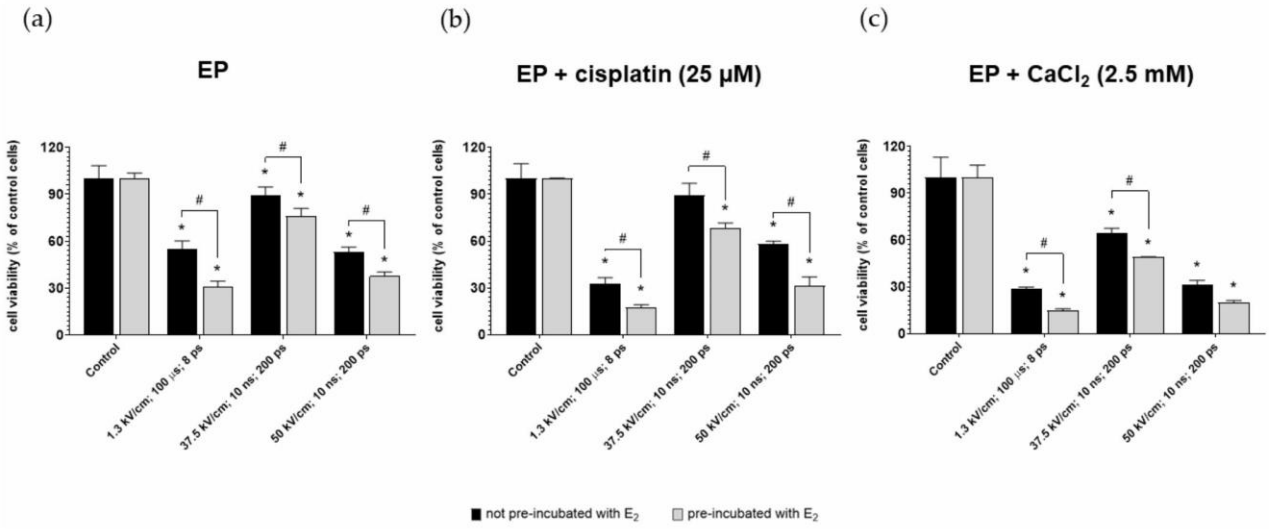

Figure 3. MDAH-2774 cells viability measured by the MTT assay after (**a**) μEP and nsEP with (**b**) 25 μM cisplatin and (**c**) 2.5 mM $CaCl_2$. Notes: (mean ± SD) $N = 3$, * $p < 0.05$ compared to control, # $p < 0.05$ compared between different pre-incubation conditions; ps—pulses.

3.3. Caspase-12 Expression

Immunocytochemical staining studies (Figure 4) revealed the expression of caspase-12 in MDAH-2774 cells after 24 h incubation with cisplatin (5 μM and 25 μM) and $CaCl_2$ (1 mM and 2.5 mM). The study was performed on two groups of cells, not pre-incubated and pre-incubated with 10 μM of 17β-estradiol (E_2) for 24 h. Controls included untreated cells. The majority of not pre-incubated cells exposed to both cisplatin concentrations remained almost unaffected. The highest level of enzyme expression was observed in cells incubated with 2.5 mM $CaCl_2$. Pre-incubation of cells with E_2 increased the expression of caspase-12 by an average of 10% in all variants of the used compounds. Cells exposed to calcium chloride showed the highest level of expression. Its significant increase was noted for the $CaCl_2$ concentration equal to 1 mM. Table 2 presents the % ratio of stained cells to the whole number of cells in the sample.

Table 2. The caspase-12 expression after exposure to cisplatin (5 μM and 25 μM) and $CaCl_2$ (1 mM and 2.5 mM) in the MDAH-2774 cell line.

	Not Pre-Incubated with E_2		**Pre-Incubated with E_2**	
	Percentage of Stained Cells	**The Intensity of Staining**	**Percentage of Stained Cells**	**The Intensity of Staining**
cisplatin				
5 μM	74%	+	81.5%	++
25 μM	80%	+	90%	++
$CaCl_2$				
1 mM	44.5%	+/−	90.5%	++
1.5 mM	95%	++	92%	++/+++

(−) negative, (+) weak, (++) moderate, or (+++) strong.

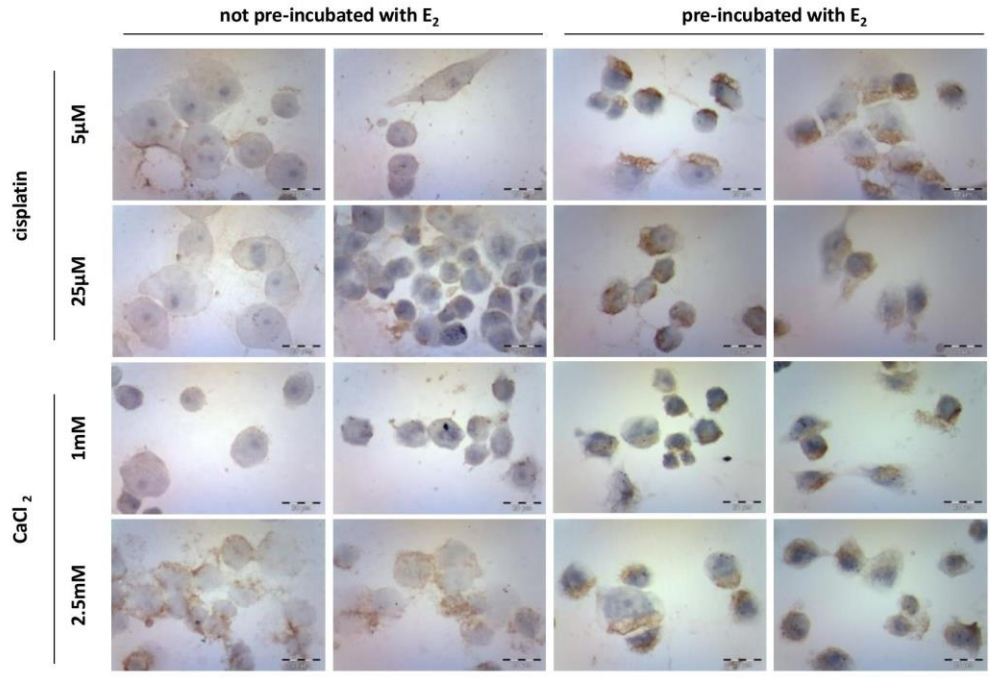

Figure 4. Caspase-12 immunocytochemical staining after exposure to cisplatin (5 μM and 25 μM) and $CaCl_2$ (1 mM and 2.5 mM) in the MDAH-2774 cell line.

3.4. Fluorescence Staining of Actin

Figures 5 and 6 present fluorescence staining of the intracellular actin in MDAH-2774 cells. The studies were obtained for two groups of cells, not pre-incubated and pre-incubated with 10 μM of 17β-estradiol (E_2) for 24 h. Cells were electroporated using μEP and nsEP protocols (Figure 6). Controls included non-electroporated and untreated samples.

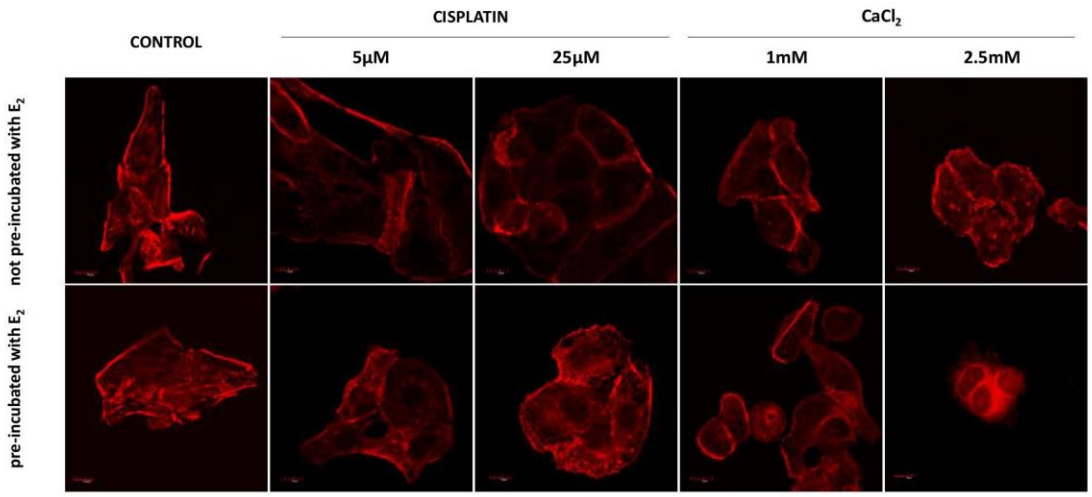

Figure 5. Immunofluorescence staining of MDAH-2774 cells (60×) after 24 h incubation with cisplatin and $CaCl_2$. Alexa Fluor™ 546 phalloidin used for actin filaments labeled. Scale bars correspond to 100 μm.

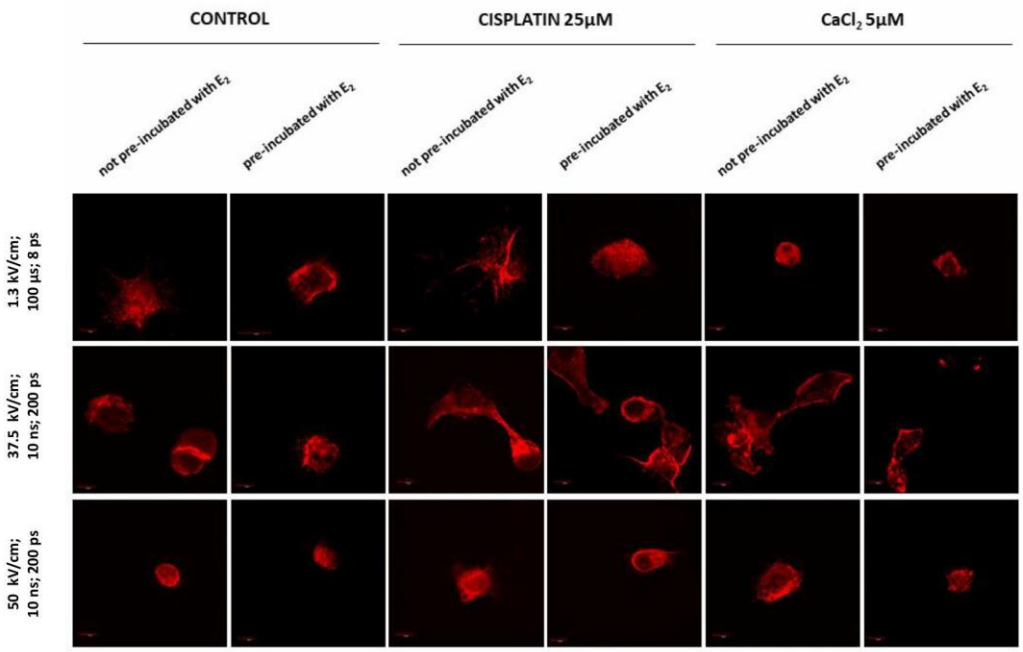

Figure 6. Immunofluorescence staining of MDAH-2774 cells (60×) after μEP and nsEP with the addition of cisplatin and $CaCl_2$. Alexa Fluor™ 546 phalloidin used for actin filaments labeled. Scale bars represent 100 μm; ps—pulses.

Control cells formed elongated-shape, well-organized F-actin filaments structure, creating a stable net with stress fibers. Both concentrations of cisplatin did not result in significant changes in the morphology of the neoplastic cells (Figure 5). The most significant changes are seen in cells pre-incubated with E_2 and then exposed to 25 μM cisplatin. The cells changed shape to oval. The actin filaments accumulated near the nucleus and on the edge of the cell in lamellipodia and filopodia. Exposure to calcium chloride-induced more substantial changes in cell morphology and the cytoskeleton. Significant shrinkage appears at a concentration of 1 mM, while at 2.5 mM $CaCl_2$, cells have an abnormal structure and probable damage to the actin cytoskeleton (pre-incubated cells).

Electroporation without the addition of any compound caused severe morphological changes (Figure 6). They are manifested in most attempts by significant cell shrinkage. The most potent effect was observed after microsecond (μEP) and nanosecond (nsEP) electroporation in particular for an electric field strength of 50 kV/cm, where cells revealed inferior assembly of actin filaments. Reorganization of actin cytoskeleton to both pre-incubated and not pre-incubated cells is visible in all these sample. Nanosecond electroporation with the intensity of 37.5 kV/cm resulted in changes in the cytoskeleton structure such as extending lamellipodia-like protrusions, which provided cell–cell contact and migration ability. However, no cell shrinkage was noted. The differences between not pre-incubated and pre-incubated cells are particularly visible in non-electroporated samples. Cells pre-incubated with E_2 are smaller, more oval, or damaged (2.5 mM $CaCl_2$).

3.5. Efficiency of Microsecond Electroporation (μEP) and Nanosecond Electroporation (nsEP)

The uptake of the Yo-Pro-1™ fluorescent dye shows the efficiency of the electroporation process (Figure 7). The dye fluorescence intensities were measured with a flow cytometer. The study included two groups of cells (not pre-incubated and pre-incubated with 10 μM 17β-estradiol for 24 h). Non-electroporated controls with and without the addition of the dye were included.

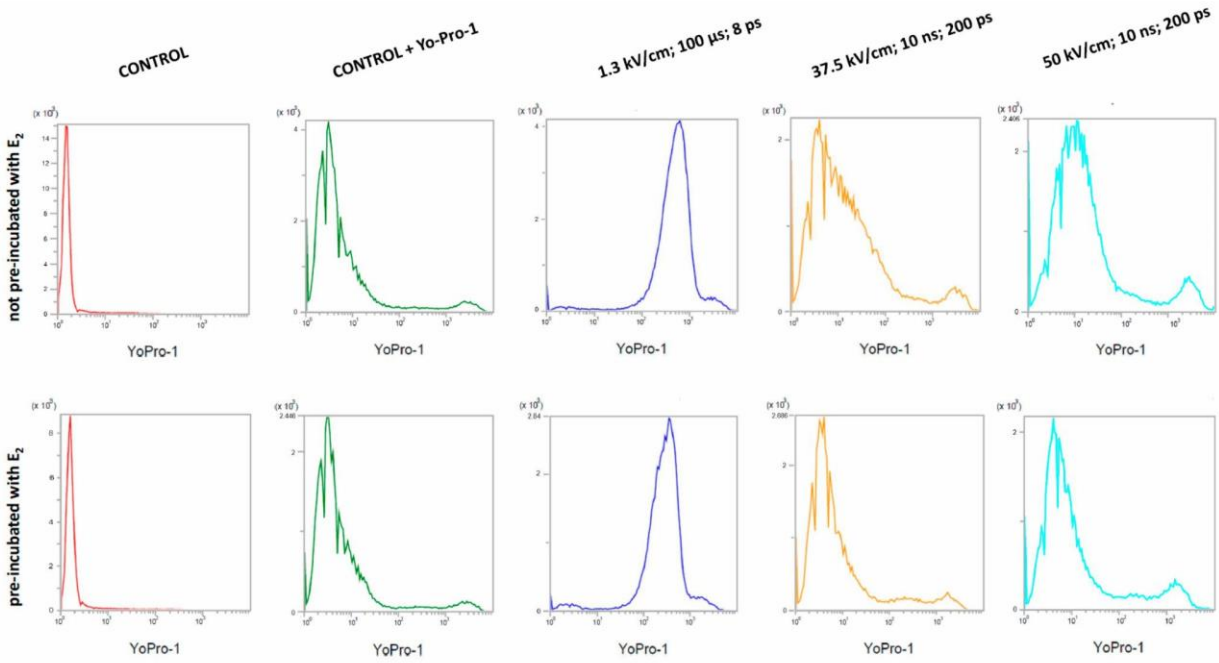

Figure 7. Yo-Pro-1^{TM} fluorescent dye absorption in MDAH-2774 cells after µEP and nsEP; ps—pulses.

The highest absorption values of the Yo-Pro-1^{TM} dye were found in cells subjected to microsecond electroporation with an electric field strength of 1.3 kV/cm. The efficiency of nsEP was much lower. At an electric field strength of 37.5 kV/cm, the mean value of fluorescence was similar to the non-electroporated control. After electroporation with the nsEP 50 kV/cm protocol, the dye absorption reached intermediate values. Pre-incubation of cells with 17β-estradiol reduced the uptake of the dye in all the tests performed. Table 3 shows the mean values of fluorescence intensity obtained during the study.

Table 3. Yo-Pro-1^{TM} fluorescence intensity in MDAH-2774 cells in arbitrary units; ps—pulses.

	Not Pre-Incubated with E_2	Pre-Incubated with E_2
Control	1.52	1.56
Control + Yo-Pro-1^{TM}	5.32	4.97
EP 1.3 kV/cm (ESOPE)	677.19	403.98
nsEP 37.5 kV/cm (10 ns, 200 ps)	23.07	6.24
nsEP 50 kV/cm (10 ns, 200 ps)	256.7	171.46

3.6. Digital Holographic Microscopy (DGM) Study

Digital Holographic Microscopy (DGM) study showed the morphological changes in the MDAH-2774 cell line after 24 h incubation with examined compounds. The experiment included two groups of cells (not pre-incubated and pre-incubated with 10 µM of 17β-estradiol for 24 h). Controls incorporated untreated samples. Figure 8 presents the obtained results. The control cells are in good condition and have a normal structure. There are no significant differences between pre-incubated and not pre-incubated controls. The most remarkable changes in cell structure are noticeable after exposure to calcium chloride at a concentration of 2.5 mM. In this case, both series of cells are visibly shrunken and take a more oval shape. Incubation with cisplatin did not provoke significant morphological changes. However, a difference can be observed between the series of cells exposed to this compound.

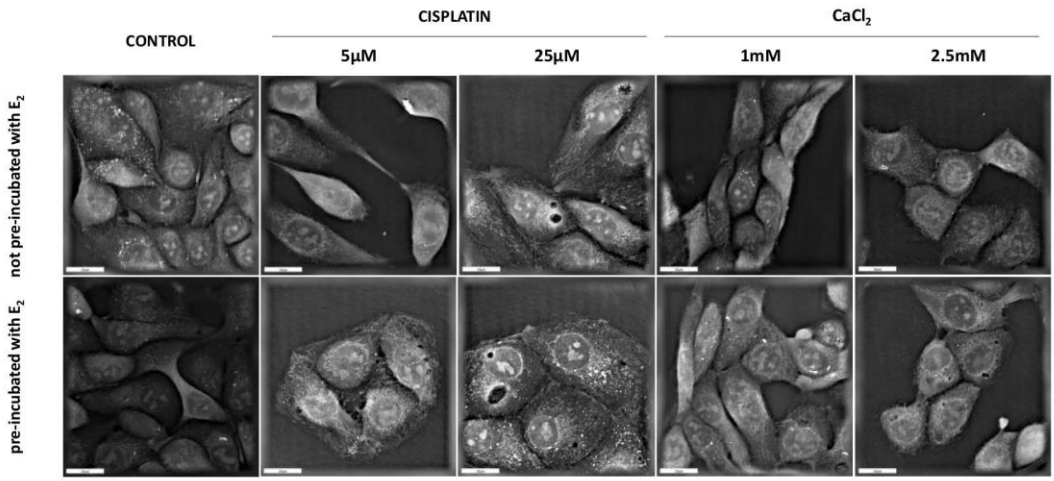

Figure 8. Holotomographic microscopy studies after 24 h with cisplatin and $CaCl_2$. Scale bars represent 20 μM.

4. Discussion

Neoplastic diseases are currently one of the most common causes of death in the world. Ovarian cancer (OC) is associated with the highest death rate out of any gynecological malignancies. The disease in the early stages of development gives no or vague symptoms. It is estimated that ~75% of women are diagnosed in stage III or IV FIGO. Apart from late diagnosis, treatment failure is caused by frequent relapses and the phenomenon of patient resistance to the drugs used. High hopes in the fight against neoplasms resistant to the currently used therapeutic strategies are given by electrochemotherapy (ECT). This method allows for an extreme increase in the absorption of cytostatics by cancer cells and, as a result, intensification of their action. ECT has already been used successfully in patients who do not respond to conventional chemotherapy [43,44].

Moreover, it is characterized by a safety profile and a lower frequency of oppressive contingent effects because of its local activity. Hitherto, ECT has been approved in the treatment of skin cancers and head and neck cancers. Additionally, it is expected that it may find application in treating deep cancers in the future. Even greater expectations are associated with calcium electroporation (CaEP), where calcium ions (Ca^{2+}) replace conventional cytostatic. Considering the low efficacy of current treatments for OC, studies on the effectiveness of different types of electrochemotherapy and their impact on the biology of ovarian cancer cells are fully justified.

The molecular mechanisms associated with ovarian cancer are closely related to the action of estrogen hormones. Their direct action on ovarian cells may affect tumor development and its progression. This is reflected in the wide range of risk factors associated with estrogens' effects (e.g., hormone replacement therapy). Estrogens, by enhancing proliferation, can cause the consolidation and accumulation of DNA damage. Apart from participation in neoplastic transformation, estrogens may have a protective effect on ovarian malignancy cells, inhibiting the apoptosis process. However, according to the studies conducted so far, estrogens' impact may differ depending on their concentration, the type of target cells, and the expression of particular estrogen receptor (ER) types. Considering the potential use of electrochemotherapy in the treatment of ovarian cancer, the above study posed the question of whether 17β-estradiol (E_2; the most active of estrogens) has a protective effect on electroporated ovarian cancer cells or, on the contrary, will intensify the toxic effects of cisplatin and calcium chloride ($CaCl_2$).

The first stage of our research was to estimate the cytotoxicity of equal concentrations of estradiol and its influence on the compounds' effectiveness in the subsequent phases during ECT. We analyzed cisplatin, a drug commonly used in OC therapy, and $CaCl_2$, a

calcium ion source for the experimental calcium electrochemotherapy. The cytotoxicity profile of the mentioned compounds was determined for 24 and 72 h of incubation.

Estradiol reduced the mitochondrial activity of MDAH-2774 cells in the concentration range of 25–200 μM. These are concentrations exceeding the physiological values. This effect was particularly visible after 72 h of incubation. No cytotoxic effect was observed at concentrations of 0.01–10 μM. Zhenga et al. pointed out that 6-day incubation of OVCAR-3 cells with 0.01–100 nM E_2 intensifies their proliferation [45]. Additionally, Langdon et al. showed analogous tendencies in PEO1 and PEO4 cell lines, and it was associated with the Erα receptor activity [46]. On the other hand, Bonavida and Bechtel demonstrated the anti-proliferative effect of estradiol at concentrations of >1 μM in HOC-7 and OVCAR-3 cells [47]. Based on the performed MTT assay, the concentration of 17β-estradiol used for the pre-incubation of cells in subsequent experiments (10 μM) was selected. This is the concentration that was not cytotoxic after 24 h of expansion and is the closest to the physiological E_2 concentration in the follicles of the ovaries [48].

To assess the influence of estradiol on the studied phenomena, all experiments were performed on two groups of cells, namely non- and pre-incubated, for 24 h with the selected concentration of E_2. Assessment of cisplatin's effect on MDAH-2774 cells was performed using a concentration range of 5–75 μM. After 24 h of incubation, cells showed no decreased mitochondrial activity over the entire range of drug concentrations. The prolongation of the exposure time caused that the cytotoxic effect was noticeable at concentrations > 5 μM. In turn, calcium chloride's action was assessed for concentrations in the range of 0.5–25 mM. 24 h incubation with $CaCl_2$ < 5 mM did not reduce cell viability. At concentrations of 10 and 25 mM, a distinct cytotoxic effect was noted. After 72 h of exposure, 5 mM cisplatin was also toxic. Pre-incubation with E_2 increased the susceptibility of cells to the action of both compounds.

Fluorescent staining of the actin cytoskeleton visualized the effects of cisplatin and $CaCl_2$ (depending on the cells' prior exposure to estradiol). It allowed evaluating changes in cell morphology caused by the action of the analyzed compounds probably. Two cisplatin concentrations (5 and 25 μM) and calcium chloride (1 and 2.5 mM) were selected for the study. Concentrations that were not toxic during the 24 h incubation were used to fix the cells to the microscope slides. Cells exposed to 5 and 25 μM of cisplatin showed slight changes in cell morphology and cytoskeleton. However, they were most prominent in cells pre-incubated and then exposed to cisplatin at a concentration of 25 μM. In turn, the action of $CaCl_2$ caused a marked shrinking of cells. Pre-incubated cells enhanced this effect.

The mechanism of 17β-estradiol action on OC cells is mostly related to the estrogen receptors (ERs). ERα and Erβ act differently, and the level of their expression depends on the type of cells. Both types are present in the ovaries' normal and cancer cells, but their quantitative ratio is changed during carcinogenesis. Chan et al. showed that the majority of patients with OC show increased expression of ERα and ERβ5 nuclear receptors and decreased expression of ERβ1 and cytoplasmic ERα receptors [49]. The activity of the ERα is associated with the phenomenon of cisplatin resistance, as shown in the studies conducted by Matrumur et al. [50].

Moreover, the ERβ may exert anti-tumor activity in response to or independently of E_2 stimulation, as reported by Treeck et al. [51]. The action of sex hormones may influence the number of ERs. Taube et al. showed that estradiol in nanomolar concentrations (nM) could lower the total amount of estrogen receptors presented by ovarian cancer cells [52]. The synergistic effect of E_2 and the compounds tested by us may be associated with a disturbance in the quantitative ratio of individual types of ERs. To confirm this hypothesis, additional experiments are required.

Another mechanism that may participate in estradiol's observed effect may be the mechanism related to the excessive production of reactive oxygen species (ROS). Moghadasi et al. have shown that estradiol in concentrations of 0.1–1000 nM increases ROS synthesis in OVCAR-3 human ovarian carcinoma cells [53].

Caspase-12 expression was assessed to determine if decreased survival of MDAH-2774 cells pre-incubated with estradiol is associated with increased ROS synthesis. Caspase-12 is involved in the process of reticular apoptosis and is activated in response to stress factors such as, for example, reactive oxygen species or high levels of calcium ions. Immunocytochemical staining showed increased caspase-12 expression in cells pre-incubated with estradiol. However, this effect differed depending on the compound used. In cisplatin (5 and 25 μM), the number of stained cells increased by ~10%. For slides with 1 mM $CaCl_2$, this value increased by 45%. Cells treated with 2.5 mM $CaCl_2$ were ~95% stained regardless of pre-incubation with estradiol. Sapkal showed that 20 and 40 nM of E_2 decreased the expression of caspase-12 in human retinal epithelial cells (ARPE-19) [54]. This effect has been linked to the antioxidant activity of 17β-estradiol. Similar results were obtained by Guo et al., studying the impact of nanomolar estradiol concentrations on the course of stress-induced apoptosis in MC3T3-E1 mouse osteoblast cells [55]. They showed that E_2 at these concentrations inhibited reticular apoptosis by increasing the expression of Grp78, which is a protein capable of binding procaspase-7 and procaspase-12. Unfortunately, there are no reports in the available literature concerning the effect of micromolar (μM) concentrations of estradiol on ovarian cancer cell lines.

The next stage of the research was to assess the effect of 17β-estradiol on the electroporation (EP) process of MDAH-2774 cells. The study was divided into two parts. In the first one, changes in the mitochondrial activity and the structure of the cytoskeleton were examined. The second part assessed the effectiveness of electroporation by checking the absorption of the Yo-Pro™-1 fluorescent dye.

Three electroporation protocols were used, plus cisplatin (25 μM) and $CaCl_2$ (2.5 mM). Calcium chloride induced a more significant decrease in cell viability than cisplatin, regardless of the used protocol. Microsecond electroporation (μEP) without any compound achieved a robust cytotoxic effect (reducing cell viability by ~50%). The addition of cisplatin and $CaCl_2$ intensified this effect. The use of nanosecond pulsed electric fields (nsEP) 37.5 kV/cm did not cause a significant decrease in survival in control without compounds and in combination with cisplatin.

In contrast, the combination with $CaCl_2$ caused a reduction in mitochondrial activity by ~30%. The nsEP 50 kV/cm protocol in control and combination with cisplatin caused a comparable decrease in the cell viability to a value ~55%. When combined with calcium chloride, the toxic effect was similar to that obtained with the μEP.

All electroporated cells pre-incubated previously with 17β-estradiol showed reduced mitochondrial activity. Fluorescent staining of actin filaments displayed morphological changes in cells electroporated using μEP and nsEP 50 kV/cm. The cells showed shrinkage and damage to the cytoskeleton. The nsEP 37.5 kV/cm did not cause such intense changes. The results obtained in this study for cells not pre-incubated with estrogen hormone are consistent with the literature data. Saczko et al. demonstrated that the combination of cisplatin with μEP significantly increases the toxicity of cytostatics on ovarian cancer cells [56].

Chenguo et al. presented that the mobilization of intracellular Ca^{2+} causes cytotoxicity to OC cells after nsEP [57]. The high concentration of $CaCl_2$ activates apoptosis. In our study, nsEP, in combination with $CaCl_2$, caused the most substantial decrease in cell viability. Unfortunately, there are currently no publications available on the effects of E_2 on EP. For this reason, the last of the experiments carried out in this study was to determine the impact of pre-incubation with estradiol on the efficiency of electroporation.

MDAH-2774 cells were also electroporated with Yo-Pro™-1. The absorption was measured using a flow cytometer. The study showed that μEP had the most substantial dye uptake. Cells pre-incubated with estradiol showed a lower dye absorption. Considering the results of MTT assay, it can be assumed that the mechanism behind the decrease in the mitochondrial activity of cells pre-incubated with E_2 is not related to changes in the permeability of the cell membrane.

5. Conclusions

The results presented in this work demonstrated that electrochemotherapy (ECT) might be a potentially effective treatment for human ovarian cancer in vitro. Pre-incubation of ovarian cancer cells with 17β-estradiol effectively enhanced the chemo- and electrochemotherapy with cisplatin and calcium chloride. Therefore, their impact should be considered when developing new schedules of treatment.

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Praca 2

Tytuł

"The influence of asymmetrical bipolar pulses and interphase intervals on the bipolar cancellation phenomenon in the ovarian cancer cell line"

Autorzy

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The influence of asymmetrical bipolar pulses and interphase intervals on the bipolar cancellation phenomenon in the ovarian cancer cell line

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ABSTRACT

The application of negative polarity electrical pulse (↓) following positive polarity pulses (↑) may induce bipolar cancellation (BPC), a unique physiological response believed to be specific to nanosecond electroporation (nsEP). The literature lacks analysis of bipolar electroporation (BP EP) involving asymmetrical sequences composed of nanosecond and microsecond pulses. Moreover, the impact of interphase interval on BPC caused by such asymmetrical pulse needs consideration. In this study, the authors utilized the ovarian clear carcinoma cell line (OvBH-1) model to investigate the BPC with asymmetrical sequences. Cells were exposed to pulses delivered in 10-pulse bursts but as uni- or bipolar, symmetrical, or asymmetrical sequences with a duration of 600 ns or 10 μs and electric field strength equal to 7.0 or 1.8 kV/cm, respectively. It was shown that the asymmetry of pulses influences BPC. The obtained results have also been investigated in the context of calcium electrochemotherapy. The reduction of cell membrane poration, and cell survival have been observed following Ca^{2+} electrochemotherapy. The effects of interphase delays (1 and 10 μs) on the BPC phenomenon were reported. Our findings show that the BPC phenomenon can be controlled using pulse asymmetry or delay between the positive and negative polarity of the pulse.

1. Introduction

Briefly exposing cells to external pulsed electric fields (PEFs) enables the temporary or permanent plasma membrane's (PM) lipid bilayer reorganization, which finally leads to aqueous pores formation [1]. This phenomenon, called electroporation (EP), improves the transport of the hydrophilic, otherwise non-permeable molecules into the cell's interior while there is no effect on intracellular organelles. Since the time of its first description by Mir et al. [2], the combination of EP with conventional chemotherapy (CT), is known as electrochemotherapy (ECT). ECT has been the subject of many studies and found clinical application in the treatment of head and neck carcinomas, but also skin, liver, and pancreatic cancers [3–9]. ECT is based on reversible electroporation (RE) when the cell, after exposure to PEFs, can rebuild its PM over time, close pores, reestablish metabolism, and survive. Clinical ECT procedures are often based on the European Standard Operative Procedure ECT (ESOPE) guidelines released in 2006 and involve the application of 100 μs × 8 pulses with a frequency equal to 1 Hz [10]. Irreversible electroporation (IRE) occurs when the disturbance presented in PM after exposure to sufficient PEFs is more extensive, the cell cannot return to its original state and enters the death pathway. However, at some point, the exact mechanisms responsible for all events occurring in the cell as a result of exposure to PEFs remain elusive. Intensive research is being done to understand and improve treatment methods based on PEF application.

Nanosecond pulsed electric fields (nsPEF) have been explored extensively in the last decade, revealing various unique bioeffects, both immediately after nsPEF exposure or days after [11]. nsPEF has been defined as high intensity (1.0–50 kV/cm) pulsed electric field with a duration limited in the sub-microsecond range [11,12]. In contrast to longer pulses, which predominantly permeabilize PM, nsPEF can affect extra- and intracellular membranes throughout the cellular volume [13], e.g., nucleus [14], mitochondria [15], and endoplasmic reticulum (ER) [16,17]. Among the effects following cell exposure to nsPEF, one

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can distinguish an increased PM's permeability for exogenous molecules, mobilization of intracellular calcium ions (Ca^{2+}) [18,19], activation or inhibition of voltage-gated channels (VGC) [20], cytoskeleton changes [21], cell swelling [22], cell death through apoptosis or necrosis [23,24] and autophagy [25]. Presently, the exact molecular influence of nsPEF on cells is heavily studied. The number and intensity of these effects may be regulated by changing the nsEP protocol parameters (e.g., pulse time duration, amplitude, or the number of pulses).

The role of the pulses shape and the effects on the polarization dynamics of cell membrane have been investigated intensively over the last decade [26]. Several studies revealed that bipolar pulses in the microsecond range (μsEP) alter cell membrane permeabilization more intensively while resulting in similar cell survival rates compared to equivalent unipolar pulse (UP) [27,28]. Kotnik et al. observed that the application of bipolar pulses allowed the achievement of permeabilization at lower pulse amplitudes than for unipolar pulses [27]. Simultaneously, they noted that the molecular uptake was higher, however, the pulse amplitude leading to cell death was practically unaltered. From a clinical point of view, it was interesting that the use of bipolar pulses reduced the electrolytic contamination from electrodes (i.e., metal ions or pH changes). Sano et al. explore intensively the role of bipolar pulses in IRE treatment. They revealed that asymmetric high-frequency IRE (H-FIRE) waveforms may create an ablation area equivalent to standard IRE treatments [28,29]. Moreover, Rolong et al. analyzed the use of H-FIRE to induce cell death of tumor-initiating cells using a mouse ovarian surface epithelial (MOSE) cancer model [29]. However, over the past decade, a nsPEFs-unique feature has been discovered called 'bipolar pulse cancellation' (BPC). Pakhomov et al. pointed out that bipolar pulse (BP), in principle, is a sequence of two unipolar pulses, which means that the influence of one unipolar pulse (cathodic pulse; ↑) on a targeted cell may be *'canceled'* by following a reverse polarity pulse (anodic pulse; ↓), even with the delivery of doubled energy and with twice the duration [20,30,31]. In the last years, studies revealed lower intracellular calcium and propidium iodide (PI) responses after exposure to bipolar nanosecond electric pulse (BP nsEP) compared to UP with the same total duration (↑300 ns + ↓300 ns and ↑600 ns) and amplitude [32]. Overall, BP nsPEF application results in lower membrane permeabilization and improved cell survival compared to UP nsPEF. What is more interesting, it has been observed that pulse width symmetry is not necessary to generate the BPC effect [12]. This effect was still observed, even when the second pulse's amplitude was reduced to 35 % compared to the PEF amplitude of the first pulse. These observations were assessed for pulses with a duration time ranging from 60 [30,32] to 900 ns [33]. The authors indicated that the analysis of the impact of asymmetrical BP EP protocol composed of nanosecond pulse and consequent microsecond pulse had not been provided.

Pakhomov et al. also indicated that interphase delay lasting < 10 μs, gradually diminished BPC as its duration increased, however, when it reached 10 μs, the effect caused by BP nsPEF (↑300 ns + ↓300 ns) was the same as after UP nsPEF (↑300 ns) application [30]. Following studies by Gianulis et al. discovered that BPC was still present with interphase intervals lasting up to 50 μs [31].

Calcium ions (Ca^{2+}) are second-order messengers involved in a wide range of cellular processes (e.g., regulation of transcription, cell proliferation, and cell death) [34]. Under physiological conditions, Ca^{2+} concentrations significantly differ between the outside (10^{-3}-10^{-2} M) and intracellular (10^{-8}-10^{-7} M) space. The calcium homeostasis is maintained by the system of ATP-dependent pumps (e.g., NCKX, NCX, PMCA). However, the rapid influx of supraphysiological Ca^{2+} concentrations into the cell interior and its intracellular overload initiates necrotic or apoptotic cell death [35]. Recently, calcium ions are also used in moderated ECT treatment methods, called calcium electroporation (CaEP), where conventional cytostatics have been replaced by calcium chloride ($CaCl_2$) solutions, which are commonly available. Simultaneously, calcium is involved in several ways of injured PMs' resealing [36]. The PM's integrity maintenance is a crucial process in terms of EP-based treatment methods. Therefore, in the presented study, the authors decided to examine the impact of extracellular Ca^{2+} on the effectiveness of calcium electrochemotherapy in the presence of the BPC effect. We have used 600 ns and 10 μs pulses with 1 and 10 μs interphase delays to study the BPC effect in the context of asymmetric pulses (both in terms of amplitude and duration). Moreover, two ESOPE-based protocols (ESOPE-1 and −2) with PEF strengths equal to 1.2 and 1.5 kV/cm, respectively, have been used for comparison.

In this study, the Authors presented new data on the interplay of positive and negative polarity pulses from different pulse duration ranges, which may be used to optimize electrochemotherapy protocols in the future.

2. Results

2.1. Calcium chloride ($CaCl_2$) concentration optimization

Fig. 1 presents the dependence of ovarian cancer cells' viability (MTT assay) on the concentration of extracellular Ca^{2+}. Cells' metabolic activity was assessed after 24 and 72 h of incubation of the cells in a growth medium including various concentrations of $CaCl_2$ (range 0.0 – 25 mM). The cells were not exposed to electric pulses.

According to the results obtained 24- and 72-hours post-incubation, the $CaCl_2$ concentration of 2.5 mM was defined as nontoxic and used for further experiments, which is in agreement with available literature reports [37,38].

2.2. Asymmetrical bipolar pulses reduce membrane permeabilization

Ovarian cancer (OvBH-1) cells' permeabilization with various PEF parameters was investigated by examination of the intracellular diffusion of the fluorescent membrane integrity marker (Yo-Pro-1™) with and without added calcium (2.5 mM). The number of fluorescent cells (Yo-Pro-1™ positive) was scaling with the electric pulse parameters and expressed as '% of permeabilized cells' in Fig. 2. The controls included OvBH-1 cells, which were not exposed to electrical pulses and additional extracellular calcium ions (CTRL black) and cells not treated with electrical pulses but with the addition of 2.5 mM $CaCl_2$ (CTRL yellow). For comparison purposes and better knowledge consolidation,

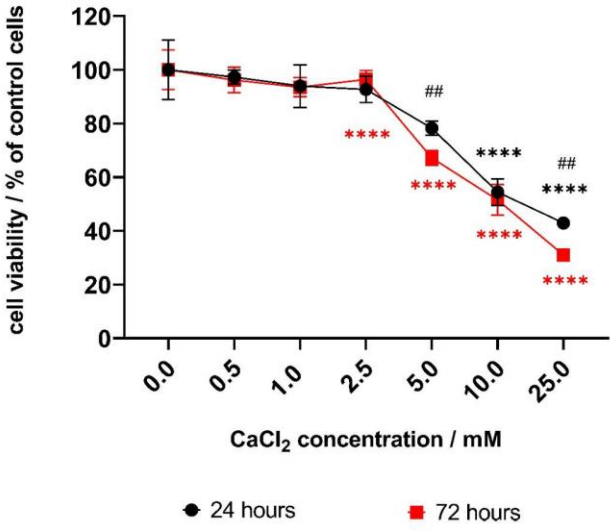

Fig. 1. The OvBH-1 cell viability was measured by the MTT assay after 24 h and 72 h incubation in increasing $CaCl_2$ concentrations. Notes: (mean ± SD) N = 3, *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ compared to control, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, #### $p < 0.0001$ compared between different incubation times.

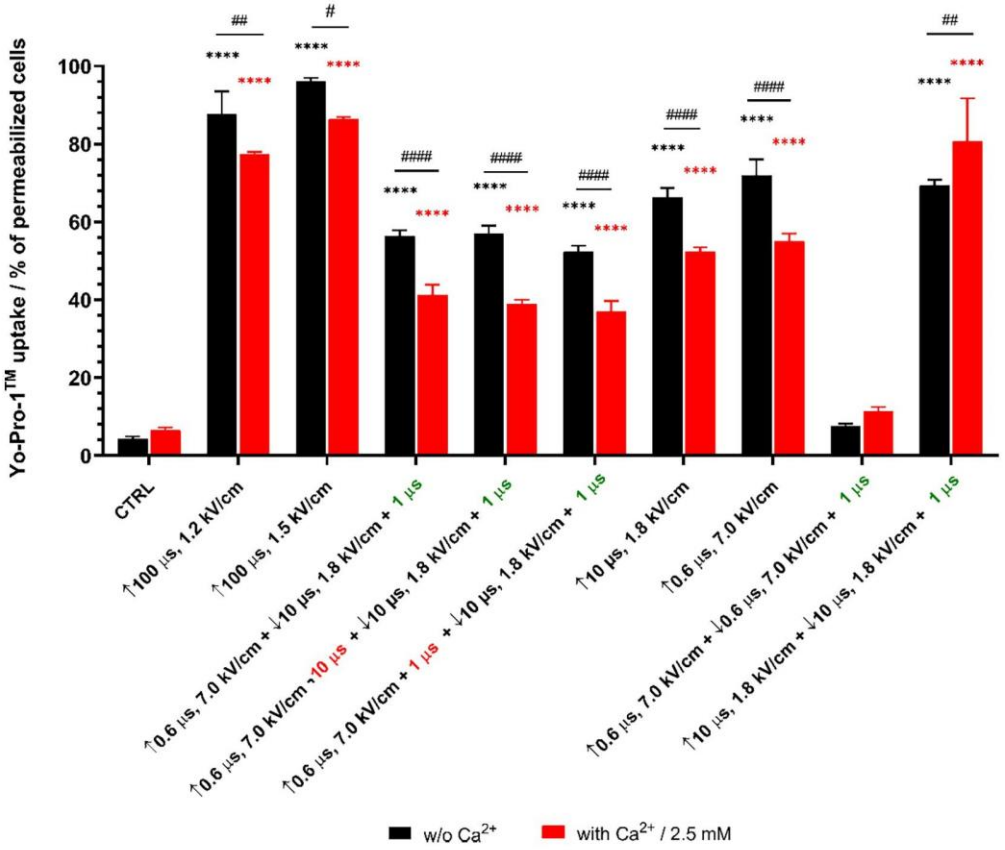

Fig. 2. Yo-Pro-1^{TM} dye uptake following exposure to different unipolar and bipolar protocols. Two ESOPE protocols (↑100 µs, 1.2 or 1.5 kV/cm) were used for comparison. ESOPE-1 and −2 pulses were delivered in a burst of 8 (1 Hz), while other protocols were delivered in a burst of 10 with predefined delays (1 or 10 µs, marked in **red**); the delay 1 µs marked by **green** color were used between consequent waveforms of short symmetrical or asymmetrical pulses. Notes: (mean ± SD) N = 3, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ *compared to control*, #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$, ####$p < 0.0001$ compared between different electroporation conditions (with or w/o $CaCl_2$); **CTRL** – control.

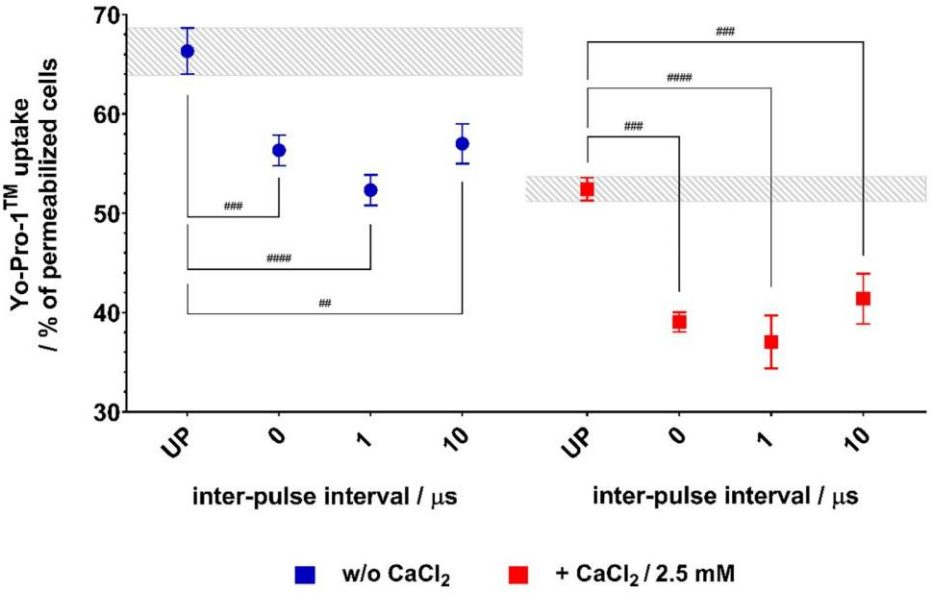

Fig. 3. Yo-Pro-1^{TM} dye uptake was measured by flow cytometer 5 min after OvBH-1 cells' exposure to bipolar protocol ↑600 ns + ↓10 µs including 0, 1, and 10 µs intervals between opposite polarity pulses. The results have been compared to the unipolar (UP) protocol (↑600 ns, 7.0 kV/cm); (**blue**) cells w/o $CaCl_2$ and the same protocol but with additional $CaCl_2$ [2.5 mM] (**red**). Notes: (mean ± SD) N = 3, #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$, ####$p < 0.0001$ compared between different protocols. Yo-Pro-1^{TM} uptake indicates the % of fluorescent cells (Yo-Pro-1^{TM} positive).

conventional ESOPE-1 and −2 protocols with PEF intensity of 1.2 and 1.5 kV/cm, respectively, have been introduced.

The exposure of cells to symmetrical BP nsPEF resulted in a significant reduction of Yo-Pro-1™ fluorescence, indicating decreased membrane permeabilization. This effect was not detected for symmetrical BP μsPEF. Nevertheless, the application of the asymmetrical BP protocol (↑600 ns + ↓ 10 μs) still resulted in detectable cancellation effects (i.e., reduced membrane permeabilization).

The addition of a 1–10 μs delay between two pulses with an opposite polarity did not affect the cancellation phenomenon dramatically. In all cases, the BPC phenomenon was detectable, resulting in statistically significant differences when compared to the unipolar (UP) sequence. A detailed comparison is shown in Fig. 3.

It can be seen (Fig. 2 and Fig. 3) that extracellular calcium results in weaker permeabilization (at the same pulsing conditions) of ovarian cancer cells for all protocols except the symmetrical μsPEF (↑10 μs + ↓10 μs) and nsPEF (↑600 ns + ↓600 ns). In these two instances, the effects of extracellular calcium on the permeabilization rate were not profound.

2.3. Cytotoxicity analysis of EP protocols

Then, the examination of the cytotoxicity of analyzed EP protocols was done. The results of the MTT assay 24- and 72 h post-electroporation are shown in Fig. 4.

Firstly, it should be noted that all reversible EP protocols w/o added $CaCl_2$ did not trigger a high cell death rate. The group exposed to EP protocols with the addition of MiliQ water corresponding to the amount of added $CaCl_2$ was included in the analysis as a control. The differences between w/o $CaCl_2$ and MiliQ groups of cells are statistically non-significant. With an increase of amplitude (i.e., 1.5 kV/cm) electroporation is partly irreversible and the ESOPE-2 protocol resulted in < 20 % cell death (24 h post-treatment) and < 50 % 72 h post-treatment. In other parametric instances, electroporation was predominantly reversible (i.e., >75 % of cells survived after 72 h). Both nsEP protocols (SEQ5: ↑600 ns and 6: ↑600 ns + ↓600 ns) triggered similar toxicity, while significantly different Yo-Pro-1™ uptake rates (Fig. 2). The use of asymmetrical BP EP (SEQ1: ↑600 ns + ↓10 μs) did not result in explicit differences when compared with other protocols. Similarly, the viability after non-ESOPE protocols is comparable with the ESOPE-1 sequence, while the differences in Yo-Pro-1™ uptake were significantly different. The BPC phenomenon did not result in a statistically significant increase in viability when bipolar pulses (both symmetrical or asymmetrical) were introduced or the differences were lost in the standard deviation of data.

The introduction of Ca^{2+} ions into the cells' environment significantly enhanced the cytotoxic activity of applied EP protocols indicating successful calcium electroporation. The BPC phenomenon was detectable for ↑600 ns + ↓600 ns nsPEF, however, it's not the case for all other protocols. Even though the YP-uptake revealed significant differences in permeabilization rates due to BPC for asymmetrical protocols (nsPEF + μsPEF), the cells still died after the treatment.

2.4. Nuclei and β-tubulin cytoskeleton staining

The fluorescent staining was performed 24 h after treatment to examine the morphological changes in OvBH-1 cells exposed to external electrical pulses and analyze the origin of the cells' viability loss. Fluorescence microscopy staining is presented in Figs. 5 and 6. The analysis has been performed for two groups of cells electroporated with or without the addition of 2.5 mM $CaCl_2$ using various EP protocols (refer to Fig. 7).

The cells in control samples formed elongated shapes and maintained a stable β-tubulin cytoskeleton. When electroporated using the ESOPE-1 (↑100 μs; 1.2 kV/cm) protocol, no changes in the cells' structure were observed. However, obtained results revealed shrinkage of cells exposed to ESOPE-2, which may be observed as a concentration of β-tubulin (white arrows) at one point. The $CaCl_2$ addition resulted in the enhancement of this effect. Moreover, due to the high toxicity of $CaCl_2$ in combination with PEFs, the number of cells reduced dramatically in each probe after its addition. The exception was noted for protocol involving 100 μs, 1.2 kV/cm pulses. Interestingly, the structure of cells differs strongly between SEQ6 and 7. However, differences in the effects of these protocols were also observed during previous experiments (i.e. MTT assay).

Microtubules (MTs) are made up of pairs of α- and β-tubulin molecules arranged in 13 linear chains known as protofilaments, which come together to form a tubular structure with an approximate diameter of 25 nm [39]. This cylindrical structure forms a hollow tube that serves as a vital component of the cytoskeleton of eukaryotic cells. MTs originate from the centrosome, a structure located in proximity to the nucleus within animal cells, and subsequently extend outwardly toward the cellular periphery in a radial fashion. MTs after PEF exposure have not been studied so extensively compared to actin. Considering that, the Authors decided to examine the structure of the β-tubulin cytoskeleton in OvBH-1 after exposure to uni- and bipolar PEF protocols.

The results obtained as a result of the cells' staining 24 h after PEFs exposure shows the different structure of β-tubulin and changes in plasma membranes. The amount of cells on observed on cover sleeps after incubation time was relevant to cells' viability result (Fig. 4a). Control (CTRL; Fig. 5) presents the proper outward radiation (polymerization) of tubulin. The addition of $CaCl_2$ does not disturb this structure. Analyzing the obtained results, for OvBH-1 cells exposed to ESOPE-1 w/o and with $CaCl_2$ (2.5 mM), the cells' shrinkage is visible. The plasma membranes are slightly fragmented, forming small aggregates.

Considering SEQ1-3, the addition of 1 and 10 μs interpulse delay, does not result in morphological changes, however, the addition of Ca^{2+} ions caused the shrinkage of cell membranes and their concentration around the cell nucleus (Figs. 5 and 6), as well as destabilization of β-tubulin fibers and their central accumulation in both SEQ2 and SEQ3 (including 10 and 1 μs interpulse delays, respectively).

Comparing the UP SEQ5 (↑600 ns) and BP SEQ6 (↑600 ns + ↓600 ns), the application of BP PEF resulted in mitigating the subtle changes that were observed with UP SEQ5. However, this trend is largely evident when considering these sequences with the addition of Ca^{2+}. In the case of SEQ6, the cells are more elongated and the membrane structure is intact compared to SEQ5. Interestingly, this effects has not been observed for SEQ4 and SEQ7. After exposure to both PEFs cells represent similar undamaged morphology.

3. Discussion

Bipolar cancellation (BPC) is a newly discovered phenomenon that needs to be analyzed more deeply. Our findings on cells' survival and Yo-Pro-1™ uptake demonstrate that the exposition of cells to opposite polarity pulses reduces the extent of plasma membrane permeabilization without affecting cells' viability. The results presented herein demonstrate that the damage to the cell membrane triggered by ↑600 nsEP application may be reduced by a following second pulse with the same amplitude and duration time but opposite polarity e.g., ↑600 ns + ↓600 ns. Results of Yo-Pro-1™ uptake clearly show a significant decrease in the dye molecules uptake entering the membrane after the introduction of the second phase of nsEP compared to UP nsEP (SEQ5 and SEQ6) (Fig. 3). High cell survival rate can be noted for both sequences when the cells are exposed to PEF only without additional calcium (Fig. 4). Confocal microscopy data show that both protocols caused morphological changes in cells, however, these changes are less profound after BP nsEP application (Fig. 6). This kind of response is typical for BPC.

The first time, BPC was observed by Pakhomov et al. [30] and confirmed by Valdez et al. [12]. The exact molecular mechanism of BPC remains unclear and lacks mechanistic explanation, however, a wide

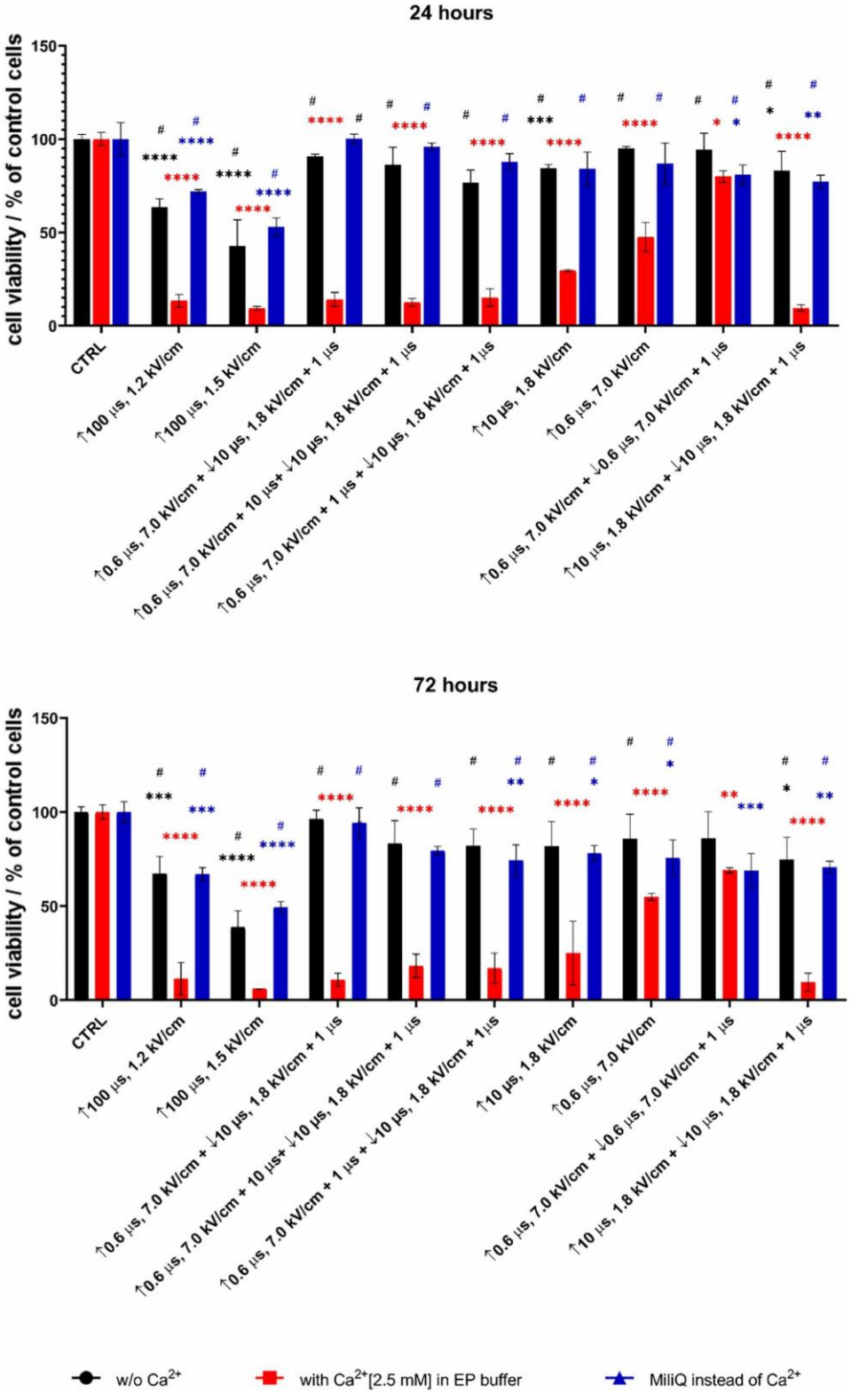

Fig. 4. The OvBH-1 cell viability was measured by the MTT assay (a) 24, and (b) 72 h after exposure to different electroporation protocols. The experiment was performed using three groups of cells, electroporated in buffer with or without $CaCl_2$ (2.5 mM) addition. The third (control) group consisted of cells electroporated in a buffer with the addition of MiliQ water corresponding to the amount of added $CaCl_2$.Two ESOPE protocols (↑100 µs, 1.2 or 1.5 kV/cm) have been used for comparison. ESOPE-1 and −2 pulses have been delivered in a burst of 8 (1 Hz), while other protocols were delivered in a burst of 10 with predefined delays (1 or 10 µs, marked in **red**); the delay 1 µs marked by **green** color have been used between consequent waveforms of short symmetrical or asymmetrical pulses. Notes: (mean ± SD) N = 3, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ *compared to control*, $\#\, p < 0.0001$ compared between w/o Ca^{2+} (**black** #) and MiliQ (**blue** #) to 'with Ca^{2+}' electroporation conditions.

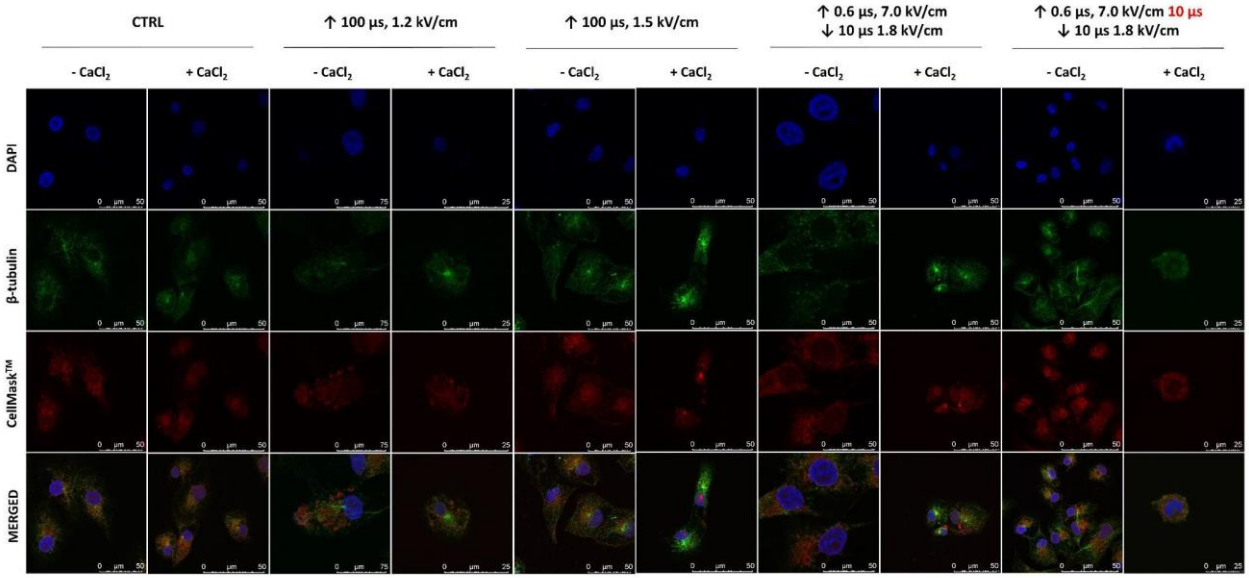

Fig. 5. β-tubulin organization in OvBH-1. After exposure to different uni- or bipolar electrical pulses cells were plated onto coverslips and fixed with 4% formaldehyde after 24 h of incubation. Cells were labeled to visualize nuclei (**blue**), β-tubulin (**green**), and plasma membrane (**red**). Merged images are shown on the lowest panel.

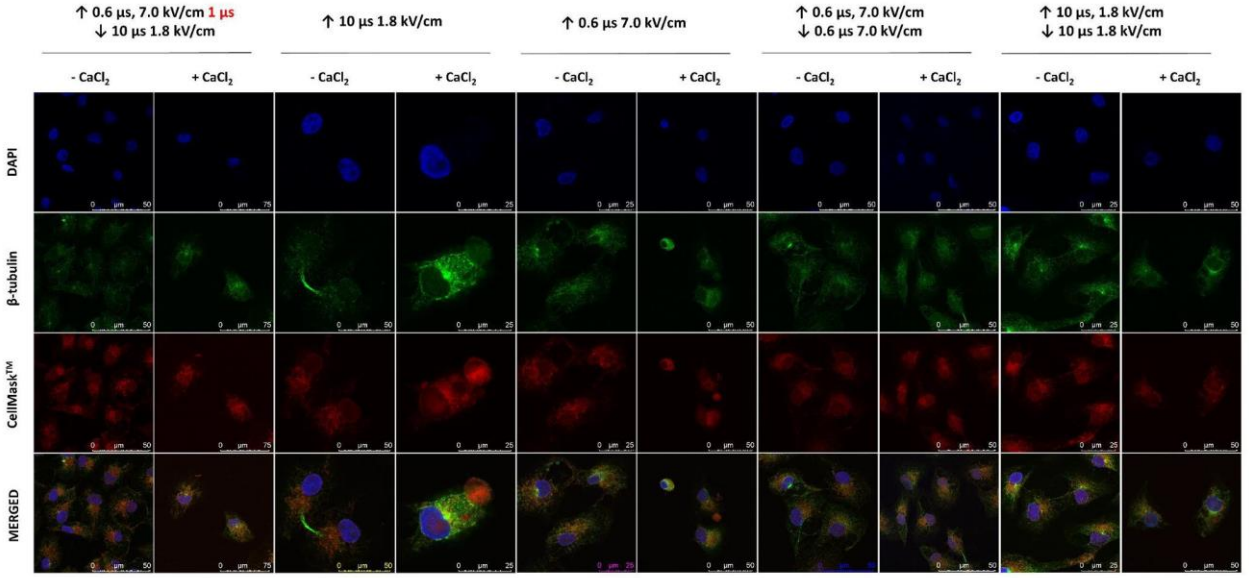

Fig. 6. β-tubulin organization in OvBH-1 cells. After exposure to different uni- or bipolar electrical pulses cells were plated onto coverslips and fixed with 4% formaldehyde after 24 h of incubation. Cells were labeled to visualize nuclei (**blue**), β-tubulin (**green**), and plasma membrane (**red**). Merged images are shown on the lowest panel.

range of studies try to explain this phenomenon e.g., using molecular modeling [40]. Our data indicate that µsBP EP (SEQ7) is as effective in causing membrane damage as UP using microsecond EP protocols (Fig. 2). Additionally, µsBP pulses resulted in significantly higher cell death (after 24 h) compared to µsUP pulses (Fig. 4), which supports the hypothesis that the BPC phenomenon is unique for nanosecond bipolar EP.

Ibey et al. pointed out that the remaining pulse duration above the indicated τ_{crit} is the time during which the number of pores increases (so-called holding time or passband) [32]. In the case of bipolar 600-ns pulses, a quick phase switch, where the membrane rapidly transitions from depolarization to repolarization, reduces the passband and, consequently membrane permeabilization intensity. Taken together, both µsEP protocols are effective in PM poration, nevertheless, the second and opposite polarity 600-ns pulse reduces the time of pores expansion in SEQ6. Elongating pulse duration to 10 µs enables long passbands to occur, which causes substantial undisturbed pores expansion [32]. However, it should be noted that according to the available knowledge, the cells start to depolarize once again with the phase switch.

Several studies describe the influence of asymmetric BP protocols on the maintenance or reduction of the BPC phenomenon [12,41].

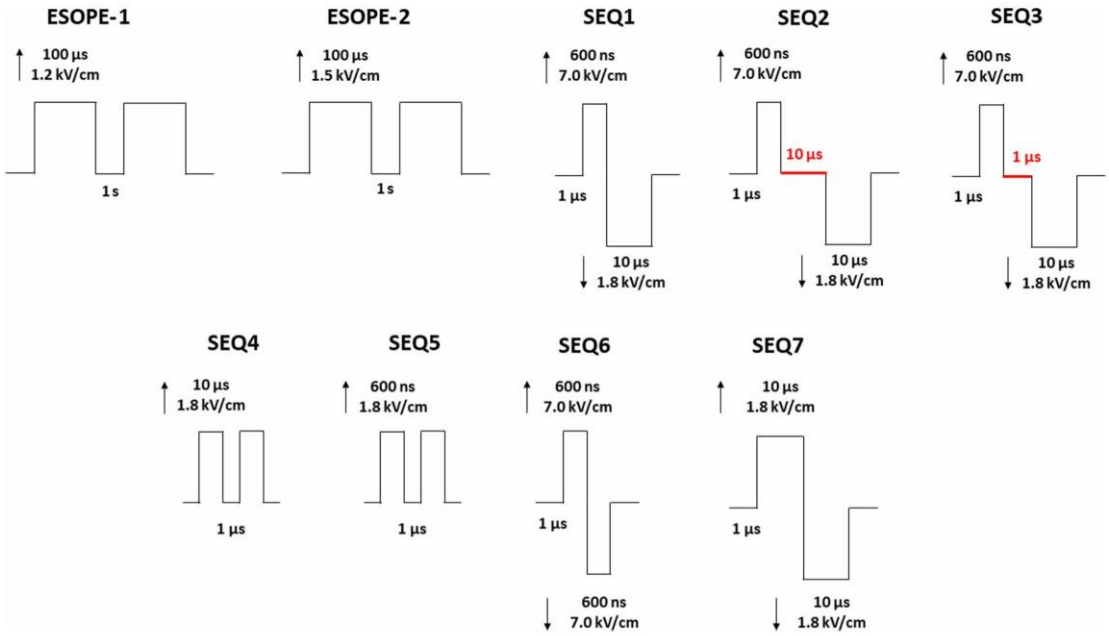

Fig. 7. The patterns of applied unipolar (UP) and bipolar (BP) pulses. The red color represents analyzed interphase intervals equal to 10 and 1 µs, respectively.

Applying a negative pulse longer than a positive one results in the reduction or complete elimination of the BPC effect. However, both articles focused on pulse duration $\leq$ 900 ns. Considering the dependencies described above, in this work, we examined the effectiveness of asymmetrical bipolar protocol conjugating nano- and microsecond duration pulses (SEQ1; ↑600 ns + ↓10 µs) on the BPC development. Obtained results indicated similar cell survival as for BP nsEP (Fig. 4). Of note, the asymmetrical SEQ1 caused statistically significant ($p < 0.005$) Yo-Pro-1^{TM} uptake reduction compared to UP EP (SEQ4 and 5) (Fig. 2). The authors assume that the delivery of a longer 10 µs pulse with opposite polarity concededly reduced the passband of the first, shorter pulse, however, the duration time of the second pulse enables the pores' expansion, manifesting in higher (compared to SEQ6) membrane poration. In conclusion, the addition of negative polarity, µs pulse may reduce the bioeffects triggered by the first positive polarity ns pulse. However, the emerging BPC phenomenon is weaker compared to those present after symmetrical BP nsEP.

During the design of the study, we speculated that the delay between the two asymmetrical pulses might have an impact on the cancellation process. Pakhomov et al. observed gradually diminishing the cancellation effects in CHO-K1 cells as the delay between pulses increased [30]. In their work, the interval between pulses were 0, 0.4, 1, and 10 µs, which allowed them to grasp the reduction of the BPC effect. As the delay duration reached 10 µs between the opposite polarity pulses (6.4 kV/cm), the PM permeabilization became about the same as in the case of a unipolar pulse. When the applied electric field increased to 7.5 kV/cm, the intracellular Ca^{2+} concentration was beyond the value obtained for the UP pulse. A similar tendency has been observed by Gianulis et al. using the same cell model, however, the authors used the 12 or 32 kV/cm electric field and observed the reduction of BPC as the interpulse interval increased up to 50 µs [31]. Moreover, Orlacchio indicated that cancellation of the Yo-Pro-1^{TM} uptake in the U87-MG cell line was observed only after bipolar pulses with no or very short delay < 30 ns (PEF up to 115 kV/cm) [42]. However, it should be noted that the studies described above have been focused on symmetrical bipolar pulses. In order to evaluate if the reversal of the effect is due to symmetry among the BP nsPEFs, we have exposed the OvBH-1 cell line to asymmetrical SEQ2 and 3, including 10 and 1 µs intervals, respectively. Obtained results indicated that BPC is present for asymmetrical electrical pulses involving both nsPEF and µsPEF. Moreover, the introduction of 1 or 10 µs delays between pulses with opposite polarity did not significantly affect this phenomenon. The same tendency has been observed for the cells electroporated with $CaCl_2$ (2.5 mM). Considering those findings, we may speculate that the duration time of applied interval between bipolar pulses may have a different impact on the BPC phenomenon occurrence depending on the type of the analyzed cells. Nevertheless, the results for symmetrical bipolar nsPEF obtained in this work show no controversy with already established knowledge about BPC [31].

As the last aspect of the presented research, we have examined the impact of the Ca^{2+} addition on the BPC effect. The intracellular transport of Ca^{2+} via electropores that occurred as a result of the application of the electrical pulse has been analyzed as an alternative for electrochemotherapy with cytotoxic drugs [34]. The addition of Ca^{2+} to EP buffer impeded the cell membrane permeabilization, except for the cells exposed to high-intensity bipolar symmetrical sequences (SEQ6 and 7). Reduced permeabilization in the presence of Ca^{2+} has been reported by Navickaite et al. [43,44] and thus agrees with established knowledge.

In the context of cell viability, a less toxic effect of BP nsPEF (SEQ6; ↑600 ns + ↓600 ns) has been detected due to BPC when compared to UP nsPEF (SEQ 5; ↑600 ns), in both analyzed groups of cells. It should be noted, that while achieving the same level of membrane permeabilization as in SEQ4 (↑10 µs; Fig. 2), SEQ5 (↑600 ns) resulted in a significantly higher rate of cell survival (Fig. 4.) We speculate that it may be caused by the different sizes of the pores occurring after the application of µsPEF and nsPEF. In the case of the µsPEF, the bigger size of the pores and a more significant electrophoretic component enables higher diffusion of Ca^{2+}, resulting in a lower survival rate.

Interestingly, in the case of SEQ1-4 and SEQ7 even though the measurements of the cells' membrane permeabilization have not been saturated, the MTT data revealed significantly lower survival rates (Fig. 4). The authors assume that the reason is the size of calcium chloride, which is considerably lower in comparison to the Yo-Pro-1 (630 Da) molecule. The main bioeffect of nsPEFs application is the nanometer-sized hydrophilic pores formation, which may be too small for detection by the uptake of conventional fluorescent dyes [45]. The results obtained in the presented study confirm this theory. In order to improve the sensitivity of cell membrane permeabilization detection,

Ca^{2+} and Ba^{2+} ions can be used in future works, as it was proposed by Bo et al. [45].

4. Materials and methods

4.1. Ovarian cancer cells culturing and preparation

The experiments were performed *in vitro* on a human ovarian clear carcinoma cell line (OvBH-1). It was established at the Department of Clinical Immunology, Wroclaw Medical University, Poland, from ascitic fluid cells of a 54-year-old patient histopathologically diagnosed with ovarian clear cell adenocarcinoma. Morphological and phenotypic characterization, as well as OvBH-1′s temperature sensitivity, has already been described by Bar et al. [46,47]. Cell culture in 25 or 75 cm^2 plastic flasks (Nunc, Roskilde, Denmark) was maintained at 37∘C, 5 % CO_2, and 95 % air humidity in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, St. Louis, MI, USA) supplemented with 10 % Fetal Bovine Serum (FBS; Sigma-Aldrich, St. Louis, MI, USA) 100 I.U./mL penicillin, and 100 I.U./mL streptomycins (Sigma-Aldrich, St. Louis, MI, USA). Cells passages were carried out once a week when confluency was about 70–80 %, washing with Dulbecco's Phosphate-Buffered Saline (DPBS; Sigma-Aldrich, St. Louis, MI, USA) and TrypLE™ Express Enzyme (GIBCO; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The culture medium was replaced 2–3 times a week.

4.2. Electric pulse exposure and PEF protocols

For electroporation, cells were trypsinized and centrifuged (5 min. × 1500 rpm; Centrifuge 5430 R, Eppendorf AG, Hamburg, Germany). Then the cells in the density of 2 × 10^6 cells/mL were suspended in HEPES EP buffer (10 mM HEPES (Lonza) × 250 mM sucrose × 1 mM $MgCl_2$ in sterile (MiliQ) water) or calcium chloride ($CaCl_2$) solution in HEPES EP buffer to 2.5 mM. The cells were placed in cuvettes with a 1 mm gap between electrodes (BTX, SyngenBiotech, Poland). BTX ECM 820 (Harvard Apparatus, Holliston, MA, USA) electric pulse generator was used to deliver ESOPE protocols, and a High-Frequency Bipolar Electroporator [48] for SEQ 1–7 was used to deliver symmetrical and asymmetrical shorter pulse sequences. ESOPE-1 and −2 protocols with an electric field equal to 1.2 and 1.5 kV/cm were used. The pulses were delivered in a burst of 8 with a repetition frequency of 1 Hz. Shorter microsecond and sub-microsecond pulses were delivered in a burst of 10 with a delay between separate waveforms of 1 μs. In the case of symmetrical and asymmetrical sequences, the delay between the positive and negative polarity of pulses was 0, 1, and 10 μs. The delays have been selected to account for the typical depolarization dynamics of cells in a low conductivity buffer, i.e., 0 μs to be in the supra-electroporation range, 1 μs to be on the edge of the typical cell depolarization time (partial depolarization), and 10 μs to grasp the BPC phenomenon when the cell had enough time to fully depolarize between the positive and negative phases of the pulse. The patterns of the applied UP and BP sequences are shown in Fig. 7.

4.3. Analysis of optimal $CaCl_2$ for OvBH-1 cell line

The cells were seeded on a 96-well plate with density 5 × 105/mL and left overnight to attach. Calcium chloride ($CaCl_2$) stock solution (100 mM) was prepared in pure Mili-Q water. The work solutions (0.5–25 mM) were prepared using a cell growth medium (DMEM). After overnight incubation, the culture medium was replaced by those including analyzed amounts of CaCl2. Cells were then incubated with the compound for 24 and 72 h, 37∘C, 5 % CO_2. Then, the MTT assay was performed to examine the activity of mitochondria as an amount of MTT dye converted to formazan crystals. Subsequently, after incubation, the culture medium was removed and replaced by 100 μL of 0.5 mg/mL MTT (Sigma-Aldrich, St. Louis, MI, USA) reagent at 37∘C, 5 % CO_2 for 2 h. Then, formazan crystals were dissolved by adding 100 μL of acidified isopropanol (38 % HCl in 99.7 % isopropanol). Finally, the absorbance was measured at a wavelength equal to 570 nm using the multiplate reader (GloMax® Discover Microplate Reader, Promega, Walldorf, Germany).

4.4. Cells permeability quantification

The efficiency of electroporation protocols was assessed by the amount of the green-fluorescent dye Yo-Pro™-1 ($\lambda_{exc}491/\lambda_{em}509$, Thermo Fisher Scientific Inc., Warsaw, Poland) absorbed by the cell. Prior to the electric pulse application, cells were trypsinized and suspended in HEPES ± $CaCl_2$ EP buffer, and 100 μL/L Yo-Pro™-1 was added to the cells' suspension. After EP, cells were incubated for 5 min., room temperature (RT), then centrifuged and suspended in 400 μL 0.9 % NaCl saline solution for measurements in polystyrene FACS tubes, 300 μL/sample were measured in triplicates. The samples were excited using the 488 nm line of the blue laser, and the fluorescence of the dye was measured with an FL-1 detector. The control samples without treatment were used as a negative control for gate definition. After permeabilization, depending on the applied protocol, a fluorescent spectrum shift due to dye uptake was observed. Flow cytometric analysis was performed using CyFlow CUBE-6 flow cytometer (Sysmex, Poland).

4.5. Analysis of cells viability after PEFs protocols application

PEFs protocols were performed according to the procedure described in paragraph 4.2. After pulsing, the cells were seeded on 96-well plates with a density of 5 × 10^5/mL, incubated at room temperature (RT) for 10 min. Then, the growth medium (DMEM) was added to all analyzed groups of cells and cells were incubated at 37∘C, 5 % CO_2, and 95 % air humidity for 24 and 72 h. After incubation, the culture medium was removed, and the MTT reagent was at 37°C, 5 % CO_2 for 2 h. The MTT assay was performed accordingly to the procedure described in section 4.3. The results have been expressed as a percentage of viable cells relative to cells untreated with electric pulses.

4.6. β-Tubulin staining

The cells were electroporated as it was described in paragraph 4.2. Subsequently, the cells were seeded on glass coverslips (2 × 10^6/mL) placed in 6 well plates (in density analyzed on the basis of cell viability results), and incubated for 10 min., RT. Then, the growth medium (DMEM) was added to all analyzed group of cells and the incubation for 24 h at 37 ∘C, 5 % CO_2 took place. After that time, the growth medium was removed and cells attached to slides were fixed using 4 % paraformaldehyde (PFA) in PBS (BioShop, dist. Lab Empire, Rzeszow, Poland) for 10 min., RT. Cells permeabilization was performed by 1 % Triton X-100 in PBS (v/v) for 10 min., RT, and blocked with 1 % Fetal Bovine Serum (FBS) in PBS for 1 h, 37 °C, 5 % CO_2. Then cells were incubated for 1 h, 37 °C, 5 % CO_2 with primary antibody, anti-β-tubulin (1:300, Cat. #ab108342, Abcam, UK) diluted in PBS. Subsequently, samples were washed with PBS three times and incubated with Goat anti-Rabbit IgG coupled to Atto 488 (1:200, Cat. #18772, Sigma-Aldrich, St. Louis, MI, USA) for 1 h at 37 °C, 5 % CO_2 followed by further three washes in PBS. For the nuclei visualization and cell mounting Fluorshield™ (Cat. #F6057, Sigma-Aldrich, St. Louis, MI, USA) with DAPI (4,6-diamidino-2-phenylindole) was applied. The samples were observed using Olympus FluoView FV1000 confocal laser scanning microscope (Olympus, Tokyo, Japan). Eight images (n = 8) for each slide (i.e. each parameter) were taken to cover the entire microscope slide area for analysis. Figs. 5 and 6 show representative images that capture the structure of 70–80 % of the cells present on each slide.

4.7. Statistical analysis

The experiments were performed in 3 replicates. The statistical

analysis was performed using GraphPad Prism 8 (GraphPad Software Inc, San Diego, CA, USA). Data are expressed as mean ± SD (standard deviation) of the mean and were analyzed by two-way ANOVA (analysis of variance), with $p < 0.05$ being considered statistically significant. Sidak's multiple comparison test for the evaluation of the difference was used when ANOVA indicated a statistically significant result and $p < 0.05$ was considered statistically significant.

5. Conclusions

In the presented study, the authors show the BPC effect presence after exposure of ovarian clear carcinoma cell line to asymmetrical (↑600 ns + ↓10 μs) PEF protocols. The obtained results enable us to confirm the presence of BPC even for pulses from different duration ranges. It implies that in the case of asymmetrical sequences, the cancellation phenomenon is not limited to nsPEF protocols. Also, it was shown that the BPC effect is present even when the cells had enough time (10 μs) to depolarize between the positive and negative phases of the symmetrical and asymmetrical pulses.

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Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: 'Julita Kulbacka reports financial support was provided by National Science Centre Poland. Vitalij Novickij reports financial support was provided by Research Council of Lithuania.'

Data availability

No data was used for the research described in the article.

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Praca 3

Tytuł

"Electroporation and electrochemotherapy in gynecological and breast cancer treatment"

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Review

Electroporation and Electrochemotherapy in Gynecological and Breast Cancer Treatment

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Abstract: Gynecological carcinomas affect an increasing number of women and are associated with poor prognosis. The gold standard treatment plan is mainly based on surgical resection and subsequent chemotherapy with cisplatin, 5-fluorouracil, anthracyclines, or taxanes. Unfortunately, this treatment is becoming less effective and is associated with many side effects that negatively affect patients' physical and mental well-being. Electroporation based on tumor exposure to electric pulses enables reduction in cytotoxic drugs dose while increasing their effectiveness. EP-based treatment methods have received more and more interest in recent years and are the subject of a large number of scientific studies. Some of them show promising therapeutic potential without using any cytotoxic drugs or molecules already present in the human body (e.g., calcium electroporation). This literature review aims to present the fundamental mechanisms responsible for the course of EP-based therapies and the current state of knowledge in the field of their application in the treatment of gynecological neoplasms.

Keywords: gynecological cancer; breast cancer; electrochemotherapy; electroporation; calcium electroporation

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1. Introduction

Despite growing awareness, screening, and intensive research into developing new therapeutic strategies, cancer remains one of the leading causes of death globally, and the number of diagnosed cases is increasing every year [1–4].

Gynecological cancers (GCs) are defined as those that originate in women's various reproductive organs and affect women mainly in the age range of 30–75 years [5,6]. The most aggressive GCs encompass cervical, ovarian, and endometrial neoplasms [6]. Each of them is characterized by individual risk factors, epidemiology, molecular pathways, symptoms, and treatment strategies. According to the significant heterogeneity of this group of malignancies, the use of different diagnostic and treatment combinations is necessary [7]. The first-line treatment for a major group of GCs includes chemotherapy (CT) which is associated with significant side effects. The fact that a wide range of patients are diagnosed when the disease is in an advanced stage of development, and the treatment methods for progressive GCs remain limited, means that gynecological neoplasms are associated with a high mortality rate (Figure 1).

It was decided to also consider therapeutic strategies for breast cancer (BC) in the presented review. This is because, despite BC not being categorized in the GC group, it has a considerable impact on women's lives. According to WHO (World Health Organization) statistical data, in 2020, BC had a worldwide incidence of ~2.3 million new cases and a mortality rate of nearly 700,000 deaths [1].

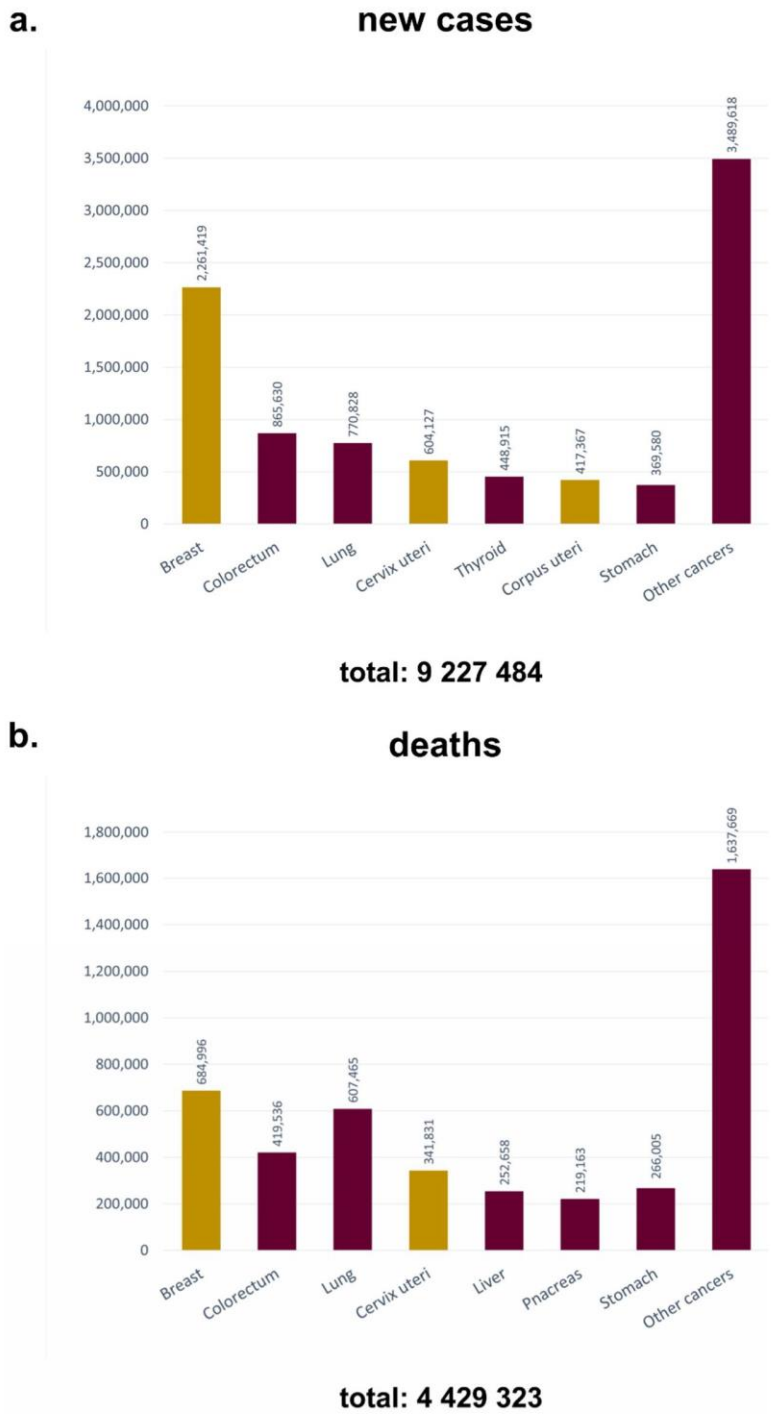

Figure 1. Graphs present reported Globocan 2020 data. (**a**) the number of new cases and (**b**) deaths in 2020 for women aged 0–85+ [8].

Forecasts for 2040 almost double these numbers. Therefore, considering all the issues referred to above, there is an urgent need to look for new and more effective solutions that will help eliminate, among others, the issue of multi-drug resistance of neoplasms and to reduce the number of side-effects affecting patients [4]. Hope has been provided by treatment methods based on the phenomenon of EP, which has been developing rapidly in recent years. EP-based therapies are already used to treat cutaneous and subcutaneous

tumors and deep-seated tumors [9–13]. Their application to gynecological and breast carcinomas is still under development and requires further investigation. However, in the presented review, we have sought to summarize current knowledge and progress made.

2. Electroporation and Electroporation-Based Treatments in Oncology

2.1. The Brief Theory of Electroporation

Electroporation (EP) is a biophysical phenomenon based on the use of pulsed electric fields (PEFs) resulting in increased plasma membrane (PM) permeability. Kotnik et al. have rightly pointed out that the narrower term 'electroporation' and the more general 'electropermeabilization' are often misused as synonyms [14]. For a detailed explanation of this issue, please refer to the mentioned review. EP has found application in a wide range of disciplines, including the food industry [15], biotechnology [16,17], and medicine [12,18–20]. In 1982, Neumann et al. demonstrated that the use of EP to temporarily increase plasma membrane permeability enabled the transport of DNA or ordinarily non-permeable molecules, e.g., cytotoxic drugs, into the cell's interior [21]. This paper initiated the medical application and broader study of EP. In later years, both techniques gained popularity under the names gene electrotransfer (GET) and electrochemotherapy (ECT), respectively [22,23]. These and other treatment solutions based on EP are precisely described in the following sections of the review.

Even though the exact mechanism of EP has not yet been fully elucidated, several hypotheses describing the events underlying this phenomenon have been proposed, including conformational changes of the phospholipid bilayer [24–28], its phase transition [29], denaturation of membrane proteins [30], and lipid oxidation affecting a wide range of PM properties [24,25,31]. However, scientists have come to a consensus that cell exposure to high-amplitude electric fields of sufficient duration results in the spontaneous formation of aqueous nanopores in PMs [14,18,32–34]. Furthermore, there is growing evidence that exposure to electrical impulses leads to chemical changes in lipids and membrane protein functions, resulting in increased membrane permeability [14].

Physiologically, every cell maintains a resting transmembrane voltage (TMV) ranging from −70 to −40 mV [14]. This term describes an electric potential difference between the inner and outer spaces of the PM, resulting from the action of the ion pumps and channels located in the PM, e.g., Na^+ and K^+ active and passive transport. When the cell is exposed to an external electric field, an induced transmembrane voltage (iTMV), denoted by $\Delta\Psi_m$, occurs that is proportional to the strength of the external electric field and exists as long as the field is present [35].

The iTMV value for regularly shaped cells (spheroids, cylinders, etc.) with a nonconductive membrane, and which are sufficiently distant from each other, can be expressed by an explicit formula, referred to as Schwan's equation [18]:

$$\Delta\Psi_m = f\,ER\cos\theta\,(1 - e^{-t/\tau})$$

where $\Delta\Psi_m$ is the induced transmembrane voltage, f is a dimensionless factor, E is the homogeneous electric field strength, R is the cell radius, θ is the angle measured from the center of the cell concerning the direction of the field, t is the time elapsed since the onset of the field, and τ is the time constant of membrane charging.

The application of a series of fields of adequate strength causes the occurrence of transmembrane voltages far exceeding the cell's physiological range [14,36,37]. The effects of this event are rearrangements in the phospholipid bilayer and the formation of nanopores, as mentioned above. Ordinarily, a series of rectangular electrical pulses are applied to permeabilize the cell membrane [38]. The general scheme of the EP process is shown graphically in Figure 2.

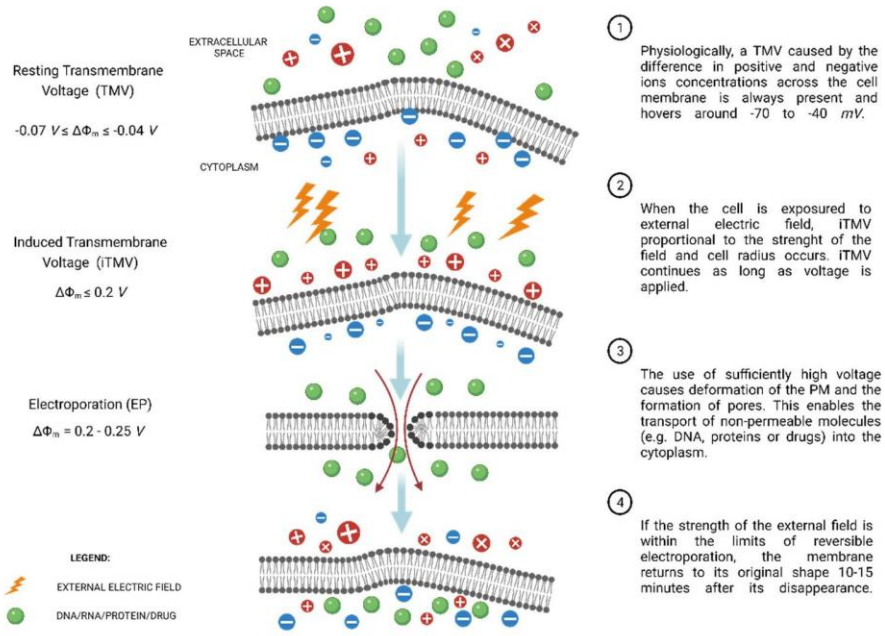

Figure 2. Conceptual scheme of electroporation/electrochemotherapy mechanism [14,39,40].

There are two types of EP, depending on pulse duration, electric field intensity, and cell features (e.g., size, membrane curvature) [25]. Aguilar et al. refer to a "two-threshold existence". The former is the iTMV, beyond which the phenomenon of reversible electroporation (RE) occurs. Cells can repair and close the formatted pores, reestablish metabolism over time and survive [41]. RE enables the enhanced transport of medications, gene material, small exogenous proteins, etc., without significantly affecting cell viability. The second critical iTMV is the transmembrane potential value beyond which RE becomes irreversible (IRE) [25]. It should be noted that the critical iTMV value is cell-specific. Disturbances occurring in the PM are irreversible for the cell, affecting its homeostasis and ultimately resulting in cell death. Both methods are used in clinical practice and are described more fully in the following subsections.

Depending on the duration of the pulses, three main types of EP protocol may be distinguished: nanosecond, microsecond, and millisecond [42]. The main principle is identical for all of them and is based on the PEFs used to increase the CM permeability. However, the protocols differ in their electric field parameters, influence on targeted cells or tissues, and their range of applications in clinical settings.

Nanosecond pulsed electric field (nsPEF), also known as nanopulse stimulation, is an intensely analyzed anticancer technology [42]. This technique is based on the use of external, ultrashort (nanosecond duration), and high-voltage (kV/cm) pulses [43,44], and is characterized by low energy and non-thermal effects [45]. The primary mechanism is similar to the other PEF methods mentioned above. However, the chain of events linking the initial events occurring after nsPEF application, e.g., membrane permeabilization, with the final effects, is still not fully understood [46]. nsPEFs lead to non-stable nanoscale pores forming in the plasma membrane [47]; however, unlike other PEF methods, nanopulse stimulation can induce extra- and intracellular membrane penetration [42]. This is caused by the fact that the pulse rise time reaches full amplitude before intracellular or intraorganellar charges can redistribute it to cancel the applied field [42]. In other words, the pulse duration is shorter than the cellular membrane charging time constant [44]. The effects of this phenomenon include the following, among others: activation of signaling pathways [48,49], calcium release from the affected endoplasmic reticulum [50], dissipation of mitochondria membrane potential [51,52], cytoskeleton destruction [46,53], cell swelling and blebbing [54], and induction of cell apoptosis or necrosis [55–58]. Some articles have

reported that nsPEFs induce platelet aggregation [59], and bacterial cells exposed to nsPEFs show lethal and sublethal effects [60].

For cells characterized by irregular shape and which are closely grouped, iTMV cannot be determined analytically, and numerical solutions must be applied [18,36,61]. An alternative involves the use of potentiometric dye [62,63]. During the application of EP-based technologies to biological tissues, their passive electric properties, such as permittivity and conductivity, should be considered [64]. Both depend on the attached electric field frequency. However, tissue permittivity is inversely proportional to the frequency value, not conductivity. Moreover, when CM reaches a state of permeabilization, its conductivity increases, due to deeper structures being electroporated by pulses of lower strength [65].

Tissues are significantly heterogeneous structures, and cells characterized by different sizes, shapes, and functions may be suspended in a more minor (e.g., epithelial tissue), or larger (e.g., bones), the volume of the extracellular matrix [64]. Moreover, tissue is surrounded by elaborate blood vessels and nerves; hence, it is difficult to anticipate their EP effects. Miklavčič et al. point out that some tissues (e.g., bone or skeletal muscle) are distinctly anisotropic; therefore, during the analysis of conductivity and permittivity values, the orientation of the electrodes relative to the tissue axis (e.g., longitudinal, transverse, or their combination) needs to be checked [64]. In fiber-organized tissues (e.g., muscles), longitudinal conductivity is significantly higher than transverse conductivity. This is because in transverse electrode orientation, the charge must overcome the extracellular matrix, which has a lower conductivity compared to cells. Moreover, tissue anisotropy is frequency-dependent, and, above a certain threshold, the anisotropic properties disappear (for muscles, in the MHz frequency range). This process, and the mechanism of tissue electrical property changes depending on illness and physiological deprivations, have been thoroughly described by Miklavčič et al. [64].

Given the circumstances described above, each aspect should be considered individually for every patient during treatment planning. Clinicians use computer simulation tools to select the appropriate electrodes, plan their placement in the targeted tissue, calculate the electric field temperature distribution, and develop the optimal protocol [66–68].

Technologies that enable the observation of electroporated tissue include electrical impedance tomography (EIT) [69,70] and magnetic resonance electrical impedance tomography (MREIT) [71]. Changes occurring after EP-based therapy can also be observed by computed tomography (CT) and magnetic resonance imaging (MRI) [72].

2.2. Irreversible Electroporation (IRE)

Irreversible electroporation (IRE) is a physical, non-thermal cancer therapy that leads to cell death via permanent membrane permeability [73] and was first proposed as a novel ablation method by Davalos et al. in 2005 [74]. As described above, the primary mechanism of IRE is based on irreversible PEF use characterized by the strength significantly exceeding a permeabilization critical threshold value [25,74]. Cells exposed to this kind of PEF are not able to restore the original plasma membrane conformation and enter the path of cell death.

Ablation areas created by IRE are characterized by clear, well-defined boundaries, which allow precise control of the ablation zone and non-ablated tissue [75]. One of the significant advantages of IRE is that it does not require the presence of chemotherapeutic drugs [74]. This is important given the current need to reduce the number of undesirable side effects and to limit the off-target toxicity associated with conventional cancer therapies. Weaver et al. point out that IRE maintains high efficiency in tumor areas conveniently located in blood vessels that provide cooling and are not limited by the heat sink effect [38,76]. As a result, IRE is a method that effectively destroys neoplastic cells that could otherwise survive treatment with other thermal ablation methods used. Moreover, literature reports suggest that, unlike other thermal ablation technologies, IRE does not lead to the destruction of connective tissue or denaturation of collagenous and other protein and/or lipid-based structures and may

be applied to the treatment of tumors localized closely to essential structures, such as bile ducts [32,77–80].

Zhang et al. have thoroughly summarized the local and systemic immune response mechanisms induced by IRE [81]. It has been shown that IRE causes the significant release of intracellular tumor antigens, becoming an "in situ tumor vaccine." This observation may be used to generate an anti-tumor immune response that destroys tumor cells after ablation. This could reduce local recurrence and would also eliminate distant metastases. In light of this, Zhang et al. suggest that IRE may be regarded as a potential immunomodulatory therapy and that its combination with immunotherapy may result in synergistic effects, potentially widening the field of application of the IRE method in the clinic [81].

It has been suggested that the mechanism underlying the ability of IRE to affect only metastatic cells membranes is related to transmembrane voltage [82–84]. The iTMV of cancer and non-cancer cells depolarizes during proliferation, reaching a value of −15 mV. The non-cancer cell TMV value returns to −70 mV after mitotic division, in contrast to cancer cells, where the value reaches −25 mV, so the TMV is required to reach iTMV, and the critical threshold of permeabilization, is lower compared to non-cancer cells. It may be caused by the disruption of PM lipids and sterol construction and the consequential influx of sodium ions (Na^+) into the interior of the cell with negative charge accumulation [84]. Almost a year later, Blackiston reported that modifications in chloride, sodium, potassium, and calcium channel activity also impact depolarized cancer cell TMV [85].

In recent years, the use of IRE in the clinic has increased significantly, which is reflected in the high number of clinical trials conducted using this technology (almost 53 studies with active status registered in the clinicaltrials.gov database). Moreover, the combination of IRE and immunotherapy has also been evaluated (#NCT04212026; #NCT04612530).

2.3. Electrochemotherapy (ECT)

The phenomenon of electrochemotherapy (ECT) was first defined by Mir et al. in 1991 [86]. ECT constitutes the intravenous or intratumoral administration of chemotherapeutic agents and the exposure of cell membranes to high-intensity, well-dosed electric pulses leading to RE [41]. The increased CM permeability enables the diffusion of low- or non-permeable drugs into the cell cytosol. Initially, ECT was intended to treat small superficial neoplastic lesions that were not amenable to surgery or radiotherapy [12,86,87]. In 2006, European guidelines for the use of ECT termed the European Standard Operating Procedures of ECT (ESOPE), and standard operating procedures (SOP), were published [88,89]. Since then, electric pulses have been delivered in a sequence of eight pulses, each 100 µs-long [90].

As a general rule, patients receiving ECT treatment first receive an intravenous or intratumoral administration of an anticancer drug to distribute over the targeted tissue, and then the application of the electric pulses takes place [41].

The main advantage of ECT is the significant reduction in chemotherapeutic dose due to the locally potentiated cytotoxic effect [41,91]. Researchers have analyzed a wide range of cytostatics, but the most satisfying results were obtained for bleomycin (BLM) and cisplatin (CSP), with toxicity increases on average of 1000- and 80-fold, respectively [86,90,92–94]. However, some articles have also reported the promising use of the natural compounds, doxorubicin, and 5-fluorouracil [95–98].

It has been shown that cells damaged after ECT release substances involving intact tumor antigen secretion [41]. Consequently, the patient's immune system activates a tumor antigen-directed immune response, and a so-called 'abscopal effect' occurs, while, as a result of ECT, a systemic immune response will be triggered against distant metastases [41,99]. Other ECT effects observed include vascular disruption, hypoperfusion, decreased blood flow, and increased drug retention time due to decreased blood flow [41]. Several studies have shown that malignant cells were much more sensitive to ECT using BLM than normal cells [88,100,101]. Notably, Frandsen et al. demonstrated that ECT did not affect the size of normal fibroblast spheroids [102].

Due to the satisfying tolerance, promising results, and technological evolution of EP tools, ECT is an increasingly widely used method in oncological treatment, with new modifications being developed (e.g., calcium electroporation) [12].

2.4. Calcium Electroporation (CaEP)

Calcium electroporation (CaEP) is a novel modification of conventional ECT, in which chemotherapeutic treatment has been replaced by supraphysiological doses of calcium ions (Ca^{2+}), as reported for the first time by Frandsen et al. in 2012 [103]. The applied EP parameters are similar to those used for ECT [104]. Solutions based on the use of non-toxic molecules are significant for clinical application, considering the side effects caused by most cytotoxic drugs (e.g., bleomycin) [102]. Moreover, EP with prior Ca^{2+} application is characterized by a long durability and lower costs [105]. CaEP's effectiveness for the treatment of different carcinomas has already been demonstrated in in vitro and in vivo studies [102,104–111]. In combination with ECT, CaEP is currently used in more than 140 clinics in Europe as an anticancer treatment modality [112].

Calcium is a ubiquitous second messenger involved in many cellular processes, from transcription regulation and proliferation to cell death [102]. Under physiological conditions, there is a 10–20,000-fold Ca^{2+} concentration gradient between the extra- (10^{-3}–10^{-2} M) and intracellular (10^{-8}–10^{-7} M) spaces, which is strictly regulated [109,112]. Monteith et al. have precisely described aspects of the Ca^{2+}-cancer signaling nexus and its role as a therapeutic target [113]. In 2012, Frandsen et al. proposed the CaEP mechanism of action [103]. In subsequent years, it has been intensively investigated, supported, and precisely described by Frandsen et al. in 2020 [112].

The primary mechanism of action is based on the sudden and massive influx of Ca^{2+} following EP, resulting in intracellular calcium homeostasis disturbances, as illustrated in Figure 3 [112]. Seeking to re-establish the balance, the cell activates the ATP-dependent: Na^+/Ca^{2+}-exchanger (NCX), $Na^+/Ca^{2+}/K^+$-exchanger (NCKX), and plasma membrane calcium ATPase (PMCA) to remove calcium excess from the cell (Figure 3). Simultaneously, the electrochemical gradient essential for ATP production is disturbed due to calcium overload, resulting in ATP production inhibition and ATP loss through pores created in the cell membrane [114]. Together, these events are the main cause of cell death in the wake of absolute ATP depletion, as reported in preclinical investigations [103,105,115]. Additional effects, such as lipase and protease activation and reactive oxygen species production (ROS), have also been reported [114].

Gibot et al. showed that, unlike ECT using BLM or CSP, CaEP does not induce genotoxicity, and its cytotoxicity is associated with ATP depletion and significant narrowing of the membrane potential [111]. Available literature sources report different types of cell death caused by CaEP therapy, depending on factors such as the cell type and morphology, time of exposure to EP, calcium concentration, etc. [112], including apoptosis, [116,117] though, necrosis is the predominant mechanism [110,115].

CaEP, as for ECT, did not affect the size of the regular spheroids and induced cell death in cancer cells more effectively [102]. Simultaneously, CaEP triggered a dramatic decrease in intracellular ATP levels in normal and malignant spheroids. Therefore, the effect of CaEP on the intracellular ATP level cannot explain the difference in cell sensitivity. However, Frandsen et al. pointed out that normal cells seem to survive this ATP depletion, whereas metabolically active malignant cells do not [103]. The differences in the vulnerability of cancer and normal cells to CaEP in other in vitro models were also previously investigated [116,117]. Notably, Frandsen et al. also indicated that normal cells could extrude the extra calcium concentration and restore it to a similar level of untreated controls approximately 4 h after treatment [110].

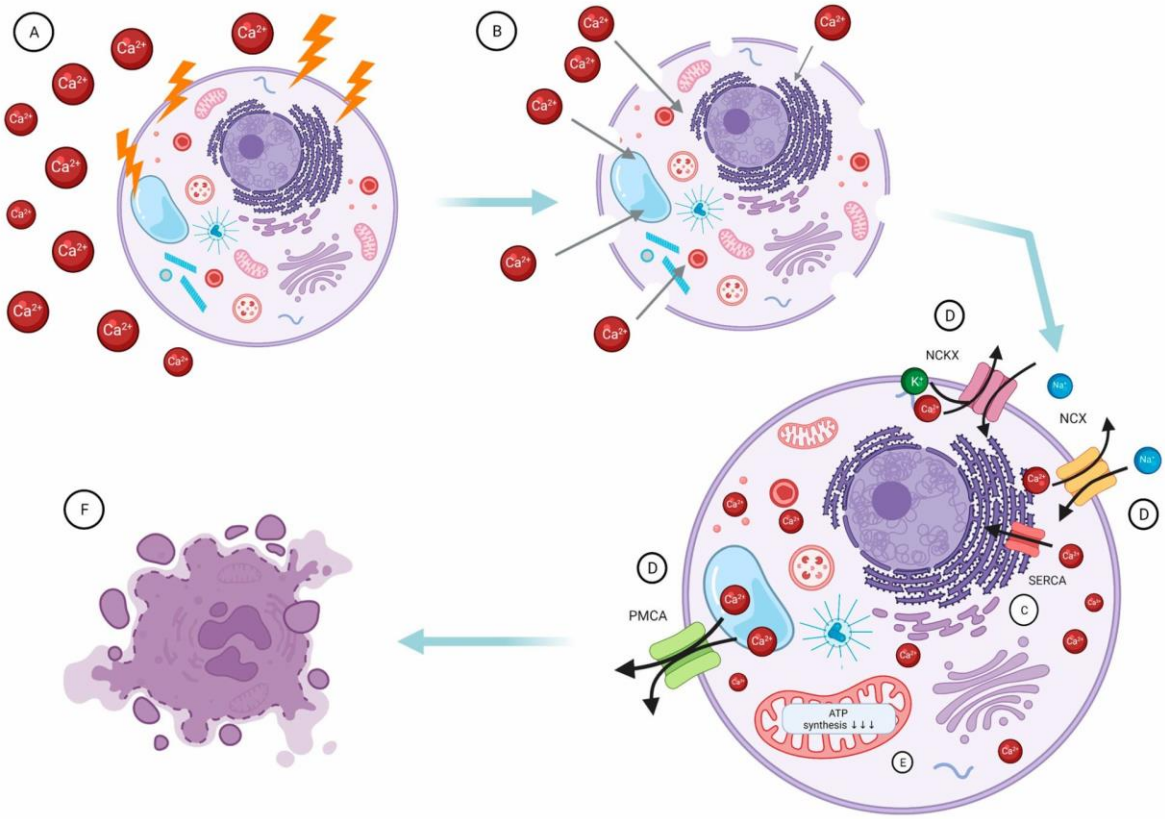

Figure 3. The general mechanism of calcium electroporation (CaEP). The concentration of calcium on both sides of the cell membrane (CM) is tightly controlled. The CaEP mechanism of action involves (**A**) application of Ca^{2+} supraphysiological concentration. (**B**) Subsequent application of electrical pulses which increases the permeability of the CM, which allows calcium introduction into the interior of the cell. (**C**) Ca^{2+} transportation to the mitochondria and the endoplasmic reticulum by sarco-endoplasmic reticulum calcium ATPase (SERCA). (**D**) Disruption of calcium homeostasis triggers the enhanced activity of the sodium-calcium potassium exchanger (NCKX), the sodium-calcium exchanger (NCX), and ATP-dependent plasma membrane calcium ATPase (PMCA) to extrude the extra Ca^{2+} from the cell. (**E**) At the same time, an increase in the Ca^{2+} concentration inhibits the process of ATP synthesis, leading ultimately to the complete use of its resources and cell death (**F**) [112,114].

In 2018, Falk et al. performed the first clinical trial using CaEP to examine its effectiveness in small cutaneous metastases therapy [106]. Currently, six trials (according to the clinicaltrials.gov database, searching for "calcium electroporation") are registered or are ongoing using CaEP, including for cutaneous and subcutaneous malignant tumors (#NCT04259658; #NCT04225767) and basal cell carcinoma (#NCT05046262). Interestingly, the use of calcium gluconate as a source of Ca^{2+} is being analyzed in a pilot study involving patients with non-curable esophageal cancer (#NCT04958044). Clinical trials involving CaEP as a therapeutical option for gynecological and breast cancer are presented in Tables 1 and 2, respectively.

2.5. Gene Electrotransfer (GET)

In recent decades, a significant evolution of gene therapies (GT) has occurred, as a result of which the treatment of hitherto non-curable diseases has become possible. GT aims at DNA or RNA transport to the targeted cell interior to genetically modify the cell by producing a protein or silencing defective or overexpressing genes [118]. This requires the genetic material to overcome several barriers, including cell membranes, nucleic barriers,

etc. Therefore, the search for vectors that would facilitate this transport was initiated. The vectors which are most widely evaluated are viral vectors, employing their ability to infect and introduce gene material into the host cell [119]. Although viral vectors show satisfactory clinical efficacy, their use is associated with several drawbacks, including pre-existing immunity and the possible risk of immunotoxicity resulting from immune response activation following viral vector injection [118,120]. This situation has forced the abandonment of specific viral vectors in some countries and limits the method's universality in using the same vector for each patient.

An alternative solution is the application of pulsed electric fields (PEFs) following naked DNA administration, termed gene electrotransfer (GET) or electrogenetherapy (EGT) [95]. Previous studies have indicated that the use of PEFs significantly enhanced gene expression compared to results obtained after naked DNA administration alone [121,122]. The GET mechanism is an elaborated and multistage process. Subsequent to PEF application, negatively charged DNA interacts with the cell membrane opposite to cathode areas [118]. PEFs, in addition to causing increased permeability of PMs, also electrophoretically push DNA towards the cell membrane. According to the available literature, the PEF strength required for DNA entrance into the cells needs to be equal to, or higher than, that required for PM electroporation [118,123,124]. In 2010, Faurie et al. indicated that DNA diffusion across the PM could take from a couple of minutes to several hours [125]. Sachdev et al., in a review, described two possible DNA entry pathways [118]. The most supported, and scientifically accepted, pathway involves the formation of DNA aggregates enclosed in the cell membrane's vesicles. In this form, the DNA is introduced into the cell via endocytosis.

However, several defects have been highlighted for the existing method. Frequently, high voltage pulses with long duration times (millisecond pulses) are required to achieve efficient genetic material delivery [121,122,126]. However, tissue damage may occur; on the other hand, reduction in voltage inhibits transfection efficiency. The conjunction of lower voltage electrotransfer with exogenously applied heat has been reported as a solution; however, it should be noted that this method requires more specialized devices (e.g., IRE laser heating).

In 2008, the first clinical trial using GET to enhance IL-12 (interleukin 12) administration in metastatic melanoma patients was conducted by Daud et al. [22]. Almost 25 clinical trials (according to the clinicaltrials.gov database, searching "electroporation" "vaccine") are currently being enrolled or are ongoing to evaluate the effectiveness of PEF-mediated delivery of DNA vaccines, including an HPV DNA vaccine for HPV16-positive cervical neoplasia (#NCT04131413), an HPV DNA vaccine (VGX-3100) for patients with HIV-positive high-grade anal lesions (#NCT03603808), a DNA vaccine against Puumala virus (PUUV) and Hantann virus (HTNV) (#NCT03718130), etc. Interestingly, three GET-based SARS-CoV-2 DNA vaccines are under investigation (#NCT05102643; #NCT04788459; #NCT04447781). Moreover, almost 11 active or recruiting studies were found in this database using the search terms "electroporation" or "immunotherapy". The analyzed immunotherapies mainly concern neoplasms, including pancreatic cancer (#NCT04835402), melanoma (#NCT04526730), hepatocellular carcinoma (#NCT03630640), acute myeloid leukemia (#NCT03083054), glioblastoma (#NCT03491683), etc.

Based on ample evidence, EP-based technologies have shown that tumors can be successfully eliminated without recurrence and with significantly lower side effects [127–129]. Table 1 presents the summarized merits and demerits of EP-based therapy methods described above. In the following subsections of this review, we summarize the most recent reports on the use of therapeutic methods based on the phenomenon of EP in the treatment of gynecological and breast cancer.

Table 1. Summarized merits and demerits of electroporation-based treatment methods.

EP-Based Method	Merits	Demerits	Ref.
Electrochemotherapy (ECT)	• applied at all stages of the cell cycle • enhanced cytotoxic drug transport into the cell interior • lower cytotoxic drug doses introduced into an organism • involving intact tumor antigen secretion	• muscle contractions • acute pain • vascular disruption • hypoperfusion • decreased blood flow • increased drug-retention time	[18,41,130,131]
Irreversible electroporation (IRE)	• non-thermal tissue ablation • applied at all stages of the cell cycle • well-defined ablation area • does not require chemotherapeutic drugs • destroys structures not sensitive to other thermal ablation methods • does not damage connective tissue, collagenous, protein, and lipid-based structures • potential immunomodulatory therapy	• muscle contraction and acute pain	[32,74,75,77,79,132]
Calcium electroporation (CaEP)	• applied at all stages of the cell cycle • does not involve cytotoxic drugs • improvement of patient's quality of life • does not involve genotoxicity • decreased toxic effects on normal cells	• muscle contraction and acute pain	[102,111,112,130]
Gene electrotransfer (GET)	• does not involve viral vectors • allowing DNA macromolecule transfer	• possible to apply only on a small area, • surgical intervention is needed when transferring to internal organs • high voltage pulses with long duration times (ms pulses) required • possible tissue damage • non-target specific • causing some vehicle damage e.g., quantum dot aggregation	[122,124,133,134]

3. The Use of EP-Based Strategies in Gynecological Malignancies

3.1. Ovarian Cancer

Ovarian cancer (OC) is the most fatal of all female reproductive cancers, with 313, 959 new cases and 207,252 deaths reported in 2020, and a 5-year survival rate of ~48% [1,135,136]. OC rarely affects women under 30, and the risk increases with age [135]. The risk increases significantly over the age of 50. In 2019, Momenimovahed et al. reviewing studies, including age at diagnosis, observed that the median age was 50–70 years old, depending on the population [137]. OC is determined as a "silent killer" because it is diagnosed when the first symptoms appear; these symptoms can be vague (often being confused by patients for gastrointestinal complaints), and, in a large number of cases (nearly 70%), appear at advanced stages III or IV of the disease [135]. Moreover, OC, together with endometrial cancer, is characterized by poor accessibility to specimen collection, which significantly hinders the early diagnosis of these diseases [7]. Therefore, as Holcakova et al. rightly pointed out, there is an urgent need to look for new biomarkers since population screening does not exist [7].

Conventional treatment of OC involves a combination of tumor debulking surgery with the surgical staging of the affected tissue and subsequent CT [138]. The overall survival of OC patients has not improved significantly despite numerous studies focusing on this disease, progress in surgical intervention, and the development of platinum-based CT and advanced molecular-targeted therapies, such as bevacizumab and olaparib [139]. For years the medical community has had to deal with patient relapses after CT, numerous side effects, and the phenomenon of multi-drug resistance [138]. Therefore, it is necessary to constantly investigate the molecular mechanisms responsible for the development of the disease and to look for new therapeutic solutions.

The effective improvement of conventional CT (with BLM) by EP on ovarian cancer CSP-resistant cell lines (OvBH-1 and SKOV-3) was observed by Saczko et al. [138]. The authors used EP protocols ranging from 0.8 to 1.0 kV/cm × 100 µs × 1 Hz × 8 pulses preceded by suspending the cells in BLM solution prepared in EP buffer. Interestingly, Saczko et al. also compared the effectiveness of ECT with CSP to ECT using 5-fluorouracil (5-FU) on the same OC cell lines [96]. The obtained results revealed significant enhancement in the transport of both drugs after EP application. This observation is all the more intriguing as both lines are resistant to standard CSP-based CT. Resistance is the main problem in CSP-based CT and it is desirable to develop new solutions.

Our previous article demonstrated the enhanced cytotoxic effects of CaEP (2.5 mM) against the MDAH-2774 OC cell line compared to ECT with CSP (25 µM) [140]. Three different EP protocols were used: (1) 1.3 kV/cm × 100 µs × 100 Hz × 8 pulses (ESOPE); (2) 37.5 kV/cm × 10 ns × 1 Hz × 200 pulses; and (3) 50 kV/cm × 10 ns × 1 Hz × 200 pulses. The introduction of PEFs improved the conventional CT therapeutic effect of OC cells. Moreover, the experimental results indicated lower cell viability after the µsPEF than for nsPEF. Yo-Pro-1^{TM} dye uptake analysis supported this result.

As mentioned in the previous subsection, IRE allows for the preservation of connective tissue surrounding the tumor. This was supported by Rolong et al. who explored the use of IRE and high-frequency irreversible electroporation (H-FIRE) to induce the death of tumor-initiating cells (TICs) [141]. The paper focused on TICs, which may play a crucial role in cancer treatment failures, including ovarian cancer (OC). It has been shown that higher frequency pulses may penetrate the epithelial layer more effectively without intensive Joule heating, causing an EP effect deeper in the target tissue. Additionally, H-FIRE reduces the intensity of muscles contractions. In the presented study, scientists using mouse ovarian surface epithelial (MOSE) cells supported the cytotoxic effect of IRE on treatment-resilient cells. The researchers applied 80 monopolar, rectangular-wave pulses with 100 µs duration at a frequency of 1 Hz with 300, 375, and 450 volts. Of particular interest, was that the results obtained indicated enhanced sensitivity of MOSE tumor-initiating variants (MOSE-L_{TICv}) and malignant, late-stage (MOSE-L) cells to H-FIRE compared to non-tumorigenic

(MOSE-E) cells. Three different H-FIRE protocols were applied: (1) 25 cycles × 2 µs pulses × 5 µs inter-pulse delay; (2) 25 cycles × 2 µs pulses × 2 µs inter-pulse delay and (3) 50 cycles × 1 µs pulses × 2 µs inter-pulse delay. Unfortunately, it is the only published study that has focused on using H-FIRE as a potential treatment for OC. According to the authors of this review, these results are so promising that it is worth undertaking a deeper analysis of the use of H-FIRE in the treatment of ovarian cancer and other gynecological cancers. The exposure of the OC cell line (SKOV-3) to IRE was also investigated by Yao et al. [142]. Notably, the authors analyzed the effectiveness of the combination of short high-voltage (SHV: 1.6 kV × 2 µs × 1 Hz × 20 pulses) pulses with long low-voltage (LLV: 0.24–0.48 kV × 100 µs × 1 Hz × 60–80 pulses) pulses. The results showed an enhanced cytotoxic effect of SHV + LLV on SKOV-3 cells than when applied alone. The animal model's enhanced ablation area after SHV + LLV therapy was also noted. The presented results are promising, and an analysis of larger pre-clinical models needs to be conducted.

Interestingly, Kobayashi et al. have used EP to load tumor suppressor miRNA (miR-199a-3p) into exosomes derived from primary-cultured omental fibroblasts of OC patients and used these constructs for miRNA replacement therapy for OC patients [143]. Treatment with miR199a-3p-loaded exosomes (miR-199a-3p-Exo) suppressed c-Met expression in CaOV3, SKOV3, and OVCAR3 cell lines, thereby inhibiting cell proliferation and invasion. In experiments using xenografts, the application of miR-199a-3p-Exo significantly disturbed c-Met expression, ERK phosphorylation, and MMP2 expression in tumors. This study presents the other type of EP application in OC treatment.

Perales-Purchat et al. applied EP as an approach to deliver a designed synthetic DNA plasmid, optimized to permit high expression of an anti-HER2 (HER2—human epidermal growth factor receptor 2) antibody (HER2dMAb) and HER2 DNA-encoded bispecific T cell engagers (HER2DBiTE), into mouse anterior tibialis muscle [144]. It was reported that HER2dMAb blocked HER2 signaling, induced antibody-dependent cytotoxicity, and delayed tumor progression for HER2-expressing ovarian and breast cancer models. The HER2DBiTE was highly cytolytic and delayed cancer progression in mice. Interestingly, it was expressed in vivo for approximately four months after a single administration, allowing for frequent dose reduction, simplifying treatment techniques, and improving expression profiles.

Unfortunately, the number of clinical trials, including with patients diagnosed with OC, is very low. The only clinical trial registered in the databases clinicaltrials.gov, PubMed, and Web of Science concerned immunotherapy alone or in combination with Il-12 DNA delivered by intramuscular EP (#NCT02960594). In 2021, Ahmed-Salim reported case series, including a patient presenting with a superficial, pre-sternal mass on a background of stage III mucinous ovarian cancer, treated with CaEP for palliation [145]. The woman underwent two debulking surgeries, radiotherapy, brachytherapy, and CT. The obtained results and CT imaging revealed resolution of the lesion and that CaEP was helpful for the reduction of distressing symptoms. Details of the CaEP used are presented in Table 2.

3.2. Vulvar Cancer

Vulvar cancer (VC) is a rare gynecological malignancy that affects women mainly after the menopause. However, the mean age of incidence has recently fallen due to the increase in human papillomavirus (HPV) infections [146,147]. It represents 5% of all malignant neoplastic gynecological lesions. The most common subtype of vulvar malignancy is squamous cell carcinoma (SCC). Other less-common histological types of VC are melanoma, Bartholin gland adenocarcinoma, extra-mammary Paget disease, basal cell carcinoma, and verrucous carcinoma, or sarcoma [148]. There is no specific screening, and the most effective strategy to reduce VC incidence is the opportune treatment of predisposing and preneoplastic lesions associated with its development. With VC progression, most women noticed vulvar pruritus pain with a lump or ulcer. Therefore, any suspicious vulvar lesion should be biopsied to exclude a malignant lesion [146]. Two primary pathological pathways lead to vulvar SCC [149].

The first pathway is associated with keratinizing changes, which usually occur in older women and are often connected with lichen sclerosis and/or differentiated vulvar intraepithelial neoplasia. The second pathway generally occurs in younger women and is caused by infection with oncogenic strains of HPV [150,151]. Currently, lesions arising from the vulva are classified into three sub-types: low-grade squamous intraepithelial lesions (LSILs), high-grade squamous intraepithelial lesions (HSILs), and differentiated vulvar intraepithelial neoplasia (dVIN) [152]. There is no determined treatment for conditions such as lichen sclerosus. Basic measures include avoiding exposure to precipitating factors, such as local irritants, moist environments, and the use of potent topical corticosteroids [153]. dVINs represent less than 5% of preneoplastic lesions of the vulvar, but these changes show a high probability of progression to squamous vulvar carcinoma and a higher recurrence rate than HSIL. Treatment of this type of lesion of the vulva is based primarily on surgical excision with 0.5–1 cm margins [154].

Treatment of VC depends mainly on histology staging. It is predominantly surgical; however, concurrent chemoradiation is commonly used, particularly for advanced tumors. Chemoradiation is a standard procedure in locally advanced VC. It allows reducing of the lesion area and performed surgical resection in 63–92% of initially inoperable tumors [155]. Surgical management should be carried out as the most conservative operation that will cure the disease and minimize treatment-related morbidity [156,157] and negative impact on the psycho-sexual condition of patients [158,159]. A combination of radio and CT is used in women with advanced VC, in whom primary surgical resection would damage central structures (anus, urethra) [159]. It has been shown that using a combination of CSP and 5-fluorouracil is effective for preserving the anal sphincter and urethra in inoperable VC treatment [160]. Effective and safe methods for reducing vulva tumors are needed to reduce the area excised during resection of the primary lesion and in palliative cases to ensure the patient's optimal comfort of living. Perrone et al. assessed the effectiveness of ECT in patients with primary vulvar neoplastic lesions in clinical trials. The main purpose of these studies was to determine whether ECT with BLM could effectively shrink the lesion before surgery and whether the resection area would be reduced with pre-operative ECT [161]. In studies carried out on nine patients, it was observed that there was a significant reduction in the tumor surface in eight cases, which made it possible to reduce the resection area. Consequently, four patients avoided a urethra resection, and two others a vaginal resection.

Furthermore, in six patients, the lesion shrinkage allowed excellent cosmetic repair after surgical resection, so the negative impact on the quality of the patient's life was reduced [161]. It has also been suggested that the inflammatory infiltrate and the immune response induced by ECT improve wound healing after resection of the lesion. At the same time, chemoradiotherapy causes tissue fibrosis, which may increase the likelihood of postoperative scar necrosis and wound dehiscence. ECT was performed only once before tumor recession in the present study. Perrone et al. suggest that because neoplastic tissue is heterogeneous, not all cells are electroporated simultaneously, which results in a suboptimal therapeutic effect. Therefore, several exposures to ECT before recession may achieve a better therapeutic effect recession than after one procedure [161].

VC recurs in about 33% of cases with an approximately 70% five-year survival rate [162]. Therapeutic options are limited in cases of relapse of VC, and quality of life is poor. In 2013, Perrone et al. published the first report on palliative ECT for patients with VC who relapsed after multimodality treatments, and for whom standard therapies were unsuitable. The results were encouraging. A complete response (CR) was observed in 62.5% of cases. Relevant symptoms, such as pain, bleeding, bad smell, and urinary discomfort subsided [132]. Based on these promising results, ECT was investigated further for the palliative treatment of VC. In studies on a group of 25 patients, local control of VC was achieved in about 80% of cases, with a 48% CR rate. A total of 7 out of 25 patients underwent a second session of ECT for disease progression, achieving a 43% CR rate [131]. Similar results were observed in another clinical study [163]. Based on these findings, several clinical centers in Italy started to treat palliative patients with VC using the method.

All data from the different centers were collected in a national database called ELECTRA. The obtained results indicate that ECT is currently the best palliative treatment method for patients with VC who cannot undergo surgery, have lesions resistant to chemotherapeutic agents, or have severe comorbidities. ECT treatment for VC is easy and quick to perform and has a favorable cost-effectiveness ratio. The side effects are minor, and most patients require only small doses of pain medications to treat pain associated with ECT. Moreover, unlike radiation therapy, it is possible to repeat several ETC cycles. However, more research is needed to assess the risk of BLM-induced pulmonary complications with multiple ECT sessions and to identify the best candidates for this treatment [164].

Ahmed-Salim et al. conducted a study involving four patients with vulval intraepithelial neoplasia (VIN) III and recurrent vulval squamous cell carcinoma treated with CaEP [145]. Details of the study are provided in Table 2.

3.3. Cervical Cancer

Cervical carcinoma (CC) ranks second globally in terms of the highest number of female deaths over the age of 60 [165]. The highest incidence is observed in less developed countries, especially South Africa and South America, where two to four times more cases are indicated than for breast cancer [166]. CC rarely shows symptoms in the initial stages of development but can be detected during a routine gynecological examination or cytology [167]. The first sign is lesions on the cervix, the cause of which is assessed using the Bethesda system [168]. Neoplastic changes do not always appear; the smear examination often indicates inflammation (up to 70%) and precancerous modifications. Fortunately, well-organized cytological screening and HPV triage, combined with vaccination programs in developed countries, have significantly reduced invasive cancer incidence and mortality [7].

The most common type of CC is squamous cell carcinoma, which accounts for almost 80% of all CC cases [167]. HPV is the most significant risk factor for developing cervical cancer. HPV DNA is present in over 90% of samples with a confirmed neoplastic lesion [169]. Due to the localization of the lesion during the initial stages of the disease, trachelectomy, pelvic lymphadenectomy, or radiotherapy are used as first-line treatment. Although they do not interfere with getting pregnant, all these methods significantly increase the risk of miscarriage. Therefore, other methods are sought that will not adversely affect a woman's fertility and will be safer for those for whom CT or radiation are contraindicated [167].

Several studies have examined the effectiveness of EP-based therapies on cervical cancer cell treatment, with IRE being the most studied. Research by Qin et al. demonstrated that IRE slows the growth of both examined cell lines (HeLa and SiHa). The survival rate estimated by the CCK-8 assay decreased from ~60% to 39.69% (HeLa) and 40.71% (SiHA). CFDA-SE assay used to assess cell proliferation showed that IRE-treated cells stop dividing. The 5th and 6th generation cells were ~7.38% for the HeLa line (control ~69.77%) and for the SiHa line ~21.72% (control 86.06%). The mechanism of cell proliferation inhibition in the G1 phase is known as cell cycle arrest, preventing cells from entering other phases and dividing. This was demonstrated by a study using propidium iodide (PI) staining involving the assessment of DNA content in cell cycle analysis. According to the study, almost 59.91% of cells in both lines were stopped in the G1 phase of the cell cycle compared to the control (44.63%) [170]. Tang et al. confirmed the inhibition of cell development in this phase and a decrease in the percentage of cells in the S phase. The theory proposed is that the regulation of cyclin D1 expression and the activity of cyclin D1-CDK4 are responsible for this. The decreased expression of cyclin D1 observed in the RT-PCR study resulted in an effect on the cell cycle and a slowdown in cell proliferation [171]. In addition to the impact on the growth of HeLa and SiHa cells, the effect of applied therapy on migration, invasiveness, and cell adhesion was confirmed, which is of great importance when assessing whether IRE can increase tumor metastasis. No significant differences were observed compared to the control, with the inference that IRE neither influenced migration nor the invasiveness or adhesiveness of HeLa and SiHa cells. This may be related to the observed excellent safety of its use in vivo [170].

Other studies have focused on the parameters of EP and its various effects. Cell survival was influenced by the electric field strength, the number of pulses used, and the time taken for the measurements, in experiments by Liu et al. Staining of cells with trypan blue was performed immediately after applying EP (0.5–2.5 kV/cm) and then after 6 and 12 h. The results showed that the higher the electric field strength, the lower the cell survival rate. Importantly, staining with trypan blue showed that the highest percentage of apoptotic cells occurred for an electric field above 1 kV/cm but less than 1.75 kV/cm. Giemsa staining and immunohistochemistry results were consistent with the flow cytometry test results. IRE significantly increased cell apoptosis and caspase-3 expression, especially at an intensity above 2 kV/cm and after 24 h from the applied therapy. Slides stained with Giemsa's reagent showed pyknosis, a characteristic of apoptosis [172].

Chai et al. investigated the effect of IRE on the whole organism using a rabbit animal model [173]. The experiment was conducted for 28 days using 90 pulses with a duration time of 70 µs and 1.5 kV/cm strength. Clinical course and histopathological examinations using hematoxylin-eosin staining first showed shedding of the mucosal epithelium and bleeding immediately after the procedure. The cervix was also swollen and painful. At the cellular level, the characteristic features of necrosis with disruption of cell membrane continuity were observed. TUNEL assay also showed significant cell apoptosis in the ablated area. In subsequent days, healing and the goal of regeneration were observed. MT staining showed increased collagen production and tissue fibrosis that increased to sizeable levels by day 28. The experiment demonstrated that the IRE treatment did not disturb the architecture of the cervix and blood vessels and nerves to such an extent that it would affect the patient's functions and fertility in the future [173].

The use of EP to improve the administration of drugs, particularly anticancer drugs, is already widely used throughout the world. In vitro studies have investigated whether ECT would be effective in the case of cervical cancer. Yabushita et al. examined bleomycin (BLM), adriamycin, cisplatin (CSP), mitomycin C and cyclophosphamide for their efficacy in treating cervical squamous cell carcinoma. The research used the CaSki cell line. It was confirmed that EP (with a strength of 25–100 V/mm) significantly enhanced the cytotoxic effect of all analyzed drugs, especially BLM. After applying ECT with BLM (the highest analyzed concentration), cell survival decreased by almost 20%. It was also observed that using the drug before EP application gave better cytotoxic results than those obtained for BLM added after EP [10]. Ramachandran et al. used the ME180 human-cervix-derived epithelial cell line (squamous cell carcinoma) and confirmed the positive results for BLM + EP treatment. When the drug was used alone, the cytotoxic rate was ~13.4%, compared to from 18.8 to 53% for ECT using different electric pulse strengths (V/cm) [174].

Clinical trials involving EP for various CC types have mainly included the administration of vaccines. Trimble et al. assessed whether VGX-3100 synthetic plasmids would positively affect the progression of cervical intraepithelial neoplasia (CIN). The goal of the nearly two-year-long experiment involving 167 patients was to eliminate HPV-16 and HPV-18, the leading causes of most cervical cancers. The study confirmed that the use of EP to facilitate the delivery of the vaccine significantly increased the level of antibodies in the human body, and the results of histopathological studies confirmed a partial regression of the disease [175]. A similar experiment is currently underway with the same vaccine in a group of women with histologically confirmed high-grade squamous intraepithelial lesions (HSIL) [176].

Table 2. Clinical trials, preliminary studies, and case reports focusing on the use of EP-based therapies in gynecological carcinomas treatment.

Gynecological Carcinoma	Trial Type	Phase	NCT Identifier (Status)	Number of Patients	Short Description	Protocol	Study Outcome	Ref.
Electrochemotherapy (ECT)								
V-SCC	Prospective	II	N/A	25	ECT in elderly (median age = 85 years) patients diagnosed with V-SCC	15,000 IU/m^2 BLM i.v. +8 pulses; 100 µs; 5 kHz 8–28 min after BLM administration	1 month after ECT: CR = 52%; PR = 28%; SD = 12%; PD = 8% 6 months after ECT SFS = 40%	[131]
V-SCC	Prospective	Preliminary study	N/A	8	safety, local efficacy, acceptability and QoL of ECT with BLM in reducing the size of tumors in patients with V-SCC	15,000 IU/m^2 BLM i.v. +8 pulses; 100 µs; 5 kHz 8–28 min after BLM administration	CR = 62.5%; PR = 12.5%; SD= 12.5%; PD = 12.5% 50% patients alive 9 months after ECT	[132]
VC	Prospective	N/A	NCT03142061 (Completed)	50	BLM + EP of cutaneous accessible tumor tissue in patients with advanced inoperable vulva carcinoma	N/A	N/A	N/A
Calcium Electroporation (CaEP)								
VIN III; V-SCC; metastatic OV	Retrospective	Case report	N/A	6	CaEP in VIN and vulvar cancer	i.t. 0.5 mL/1 cm^3 $CaCl_2$ solution (10 mL of a stock 10 mmoL/10 mL solution of $CaCl_2$ with 35 mL of 0.9% NaCl) +2 µs bipolar pulses; 1.3 kV/cm (520 V each polarity); 166 kHz 30 'trains' of pulses	CaEP applied 10 times CR = 50%; PR = 40% For 8 episodes, symptoms improved within 6 weeks Beyond 6 weeks, symptoms eventually recurred in all patients, and 4 patients required more than one CaEP procedure.	[145]
Gene Eectrotransfer (GET)								
CC	Non-randomized	I, II	NCT02172911 (Completed)	10	safety and tolerability of a therapeutic DNA vaccination against HPV16 and HPV18 E6/E7 oncogenes after chemoradiation for cervical cancer	DNA-based vaccine against HPV-16/18 coinjected with an IL-12 plasmid	8/10 patients had detectable cellular or humoral immune responses against HPV antigens after chemoradiation and vaccination; 6/10 patients generated anti-HPV antibody responses 6/10 patients generated IFNγ-producing T cell responses	[177]

Table 2. *Cont.*

Gynecological Carcinoma	Trial Type	Phase	NCT Identifier (Status)	Number of Patients	Short Description	Protocol	Study Outcome	Ref.
VC CC	N/A	II	NCT03439085	21	MEDI0457 and durvalumab for patients with recurrent/metastatic HPV-associated cancers.	7 mg IL-12/HPV DNA plasmid i.m. and via EP at W 1, 3, 7, and 12 starting W 12, cycles repeat every 8 weeks+1500 mg Durvaluma i.v. at W 4, 8, and 12 starting W 12, cycles repeat every 4 weeks up to 13 doses	21 patients were evaluated for toxicity and 19 for a response. ORR = 21%; DCR = 42%; CR = 5.3%; PR = 15.8%; SD = 21%	[178]
CC	Non-randomized	I	NCT00685412 (Completed)		evaluate the safety and tolerability of a therapeutic DNA vaccination (VGX-3100) against HPV16 and HPV18 E6/E7	i.m. injection of 3 doses 0.6/2/6 mg VGX-3100 +EP D 0, M 1 and 3	N/A	N/A
CC	Randomized	II	NCT01304524 (Completed)		a.a	i.m. injection 1 mL VGX-3100 +EP D 0, W 4 and 12	49.5% recipients and 30.6% placebo recipients (in the per-protocol analysis) 48.2% recipients and 30% placebo recipients (in the modified intention-to-treat analysis) had histopathological regression	[175,179]
CC	N/A	I	NCT01188850 (Completed)	14	evaluate the safety, tolerability, and immunogenicity of the fourth dose of Human papillomavirus (HPV) DNA plasmid (VGX-3100) + electroporation (EP) in adult females previously immunized with VGX-3100	i.m. injection of 6 mg VGX-3100 +EP in D 0	Increased immune reactivity after bosting vaccination	[180]
CC	Non-Randomized	I	NCT01634503 (Completed)	9	evaluate the safety and tolerability of DNA-based vaccine (GX-188E) administrated via EP in patients with HPV-16 or HPV-18 associated CIN III	i.m. administration of 1/2/4 mg GX-188E via EP	N/A	N/A
CC	Randomized	II	NCT02139267 (Completed)	72	a.a	i.m administration of 1 or 4 mg GX-188E via EP in W 0, 4 and 12	64 patients were included in the per-protocol analysis (V7) and 52 in extension analysis (V8) V7: 52% (33/64) V8: 67% (35/52) presented histopathologic regression after GX-188E injection 73% (V7) and 77% (V8) of the patients with histologic regression showed HPV clearance	[181]

Table 2. *Cont.*

Gynecological Carcinoma	Trial Type	Phase	NCT Identifier (Status)	Number of Patients	Short Description	Protocol	Study Outcome	Ref.
OC	Non-randomized	I	NCT02960594 (Completed)	93	Immunotherapy alone or in combination with Il-12 DNA delivered by IM EP in solid tumors therapy	hTERT (2/8 mg) + i.m. EP +/− IL-12 (0.5/2 mg) +/− SynCon® TERT (2/8 mg) D 0, W 4, 8 and 12	hTERT immunotherapy induced a de novo cellular immune response or enhanced pre-existing cellular responses to native hTERT in 96% (88/92) of patients	[182]
Irreversible Electroporation (IRE)								
CC	Randomized	I, II	NCT02430610 (Completed)	30	safety and efficacy of IRE for unresectable uterine cervical neoplasms	N/A	N/A	N/A

a.a—as above; **N/A**—not applicable; **D**—day; **W**—week; **M**—month; **mg**—milligram; **mL**—milliliter; **ms** -millisecond; **µs**—microsecond; **kV**—kilovolts; **cm**—centimeter; **i.v**.—intravenous; **i.t.**—intratumoral; **i.m.**—intramuscular; **PI**—pulse interval; **EP**—electroporation; **OR**—objective response; **CR**—complete response; **SD**—stable disease; **PD**—progressive disease; **ORR**—overall response rate; **DCR**—disease control rate; **DFS**—disease-free survival; **SAE**—serious adverse events; **SFS**—symptom-free survival; **CC**—cervical cancer; **OC**—ovarian cancer; **VC**—vulvar cancer; **V-SCC**—squamocellular vulvar cancer; **CIN III**—cervical intraepithelial neoplasia grade III; **IL12**—interleukin 12; **TIL**—tumor-infiltrating lymphocyte; **hTERT**—human telomerase reverse transcriptase.

3.4. Breast Cancer

Breast cancer (BC) is the most commonly diagnosed cancer and the leading cause of death among women worldwide (Figure 1) [1]. According to data collected in the GLOBOCAN database, almost 2.3 million new cases of BC were estimated in 2020; thereby, BC has surpassed lung cancer. Forecasts show that this number is expected to increase to ~3.19 million by 2040 [183]. Current treatment includes a combination of surgical resection via mastectomy or lumpectomy, irradiation, and adjuvant CT [184]. Unfortunately, these surgical solutions remain associated with significant scarring and disfigurement, which may complicate monitoring for residual tumors and recurrence of malignancy [185]. CT is not satisfactorily effective according to patient relapses, and a wide range of side effects affect patients' quality of life [2].

Moreover, the multidrug resistance (MDR) phenomenon is a growing problem in most cases. Another difficulty associated with BC treatment is planning the appropriate therapy, taking into account histological variety, hormonal dependence, eventual overexpression of estrogen receptor α (ERα), estrogen receptor β (ERβ), human epidermal growth factor receptor 2 (HER2), and resistance against conventional treatment [186]. Therefore, there is an urgent need to develop new approaches enabling earlier detection, which will be more effective, less toxic, and associated with fewer side effects.

Rembiałkowska et al. examined the ECT effectiveness of applying doxorubicin with EP in MCF-7/WT and MCF-7/DOX breast cancer cells sensitive and resistant to doxorubicin (DOX), respectively [95]. Interestingly, increased cytotoxicity of DOX was noted in MCF7/DOX when drug administration was combined with EP. The resistant cell line was shown to be more sensitive to electric pulses. It has been suggested that EP-based methods might be attractive for cancer treatment in human BC, especially those with developed resistance. EP enables a reduction in drug doses and exposure time in this type of cancer, diminishing the side effects of systemic therapy. Interesting changes were observed in analyzed cell lines using the electron microscope. In the case of electric pulses together with DOX, there were many differences in lysosomes. Secondary lysosomes and vacuoles with more irregular shapes were obtained from heterogeneous material. Analysis of the cellular ultrastructure showed that MCF-7/WT cells were more sensitive to electric fields and DOX than MCF-7/DOX cells.

Due to the lack of the three main receptors, estrogen ER, progesterone, PR, and HER2, triple-negative breast cancer (TNBC) is mainly resistant to standard CT [187]. ECT might be a promising alternative method to treat TNBC. The impact of EP without anticancer drugs on MDA-MB-231 TNBC and human colon cancer (SW-480 and HCT-116) in comparison to human fibroblast cell line (MRC-5), primary human aortic smooth muscle cells (hAoSMC), and human umbilical vein endothelial cells (HUVEC) has been evaluated. The inhibition of cell proliferation after EP in a dose-dependent manner was observed. Electric pulses of strength 375–437.5 V/cm induced the IRE of cancer cells and RE of normal human cells. The lower voltage induced apoptosis as the predominant type of cell death in contrast to higher voltages, which mainly led to necrosis in human cancer cell lines. Considering the results obtained, EP might be a promising method for use in TNBC human cell lines [188].

Mittal et al. considered the mechanism of ECT using electrical pulses and CSP on an MDA-MB-231 cell line by quantitative proteomic analysis which correlated well with cell viability, western blot (WB), and quantitative polymerase chain reaction (qPCR) data [189]. EP with CSP was found to be involved in regulating 14 essential glycolysis proteins. EP with CSP-induced pathways also led to oxidative imbalance, increased reactive oxygen species, and apoptotic cell death. These results indicated the potential role of EP + CSP against TNBC cells. The studies were confirmed by others [190,191].

Combining calcium with EP has been tested as a new, EP-based cancer treatment modality (CaEP). In vitro study showed that an increase in supraphysiological doses of calcium ions (Ca^{2+}) into cells primarily caused necrotic cell death associated with acute and critical energy depletion [102,192]. CaEP had a similar effect to ECT with anticancer drugs on breast and ovarian cancer [140,193]. Kulbacka et al. demonstrated an enhanced

antiproliferative effect in MCF-7 and MCF-7/DX cells electroporated using nsPEF protocols in combination with Ca^{2+} [194]. Furthermore, it was observed that the combination of nsPEF with calcium may be used as a temporary MDR controlling tool to obtain better drug uptake. The results obtained showed that nsPEF + Ca^{2+} triggered decreased MDR1 activity, which, consequently, may disrupt cancer cells' MDR resistance mechanism.

IRE has also found applications in the treatment of breast cancer. Babikr et al. considered the combination of IRE alone or with Toll-like receptor (TLR)3/9 agonists (poly I:C/CpG) (IRE + pIC/CpG), PD-1 blockade (IRE +PD-1 blockade), and their combination (IRE + Combo) [195]. The results revealed effective therapeutic outcomes for IRE + Combo on two mouse breast cancer models (Tg1-1 and 4T1), suggesting that this method may represent a promising improvement for IRE ablation in cancer treatment. Zhang et al. evaluated the effects of combining IRE and photodynamic therapy (PDT) in BC cells in vitro (MCF-7 cell line) and in vivo (BALB/C mice) [196]. They reported that combining IRE and PDT enhanced anti-tumor effects in BC and that apoptosis was the primary mechanism responsible. Compared with controls, the IRE + PDT group exhibited lower levels of VEGF, CD31, TGF-β, and Ki67 indicators. Moreover, the in vivo tumor suppression rate for IRE (1200 V) + PDT (10 mg/kg) was 68.3%.

Bazzolo et al. investigated the response of breast cancer cell (HCC1954) culture on electrospun poly (ϵ-caprolactone) (PCL) fibrous scaffolds to ECT therapy with bleomycin (1.0 kV/cm ± 10 μM bleomycin) to evaluate it as a potential tool for the study of EP and its applications [197]. The described three-dimensional (3D) additive manufactured PCL scaffolds revealed their potential in breast reconstruction improvement [198]. Moreover, the authors analyzed the extracellular matrix production in this kind of 3D culture. They rightly pointed out that most of the anticancer drugs selected with the traditionally used two-dimensional (2D) cell cultures have been shown to be ineffective in in vivo models. This is because 2D cultures are not able to precisely mimic the cancer environment including, for example, cell-matrix interactions. The results revealed lower sensitivity of PCL-based cultures to doxorubicin and EP/bleomycin than adherent cell cultures. The authors argue that this effect may be caused by the increased level of cancer stem cells (CSCs) detected in the proposed 3D cultures. The use of electrospun PCL cultures has also been characterized by mucopolysaccharide production and enhanced CD44 expression. Of course, further studies are essential to better understand the proposed in vitro model; however, the results obtained to date are promising.

ECT has also been shown to effectively treat BC in clinical practice. The minor side effects of the therapy and the low intraoperative duration of cure make it possible to admit patients to the hospital for only a short time. Thus, the repeated use of ECT has enabled an increase in the rate of complete remissions. In 2013, this form of treatment, which consists of a low-dose cytostatic and EP, was also included in the Working Group for Gynaecological Oncology (AGO) mammary guidelines and the German Cancer Society (DKG). The favorable cost-benefit ratio makes this method of treatment interesting in clinical practice, and, as a result, it is already being used successfully in many German hospitals.

The first clinical trial using RE combined with a chemotherapeutic drug (ECT) was conducted in 1990–1991 [199]. Since then, numerous clinical trials applying EP-based technologies have been performed to treat small tumors, such as cutaneous and subcutaneous metastases, and larger tumors (e.g., chest wall breast cancer) [200,201]. The clinical observations using ECT with BLM in patients with BC are exciting. This study showed that small tumor BC in the absence of visceral metastasis, ER positivity, and low Ki67 index had produced a complete response to ECT with BLM. Thus, ECT was effective in BC patients. Larkin et al. noted 60% regression after ECT with BLM [202].

The first clinical trial on CaEP in breast cancer noted that CaEP could be an effective and safe treatment option [106]. Another clinical study has shown that CaEP is not inferior or less effective in comparison to BLM-based ECT, especially for cutaneous metastasis of breast cancer [108]. Details relevant to clinical trial reports the use of EP-based treatment methods in BC therapy has been shown in Table 3.

Table 3. Clinical trials and case reports focusing on the use of EP-based therapies in breast carcinoma treatment.

Type of Therapy	Trial Type	Phase	NCT Identifier (Status)	Number of Patients	Short Description	Protocol	Study Outcome	Ref.
ECT	Prospective	N/A	N/A	39	ECT for patients with cutaneous or subcutaneous metastases with palliative intent	i.v. BLM (15,000 IU/m^2) + EP 8 pulses; 1.0 kV/cm; PD: 100 µs; 5 kHz; needle electrodes	No SEAs were observed; ORR = 66.6%	[203]
GET	N/A	I	NCT02531425 OMS-I140 (Completed)	10	IL12 plasmid (Tavo) deliver by msEP in the TNBC treatment	Tavo (0.5 mg/mL); dose $\frac{1}{4}$ tumor volume; +i.t. EP 6 pulses; 1.5 kV/cm; pulse duration: 100 ms; PI: 300 ms 6 needle electrodes; 1.0/0.5 cm diameter	enhanced antigen presentation; enhancement of CD8+ T-cell infiltration	[204]
GET + ECT	Non-Randomized Multi-Cohort	II	NCT03567720 (Recruiting)	65	IL12 plasmid (Tavo) delivered by msEP in the TNBC treatment combined with immune- and CT therapy	Tavo + i.t. EP (every 6 W) +i.v. injected Pembrolizumab (3 weekly) +/− i.v. Abraxane® (4 weekly)	N/A	N/A
ECT	Randomized	N/A	N/A	38	ECT for breast cancer metastasis to the skin and subcutaneous tissue treatment	15,000 IU/m^2 BLM i.v. +8 pulses; pulse duration: 0.1 ms; 1.0 kV/cm 5 hHz 8–28 min after BLM administration	CR = 42% and PR = 29% 12 weeks after	[205]
GET	Randomized	I	NCT03199040 (Active, not recruiting)	13	Neoantigen DNA vaccine delivered by EP in the TNBC treatment + Durvalumab (anti-PD-L1 antibody),	Vaccine 2 i.m. EP in 2 different sites, 3 M after the standard of care (D 1) and then D 29, 57, 85, 113 and 141 +/− Durvalumab 1.5 mg every 4 W, at D 85	minimal adverse events reported;	[206,207]
GET	N/A	I	NCT02348320 (Completed)	18	Polyepitope, neoantigen DNA vaccine, delivered by EP in the TNBC treatment +/− Durvalumab after completion of a standard of care therapy	Vaccine (4 mg) + i.m. EP at D 1, 29 and 57	N/A	[207,208]

Table 3. *Cont.*

Type of Therapy	Trial Type	Phase	NCT Identifier (Status)	Number of Patients	Short Description	Protocol	Study Outcome	Ref.
CaEP + ECT	Randomized	II	NCT01941901 (Completed)	7	The comparison between CaEP and ECT in BC treatment.	i.t. CaEP ($CaCl_2$; 9 mg/mL; total dose: 0.5 mL/cm^3 tumor volume)or BLM (1000 IU/mL; total dose: 0.5 mL/cm^3 tumor volume) + i.t. EP 8 pulses; 0.4 kV/cm; pulse duration: 0.1 ms; 5 kHz	CaEP: OR = 72% (13/18); CR = 66% (12/18); ECT: OR = 84% (16/19) CR = 68% (13/19) no significant difference between the two treatments (p = 0.5) ulceration, itching and exudation reported after ECT	[106]
GET	Non-Randomized	I	NCT02960594 (Completed)	93	Immunotherapy alone or in combination with Il-12 DNA delivered by IM EP in solid tumors therapy	hTERT (2/8 mg) + i.m. EP +/− IL-12 (0.5/2 mg) +/− SynCon® TERT (2/8 mg) D 0, W 4, 8 and 12	hTERT immunotherapy induced a de novo cellular immune response or enhanced pre-existing cellular responses to native hTERT in 96% (88/92) of patients	[182]
GET	Non-Randomized	I	NCT02204098 (Recruiting)	56	Mammaglobin-A DNA vaccine delivered by EP for ER+, HER2-BC patients undergoing neoadjuvant endocrine therapy or CT	+/− Neoadjuvant endocrine therapy +/− mammaglobin-A DNA vaccine (4 mg) D 28, 56, and 84 +/− EP +/− Neoadjuvant CT	N/A	N/A

N/A—not applicable; **D**—day; **W**—week; **M**—month; **mg**—milligram; **mL**—milliliter; **ms**—millisecond; **µs**—microsecond; **kV**—kilovolts; **cm**—centimeter; **i.v.**—intravenous; **i.t.**—intratumoral; **i.m.**—intramuscular; **CT**—chemotherapy; **EP**—electroporation; **ECT**—electrochemotherapy; **CaEP**—Calcium electroporation; **GET**—gene electrotransfer; **OR**—objective response; **CR**—complete response; **PR**—partial response; **ORR**—overall response rate; **DFS**—disease-free survival; **SAE**—serious adverse events; **BC**—breast cancer; **TNBC**—triple-negative breast cancer; **IL12**—interleukin 12; **TIL**—tumor-infiltrating lymphocyte; **hTERT**—human telomerase reverse transcriptase; **BLM**—bleomycin.

To conclude, the application of ECT and CaEP appears to be a more effective and safer treatment for breast carcinoma in vitro and in vivo. ECT has found application in the treatment of breast cancer and its metastases. Palliative effects of ECT have also been demonstrated, and pain reduction has been observed in patients.

4. Conclusions

Considering increasing gynecological and breast cancer incidence, there is an urgent need to look for new, more effective, and less toxic treatment modalities. Electroporation has been investigated for the last two decades. Understanding of the basic mechanisms responsible for this phenomenon has expanded the scope of its application. EP-based therapies offer opportunities for patients who would otherwise be deprived of any alternative cancer treatment. The discovery of the stimulating effect of electroporation on the effectiveness of conventional cytostatics, which was then called electrochemotherapy (ECT), has been significant for oncology. The addition of EP to CSP-based CT enabled a significant therapeutic effect even in CSP-resistant cell lines (e.g., OvBH-1 and SKOV-3) [138,140]. The results presented in our review have also indicated that ECT and CaEP could be used as palliative therapy, its use reducing the number of side effects and improving the comfort of patients' life [132,145]. Other EP-based methods have also shown promising results to date. As a non-thermal ablation therapy, IRE has recently found application as an alternative method of cervical and breast cancer treatment. The advantage of eP-based methods lies in the fact that they do not require the application of cytostatics and are based on naturally occurring molecules in the human body (e.g., CaEP).

Furthermore, it was indicated that most of these methods (e.g., CaEP or IRE) do not affect healthy tissue/cells at a significant level. The other important advantage is the low cost of these methods. The construction of the electroporator and electrodes does not require large financial outlays, which can significantly support oncological treatment in less affluent countries [112], especially considering that, as described above, expensive cytotoxic drugs can easily be omitted or replaced with calcium chloride ($CaCl_2$). According to in vitro and in vivo studies undertaken already, this will not reduce the effectiveness of the therapy. Moreover, the EP-based therapies' immunomodulatory properties should be more precisely investigated. Babikr et al. have rightly pointed out that tools which enable IRE-induced therapeutic immunity improvement are little known and require further investigation [195].

Each of the treatment options described above has shown promising results to date, although they still require careful evaluation. A significant breakthrough in in vitro research of EP-based therapies may be the wider use of 3D cell cultures, for example, on electrospun PCL [197]. The use of this type of synthetic polymer enables scientists to mimic the tumor microenvironment more accurately in the laboratory. This will help to more precisely predict how a tested drug or treatment will perform in vivo.

Despite a variety of advantages, EP-based therapies are still associated with some side effects. Namely, electric pulses used in IRE or ECT, for example, stimulate excitable tissues and nerves, causing pain and muscle contractions [130]. Preliminary studies have shown the potential of nanosecond-range electrical pulses to overcome this problem, but these need to be studied more precisely. Future research should focus on the search for EP protocols that will enable minimizing the doses of cytotoxic drugs or achieving similar therapeutic effects while completely eliminating them. The molecular mechanisms underlying CaEP in gynecological cancers should also be explored in more detail, and EP protocols designed to reduce or eliminate muscle contractions and acute pain that currently accompany these therapies. It should also be pointed out that the number of studies focusing on the use of EP in the treatment of gynecological and breast cancers and its effects is still negligible compared to other cancers. This is an area of oncology that deserves attention.

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11. Informacje o źródłach finansowania badań

Badania składające się na rozprawę doktorską zostały sfinansowane przez:

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12. Oświadczenia współautorów prac będących podstawą cyklu

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Z głębokim żalem informujemy o śmierci **prof. dr hab. Jolanty Saczko**, która pełniła rolę pierwszego Promotora niniejszej pracy doktorskiej w latach 2020-2023. Pani Prof. Saczko jest współautorem każdej z zawartej w cyklu publikacji naukowej, a jej wkład polegał na budowaniu koncepcji prac naukowych, nadzorowaniu przeprowadzonych eksperymentów, pomocy w analizie i interpretacji wyników oraz pomocy w przygotowywaniu każdego z manuskryptów.

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Julita Kulbacka
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Imunologijos ir
bioelektrochemijos
skyriaus vadovas
Vitalij Novickij

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Niniejszym oświadczam, że mój udział w artykule naukowym autorstwa Zofia Łapińska, Vitalij Novickij, Nina Rembiałkowska, Anna Szewczyk, Magda Dubińskiej-Magiera, Julita Kulbacka, Jolanta Saczko, pt. „**The influence of asymmetrical bipolar pulses and interphase intervals on the bipolar cancellation phenomenon in the ovarian cancer cell line**" opublikowanym w czasopiśmie Bioelectrochemistry w 2023 roku vol. 153; DOI:10.1016/j.bioelechem.2023.108483, polegał na opracowaniu koncepcji pracy, wykonaniu elektroporacji komórek i przygotowaniu ich do testów przeżywalności oraz edycji manuskryptu.

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Wydział Farmaceutyczny
Uniwersytet Medyczny im. Piastów Śląskich we Wrocławiu

OŚWIADCZENIE

Oświadczam, iż w pracy autorstwa Zofii Łapińskiej, Urszuli Szwedowicz, Anny Choromańskiej i Jolanty Saczko pt. **„Electroporation and electrochemotherapy in gynecological and breast cancer treatment"** opublikowanej w czasopiśmie *Molecules* w 2022 roku vol. 27; art. 2476 mój udział polegał na dokonaniu przeglądu literatury oraz przygotowaniu manuskryptu w części *3.3 Cervical Cancer*.

Urszula Szwedowicz
.....
(podpis współautora)

.....
(podpis promotora)

Wrocław, 26.08.2024

dr hab. Anna Choromańska, prof. UMW

Katedra i Zakład Biologii Molekularnej i Komórkowej
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Anna Choromańska
.....
(podpis współautora)

.....
(podpis promotora)

13. Życiorys oraz dorobek naukowy

Dane osobowe:

- **Imię i nazwisko:** Zofia Łapińska
- **Data i miejsce urodzenia:** 18.12.1996 r. Łapy
- **Adres korespondencyjny:** Łapy-Szołajdy 31, 18-100 Łapy

Aktualne miejsce i stanowisko pracy: brak

Edukacja:

- **2020-2024** Doktorantka w Szkole Doktorskiej Uniwersytetu Medycznego im. Piastów Śląskich we Wrocławiu; Katedra i Zakład Biologii Molekularnej i Komórkowej
- **02.2024 – 11.2024** Stażystka w grupie Cancer and Genome Instability, wydział Cancer Biology, The Institute of Cancer Research, Londyn, Zjednoczone Królestwo Wielkiej Brytanii i Irlandii Północnej (konkurs Nagroda Naukowa); **opiekun:** dr Jadwiga Nieminuszczy, prof. Wojciech Niedźwiedź
- **2018 – 2020** Studia magisterskie na kierunku biotechnologia Wydziału Biotechnologii Uniwersytetu Wrocławskiego, spec. biologia molekularna. Praca magisterska realizowana w Zakładzie Biotransformacji; **tytuł pracy:** „Rola mutacji punktowych genu *ERG11* w oporności grzybów *Candida albicans* na flukonazol."
- **2015 – 2018** Studia licencjackie na kierunku biotechnologia Wydziału Biotechnologii Uniwersytetu Wrocławskiego. Praca licencjacka realizowana w Zakładzie Biotransformacji; **tytuł pracy:** „Rola receptora cukrów Mig1p u *Candida albicans.*"
- **2012 – 2015** I Liceum Ogólnokształcące im. Adama Mickiewicza w Białymstoku

13.1 Prace pełnotekstowe w czasopismach naukowych

Liczba rozdziałów: 10; **Łączna punktacja: IF:** 43,233; **MNiSW:** 1170 pkt.

1) Kowalski Szymon, Karska Julia, **Łapińska Zofia**, Hetnał Bartosz, Saczko Jolanta, Kulbacka Julita: An overview of programmed cell death: apoptosis and pyroptosis-mechanisms, differences, and significance in organism physiology and pathophysiology, *Journal of Cellular Biochemistry*, 2023, vol. 124, nr 6, s. 765-784, DOI:10.1002/jcb.30413 **IF:** 3,0; **MNiSW:** 100 pkt.

2) Radzka Justyna, **Łapińska Zofia**, Szwedowicz Urszula, Gajewska-Naryniecka Agnieszka, Gizak Agnieszka, Kulbacka Julita: Alternations of NF-κB signaling by natural compounds

in muscle-derived cancers, *International Journal of Molecular Sciences*, 2023, vol. 24, nr 15, art.11900 [15 s.], DOI:10.3390/ijms241511900 **IF:** 4,9; **MNiSW:** 140 pkt. Gajewska-Naryniecka Agnieszka, Szwedowicz Urszula, **Łapińska Zofia**, Rudno-Rudzińska Julia, Kielan Wojciech, Kulbacka Julita: Irreversible electroporation in pancreatic cancer - an evolving experimental and clinical method, *International Journal of Molecular Sciences*, 2023, vol. 24, nr 5, art.4381 [19 s.], DOI:10.3390/ijms24054381 **IF:** 4,9; **MNiSW:** 140 pkt.

3) **Łapińska Zofia**, Novickij Vitalij, Rembiałkowska Nina, Szewczyk Anna, Dubińska-Magiera Magdalena, Kulbacka Julita, Saczko Jolanta Ewa: The influence of asymmetrical bipolar pulses and interphase intervals on the bipolar cancellation phenomenon in the ovarian cancer cell line, *Bioelectrochemistry*, 2023, vol. 153, art.108483 [10 s.], DOI: 10.1016/j.bioelechem.2023.108483 **IF:** 4,8; **MNiSW:** 100 pkt.

4) Urbanek Aneta K.*, **Łapińska Zofia***, Derkacz Daria, Krasowska Anna: The Role of ERG11 point mutations in the resistance of Candida albicans to fluconazole in the presence of lactate, *Pathogens*, 2022, vol. 11, nr 11, art.1289 [15 s.], DOI:10.3390/pathogens11111289 **IF:** 3,7; **MNiSW:** 100 pkt.

***** *Autorzy ci w równym stopniu przyczynili się do powstania tej pracy.*

5) **Łapińska Zofia**, Szwedowicz Urszula, Choromańska Anna, Saczko Jolanta: Electroporation and electrochemotherapy in gynecological and breast cancer treatment, *Molecules*, 2022, vol. 27, nr 8, art.2476 [32 s.], DOI:10.3390/molecules27082476 **IF:** 4,6; **MNiSW:** 140 pkt.

6) Szwedowicz Urszula, **Łapińska Zofia**, Gajewska-Naryniecka Agnieszka, Choromańska Anna: Exosomes and other extracellular vesicles with high therapeutic potential: their applications in oncology, neurology, and dermatology, *Molecules*, 2022, vol. 27, nr 4, art.1303 [34 s.], DOI:10.3390/molecules27041303 **IF:** 4.6; **MNiSW:** 140 pkt.

7) **Łapińska Zofia**, Dębiński Michał, Szewczyk Anna, Choromańska Anna, Kulbacka Julita, Saczko Jolanta: Electrochemotherapy with calcium chloride and 17β-estradiol modulated viability and apoptosis pathway in human ovarian cancer, *Pharmaceutics*, 2021, vol. 13, nr 1, art.19 [17 s.], DOI:10.3390/pharmaceutics13010019 **IF:** 6,525; **MNiSW:** 100 pkt.

8) Kulbacka Julita, Choromańska Anna, **Łapińska Zofia**, Saczko Jolanta: Natural polymers in photodynamic therapy and diagnosis, *Polimery w Medycynie*, 2021, vol. 51, nr 1, s. 33-41, DOI:10.17219/pim/139587 **IF:** 0,0; **MNiSW:** 70 pkt.

9) Szlasa Wojciech, Kiełbik Aleksander, Szewczyk Anna, Novickij Vitalij, Tarek Mounir, **Łapińska Zofia**, Saczko Jolanta, Kulbacka Julita, Rembiałkowska Nina: Atorvastatin modulates the efficacy of electroporation and calcium electrochemotherapy, *International*

Journal of Molecular Sciences, 2021, vol. 22, nr 20, art.11245 [21 s .], DOI:10.3390/ijms222011245 **IF:** 6,208; **MNiSW:** 140 pkt.

13.2 Rozdziały w monografiach naukowych i inne

Liczba rozdziałów: 2; **Łączna punktacja: IF:** 2,1; **MNiSW:** 90 pkt.

1) **Łapińska Zofia**, Michel Olga, Szlasa Wojciech, Saczko Jolanta: Hormonotherapy in gynecological cancers, W: Advances in biomedical research: from cancer prevention to treatment, (red.) Izabela Młynarczuk-Biały, Łukasz Biały, Lublin; Warszawa 2020, Warszawski Uniwersytet Medyczny, s. 130-142, ISBN 978-83-66489-45-5, 978-83-7637-551-9 **IF:** 0,0; **MNiSW:** 20 pkt.
2) **Łapińska Zofia**, Saczko Jolanta: Novel electroporation-based treatments for breast cancer [editorial], Advances in Clinical and Experimental Medicine, 2022, vol. 31, nr 11, s. 1183-1186, DOI:10.17219/acem/156058 **IF:** 2,1; **MNiSW: 7**0 pkt.

13.4 Streszczenia zjazdowe

1) **Łapińska Zofia**, Novickij Vitalij, Rembiałkowska Nina, Radzevičiūtė-Valčiuke Eivina, Szewczyk Anna, Dubińska-Magiera Magda, Kulbacka Julita, Saczko Jolanta, Baczyńska Dagmara; Modulating electrochemotherapy efficacy in ovarian carcinoma with bipolar nsPEFs: Insights into cell membrane permeabilization and reactive oxygen species levels;
2) **Łapińska Zofia**, Novickij Vitalij, Rembiałkowska Nina, Radzevičiūtė-Valčiuke Eivina, Szewczyk Anna, Dubińska-Magiera Magda, Kulbacka Julita, Saczko Jolanta, Baczyńska Dagmara; The effect of asymmetrical pulsed electric fields consists of micro- and nanosecond-range pulses and interphase delays on bipolar cancellation phenomenon; W: XXVII Gliwice Scientific Meetings. Gliwice, November 16-17, 2023. Conference materials [online] 2023, 124 poz. [VI-5]
3) **Łapińska Zofia**, Szwedowicz Urszula, Kulbacka Julita; Nutraceutyki w walce z kancerogennym działaniem smogu; V Ogólnopolska Konferencja Naukowa „Ochrona środowiska - rozwiązania i perspektywy". Lublin, 25 maja 2023 r. Abstrakty, red. Paulina Szymczyk i Monika Maciąg, Fundacja na rzecz promocji nauki i rozwoju TYGIEL, 2023, s. 22–23.
4) **Łapińska Zofia** Novickij Vitalij, Rembiałkowska Nina, Radzevičiūtė-Valčiuke Eivina, Szewczyk Anna, Dubińska-Magiera Magda, Kulbacka Julita, Saczko Jolanta, Baczyńska Dagmara; The influence of asymmetrical electric fields with calcium ions or bleomycin on ovarian carcinoma; 5th International Wroclaw Scientific Meetings. Wrocław, Poland,

October 19-21, 2023; Advances in Clinical and Experimental Medicine, Uniwersytet Medyczny im. Piastów Śląskich we Wrocławiu, vol. 32, no. 3 spec., 2023, pp. 31

5) Bieżuńska-Kusiak Katarzyna, Gajewska-Naryniecka Agnieszka, Szwedowicz Urszula, **Łapińska Zofia**: The influence of microplastic particles on the development of breast cancer cells; 5th International Wroclaw Scientific Meetings. Wrocław, Poland, October 19-21, 2023; Advances in Clinical and Experimental Medicine, Uniwersytet Medyczny im. Piastów Śląskich we Wrocławiu, vol. 32, no. 3 spec., 2023, pp. 51

6) Śmietańska Aleksandra, Szlasa Wojciech, **Łapińska Zofia**, Saczko Jolanta: Evaluation of the expression of folic acid receptors in ovarian cancer after chemotherapy: In vitro research; 5th International Wroclaw Scientific Meetings. Wrocław, Poland, October 19-21, 2023; Advances in Clinical and Experimental Medicine, Uniwersytet Medyczny im. Piastów Śląskich we Wrocławiu, vol. 32, no. 3 spec., 2023, pp. 112

7) Kulbacka Julita, Rossowska Joanna, Chwiłkowska Agnieszka, Choromańska Anna, **Łapińska Zofia**, Rembiałkowska Nina: Antitumor effects of nanosecond PEFs with calcium ions in colon cancer in vitro and in vivo, In: 4th World Congress on Electroporation and Pulsed Electric Fields in Biology, Medicine, and Food & Environmental Technologies. Copenhagen, Denmark, 9 - 13 October 2022. Book of abstracts / Gehl Julie, Frandsen Dobbelaar Stine, Mahnič-Kalamiza Samo (eds.), 2022, 108 poz. OR-164

8) **Łapińska Zofia**, Szewczyk Anna, Kulbacka Julita, Saczko Jolanta: Electroporation-based modalities fused with 17β-estradiol in ovarian cancer therapy in vitro, In: 4th World Congress on Electroporation and Pulsed Electric Fields in Biology, Medicine, and Food & Environmental Technologies. Copenhagen, Denmark, 9 - 13 October 2022. Book of abstracts / Gehl Julie, Frandsen Dobbelaar Stine, Mahnič-Kalamiza Samo (eds.), 2022, 175 poz.PO-021

9) Bieżuńska-Kusiak Katarzyna, Gajewska-Naryniecka Agnieszka, **Łapińska Zofia**, Szwedowicz Urszula: Rodzaje śmierci komórkowej ze szczególnym uwzględnieniem apoptozy po zastosowaniu elektroporacji w obecności jonów wapnia w komórkach gruczolakoraka gruczołu sutkowego MCF-7/WT i MCF-7/DOX, In: VI Ogólnopolska Konferencja Naukowa "Współczesne zastosowanie metod analitycznych w farmacji i medycynie". Wrocław, 03 grudnia 2021 r. Książka abstraktów, 2021, pp. 5

10) Gajewska-Naryniecka Agnieszka, **Łapińska Zofia**, Szwedowicz Urszula, Rudno-Rudzińska Julia, Kulbacka Julita: Biomarkers for early detection of pancreatic cancer - what is new, In: VI Ogólnopolska Konferencja Naukowa "Współczesne

zastosowanie metod analitycznych w farmacji i medycynie". Wrocław, 03 grudnia 2021 r. Książka abstraktów, 2021, pp. 16

11) **Łapińska Zofia**, Szlasa Wojciech, Szewczyk Anna, Szwedowicz Urszula, Gajewska-Naryniecka Agnieszka, Bieżuńska-Kusiak Katarzyna, Kulbacka Julita, Saczko Jolanta: Elektroporacja z jonami wapnia i 17β-estradiolem jako potencjalna metoda leczenia raka jajnika in vitro, In: VI Ogólnopolska Konferencja Naukowa "Współczesne zastosowanie metod analitycznych w farmacji i medycynie". Wrocław, 03 grudnia 2021 r. Książka abstraktów, 2021, pp. 9
12) Szwedowicz Urszula, **Łapińska Zofia**, Bieżuńska-Kusiak Katarzyna, Gajewska-Naryniecka Agnieszka, Kulbacka Julita, Saczko Jolanta, Chwiłkowska Agnieszka, Choromańska Anna: Wpływ elektroporacji na uwalnianie mikropęcherzyków błonowych z komórek nowotworowych, In: VI Ogólnopolska Konferencja Naukowa "Współczesne zastosowanie metod analitycznych w farmacji i medycynie". Wrocław, 03 grudnia 2021 r. Książka abstraktów, 2021, pp. 10
13) **Łapińska Zofia**, Szlasa Wojciech, Gajewska-Naryniecka Agnieszka, Szwedowicz Urszula, Kulbacka Julita, Choromańska Anna: In vitro study of calcium electroporation (CaEP) fused with 17β-estradiol in ovarian cancer treatment, In: II Interdisciplinary Conference of Doctoral Students of Medical Universities "DocUMed" under the slogan "Libertas et ipsa scientia". Łódź, October 22-24, 2021. Book of abstracts / Duda Łukasz (eds.), 2021, ISBN 978-83-963099-5-2, pp. 28
14) Szwedowicz Urszula, Gajewska-Naryniecka Agnieszka, **Łapińska Zofia**, Kulbacka Julita, Saczko Jolanta: Electroporation-based applications: extracellular vesicles, In: II Interdisciplinary Conference of Doctoral Students of Medical Universities "DocUMed" under the slogan "Libertas et ipsa scientia". Łódź, October 22-24, 2021. Book of abstracts / Duda Łukasz (eds.), 2021, ISBN 978-83-963099-5-2, pp. 32
15) **Łapińska Zofia**, Szwedowicz Urszula, Gajewska-Naryniecka Agnieszka, Szewczyk Anna, Saczko Jolanta: Calcium electroporation (CaEP) fused with 17β-estradiol in ovarian cancer treatment in vitro, In: XXVIth International Symposium on Bioelectrochemistry and Bioenergetics of the Bioelectrochemical Society. Cluj-Napoca, Romania, 9-13 May 2021. Abstract book, 2021, 207 poz. S3-P-06
16) **Łapińska Zofia**, Kulbacka Julita, Saczko Jolanta: Calcium electroporation as a potential ovarian cancer treatment modality, In: Proceedings and workbook of the electroporation-based technologies and treatments: International Scientific Workshop and Postgraduate

Course. Ljubljana, Slovenia, November 16-December 4, 2020 / Kramar Peter, Miklavcic Damijan, **2020**, Založba FE, ISBN 978-961-243-410-6, pp. 23

13.4 Nagrody i wyróżnienia

1) **Laureatka** konkursu **Nagroda Naukowa** organizowanego przez firmę **AstraZeneca**, **The Institute of Cancer Research (ICR)** oraz **Porozumienie Akademickich Centrów Transferu Technologii (PACTT)** – stypendium na 10-miesięczny staż badawczy w ICR, Londyn, Anglia, I edycja, 2023
2) **Nominacja** do nagrody **Naukowiec Przyszłości 2023** w kategorii **Kobieta nauki, która zmienia świat** organizowanej przez **Forum Inteligentnego Rozwoju**
3) **I nagroda** Komitetu Naukowego VI Ogólnopolskiej Konferencji Naukowej *„Współczesne zastosowanie metod analitycznych w farmacji i medycynie”*, 03.12.2021, Wrocław, Polska
4) **I nagroda** Komitetu Naukowego Konferencji II Interdyscyplinarna Konferencja Doktorantów Uczelni Medycznych „DocUMed” pod hasłem: *Libertas et ipsa scientia* **za najlepsze wystąpienie ustne**, 22-24.10.2021, Łódź, Polska

13.5 Udział w projektach naukowych

1) **2020/38/L/NZ7/00342** (DAINA 2 – NCN) *"Manipulacja oporności lekowej w komórkach nowotworowych poprzez nanosekundowe, asymetryczne sekwencje impulsów"*; **kierownik projektu:** prof., dr hab. Julita Kulbacka; **okres działalności Doktorantki:** 01.09.2022-31.05.2024; **funkcja:** wykonawca (stypendysta)
2) **SKN.D260.22.001** (Studenckie Koła Naukowe Tworzą I nnowacje – MNiSW) *„Nutraceutyki w walce z kancerogennym działaniem smogu”*; **kierownicy projektu:** prof., dr hab. Julita Kulbacka (opiekun), mgr Urszula Szwedowicz, mgr Zofia Łapińska; **okres działalności Doktorantki:** 19.04.2022 – 18.04.2023; **funkcja:** kierownik, główny wykonawca
3) **2016/22/E/NZ5/00671** (Sonata Bis 6 – NCN) *"Udział ultrakrótkich impulsów elektrycznych w indukcji stresu oksydacyjnego oraz ocena przeciwnowotworowego potencjału elektroporacji nanosekundowej (nsPEF) w nowotworach jelita grubego na modelu in vitro i in vivo"*; **kierownik projektu:** prof., dr hab. Julita Kulbacka; **okres działalności Doktorantki:** 01.09.2021 – 28.02.2022; **funkcja:** wykonawca (stypendysta)
4) **STM.A040.18.013** (Projekty Młodych Naukowców – MNiSW) *"Wpływ modulatorów ekspresji receptorów retinoidowych i naturalnych substancji przenikających barierę krew mózg na ludzkie nowotworowe i prawidłowe komórki nerwowe."*; **kierownik projektu:**

dr hab. Anna Choromańska, prof. UMW; **okres działalności Doktorantki:** 11.2020 – 31.12.2021, **funkcja:** wykonawca

13.6 Udział w warsztatach i szkoleniach

1) **3D Bioprinting and live cell microscopy workshops**; 02.12.2022 r.; Katedra i Zakład Biologii Molekularnej i Komórkowej, Wydział Farmaceutyczny Uniwersytetu Medycznego im. Piastów Śląskich we Wrocławiu
2) **4th Annual Flow Cytometry Conference. Fundamentals of Flow Cytometry**; 22-24.09.2021 r.; Laboratorium Cytometrii Przepływowej przy Katedrze i Zakładzie Podstaw Nauk Medycznych, Wydział Farmaceutyczny, Uniwersytet Medyczny im. Piastów Śląskich we Wrocławiu
3) **Kurs dla osób uczestniczących w wykonywaniu procedur doświadczalnych z udziałem zwierząt**; 14.06-28.06.2021 r.; Instytut Immunologii i Terapii Doświadczalnej PAN oraz PolLASA Polska
4) **Podstawy analizy statystycznej w medycynie z wykorzystaniem oprogramowania Statistica**; 27.02 i 06.03. 2021 r.; StatSoft Polska
5) **Electroporation-based Technologies and Treatments (EBTT) – International Scientific Workshop and Postgraduate Course**, 16.11-4.12.2020 r.; Laboratorium Cybernetyki, Wydział Elektryczny, Uniwersytet w Lublanie (Słowenia)

13.7 Dodatkowa działalność Doktorantki

- Członkostwo w Studenckim Kole Naukowym (SKN) Biologii Komórki Nowotworowej; Doktorantka pełniła funkcję Sekretarza SKN w latach 2020-2023
- Członkostwo w Radzie Doktorantów UMW w latach 2021-2023
- Członkostwo w Kierunkowym Zespole ds. Jakości Kształcenia przy Wydziale Lekarskim UMW w latach 2022-2023