UNIVERZA V LJUBLJANI
BIOTEHNIŠKA FAKULTETA

Tamara POLAJŽER

PRIMERJAVA UČINKOV VISOKOFREKVENČNIH BIPOLARNIH IN NIZKOFREKVENČNIH MONOPOLARNIH ELEKTRIČNIH PULZOV NA PREŽIVETJE CELICE

DOKTORSKA DISERTACIJA

Ljubljana, 2022

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DOKTORSKA DISERTCIJA

THE EFFECTS OF HIGH-FREQUENCY BIPOLAR AND LOW-FREQUENCY MONOPOLAR ELECTRIC PULSES ON CELL SURVIVAL

DOCTORAL DISERTATION

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AI Elektroporacija je pojav, kjer izpostavljenost celice zunanjemu električnemu polju povzroči povečano prepustnost membrane, zato v celico lahko vstopijo molekule, za katere je drugače membrana neprepustna. Če po prenehanju delovanja električnega polja celica preživi govorimo o reverzibilni elektroporaciji, o ireverzibilni elektroporaciji pa, če celica umre. Pred kratkim se je pojavila nova oblika ireverzibilne elektroporacije - visokofrekvenčna ireverzibilna elektroporacija, pri kateri naj ne bi prišlo do nenadzorovanega mišičnega krčenja in občutka bolečine, kot smo tega navajeni pri monopolarnih pulzih. Namen doktorske disertacije je bilo preučiti učinek nove oblike elektroporacijskih pulzov in ga primerjati z učinkom uveljavljenih monopolarnih pulzov, ovrednotiti učinkovitost ponovnega zdravljenja z elektroporacijo v primeru delnega odgovora tumorja ali ponovnem pojavu tumorja in preučiti vzrok za aktivacijo imunskega sistema po elektroporaciji. Rezultati kažejo, da je učinkovitost visokofrekvenčnih bipolarnih pulzov ob povečanju amplitude primerljiva z monopolarnimi pulzi. Vzrok za potrebovano višjo amplitudo bi se lahko skrival v izničevalnemu učinku. Ta je odvisen od elektroporacijskega pufra. Zdravljenje z elektroporacijskim metodami lahko uporabimo večkrat, učinek vsakega nadaljnjega zdravljenja pa bo primerljiv s prvotnimi zdravljenjem. Celice pri elektroporaciji umrejo tudi po poti imunogene celične smrti, ki preko sproščanja DAMP molekul lahko aktivirajo imunski sistem in s tem pripomorejo k boljšem izidu zdravljenja tumorjev z elektroporacijo.

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AB Electroporation is a phenomenon in which exposure of a cell to an external electric field causes the formation of pores in the membrane. This allows molecules to which the membrane is otherwise impermeable to enter the cell. If the cell survives, this is called reversible electroporation; if the cell dies, this is considered irreversible electroporation. Recently, a new form of irreversible electroporation has emerged - high-frequency irreversible radiofrequency electroporation. These pulses do not cause involuntary muscle contractions or pain, which is inevitable when using established monopolar pulses. The aim of this dissertation is to compare the efficacy of the novel pulses with monopolar pulses, to evaluate the efficacy of repeat treatment with electroporation in case of partial response or tumor recurrence, and to understand the cause of immune system activation after electroporation. The results show that the efficacy of high-frequency bipolar pulses is comparable to that of monopolar pulses, although a higher amplitude is required. Possible reason for the higher amplitude is the cancellation effect. However, it seems that this effect depends on the electroporation buffer. Electroporation therapies with long monopolar pulses can be applied multiple times, achieving the same treatment efficacy as the original treatment. Cells killed by electroporation may die by immunogenic cell death, in which the release of DAMP molecules may activate the immune system and affect the outcome of electroporation therapy.

KAZALO VSEBINE

KAZALO ZNANSTVENIH DEL

Polajžer T., Dermol–Černe J., Reberšek M., O'Connor R., Miklavčič D. 2020. Cancellation effect is present in high-frequency reversible and irreversible electroporation. Bioelectrochemistry, 132: 1-11

Polajžer T., Miklavčič D. 2020. Development of adaptive resistance to electric pulsed field treatment in CHO cell line in vitro. Scientific Reports, 10: 9988

Polajžer T., Jarm T., Miklavčič D. 2020. Analysis of damage-associated molecular pattern molecules due to electroporation of cells in vitro. Radiology and Oncology, 54: 317-328

Batista Napotnik T., **Polajžer** T., Miklavčič D. 2021. Cell death due to electroporation – A review. Bioelectrochemistry, 141: 107871

KAZALO SLIK

KAZALO PRILOG

OKRAJŠAVE IN SIMBOLI

GET	Genska elektrotransfekcija (angl. Gene Electrotransfer)
DNA	Deoksiribonukleinska kislina
CAR	Himerni receptorji T celic (angl. chimeric antigen receptors)
CRISPR/Cas9	Kompleks gruč enakomerno prekinjenih kratkih palindromnih ponovitev (angl. clustered regularly interspaced short palindromic repeats) in encima CRISPR 9 (angl. CRISPR-associated 9)
COVID-19	Coronavirus disease 2019
IRE	Ireverzibilna elektroporacija (angl. irreversible electroporation)
PVI	Izolacija pljučnih ven (angl. pulmonary vein isolation)
PFA	Ablacija pulzirajočega polja (angl. Pulsed field ablation)
HFIRE	Visokofrekvenčna ireverzibilna elektroporacija (angl. high frequency irreversible electroporation)
ATP	Adenozintrifosfat
ROS	Reaktivne kisikove spojine
ESCRT	Kompleks endosomov (angl. endosomal sorting complexes required for transport)
ICD	Imunogena celična smrt (angl. immunogenic cell death)
DAMP	Molekulski vzorci povezani s poškodbo/nevarnostjo (angl. damage-associated molecular patterns/danger-associated molecular patterns)
PRR	Vzorčno prepoznavni receptorji (angl. pattern recognition receptors)
CRT	Kalretikulin (angl. calreticulin)
HMGB1	Visoko mobilen protein 1 (angl. high mobility group box 1 protein)
PI	Propidijev jodid

1 UVOD S PREDSTAVITIVJO PROBLEMATIKE, CILJEV IN HIPOTEZ/ZNANSTVENIH VPRAŠANJ

1.1 ELEKTROPORACIJA

Osnovni gradnik vseh živih bitij je celica, ki je obdana z membrano iz fosfolipidnega dvosloja. Membrana ločuje notranjost celice od okolja in hkrati zaradi njene selektivne prepustnosti določa sestavo celične notranjosti. Selektivna prepustnost onemogoča prosto prehajanje večine ionov in bioloških molekul, zato je njihov prehod odvisen od membranskih proteinov, ki gradijo ionske kanale in črpalke. Zaradi različnih koncentracij ionov na obeh straneh membrane, se na membrani ustvari električna napetost, imenovana mirovna membranska napetost oziroma mirovni membranski potencial (Alberts in sod., 2002; Reece in sod., 2014). Kadar celico oziroma celično membrano izpostavimo zunanjemu električnemu polju, ob tem nastane vsiljena membranska napetost, ki se prišteje vrednosti mirovnega membranskega potenciala. Določeno povečanje membranske napetosti lahko membrana prenese, vendar pa nekje pride do točke, imenovane kritična napetost (približno 200 mV ali več, odvisno do trajanja pulza). Pri tem membrana postane nestabilna, zaradi česar se spremeni prepustnost membrane za molekule in ione, ki drugače ne ali težko prehajajo membrano (Glaser in sod., 1988; Kotnik in sod., 2012; Neumann in Rosenheck, 1972; Rems in Miklavčič, 2016; Tarek, 2005; Weaver in sod., 1996). Zaradi povečane prepustnosti (angl. permeability) pojav imenujemo elektropermeabilizacija ali elektroporacija. Slednji izraz izhaja iz prepričanja, da se ob izpostavljenosti zunanjemu električnemu polju v membrani pojavijo pore. Vodne molekule, naj bi namreč pod vplivom električnega polja vdrle v lipidni dvosloj, zaradi prerazporeditve lipidnih glav pa naj bi se oblikovale pore (Kotnik in sod., 2019; Tarek, 2005).

Elektroporacija oziroma pojav povečane prepustnosti membrane, je lahko reverzibilna/povratna ali ireverzibilna/nepovratna, odvisno od parametrov električnih pulzov, kot so električna poljska jakost, dolžina, število in ponavljalna frekvenca pulzov (Rems in Miklavčič, 2016). Če po prenehanju delovanja električnega polja celica ponovno vzpostavi fiziološke procese govorimo o reverzibilni elektroporaciji (Rols in Teissié, 1990a). O ireverzibilni elektroporaciji pa govorimo, ko po izpostavitvi električnemu polju celica ne uspe zaceliti membrane (Rubinsky, 2007) ali kljub zaceljeni membrane ne uspe vzpostaviti celične homeostaze in s tem fizioloških procesov zato celica umre (Corrotte in Castro-Gomes, 2019).

Elektroporacija se uporablja v širokem naboru postopkov v živilski tehnologiji, biotehnologiji in medicini. Zaradi netermičnega delovanja elektroporacija nima vpliva na okus, barvo in hranilno vrednost tkiv. Ravno zato je primerna za uporabo v živilski tehnologiji, kjer omogoča lažje in učinkovitejše pridobivanje rastlinskega soka iz rastlinskih tkiv, dehidracijo/sušenje rastlinskega tkiva, učinkovitejšo ekstrakcijo celičnih komponent (pigmentov, antioksidantov, metabolitov, sladkorjev in lipidov), nastanek boljšega vina ter

učinkovitejše zamrzovanje hrane (Gagneten in sod., 2019; Gómez in sod., 2019; Mahendran in sod., 2019; Ozturk in Anli, 2017; Thamkaew in Gómez Galindo, 2020). Poleg tega metoda elektroporacije omogoča inaktivacijo in uničenje bakterij v odpadnih vodah, pasterizacijo tekočin (sadni sokovi, mleko, pivo) in čvrstih prehrambnih produktov (meso) (Bermúdez-Aguirre in sod., 2012; Evrendilek in sod., 2019; Gómez in sod., 2019; Montanari in sod., 2019). V biotehnologiji se elektroporacija uporablja za ekstrakcijo farmacevtsko ali medicinsko zanimivih molekul in plazmidov iz mikroorganizmov (Kotnik in sod., 2015). Elektroporacija ima pomembno vlogo tudi pri sušenju zelene biomase, ki služi kot vir za biogorivo (Golberg in sod., 2016). V nasprotju z biotehnologijo in živilsko tehnologijo, v medicini uporabljajo elektroporacijo za vnos molekul v notranjost celice, kot je to pri elektrokemoterapiji (Campana in sod., 2019b), genski elektrotransfekciji (genski terapiji z elektroporacijo) (Sachdev in sod., 2022) ter vnosu učinkovin v in skozi kožo (Zorec in sod., 2015). Poleg tega elektroporacija omogoča tudi zlivanje celic (Rems in sod., 2013) in netermično odstranjevanje oziroma uničenje tkiva (Aycock in Davalos, 2019; Reddy in sod., 2019).

V doktorski disertaciji sem se osredotočila predvsem na metode elektrokemoterapije in genske elektrotransfekcije z reverzibilno elektroporacijo ter netermičnega odstranjevanja tkiva z ireverzibilno elektroporacijo. Te metode so podrobneje predstavljene v naslednjih poglavjih.

1.1.1 Reverzibilna elektroporacija

1.1.1.1 Elektrokemoterapija

Zdravljenje s klasično kemoterapijo poteka s sistemsko ali lokalno dovedenim kemoterapevtikom. Uspešnost zdravljenja je odvisna od uspešnosti vstopa kemoterapevtika v celico, kjer se ta veže na genski material tumorske celice in s tem aktivira svoje delovanje. Vstop kemoterapevtikov v tumorsko celico omejuje celična membrana, zato je učinkovitost takšnega zdravljenja nizka. Lahko pa kemoterapevtiku dodamo elektroporacijo zaradi česar se prepustnost membrane poveča in kemoterapevtik vstopi v notranjost celice v veliko večji koncentraciji. S tem se poveča učinkovitost zdravljenja in hkrati je koncentracija uporabljenega kemoterapevtika lahko manjša, kar omili stranske učinke zdravljenja (Grošelj in sod., 2018; Miklavčič in sod., 2014). Tako zdravljenje imenujemo elektrokemoterapija. Pri elektrokemoterapiji gre torej za lokalno zdravljenje tumorja s kombinirano uporabo kemoterapevtikov in elektroporacijo (Campana in sod., 2019b; Mir in sod., 1991; Okino in Mohri, 1987). Ključ za uspešno elektrokemoterapijo je dovolj veliko električno polje in zadostna količina kemoterapevtika (Čemažar in sod., 2001, 1998; Miklavčič in sod., 1998; Miklavčič in sod., 2006). Najpogosteje uporabljena kemoterapevtika pri elektrokemoterapiji sta cisplatin in bleomicin (Gehl in sod., 1998). Pri elektrokemoterapiji tarčnega tkiva ne smemo poškodovati, zato se najpogosteje uporablja osem 100 µs dolgih monopolarnih pulzov, dovedenih pri poljski jakosti v območju reverzibilne elektroporacije (Jaroszeski in

sod., 2000; Mir in sod., 1991; Serša in sod., 1995). Priporočila za izbor elektrod, kemoterapevtika, način vnosa kemoterapevtika ter anestezijo glede na velikost, lokacijo in število tumorjev so zapisana v zbirki o standardnih protokolih (angl. standard operation procedures) (Campana in sod., 2019b; Gehl in sod., 2018; Mir in sod., 2006) .

Poleg povečanja prepustnosti membrane za kemoterapevtike, se pri elektrokemoterapiji pojavijo še drugi pozitivni učinki zdravljenja, kar še poveča uporabno vrednost oziroma učinkovitost elektrokemoterapije. Elektrokemoterapija povzroči tudi lokalne poškodbe oz. zoženje žil tumorskega ožilja, s čimer se zmanjša pretok krvi skozi tumor. To vpliva na zmanjšano dostavo hranil, hkrati pa se zmanjša tudi spiranje kemoterapevtika iz tumorja (Jarm in sod., 2010). Novejše študije elektrokemoterapiji pripisujejo tudi aktivacijo imunskega sistema, ki vpliva na preostale tumorske celice in oddaljene metastaze (Calvet in sod., 2014; Calvet in Mir, 2016; Di Gennaro in sod., 2016; Serša in sod., 2015).

Elektrokemoterapija je učinkovita in relativno uveljavljena terapija za zdravljenje kožnega raka (Campana in sod., 2019a; Campana in sod., 2019b; Mali in sod., 2013). V zadnjih letih so učinkovitost elektrokemoterapije potrdili tudi pri zdravljenju globlje ležečih tumorjih mehkega tkiva (Bianchi in sod., 2016; Campana in sod., 2019b; Edhemovic in sod., 2011; Edhemovic in sod., 2014; Gargiulo in sod., 2012; Gasbarrini in sod., 2015; Linnert in sod., 2012; Tarantino in sod., 2017). Zaradi razmeroma nizke koncentracije uporabljenega kemoterapevtika je elektrokemoterapija opredeljena kot varna terapija z malo stranskih učinkov (Marty in sod., 2006).

1.1.1.2 Genska elektrotransfekcija

Reverzibilna elektroporacija se uporablja tudi pri genski terapiji za vnos tujega genskega materiala v celico. Celični mehanizem nato s prepisom in sintezo tujega genskega materiala omogoči nastanek izbranega proteina ali utišanje gena (Anguela in High, 2019; Ginn in sod., 2018). Za uspešno gensko terapijo mora tuj genski material prepotovati ovire kot so celična membrana, citoplazma in jedrni ovoj. Vnos tujega genskega materiala v celico poteka preko virusnih in ne-virusnih vektorjev. Uporaba virusnih vektorjev pogosto povzroči nezaželene reakcije kot je aktivacija imunskega odziva (Zhao in sod., 2022), zato v zadnjem času narašča zanimanje za ne-virusne vektorje, ki pa so lahko kemični ali fizikalni (Yin in sod., 2014). Ne-virusna metoda, ki kaže velik potencial za vnos genskega materiala je genska terapija z elektroporacijo oziroma genska elektrotransfekcija (angl. Gene Electrotransfer, GET) (Lambricht in sod., 2016).

Pri tej metodi električno polje poleg povečanja prepustnosti membrane povzroči tudi elektroforezno silo, ki omogoči gibanje negativno nabitega genskega materiala (DNA) v bližino celične membrane. Natančen mehanizem metode še ni v celoti znan. V i*n vivo* naj bi bil prvi korak prerazporeditev DNA iz mesta injiciranja do tarčnih celic (Sachdev in sod., 2022), naslednji koraki pa podobni kot v *in vitro*. Tam naj bi DNA potekal v petih korakih:

interakcija izbrane DNA s celično membrano, transport DNA čez membrano, premik DNA preko citoplazme do jedra, vstop v jedro in izražanje genov. Glede vstopa DNA v celico obstajata dve teoriji. Pri prvi, bolje sprejeti teoriji, pride ob elektroporaciji, do interakcije celične membrane z DNA, ki kasneje z endocitozo vstopi v notranjost celice (Escoffre in sod., 2011; Faurie in sod., 2010). Po drugi, manj sprejeti teoriji pa naj bi DNA z elektroforezno silo vstopila v celico preko ob elektroporaciji nastalih por v membrani (Breton in sod., 2012). Vnesena DNA naj bi nato po citoplazmi celice potovala preko aktinskega omrežja citoskeleta do bližine jedra (Dauty in Verkman, 2005). Prehod DNA preko jedrnega ovoja pa naj bi bil najuspešnejši v času delitve celice, ko je jedrni ovoj začasno odsoten (Brunner in sod., 2002).

V onkologiji se genska elektrotransfekcija uporablja za zdravljenje tumorskih tkiv in za preventivno zdravljenje oziroma cepljenje (Algazi in sod., 2020; Bhatia in sod., 2020; Heller in Heller, 2010; Heller in Heller, 2015). Več desetletne študije izpostavljajo gensko elektrotransfekcijo kot varno in učinkovito (Sachdev in sod., 2022). Pred kratkim so pokazali, da se genska elektrotransfekcija lahko uporablja tudi pri zdravljenju tumorjev preko *ex vivo* genskega inženiringa, kjer omogoči vstop kompleksa za preurejanje genoma v T celice. T celice s spremenjenim genomom začnejo izražati specifične receptorje (angl. chimeric antigen receptors, CARs), preko katerih se vežejo na antigene tumorskih celic in jih označijo za uničenje (Alzubi in sod., 2021). Klinične študije genski elektrotransfekciji pripisujejo tudi vnos CRISPR/Cas9 kompleksov za urejanje genskih zapisov (Fajrial in sod., 2020) in vnos gole DNA kot metodo cepljenja v luči trenutne epidemije COVID-19 (Folegatti in sod., 2020; Morrow in sod., 2016; Topol, 2021).

1.1.2 Ireverzibilna elektroporacija/uničenje tkiva

Ireverzibilna elektroporacija (IRE) je ablacijska metoda, pri kateri pride do celične smrti oz. uničenja bolezenskega in tumorskega tkiva brez uporabe kemoterapevtika. Sprva je IRE veljala za neželen stranski učinek reverzibilnih elektroporacijskih pulzov. Šele leta 2005 so IRE predlagali kot potencialno samostojno metodo za uničenje mehkih tkiv (Davalos in sod., 2005). IRE predstavlja netermično obliko ablacije, pri kateri uničenje tkiva ne temelji na visoki temperaturi kot radiofrekvenčna in mikrovalovna ablaciji ali nizki temperaturi kot krioablacija (Cho in sod., 2011; Davalos in sod., 2005; Scheffer in sod., 2014; Swan in sod., 2013; Tondo in sod., 2018).

V primerjavi z elektrokemoterapijo IRE zahteva večje električno polje in daljšo izpostavitev (daljše pulze ali večje število pulzov). Najpogosteje uporabljajo 90 ali več visokonapetostnih (1–3 kV) pulzov z dolžino 70-100 µs (Aycock in Davalos, 2019; Cho in sod., 2011; Davalos in sod., 2005; Scheffer in sod., 2014; Swan in sod., 2013). Ustrezna postavitev elektrod je skrbno načrtovana za vsakega pacienta posebej in zagotavlja natančno pokritje celotnega tumorja in minimalen dvig temperature (Aycock in Davalos, 2019). To pripomore tudi k

ohranitvi strukture tkiva, ki se nahaja v bližini zdravljenega tkiva kot so žile, živci in sečevod. To je še posebej zaželeno pri zdravljenju kirurško težko dostopnih predelov (Chen in sod., 2015; Maor in sod., 2007). Danes je IRE relativno uveljavljena elektroporacijska metoda za uničenje tumorjev mehkega tkiva jeter, prostate, ledvic in trebušne slinavke (Bower in sod., 2011; Geboers in sod., 2020; Guenther in sod., 2019; Lee in sod., 2007; Pech in sod., 2011).

IRE bi se lahko uveljavila tudi kot metoda za odstranjevanje tkiva, ki povzroča nastanek atrijske fibrilacije (Bradley in Haines, 2020; Stewart in sod., 2019, 2021). Slednja velja za najpogostejšo motnjo srčnega ritma in vodi v razvoj številnih srčnih bolezni, ki so poleg raka najpogostejši vzrok smrti človeka v 21. stoletju (Ahmad in Anderson, 2021). Pri zdravem srčnem tkivu signal za krčenja srca nastane v sinoatrialnem vozlu, od koder signal potuje do prekatov. Pravilno širjenje signala je ključno za pravilno krčenje srčne mišice in črpanje krvi. Pri atrijski fibrilaciji se v atriju pojavi dodaten signal, ki največkrat izhaja iz pljučne vene. Ta signal potuje preko stika med pljučnimi venami in levim atrijem. V tem stiku je zgradba tkiva zapletena in drugačna kot v drugih delih srca - srčna mišična vlakna se spontano depolarizirajo, prevajanje akcijskega potenciala pa je zelo počasno. Zaradi spremenjene električne aktivnosti srca pride do neurejenega krčenja atrijev (Štublar in sod., 2021). To povzroči zastajanje krvi v atriju, s tem pa se povečuje nevarnost možganske kapi (Oladiran in Nwosu, 2019). Poleg tega atrijska fibrilacija povzroča še druge simptome, kot so občutek razbijanja v prsnem košu, utrujenost, slabo počutje, oslabelost in omotica, ki vplivajo na kakovost življenja pacienta (Lip in sod., 2016). Zdravljenje atrijske fibrilacije poteka z antiaritmični zdravili ali ablacijo tkiva ki generira neustrezne signale. Ker te signale v veličini primerov generira tkivo, ki leži v pljučni veni, poseg imenujejo tudi izolacija pljučnih ven (angl. pulmonary vein isolation, PVI), s katero prekinemo signal med pljučno veno in atrijem (Štublar in sod., 2021). Trenutno najbolj razširjena ablacijska metoda za zdravljenje atrijske fibrilacije je radiofrekvenčna ablacija (Štublar in sod., 2021). Pri radiofrekvenčni ablaciji skozi konico ablacijskega katetra dovedejo visokofrekvenčni izmenični električni tok, ob tem pa se v tarčno tkivo sprosti energija v obliki toplote. Če nastala toplota preseže 50°C to povzroči poškodbo tkiva oz. srčnomišične stene (Raatikainen in sod., 2017). Poškodba v globini nekaj mm zadošča za poškodbo vseh plasti (transmuralnost) srčnomišične stene od endokarda do epidakrada, s čimer se trajno prekine prevajanje signala (Kumar in sod., 2015). Ključnega pomena za uspešno zdravljenje je torej zadostna globina in neprekinjenost izolacijske linije (Miller in sod., 2012). Glavna pomanjkljivost radiofrekvenčne ablacije je ozko temperaturno območje delovanja, zato metodo lahko spremljajo nezaželeni stranski učinki, kot so nezadostna poškodba tarčnega tkiva ali poškodba okoliškega tkiva, kot sta požiralnik in frenični živec (Calkins in sod., 2017; Singh in sod., 2013).

Pred kratkim se je pojavila nova metoda ablacije, ki za razliko od radiofrekvenčne ablacije, ne temelji na termičnem poškodovanju tkiva, temveč na elektroporaciji tkiva, imenovana

ablacija pulzirajočega polja (angl. pulsed field ablation, PFA). Trenutno je PFA predmet številnih predkliničnih in kliničnih študij, kjer potrjujejo varnost in učinkovitost PFA ablacije (Koruth in sod., 2019; Reddy in sod., 2019, 2021). V nobeni študiji niso zaznali tipičnih nezaželenih stranskih učinkov oziroma poškodb okoliškega tkiva, ki navadno spremljajo zdravljenje s termično radiofrekvenčno ablacijo (Koruth in sod., 2019; Maor in sod., 2019; Stewart in sod., 2019, 2021). Vzrok za to bi bila lahko različna celična/tkivna občutljivost na elektroporacijske pulze. Domnevajo, da naj bi bil elektroporacijski prag srčnega tkiva namreč nižji od elektroporacijskega praga drugih tkiv (Kaminska in sod., 2012). Poleg tega zdravljenje z PFA v primerjavi z radiofrekvenčno ablacijo dosega boljšo transmuralnost in je vsaj za tretjino časa krajši (Bradley in Haines, 2020; Reddy in sod., 2019b). Ob nadaljnjem izboljševanju metode PFA, bo slednja verjetno postala prevladujoča ablacijska metoda atrijske fibrilacije v klinični praksi, vendar pa je za popolno uveljavitev metode potrebno zagotoviti večje razumevanje te metode, njene prednosti in omejitve (Haines, 2022).

1.2 VISOKOFREKVENČNI BIPOLARNI PULZI

Trenutno največkrat uporabljeni pulzi v medicini so monopolarni. Učinkovitost elektroporacije je odvisna od parametrov električnih pulzov kot so amplituda, ponavljalna frekvenca, število in dolžina, ki pa lahko meri vse od nanosekund pa do milisekund (ms) (Rems in Miklavčič, 2016). Tako elektrokemoterapija kot IRE za svoje delovanje uporabljata 100 µs dolge pulze, v različnem številu in amplitudi. Kljub visoki učinkovitosti elektroporacije pa ima zdravljenje z monopolarni pulzi tudi določne omejitve oziroma težave. Dovajanje pulzov je potrebno uskladiti elektrokardiogramom, v izogib nastanku motenj srčnega ritma (Ball in sod., 2010). Pacient pulze občuti kot kratko ostro bolečino, ki jo spremlja nenadzorovano krčenje mišic. Zaradi nenadzorovanega krčenja mišic lahko pride do premika elektrod in potencialnih poškodb tkiva (Aycock in Davalos, 2019; Scheffer in sod., 2014). Pri dovajanju 100 µs dolgih pulzov, naj bi do vzdraženja živčnih vlaken prišlo pri veliko nižji amplitudi, kot je potrebna za IRE, kar pomeni, da do vzdraženja živčnih vlaken pride v večjem področju okoli elektrod (Mercadal in sod., 2017). Poleg neprijetnosti ob zdravljenju, ki jih pacient občuti, pa so prisotne tudi drugi stranski učinki zdravljenja. V okolici elektrod zaradi elektrokemijskih reakcij pride do sprememb pH-ja (Klein in sod., 2019; Olaiz in sod., 2014) in nastanka mehurčkov (Mahnič-Kalamiza in Miklavčič, 2020). Res je, da uporaba mišičnih relaksantov in anestezija, v primeru globlje ležečih tumorjev, pripomoreta k boljšem/prijetnejšem zdravljenju, vendar bi bila najbolj optimalna rešitev minimiziranje vzdraženja živčnih vlaken in elektrokemijskih reakcij (Gudvangen in sod., 2022).

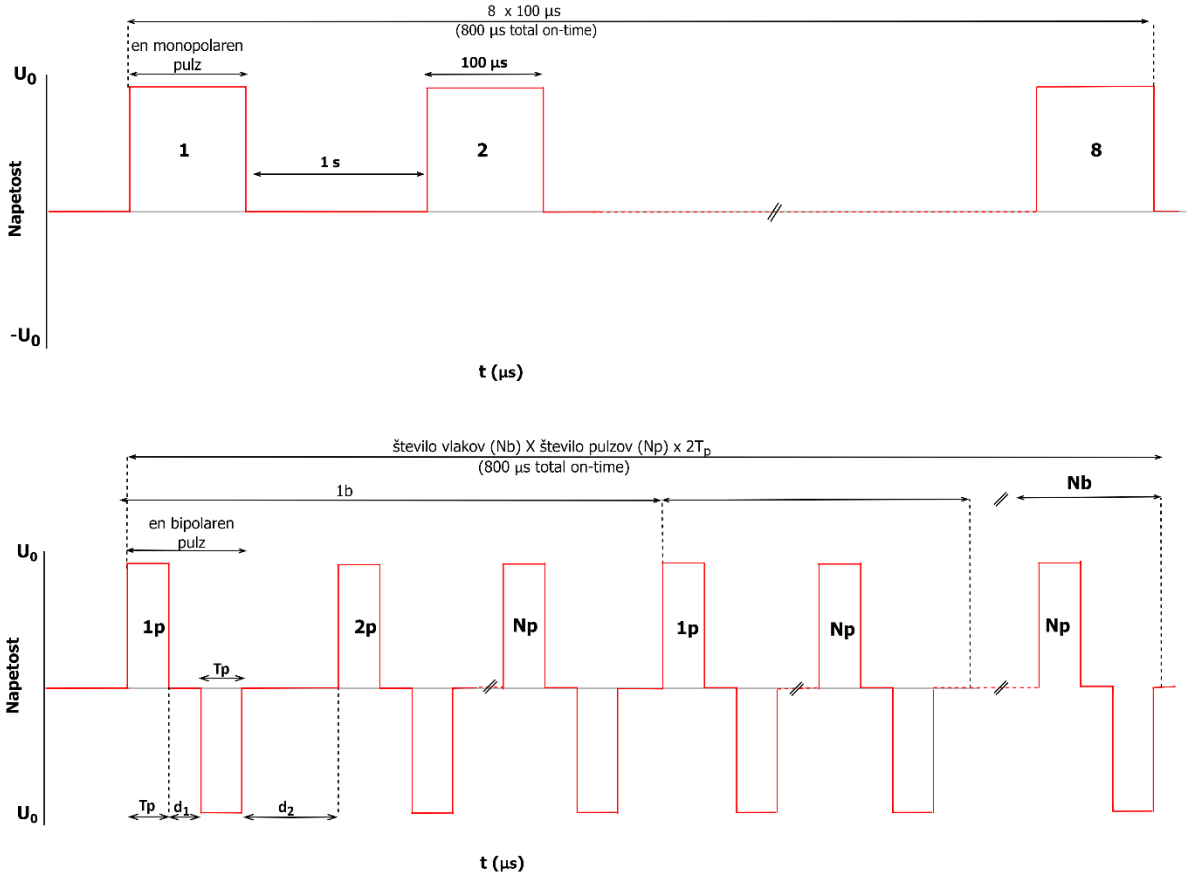
Pri iskanju pacientu prijaznejših pulzov, so ugotovili, da višja frekvenca pulzov in uporaba zelo kratkih pulzov zmanjša mišično krčenje. Ob povečanju ponavljalne frekvence iz 1 Hz na 5 kHz, je bil občutek bolečine sicer manjši kot prej, vendar še vedno neprijeten (Miklavčič in sod., 2005; Zupanič in sod., 2007). Na podlagi *in vitro* študij so ugotovili, da imajo kratki

nanosekundi pulzi zelo nizko učinkovitost pri depolarizaciji vzdražnih celic (Azarov in sod., 2019; Bagalkot in sod., 2019; Bowman in sod., 2010; Hristov in sod., 2018; Wang in sod., 2009). Prag elektroporacije vzdražnih celic z nanosekundnimi pulzi naj bi bil nižji kot je prag za nastanek akcijskega potenciala. Akcijski potencial, ki sledi elektroporaciji oziroma povečani prepustnosti membrane z nanosekundnimi pulzi, naj bi nastal kot posledica izgube mirovnega membranskega potenciala po elektroporaciji (Azarov in sod., 2019; Pakhomov in sod., 2017; Wang in sod., 2009), medtem ko naj bi pri daljših pulzih (100 μs) prišlo do nastanka akcijskega potenciala pred elektroporacijo oziroma povečano prepustnostjo membrane, saj je prag za nastanek akcijskega potenciala nižji od pragu elektroporacije. Vzrok za drugačno dinamiko celic po izpostavitvi različnim pulzom si lahko razlagamo z minimalnimi časovnim intervalom, ki je potreben za odpiranje napetostno občutljivih ionskih kanalov (Pakhomov in Pakhomova, 2020).

Na področju ireverzibilne elektroporacije v zadnjih letih opisujejo novo obliko elektroporacijskih pulzov, pri kateri bi bil lahko postopek zdravljenja z elektroporacijo pacientom bolj prijazen kot trenutna uveljavljena (monopolarna) oblika pulzov (Arena in sod., 2011). Takšen način zdravljenja so poimenovali visokofrekvenčna ireverzibilna elektroporacija (ang. high frequency irreversible electroporation, HFIRE). Visokofrekvenčne bipolarne pulze sestavljajo vlaki zelo kratkih pulzov, ki si sledijo v izmenjujoči se polariteti. Nova oblika pulzov odpravlja zgoraj navedene pomanjkljivosti monopolarnih pulzov, hkrati pa je učinkovitost visokofrekvenčnih bipolarnih pulzov ob povišanju amplitude ali števila pulzov enakovredna učinkovitosti monopolarnih pulzov (Arena in sod., 2011; Siddiqui in sod., 2016, 2017). Glavna posebnost visokofrekvenčnih bipolarnih pulzov je torej odsotnost nenadzorovanega krčenja mišic. V nasprotju s klasično IRE, pri terapiji z visokofrekvenčnimi bipolarnimi pulzi tako ne potrebujemo mišičnih relaksantov in sinhronizacije z srčnim utripom, kar močno olajša sam proces in trajanje terapije (Siddiqui in sod., 2016). Potencialno uporabo visokofrekvenčnih bipolarnih pulzov so v zadnjih letih prepoznali tudi za ablacijo srčne mišice, kjer je odsotnosti vzdraženja napetostno občutljivih mišičnih celic, in odsotnost nastanka mehurčkov v krvi srca še posebej pomembno. V nasprotju z drugimi ablacijski metodami lahko pri PFA tudi spreminjajo veliko več parametrov, s čimer vplivajo na samo učinkovitost ablacije (Reddy in sod., 2018, 2019). Neustrezno spreminjanje parametrov pa lahko povzroči tudi termične poškodbe, zato je poznavanje delovanja visokofrekvenčnih bipolarnih pulzov, tako parametrov in posebnosti, ki jih prinaša ta oblika pulza, ključna za čim uspešnejšo uporabo v kliniki.

V primerjavi s klasičnimi monopolarnimi pulzi imajo visokofrekvenčni bipolarni pulzi kompleksnejšo strukturo in večje število parametrov, saj lahko poleg dolžine, števila, frekvence in amplitude pulzov nadzorujemo tudi časovni zamik med pozitivno in negativno fazo bipolarnega pulza, ponavljalno frekvenco, število pulzov v vlaku in zamik med vlaki pulzov. Poleg tega imata lahko pozitivna in negativna faza različne amplitude in dolžine

(Pirc in sod., 2021). Posledično to pomeni več spremenljivk, ki vplivajo na učinek elektroporacije (Slika 1).



Slika 1: Shema monopolarnih pulzov (zgoraj) in visokofrekvenčnih bipolarnih pulzov (spodaj). Pri visokofrekvenčnih pulzih so vlaki bipolarnih pulzov (b) ločeni z ponavljano frekvenco 1 Hz, en vlak pulzov pa je sestavljen iz številih (Np) bipolarnih pulzov (p), ki so med seboj ločeni s časovnim zamikom v dolžini d_2. Vsak bipolaren pulz pa je sestavljen iz pozitivne in negativne faze pulza dolžine Tp, med njima pa je kratek časovni zamik (d_1).

Visokofrekvenčne bipolarne pulze si lahko predstavljamo kot sestav enostavnejših oblik pulzov, ki pa so jih v preteklosti že raziskovali z namenom bolšjega razumevanja delovanja elektroporacije. Ali se posebnosti teh enostavnejših pulzov in njihove posebnosti odražajo tudi v obliki visokofrekvenčnih bipolarnih pulzov in v kolikšni meri to vpliva na učinkovitost elektroporacije z visokofrekvenčnimi bipolarnimi pulzi pa zaenkrat ostaja neraziskano. Prva posebnost je tako imenovan izničevalni učinek (angl. cancellation effect), kjer si pozitivna in negativna faza pulza sledita zelo hitro, zato drugi pulz (t.i. negativna faza) izniči učinek prvega (t.i. pozitivne faze) (Ibey in sod., 2014; Pakhomov in sod., 2014; Schoenbach in sod., 2015). Pojav so odkrili pri nanosekundnih pulzih, ob primerjavi učinkovitosti posameznega bipolarnega pulza v primerjavi z monopolarnim pulzom enake dolžine (seštevek dolžine negativne in pozitivne faze bipolarnega pulza je enak dolžini monopolarnega pulza). Kljub

enaki količini dovedene energije obeh oblik pulzov, je učinek bipolarnega pulza zaradi izničevalnega učinka manjši kot pri monopolarnem pulzu (Ibey in sod., 2014; Pakhomov in sod., 2014; Schoenbach in sod., 2015). Vse kaže, da se izničevalni učinek z večjo dolžino pulza izgubi, saj imajo daljši bipolarni pulzi enako ali celo boljšo učinkovitost kot monopolarni pulzi kljub enaki količini dovedene energije (Kotnik in sod., 2003; Kotnik in sod., 2001; Kotnik in sod., 2001).

Naslednja posebnost je preučevanje učinka vlakov monopolarnih pulzov oz. deljenje enega daljšega vlaka pulzov na več krajših vlakov pulzov. Elektroporacija celic z enim daljšim monopolarnim pulzom, naj bi bila manj uspešna, kot če so ti pulzi razdeljeni na več krajših vlakov monopolarnih pulzov. Elektroporacija v primeru delitve na več vlakov poteka dlje časa ob enako veliki dovedeni energiji, kar pripomore tudi k manjšemu segrevanju tarčnega tkiva. Pri tem naj bi prvi vlak pulzov povečal občutljivost celic na električne pulze, zato naj bi bile celice ob naslednjem vlaku nanj bolj občutljive. Podaljševanje časa med vlaki torej povečuje učinkovitost elektroporacije monopolarnih pulzov. Opisan pojav so imenovali elektrosenzitizacija (Jiang in sod., 2014; Pakhomova in sod., 2011; Pakhomova in sod., 2013). Nadaljnje študije so pokazale, da so ti fenomeni odvisni od elektroporacijskega pufra (Dermol in sod., 2016).

Eden od pomembnih parametrov, ki verjetno vpliva na elektroporacijo je elektroporacijski pufer. Znano, je da ima lahko elektroporacijski pufer, zaradi sestave, osmolarnosti ter prevodnosti pomemben vpliv na učinek elektroporacije *in vitro* (Barrau in sod., 2004; Pucihar in sod., 2001; Rols in Teissie, 1989; Rols in Teissié, 1990b). Sicer velja, da je preučevanje delovanja in posebnosti pulzov do določene mere najlažje *in vitro*, je pri tem potrebno upoštevati posebnosti, ki pa jih v *in vivo* pogojih ni, kot na primer elektroporacijski pufer. Posledično, ne vemo ali so od elektroporacijskega pufra odvisni učinki elektroporacije, kot na primer izničevalni učinek in elektrosenzitizacija, prisotni tudi v *in vivo* pogojih in če so, do kakšne mere. Kljub temu da na podlagi raziskav opravljenih v različnih pufrih ne dobimo vedno uporabnih informaciji preko katerih lahko sklepamo o dogajanju *in vivo*, pa so ti podatki pomembni za optimizacijo pogojev *in vitro* študij (Mattes, 2020).

1.3 VEČKRATNO ZDRAVLJENJE Z ELEKTROPORACIJO

Pri elektrokemoterapiji in ireverzibilni elektroporaciji gre večinoma za enkratno terapijo. V primeru delnega/nepopolnega odgovora pa je potrebno terapijo ponoviti. Pri tem se pojavi vprašanje učinkovitosti, ali le-ta ob ponovitvi ostane enaka ali je slabša. Medtem ko je učinek različnih parametrov pulzov raziskovan tako v *in vivo* kot *v vitro* študijah, pa se o učinkih ponovnega zdravljenja oziroma ponovne izpostavitve celic električnim pulzom, ne ve veliko. Niti, če lahko celice razvijejo odpornost na električne pulze in s tem postanejo nanje manj občutljive, kar bi lahko pomenilo manjšo učinkovitost ponovnega zdravljenja.

Obstoječe raziskave so bili izvedene na tumorskih celicah in bakterijah. Tumorske celice naj bi že po enkratni izpostavljenosti električnemu polju postale manj občutljive na nadaljnje izpostavitve električnemu polju. Pri tem pa je celotna študija zajemala zgolj dve generaciji (Shao in sod., 2017). Bolj natančno preverjanje razvoja odpornosti na električne pulze so izvedli na bakterijah *Pseudomonas putida,* ki so v velikem številu prisotne v odpadnih vodah. Bakterije so, izpostavljali električnemu polju kar 30 generacij. V vsem tem času niso zaznali, da bi prišlo do razvoja odpornosti bakterij na elektroporacijske pulze (Gusbeth in sod., 2009). Dosedanje študije večkrat izpostavljenih celic električnim pulzov so si nasprotujoče, prekratke ali za namen zdravljenja tumorskega tkiva neustrezne. Za boljšo uporabo elektroporacije v medicini, tako iz vidika reverzibilne elektroporacije kot ireverzibilne elektroporacije, ob ponovni rasti tumorja, ob ponovni vzpostavitvi atrijske fibrilacije, kot tudi ob večkratni genski elektrotransfekciji, je torej nujno razumevanje učinkovitosti večkratnega zdravljenja z elektroporacijo.

1.4 AKTIVACIJA IMUNSKEGA SISTEMA PO ELEKTRPORACIJI

Aktivacija imunskega sistema pacienta, naj bi izboljšala izid zdravljenja z reverzibilno (Calvet in sod., 2014; Calvet in Mir, 2016; Serša in sod., 2015; Serša in sod., 1997) kot tudi z ireverzibilno elektroporacijo (Bulvik in sod., 2016; José in sod., 2012; Pandit in sod., 2019; Scheffer in sod., 2019; Vogl in sod., 2009; White in sod., 2018). Poleg uničenega tarčnega tumorskega tkiva, aktiviran imunski sistem lahko vpliva tudi na oddaljene metastaze. Aktivacija imunskega sistema pri elektroporaciji bi bila lahko posledica imunogene celične smrti (angl. immunogenic cell death, ICD) elektroporiranih celic. Pod ICD se šteje kakršna koli oblika celične smrti (programirana ali neprogramirana), ki povzroči aktivacijo imunskega sistema. Ob ICD se iz celic sproščajo posebne signalne molekule imenovane molekulski vzorci s povezani poškodbo/nevarnostjo (angl. damage-associated molecular patterns or danger-associated molecular patterns, DAMP) (Chaplin, 2010; Diercks in Kluin, 2016; Kellie in Al-Mansour, 2017). Gre za signalne molekule, ki se sproščajo iz umirajočih celic in delujejo kot notranji signal za poškodbe/nevarnost. Pri nepoškodovanih celicah se večina DAMP molekul nahaja v celični notranjosti (Roh in Sohn, 2018), kjer opravljajo specifične funkcije. Ob poškodbi pa pride do aktivnega ali pasivnega prenosa DAMP molekul v izvencelični prostor oziroma na površino celic, kjer prevzamejo vlogo signalne molekule (Gong in sod., 2019; Kato in Svensson, 2015; Obeid in sod., 2007; Patel, 2018). Kot signalna molekula se vežejo na vzorčno prepoznavne receptorje (angl. pattern recognition receptors, PRR), ki jih nosijo celice imunskega sistema, natančnejše fagociti (Kato in Svensson, 2015; Rock in sod., 2011). Fagociti, kot so makrofagi, ob fagocitozi tujih molekul oziroma DAMP molekul izločajo vnetne mediatorje, ki sprožijo vnetno reakcijo. Stopnjo poškodbe oz. stresa odraža količina DAMP molekul, saj v odvisnosti od koncentracije DAMP molekul lahko pride do zdravljenja preko blage vnetne reakcije ali uničenja oz. fibroze tkiva preko močnega vnetja (Stoecklein in sod., 2012). Fagociti nato ustrezno razgrajene tuje molekule predstavijo celicam T pomagalkam in s tem sprožijo specifičen imunski odziv. Razvijejo se limfociti B in T, ki se diferencirajo v spominske

limfocite. Slednji se ob vztrajanju tujka/tumorskih celic ali njegovi ponovni izpostaviti preoblikujejo v efektorske celice, ki odstranjujejo tujke (Kreiger in sod., 2011). Umirajoče tumorske celice, podvržene imunogeni celični smrti, naj bi tako delovale kot in situ cepivo, in tako pripomogle k uničenju preostalih tumorskih celic v telesu in vzpostavitvi dolgoročne proti-tumorske imunosti (Serša in sod., 2015).

Preučevanju imunogene celične smrt *in vitro* poteka preko detekcije številnih DAMP molekul v izvenceličnem prostoru, med katerimi so najbolj znane adenozintrifosfat (angl, adenosine triphosphate, ATP), kalretikulin (angl. calreticulin, CRT) in protein HMGB1 (angl. high mobility group box 1 protein) (Roh in Sohn, 2018; Zhou in sod., 2019). Elektroporacija s povečanjem prepustnosti membrane omogoča prehod molekulam, ki drugače ne prehajajo membrane, vključno z DAMP molekulami. Izvencelični ATP je v zgodnjem raziskovanju elektroporacije služil kot molekula za potrditev povečane prepustnosti membrane (Rols in sod., 1998; Rols in Teissié, 1990a; Sixou in sod., 1991; Volker in sod., 1989). Šele študije zadnjih let ATP molekulo pri elektroporaciji prištevajo med DAMP molekule, ključno za aktivacijo imunogene celične smrti (Calvet in sod., 2014). V splošnem je potek imunogene celične smrti povzročen z elektroporacijo neznan, saj je razen iztekanja ATPja, izpust ostalih DAMP molekul v odvisnosti od elektroporacijskih pulzov slabo raziskan.

Trenutno razumevanje signalnih poti vpletenih odziv imunskega sistema je slabo. V prihodnosti bi lahko znanje o teh signalnih poteh potencialno izboljšalo uporabe elektroporacije v kliniki. Razumevanje prepletenosti ireverzibilne elektroporacije in aktivacije imunskega odziva je pomembno tudi iz vidika nastanka fibroznega tkiva. Potencial pa kaže tudi imuno terapija v kombinaciji z elektroporacijo (Imran in sod., 2022). Ker bi vzrok za aktivacijo imunskega odziva pri zdravljenju z elektroporacijo lahko ležal v mehanizmu celične smrti pri elektroporaciji bo potrebno posebno pozornost posvetiti tudi raziskovanju celične smrti, ki po povzročijo elektroporacijski pulzi sami in v kombinaciji z kemoterapevtiki.

Načeloma velja, da je preživetje celic odvisno od njihove sposobnosti ohranjanja homeostaze ob stalnem prilagajanju na zunanje in notranje dražljaje. Ko je intenziteta dražljaja prevelika in se celice nanj ne morejo več prilagoditi, nastane celična poškodba, ki povzroči porušenje celične homeostaze (Miller in Zachary, 2017). Prav to se zgodi tudi pri elektroporaciji, kjer dražljaj povzroči nastanek poškodb oz. por v membrani in tako poruši celično homeostazo. Rezultat poškodbe celice je reverzibilna ali ireverzibilna elektroporacija, odvisno od celičnih popravljalnih mehanizmov. Elektroporacija povzroči prehod celice skozi tri faze - nastanek poškodbe, popravljanje in celična smrt. Mehanizem vsakega od teh ob elektroporaciji je zaenkrat slabo raziskan, vendar pa zaradi vse večjega zanimanja uporabe ireverzibilne elektroporacije v medicini vedno bolj pomemben.

V literaturi je opisanih nekaj celičnih poškodb, ki nastanejo ob elektroporaciji in potencialno vplivajo na potek celične smrti pri elektroporaciji. Elektroporacija primarno povzroči poškodbo plazemske membrane nastanka pojava por v membrani. Celica je zato podvržena osmotskemu neravnotežju. Ca^{2+} ioni vdrejo v celico in poruši se znotrajcelična homeostaza. ATP izteče iz celic, kar vodi do pomanjkanja energetsko bogatih molekul znotraj celice (Gibot in sod., 2020; Pakhomova in sod., 2014; Rols in Teissié, 1990a). Nastanejo tudi kisikove reaktivne spojine ter oksidativne poškodbe nenasičenih lipidov v membrani (Teissie, 2017; Wiczew in sod., 2020). Signalne poti bioloških molekul (Ca^{2+}, ATP in ROS) na katere vpliva elektroporacija se v celici močno prepletajo, zato je primaren vzrok poškodbe pri elektroporaciji težko določiti (Brookes in sod., 2004). Elektroporacijski pulzi pa lahko vplivajo tudi na večje molekule. Spremenijo lahko konformacijo in strukturo proteinov, posredno pa povzročijo tudi poškodbe DNA (Chafai in sod., 2019; Hekstra in sod., 2016). Posebno skupino pulzov predstavljajo nanosekundi pulzi, ki povzročijo tudi poškodbe membran celičnih organelov, kot je mitohondrij (Beebe, 2017; Napotnik Batista in sod., 2012).

Ključnega pomena za preživetje celice je aktivacija popravljalnih mehanizmov, ki s celjenjem membrane celici omogočijo ponovno vzpostavitev celične homeostaze in s tem njeno preživetje (Corrotte in Castro-Gomes, 2019). Velikost por, ki nastanejo v plazemski membrani pri elektroporaciji je ocenjena na velikost do 100 nm (Bowman in sod., 2010). Medtem, ko se pri sobni temperaturi ali optimalnih 37°C membrana po elektroporaciji povrne v prvotno stanje že v nekaj minutah po elektroporaciji, pri nizkih temperaturah (4°C) za svojo zacelitev membrana potrebuje tudi ure (Kotnik in sod., 2019). Poleg temperature imajo na popravljalne mehanizme vpliv tudi parametri pulzov (Gabriel in Teissie, 1994; Jakstys in sod., 2020; Rols in Teissié, 1990a; Saulis in Saule, 2012), koncentracija Ca^{2+} v elektroporacijskim pufru (Ciobanu in sod., 2018), sestava elektroporacijskega pufra (Gabriel in Teissie, 1994; Rols in Teissié, 1990b) in nastanek ROS (Gabriel in Teissie, 1994). Natančni popravljalni mehanizem elektroporirane membrane zaenkrat ostaja neznan, vendar pa obstaja nekaj teorij. Najmanjše poškodbe membrane velikosti nekaj nanometrov, naj bi se zacelile spontano (Cooper in McNeil, 2015; Levine in Vernier, 2010). Pri večjih poškodbah pa naj bi se uvihki poškodovanih delov membrane odstranili preko endocitoze, eksocitoze lizosomov (Hai in Spira, 2012) ali preko ESCRT (angl. endosomal sorting complexes required for transport) kompleksa (Ding in sod., 2017). Medtem ko predvidevajo, da naj bili pri celjenju membrane poškodovane z mikrosekundnimi in milisekundnimi pulzi vpleteni enaki ali podobni popravljalni mehanizmi pri katerih imajo pomembno vlogo lizosomi (Silve in sod., 2014), pa to ne velja za nanosekunde pulze. Ti namreč povzročijo prekinitve mreže mikrotubolov, zaradi česar je notrajcelična migracija lizosomov onemogočena (Thompson in sod., 2018; Thompson in sod., 2014).

Kljub uspešnemu celjenju membrane pa včasih celice ne uspejo vzpostaviti homeostaze, zato celica umre. Elektroporacija lahko sproži različne oblike celičnih smrti v odvisnosti od

parametrov pulzov, tipa celic ali tkiva in ostalih dejavnikov. Apoptoza je najpogosteje opisana celične smrti, ki jo sproži elektroporacija tako pri *in vivo* (Arena in sod., 2011; Chai in sod., 2017; Faroja in sod., 2012; Mercadal in sod., 2020; Ringel-Scaia in sod., 2019; Scheffer in sod., 2014) kot pri *in vitro* študijah ob uporabi nanosekundnih (Beebe in sod., 2003; Beebe in sod., 2013; Ren in Beebe, 2011), mikrosekundnih (Kaminska in sod., 2012; Mercadal in sod., 2020; W. Zhou in sod., 2012) in milisekundnih pulzov (Matsuki in sod., 2010). Poleg apoptoze so pri elektroporaciji potrdili tudi nekrozo celic. V neposredni bližini elektrod je namreč električno polje najvišje, kar se lahko odraža tudi v termičnih poškodbah celic, ki povzročijo nekrozo celic (Žmuc in sod., 2019).

Nekroza je dolga leta velja za neprogramirano celično smrt, vendar pa novejše študije opisujejo aktivacijo določenih signalnih poti, kar kaže da je nekroza deloma programirana celična smrt (Festjens in sod., 2006; Proskuryakov in sod., 2003). V zadnjem času se je pojavila nova nomenklatura celične smrti, ki celičnih smrti ne deli več na nekrozo, apoptozo ter avtofagijo, temveč na podlagi morfološkega, biokemijskega in funkcijskega vidika definira številne nove poti celične smrti (Galluzzi in sod., 2018). Na področju elektroporacije so do sedaj *in vitro* potrdili dve novi poti celične smrti in sicer nekroptozo (angl. necroptosis) in piroptozo (angl. pyroptosis). Za obe pa je značilno sproščanje DAMP molekul oziroma imunogena celična smrt (Calvet in sod., 2014; Mercadal in sod., 2020; Ringel-Scaia in sod., 2019).

1.5 HIPOTEZE

V okviru problematike in načrtovanega raziskovalnega dela doktorske disertacije smo postavili naslednje hipoteze, katerih potrditev ali zavrnitev bo prispevala k izboljšanju metod elektroporacije v medicini.

- Učinek elektroporacije visokofrekvenčnih bipolarnih pulzov je manjši od nizkofrekvenčnih monopolarnih pulzov pri enaki dovedeni energiji in amplitudi ter odvisen od elektroporacijskega pufra.

- Večkratna izpostavljenost električnemu polju povzroči razvoj rezistence na električne pulze.

- Parametri električnih pulzov vplivajo na iztek molekulskih vzorcev povezanih z nevarnostjo (DAMP) iz celic.

2 ZNANSTVENA DELA

2.1 OBJAVLJENA ZNANSTVENA DELA

2.1.1 Izničevalni učinek visokofrekvenčnih bipolarnih pulzov je prisoten pri reverzibilni in ireverzibilni elektroporaciji

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Uporaba visokofrekvenčnih bipolarnih pulzov naj bi bila pri zdravljenju z elektrokemoterapijo kot tudi pri ablaciji tkiva z elektroporacijo pacientu prijaznejša, saj ti ne povzročajo bolečine in nenadzorovanega krčenja mišic. Učinkovitost visokofrekvenčnih bipolarnih pulzov, tako iz vidika povečane prepustnosti membrane kot vpliva na preživetje celic, je ob povečanju amplitude primerljiva z monopolarnimi pulzi (8x100 µs), ki so trenutno uveljavljeni za uporabo v medicini, vendar je pri tem potrebno povečati amplitudo. Vzrok za višjo amplitudo pulzov visokofrekvenčnih bipolarnih pulzov bi lahko bil v njihovi obliki, kjer si veliko število zelo kratkih pulzov sledi v izmenjujoči se polariteti. Znotraj visokofrekvenčnih bipolarnih pulzov bi lahko prišlo do tako imenovanega izničevalnega učinka, kjer si pulza različnih polaritet sledita zelo hitro in ob tem učinek prvega pulza izniči učinek z drugega pulza. V študiji smo na celični liniji CHO v različnih elektroporacijskih pufrih prisotnost izenačevalnega učinka visokofrekvenčnih bipolarnih pulzov potrdili pri ireverzibilni elektroporaciji oziroma vplivu na preživetje celice. Pri preučevanju povečane prepustnosti membrane, je bil izničevalni učinek visokofrekvenčnih bipolarnih pulzov v nizkoprevodnem elektroporacijskem pufru odsoten, ob višanju prevodnosti pufrov pa se izničevalni učinek spet pojavi. Moč izničevalnega učinka visokofrekvenčnih bipolarnih pulzov je pogojena tako z dolžino pulzov pozitivne in negativne faze bipolarnega pulza (1-10 µs), kot tudi z dolžino časovnega zamika med pozitivno in negativno fazo bipolarnega pulza (0,5 µs – 10 ms). V splošnem velja, da daljši pulzi in daljši zamik povzročita manj izrazit izničevalni učinek. Vzrok za nastanek izničevalnega učinka lahko deloma pojasnimo z aktivnim praznjenjem celične membrane (ang. assisted discharge). Ker izničevalni učinek še ni v celoti pojasnjen smo testirali tudi hipotezo o hiperpolarizaciji celice zaradi vdora kloridnih ionov, ki pa je nismo uspeli potrditi.

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Cancellation effect is present in high-frequency reversible and irreversible electroporation

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ABSTRACT

It was recently suggested that applying high-frequency short biphasic pulses (HF-IRE) reduces pain and muscle contractions in electrochemotherapy and irreversible ablation treatments; however, higher amplitudes with HF-IRE pulses are required to achieve a similar effect as with monophasic pulses. HF-IRE pulses are in the range of a microseconds, thus, the so-called cancellation effect could be responsible for the need to apply pulses of higher amplitudes. In cancellation effect, the effect of first pulse is reduced by the second pulse of opposite polarity. We evaluated cancellation effect with high-frequency biphasic pulses on CHO-K1 in different electroporation buffers. We applied eight bursts of 1–10 μs long pulses with inter-phase delays of 0.5 μs – 10 ms and evaluated membrane permeability and cell survival. In permeability experiments, cancellation effect was not observed in low-conductivity buffer. Cancellation effect was, however, observed in treatments with high-frequency biphasic pulses looking at survival in all of the tested electroporation buffers. In general, cancellation effect depended on inter-phase delay as well as on pulse duration, *i.e.* longer pulses and longer interphase delay cause less pronounced cancellation effect. Cancellation effect could be partially explained by the assisted discharge and not by the hyperpolarization by the chloride channels.

1. Introduction

Electroporation is a phenomenon in which cells exposed to pulsed electric fields of a sufficient intensity form nanoscale defects referred to as pores in the cell membrane, where lipids are chemically modified and the function of membrane proteins is modulated [1]. Consequently, membrane permeability increases and allows molecules for which the membrane is usually impermeable to cross the cell membrane. If cells recover after treatment and survive, this is termed reversible electroporation. However, when damage is more extensive, and cells die, this is termed irreversible electroporation (IRE). Electroporation is used in medicine, *i.e.* electrochemotherapy (ECT) [2–5], gene therapy [6,7], DNA vaccination [8,9], and tumor [10,11] or cardiac ablation [12–15] by irreversible electroporation, in biotechnology [16–18], and food processing [16,19].

In ECT and IRE treatments, 50–100 μs long monophasic pulses are traditionally applied at approximately 1 Hz repetition frequency, synchronized with the heart rhythm [4,11,20]. Low repetition frequency results in separate muscle contractions, *i.e.* individual multiple muscle twitches associated with every pulse delivered [21]. Since pulses cause electrical stimulation of excitable tissue, also sensory nerves are stimulated, therefore the procedure is also painful for the patients [22,23]. Therefore, general anesthesia [24], synchronization with electrocardiogram [25–27] and administration of muscle relaxants are needed during the treatment to prevent painful muscle contraction.

It was previously suggested that unpleasant sensations could be reduced by increasing the pulse repetition frequency [23,28]. Although *in vitro* results were promising, as the obtained molecular uptake remained similar up to 8.3 kHz repetition frequency, only slightly higher voltages had to be applied with higher repetition frequencies [29]. *In vivo*, at 1 Hz a higher percentage of complete tumor regression was observed than at 5 kHz repetition frequency, especially when using sub-optimal drug concentrations [28,30]. Another suggestion to reduce muscle and nerve excitation and elevate pain was to use specially designed electrodes, *i.e.* insulated needle electrodes [31] or "current cage" electrode placement [32]. Another recent approach was to replace the standard 50–100 μs monophasic pulses by bursts of short biphasic pulses, the so-called high-frequency irreversible electroporation (HF-IRE). When HF-IRE pulses were applied *ex-vivo* and *in vivo* to several animal models [33–35] as well as to humans in the first human

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study on prostate cancer [36], there were fewer muscle contractions observed and less muscle relaxants needed than in standard IRE treatments. The efficiency of the HF-IRE treatment was comparable to the IRE treatment; however, higher amplitudes of electric pulses had to be in the HF-IRE treatments than when using standard IRE pulses. Nevertheless HF-IRE pulse treatments could potentially improve the procedural safety for patients by obviating the need for neuromuscular blockage and general anesthesia.

In addition to IRE it was also demonstrated that high-frequency electroporation, *i.e.* HF-EP with bursts of short biphasic pulses could be used to increase membrane permeability to fluorescent dyes [37] and recently, also to chemotherapeutic cisplatin in electrochemotherapy *in vitro* [38]. However, in this case higher electric pulses had to be delivered with HF-EP than with classical 8 × 100 μs ECT to achieve a comparable effect for equal pulse duration.

It was previously reported that biphasic pulses were at least as efficient as monophasic pulses. *In vitro*, higher DNA transfection efficiency was obtained with biphasic pulses than with monophasic pulses [39]. Presumably, biphasic pulses induced cell membrane permeabilization on both sides of the membrane facing the electrodes and not only on one side, as would be expected with monophasic pulses. Improved efficiency of permeabilization with biphasic pulses was later confirmed also by increased membrane permeability [40], while the electrolytic contamination with biphasic pulses was lower than with monophasic pulses [41]. *In vivo*, monophasic (100 μs) and biphasic pulses (50 + 50 μs) were reported to be of similar efficiency in electrochemotherapy [42], whilst with 20 ms long biphasic pulses a higher transgene expression in liver tissue was obtained than with unipolar pulses (monophasic) [43]. In another study, no difference was seen in gene transfer of skin between applying monophasic and biphasic pulses [44]. In all studies mentioned above, however, longer pulses were applied than those used in HF-IRE (>10 μs).

Over time, the development of new pulse generators has made it possible to deliver even shorter pulses in the nanosecond time range [45]. Interesting new observations were made using biphasic nanosecond pulses suggesting they were less efficient in permeabilizing and killing cells than monophasic nanosecond pulses [46], *i.e.* the so-called cancellation effect was observed which challenged the existing knowledge. Briefly, a cancellation effect was reported in which the effect of the first pulse was cancelled (or reduced) by the effect of the second pulse of the opposite polarity, although applying asymmetrical biphasic pulses (in voltage [47] and time [48]) decreases the extent of the cancellation effect. This cancellation effect was observed for one or more biphasic pulses with the duration of the positive or the negative pulse between 60 and 900 ns and the delay between the positive and the negative pulse up to 10 ms [46–51]. It was detected via calcium influx, the influx of fluorescent dyes, phosphatidylserine externalization, metabolic assays of survival, and membrane conductance measurements. The reason(s) for this cancellation effect have not yet been identified; however, different theories and models were proposed [52,53]. The mechanisms suggested are: assisted membrane discharge; reversed electrophoretic ion transport; two-step oxidation of membrane phospholipids [49]; localized charging and discharging events across the membrane [48]; and reversed elongation forces due to electrodeformation [51,54]; but evidence supporting each of these mechanisms are lacking. Here, we investigated a new hypothesis - a hyperpolarization of chloride channels.

Chloride channels (CLC) are responsible for the movement of Cl^- ions necessary in neuronal, muscular, cardiovascular, and epithelial function [55]. CLC channels are dimers with each of the subunits forming 'protopores' that combined together leads to two types of gating, slow and fast [56]. Unlike most other types of voltage-gated ion channels, their structure does not include an S1-S4 transmembrane voltage-sensing motif. Instead, their fast gating voltage dependence arises from the movement of the permeant Cl^- ion through the transmembrane electric field, which interestingly can be activated by either hyperpolarization or depolarization. Consequently, CLC channels can exhibit bidirectional ultrafast gating of Cl^- in the μs range that is dependent on the concentration of extracellular Cl^-. We hypothesized that the transit of Cl^- in the pores of CLC channels might therefore be sensitive to the rapid reversal of electric field in biphasic pulses, leading to the cancellation effect.

Since pulses, usually applied in HF-IRE treatments are biphasic and 1 μs long, they are already in the time range of the cancellation effect. Thus, in our study, we aimed to determine if the cancellation effect is also present in HF-IRE treatments *in vitro*. The cancellation effect could partially explain why higher voltages must be applied with HF-IRE pulses than with IRE pulses to achieve a comparable effect. We evaluated irreversible as well as reversible electroporation, and thus we call our protocol high-frequency electroporation (HF-EP). We varied pulse duration between 1 and 10 μs, while the inter-phase delay was varied between 0.5 μs – 10 ms. We compared the effect of HF-EP pulses to standard IRE or ECT pulses (*i.e.* 100 μs monophasic pulses) with the same total pulse duration. Experiments were performed in three different electroporation buffers, as it was already shown that electroporation buffers significantly influence electroporation experiments [57]. We also performed calculations where we evaluated the effect of buffer conductivity on membrane charging and discharging. Our results show that the cancellation effect is present in HF-EP treatments and shows its complex dependency on the electroporation buffer.

2. Materials and methods

2.1. Electroporation buffers

Three different electroporation buffers were used (Table 1). A standard low-conductivity potassium-phosphate (KPB) buffer is often used in *in vitro* experiments due to current limitations of pulse generators. To obtain the high-conductivity buffer, we *iso*-osmotically replaced the sucrose by NaCl as sucrose is physiologically not present at high concentrations. To obtain the buffer without chloride, MgCl was replaced by magnesium D-gluconate hydrate and NaCl by sodium gluconate. We eliminated all chloride ions to test a hypothesis that the cancellation effect could be explained by hyperpolarization of the cell membrane caused by the activation of chloride channels. All buffers were *iso*-osmotic (300 mOsm/kg), as determined by freezing point depression method with Knauer cryoscopic unit (model 7312400000, Knauer,

Table 1
Composition and electrical conductivity of three electroporation buffers, used in our study.

Electroporation buffer	Composition	Electrical conductivity (mS/cm)
Low-conductivity buffer	10 mM K_2HPO_4/KH_2PO_4 in ratio 40.5:9.5, 1 mM $MgCl_2$, 250 mM $C_{12}H_{22}O_{11}$	1.76 [57]
High-conductivity buffer	10 mM K_2HPO_4/KH_2PO_4 in ratio 40.5:9.5 1 mM $MgCl_2$, 150 mM NaCl	19.12 [57]
Buffer without chloride	10 mM K_2HPO_4/KH_2PO_4 in ratio 40.5:9.5, 1 mM $C_{12}H_{22}MgO_{14}\,xH_2O$, 150 mM $NaC_6H_{11}O_7$	9.57*

* Measured with the conductometer S230 SevenCompact (Mettler Toledo, Switzerland) at room temperature (24 °C).

Germany). All chemicals were from Sigma Aldrich, Germany, except for KH_2PO_4, which was from Merck, Germany.

2.2. Cell preparation

Chinese hamster ovary (CHO-K1) cells purchased from the European Collection of Authenticated Cell Cultures were grown in HAM F-12 growth medium (PAA, Austria) in culture flasks (TPP, Switzerland) in an incubator (Kambič, Slovenia) at 37 °C with a humidified 5% CO2. The growth medium was supplemented with 10% fetal bovine serum (Sigma-Aldrich, Germany), L-glutamine (StemCell, Canada), antibiotics penicillin/streptomycin (PAA, Austria) and gentamycin (Sigma-Aldrich, Germany) (*i.e.*, full HAM-F12). After 2–3 days when 70% confluency was reached, cells were detached by 10x trypsin-EDTA (PAA, Austria), diluted 1:9 in Hank's basal salt solution (StemCell, Canada), which was inactivated after 2 min by addition of fresh full HAM F-12. The cell suspension was centrifuged at 180 g and 22 °C for 5 min. The supernatant was removed, and the cell pellet was re-suspended in the chosen electroporation buffer at the cell density 2×10^6 cells/ml.

2.3. Pulse generation

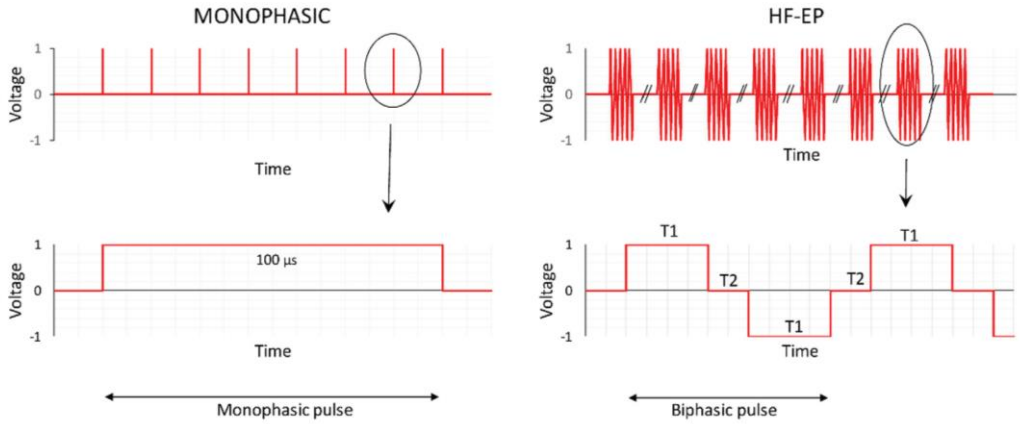
Pulses were applied by a laboratory prototype pulse generator (University of Ljubljana), based on H-bridge digital amplifier with 1 kV MOSFETs (DE275-102N06A, IXYS, USA) [37]. Two types of pulses were delivered –monophasic pulses and bursts of biphasic pulses (HF-EP treatment). We adopted the nomenclature from the field of cardiac ablation where bipolar pulses are called biphasic pulses and the delay between the pulses is the inter-phase delay. Pulses were delivered between stainless steel 304 plate electrodes with 2 mm interelectrode distance. Between samples, electrodes were cleaned in experimental electroporation buffer and dried with sterile gauze. Control sample was subjected to the same procedure of the exposed sample in absence of pulses, *i.e.* 0 V/cm amplitude. Monophasic square pulse treatment consisted of eight 100 μs long pulses of 100–1000 V (resulting in voltage-to-distance ratio: 0.5–5 kV/cm) delivered in a step of 100 V at 1 Hz pulse repetition frequency and was used as a reference for standard electroporation protocols. In HF-EP treatment (Schematic 1, Table 2), eight bursts were applied at 1 Hz repetition frequency. Each burst consisted of several biphasic pulses. One biphasic pulse consisted of a negative and a positive pulse, both of lengths 1, 5 or 10 μs (T1) and voltage 100 – 1000 V, delivered in a step of 100 V. The inter-phase delay and the delay between biphasic pulses was 0.5 μs – 10 ms (T2) (see Table 2), *i.e.* delay lengths already studied in cancellation effect [49,50]. The number of biphasic pulses and their duration in one burst was adapted to obtain a total on-time in one burst (the time when the voltage was different from zero) of 100 μs (see Table 2). The total on-time of HF-EP pulse treatment was thus equivalent to the monophasic pulse treatment (Σ = 800 μs). The delivered voltage and current was measured with an oscilloscope, Wavesurfer 422, 200 MHz, using a differential probe (ADP305) and current probe (CP030) (all from LeCroy, USA) (see Fig. 1). The current in low-conductivity buffer was measured up to 1000 V, however, in high-conductivity buffer and in buffer without chloride the highest measured voltage was 700 V, as higher voltages resulted in currents above 30 A which could damage the current probe.

2.4. Permeabilization assay

Just before electric pulses were applied, 50 μl of cells suspension was mixed with propidium iodide (PI, Life Technologies) to obtain a final concentration of 100 μg/ml. The sample was transferred between electrodes, and electric pulses were applied. The sample was transferred to a 1.5 ml tube and incubated at room temperature for three minutes. Afterwards, 150 μl of electroporation buffer was added to obtain a high-enough volume for measurement. The uptake of PI was detected by flow cytometry (Attune NxT; Life Technologies, Carlsbad, CA, USA). Samples were excited with a blue laser at 488 nm and emitted fluorescence was detected through a 574/26 nm band-pass filter. 10,000 events were obtained, and data were analyzed using the Attune Nxt software. On the dot-plots of forward-scatter and side-scatter, single cells were separated from debris and clusters. The percentage of PI permeabilized cells was obtained from the PI fluorescence intensity histogram, by gating permeabilized from non-permeabilized cells. Each data point was repeated three times.

2.5. Viability assay

50 μl samples of cell suspension were transferred between the electrodes, and electric pulses were applied. Afterwards, 40 μl of the electroporated cell suspension was diluted in full HAM-F12 growth media to obtain cell density 2×10^4 cells/100 μl. 100 μl



Schematic 1. Scheme of the pulses applied in experiments. On the left is the pulse shape of standard monophasic pulse treatment (8 monophasic pulses of 100 μs) and on the right is the pulse shape of HF-EP pulse treatment. One burst consists of several biphasic pulses. We varied pulse length (T1), inter-phase delays (T2), while the on-time of each burst was fixed to 100 μs by number of pulses in one burst.

Table 2
Description of the HF-EP pulse parameters. Total on-time in one burst was the same as in one 100 μs monophasic pulse since *pulse length × 2 (positive and negative part of a biphasic pulse) × number of biphasic pulses* = 100 μs.

Pulse length (μs) – T1	Inter-phase delay (μs) – T2	Number of biphasic pulses in one burst (–)	Applied voltage (V)	Total time (μs)
1	0.5, 1, 5, 10, 100, 1000, 10,000	50	100–1000 V in a step of 100 V	100
5	0.5, 1, 5, 10, 100, 1000, 10,000	10	100–1000 V in a step of 100 V	100
10	0.5, 1, 5, 10, 100, 1000, 10,000	5	100–1000 V in a step of 100 V	100

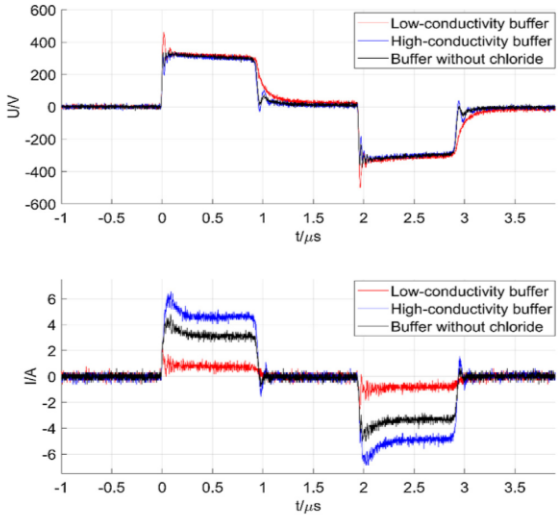


Fig. 1. Measured voltage and current of pulses in different buffers. The waveform of one biphasic pulse with the duration T1 of 1 μs and inter-phase delay T2 of 1 μs 320 V (1.6 kV/cm) were applied. Waveforms in different buffers are shown in correspondent colors (low-conductivity buffer in red, high-conductivity in blue and buffer without chloride in black). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

of cell suspension was then transferred (in three technical repetitions) to wells in the 96-well plate and incubated at 37 °C and humidified 5% CO_2 atmosphere. MTS assay (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega, USA) was used to assess cell viability 24 h after electric pulses were applied. 20 μl of MTS tetrazolium reagent was added to the samples, and the 96-well plate was returned to the incubator for 2.5 h. The absorbance of formazan (reduced MTS tetrazolium compound) was measured with a spectrofluorometer (Tecan Infinite M200, Tecan, Austria) at 490 nm. Each data point was repeated three times. Background absorbance was subtracted for all the samples and control and the percentage of viable cells was calculated by subtracting the background and normalizing the absorbance of the samples to the absorbance of the control (0 V/cm).

2.6. Temperature measurement

50 μl of all three electroporation buffers was transferred between the stainless steel plate electrodes (d = 2 mm). Experiments were performed at room temperature (24 °C). Temperature changes were measured by the fiber optic sensor system (opSens, Québec, Canada), with a temperature probe (ProSens, opSens), which consisted of ProSens signal conditioner and a fiber optic temperature sensor OTG-M170 with a diameter of 0.17 mm. The sensor was placed in the drop between the electrodes. The temperature was measured before, during and 10 s after electric pulse delivery. With biphasic pulses, we tested bursts of 1, 5 and 10 μs long pulses and applied 320 V (voltage in permeability experiments), 600 V (voltage in survival experiments) and 1000 V (maximal applied voltage) with the same pulse and burst number as in permeability and survival experiments. We chose 0.5 μs as the inter-phase delay to have the smallest heat dissipation between pulses, *i.e.* the worst-case scenario. We also applied eight 100 μs long monophasic pulses at 320 V, 600 V and 1000 V.

2.7. Statistical analysis

Statistical analysis was performed using Graphpad Prism 7 (Graphpad software, San Diego, USA). The results are expressed as mean ± SD, and statistically significant differences ($^{*}p < 0.05$, $^{**}p < 0.01$,$^{***}p < 0.001$) were determined by one-way ANOVA with Tukey's multiple comparisons test.

2.8. Calculation

We conducted a theoretical analysis of the time course of transmembrane voltage induced by square pulses, as described in [58]. For the induced transmembrane voltage ($\Delta\Phi_m$) in response to a step turn-on of the DC field, we presumed the exponential shape of increase in the ITV on a single spherical cell:

$$\Delta\Phi_m(t) = fER\cos\theta\left(1 - \exp\left(-\frac{t}{\tau}\right)\right) \quad (1)$$

With the time constant τ being defined as

$$\tau = \frac{RC_m}{\frac{2\lambda_0\lambda_i}{2\lambda_0+\lambda_i} + \frac{R}{d}\lambda_m} \quad (2)$$

where λ_o, λ_i, and λ_m are extracellular, intracellular and membrane conductivity, respectively, R cell radius (flow cytometry analysis showed no difference in FSC scatter of cells in low-conductivity buffer, high-conductivity buffer or buffer without chloride, *i.e.* buffers did not cause cells to shrink or swell), d cell membrane thickness, C_m membrane capacitance, E applied electric field, f the geometrical factor (approx. 1.5), θ the angle between the electric field from the center of the sphare to a point on the cell membrane.

Square pulses consist of two steps (turn-on and turn-off), and thus the response is a superposition of the two separate responses. If several pulses are applied, the response is a superposition of responses to each pulse separately (Fig. 7 in [58]). For the calculations, we used equations (9a-f), (A6d) and (A8) from [58], and the reader is advised to search there for the details of our calculation. Calculations were performed in Matlab R2017 (Mathworks, USA).

In our experiments, cells were electroporated in three different buffers. From Eq. (2) we can see that the time constant also depends on the electric conductivity of the extracellular liquid (λ_o). The parameters used in our calculations and their values are given in Table 3. The values (except for the extracellular conductivity, which was determined experimentally in the scope of our study) were all taken from [58]. The results are reported as ITV normalized to f ER.

Table 3
Parameters used in our calculations, their symbols and values.

Parameter	Symbol	Value
Cell radius	R	10 μm
Membrane capacitance	C_m	ε_m/d = 8.8 F/m^2
Cytoplasmic conductivity	Λ_i	0.3 S/m
Cytoplasmic permittivity	ε_i	7.1 × 10^{-10} As/Vm
Membrane conductivity	Λ_m	3 × 10^{-7} S/m
Membrane permittivity	ε_m	4.4 × 10^{-11} As/Vm
Membrane thickness	d	5 nm
Conductivity of the electroporation buffer	λ_o	λ_1 = 1.76 mS/cm λ_2 = 19.12 mS/cm λ_3 = 9.57 mS/cm

3. Results

3.1. Cancellation effect as a function of the amplitude of the electric field

First, we focused on cell survival (Fig. 2) as HF-IRE pulses are predominantly used to achieve irreversible electroporation. Then, we determined cell membrane permeability (Fig. 3), as we aim to use HF-EP also to achieve reversible electroporation. Both cell survival and cell membrane permeabilization were evaluated as a function of electric field amplitude for monophasic pulses and for HF-EP pulses when pulse length (T1) was 1 μs, and the inter-phase delay (T2) was 1 μs or 10 ms. Three different electroporation buffers were used to observe buffer dependency.

Monophasic pulses were more efficient than biphasic pulses in survival and permeability experiments, as they caused higher cell membrane permeabilization and lower survival at the same electric fields (Figs. 2 and 3).

In Fig. 2 we can see that the cancellation effect was consistently observed in all three buffers when cell survival was evaluated. Namely, when the inter-phase delay was 10 ms (dashed lines), pulses were more effective at decreasing cell survival, and lower electric fields were needed to achieve the same cell death than with 1 μs (solid lines) inter-phase delay.

On the contrary in permeability experiments, we observed either a cancellation or a sensitization effect (Fig. 3). A cancellation effect was present in the high-conductivity buffer and the buffer without chloride, as HF-EP pulse treatment with a longer inter-phase delay (10 ms) was more efficient than HF-EP pulse treatment with a shorter inter-phase delay (1 μs) between pulses at the same electric field. However, in the low-conductivity buffer, we observed that HF-EP pulse treatment with a shorter inter-phase delay (1 μs) between pulses was more efficient than HF-EP pulse treatment with a longer inter-phase delay (10 ms) between pulses at the same electric field, *i.e.* we observed the 'sensitization' effect, similar as in [57].

3.2. Cancellation effect as a function of pulse duration and inter-phase delay

The cancellation effect in HF-EP treatment was studied in detail by applying 1, 5 and 10 μs long pulses (T1) with several different inter-phase delays (0.5 μs – 10 ms) (T2) (Fig. 4), again by evaluating cell survival and cell membrane permeabilization. We applied a fixed voltage of 600 V, *i.e.* 3000 V/cm between the electrodes in survival experiments and 320 V *i.e.* 1600 V/cm in permeability experiments. As our hypothesis about the contribution of voltage-gated chloride ions was disproved, these experiments were performed only in the low- and high-conductivity buffer.

Longer pulses (10 μs) were more efficient than shorter pulses (1 μs) in achieving cell membrane permeabilization and cell death, irrespective of the inter-phase delay T2 and the buffer.

In the low-conductivity buffer, no cancellation effect was present in permeabilization or survival. On the contrary, for pulse duration T1 = 1 μs, cell permeabilization was less efficient when the inter-phase delay was increased, *i.e.*, we observed a sensitiza-

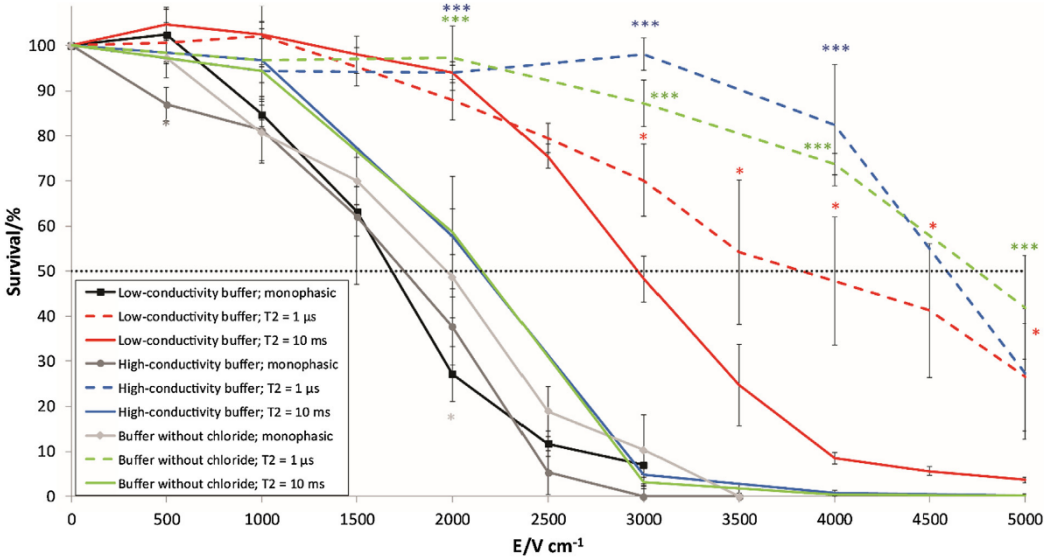


Fig. 2. Cell survival as a function of the electric field in three different buffers. In HF-IRE treatment, pulse duration was 1 μs, and the inter-phase delay was either 1 μs or 10 ms. Monophasic pulses are shown in solid black, dark grey and light grey lines for low-conductivity buffer, high- conductivity buffer and buffer without chloride. HF-IRE pulses with T1 = 1 μs are shown in red, blue and green for low-conductivity buffer, high- conductivity buffer and buffer without chloride, respectively. Dashed lines (– – –) are used for pulses with 1 μs inter-phase delay and solid lines (————) for 10 ms. 50% survival is shown in a dotted line (. . . . .). Results are shown as mean ± standard deviation. The asterisks (*) mark $p < 0.05$*, $p < 0.01$**, $p < 0.001$*** and show statistically significant differences between monophasic pulses and different T2 of the same buffer in HF-IRE pulses. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

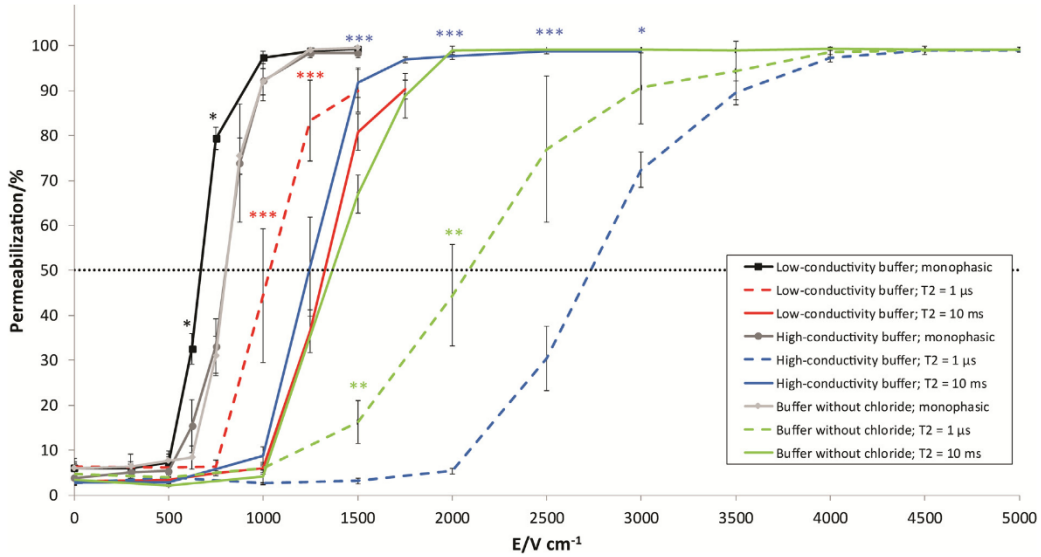


Fig. 3. Cell membrane permeabilization as a function of the applied electric field in different buffers. In HF-EP treatment, pulse duration was 1 µs, and the inter-phase delay was either 1 µs or 10 ms. Monophasic pulses are shown in solid black, dark grey and light grey lines for low-conductivity buffer, high- conductivity buffer and buffer without chloride. HF-EP pulses with T1 = 1 µs are shown in red, blue and green, for low-conductivity buffer, high- conductivity buffer and buffer without chloride, respectively. Dashes lines (– – –) are used for pulses with 1 µs inter-phase delay and solid lines (–––) for 10 ms. 50% permeabilization is shown in a dotted line (.....). Results are shown as mean ± standard deviation. The asterisks (*) mark $p < 0.05^{*}$, $p < 0.01^{**}$, $p < 0.001^{***}$ and show statistically significant differences between monophasic pulses and between different T2 of the same buffer. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

tion effect (Fig. 4b). For cell membrane permeabilization with 5 and 10 µs long pulses, the sensitization effect was observed only with longer inter-phase delays of 1 ms and 10 ms. In high-conductivity buffer, however, longer inter-phase delays (T2) were more efficient in permeabilizing and killing cells than shorter, *i.e.* a cancellation effect was present and decreased with longer T2 (statistical difference between T2 = 0.5 µs and T2 = 1000 µs or more is $p < 0.001$) (Fig. 4d). The extent of the cancellation was reduced with an increase in pulse duration (T1). Interestingly, the cancellation effect in survival assay was still observed at 10 µs, the longest pulse length tested (Fig. 4c).

3.3. Temperature measurements

Fig. S1 shows the time dependency of temperature when HF-EP or ECT pulses are applied. We can see that in high-conductivity buffer and in buffer without chloride during each burst, the temperature increases and during the delay between bursts, it decreases.

In Table S1, the maximal temperature is shown for various combinations of pulse parameters and electroporation buffers. We can see that the maximal increase was 23 °C when 1000 V was applied.

3.4. Calculation of the assisted discharge

Assisted discharge was suggested as one of the possible mechanisms responsible for the cancellation effect [49]. The time-constant of the membrane depends, amongst other factors, on the extracellular conductivity, which varied between our buffers (Table 1). Fig. S2 shows the membrane time constant tau (calculated according to Eq. (1)) as a function of extracellular conductivity with marked time constants for the three buffers, used in our study.

In Fig. 5, we can see that with longer inter-phase delays (5 µs or more) the membrane discharges completely and there is no contribution of the assisted discharge. In Fig. 6, we can see that with 5 µs pulses, we reach stationary transmembrane voltage during the pulse application, *i.e.* the membrane charges completely. Similarly as in Fig. 5 with 1 µs pulses, we can see that with the inter-phase delays of 0.5 and 1 µs the membrane does not discharge completely, but with inter-phase delays of 5 µs it does. From Figs. 5, 6 and Table 4 we can observe that cell membranes charge and discharge slowest in the low-conductivity buffer and in all three buffers, it takes maximally 5 µs to charge/discharge.

4. Discussion

In our study, we evaluated the cancellation effect in *in vitro* experiments with high-frequency short biphasic pulses, *i.e.*, high-frequency electroporation (HF-EP), by determining cell survival and cell membrane permeability in three different electroporation buffers across a wide range of pulse parameters. In HF-EP, we applied eight bursts of 1, 5 and 10 µs long pulses (T1) with inter-phase delay of 0.5 µs to 10 ms (T2) and an on-time of 800 µs. We compared the effect of HF-EP to the standard ECT pulses, *i.e.* eight 100 µs long pulses.

4.1. Monophasic vs HF-EP pulse treatment

We compared the efficiency of a monophasic and HF-EP pulse treatment in three different buffers. We determined that short biphasic pulses were less efficient in decreasing cell survival and increasing cell membrane permeability than monophasic pulses as ~~es~~ higher electric field had to be applied to achieve a similar effect (Figs. 2 and 3), which is in agreement with the existing HF-IRE and HF-EP studies [34,37,38,59].

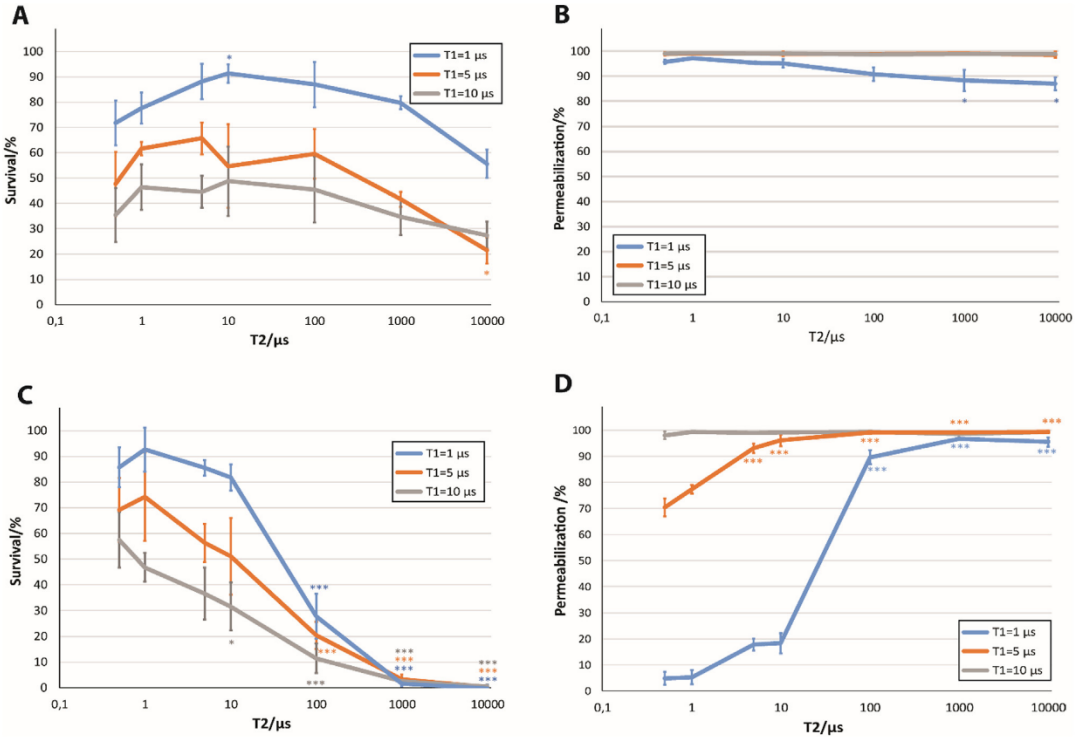


Fig. 4. Cancellation effect in survival and permeabilization after HF-EP pulse treatment in two different electroporation buffers as a function of pulse duration. In survival experiments, samples were exposed to 600 V (3000 V/cm) and in permeabilization experiments, to 320 V (1600 V/cm). Graphs A and B show results in low-conductivity buffer and graphs C and D in buffer 2 high-conductivity buffer. The asterisks (*) mark $p < 0.05$*, $p < 0.01$**, $p < 0.001$*** and show statistically significant differences between T2 = 0.5 µs (the shortest inter-phase delay) and Δ T2. Differences in each pulse length (T1) are shown in correspondent colors (blue 1 µs, orange 5 µs and gray 10 µs). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

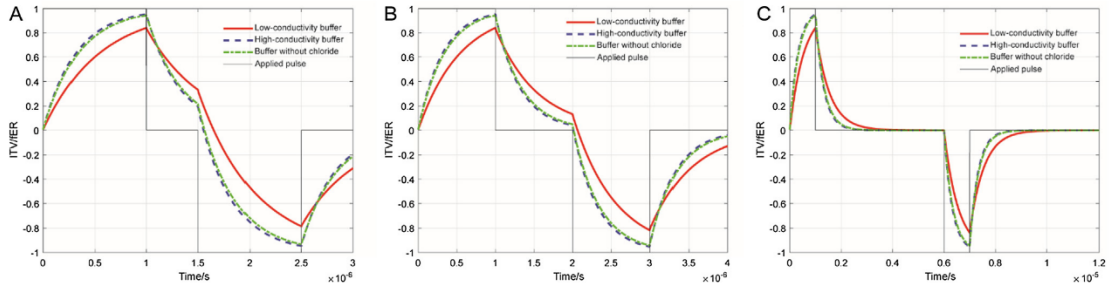


Fig. 5. Time constant of membrane charging as a function of the electric conductivity of the extracellular medium as a response to a step. We can see that in our experiments, time constant was in the range of 0.3 µs – 0.5 µs for all three buffers. (A) The inter-phase delay was 0.5 µs or (B) 1 µs. (C) The inter-phase delay of 5 µs was already enough for membranes to completely discharge and for the negative pulse to be applied to a membrane at 0 V. Solid black line shows the shape of the applied biphasic pulse. The induced transmembrane voltage was normalized to geometrical factor f, applied electric field E and cell radius R. Red, blue and green lines show the normalized calculated time dependence of transmembrane voltage for low-conductivity, high-conductivity buffer and buffer without chloride, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4.2. Survival in HF-IRE pulse treatments

First, we focused on cell survival, as HF-IRE pulses are predominantly applied to obtain irreversible electroporation. We applied 1 µs long pulses with two different inter-phase delays (1 µs and 10 ms) in three electroporation buffers as a function of the electric field. A cancellation effect was observed in all three buffers at a high-enough electric field (from 3 kV/cm in the low-conductivity buffer, from 1.5 kV/cm in the high-conductivity buffer and the buffer without chloride) (Fig. 2) which is in agreement with already published studies where the cancellation effect was observed in assessing cell survival [46,49]. Interestingly, cells responded similarly in the high-conductivity buffer and the buffer without chloride; there was no significant difference at any of the tested

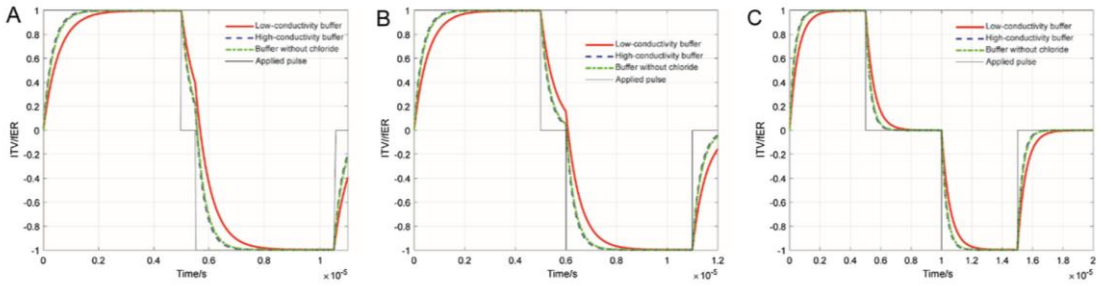


Fig. 6. Charging and discharging of the cell membrane when there is 1 0.5–5 μs inter-phase delay between 5 μs long pulses in three different electroporation buffers. We can see that the stationary value of induced transmembrane value was reached during the pulse. (A) The inter-phase delay was 0.5 μs or (B) 1 μs. (C) The inter-phase delay of 5 μs was already enough for membranes to completely discharge and for the negative pulse to be applied to a membrane at 0 V. Solid black line shows the shape of the applied biphasic pulse. The induced transmembrane voltage was normalized to geometrical factor f, applied electric field E and cell radius R. Red, blue and green lines show the normalized calculated time dependence of transmembrane voltage for low-conductivity, high-conductivity buffer and buffer without chloride, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 4
Fraction of Induced transmembrane voltage normalized to geometric factor f, applied electric filed E and cell radius R [ITV/f ER]. After the voltage is turned off, the transmembrane voltage decreases exponentially and depends on the time constant of the membrane, except when assisted (T2 < 1 μs).

	0.5 μs	1 μs	5 μs	10 μs	100 μs	1 ms	10 ms
Low-conductivity buffer	3.97×10^{-1}	1.58×10^{-1}	9.74×10^{-5}	9.49×10^{-9}	0	0	0
High-conductivity buffer	2.05×10^{-1}	4.21×10^{-2}	1.32×10^{-7}	1.75×10^{-14}	0	0	0
Buffer without chloride	2.28×10^{-1}	5.21×10^{-2}	3.85×10^{-7}	1.48×10^{-13}	0	0	0

electric fields which indicates that extracellular chloride ions do not play a major role in cell survival after electroporation.

In Fig. 4, a wider range of inter-phase delays was tested at a fixed electric field (3 kV/cm). In the low-conductivity buffer, no cancellation effect was observed when we compared different T2 to the shortest T2 = 0.5 μs used. A statistically significant difference between T2 = 1 μs and T2 = 10 ms was observed, as seen in Fig. 2 and also in Fig. 4 ($p < 0.05$). In the high-conductivity buffer, cell survival increased with decreasing inter-phase delay, *i.e.* the cancellation effect was observed. With longer pulses (10 μs), lower electric fields were already sufficient to achieve a similar decrease in cell survival than with shorter pulses (1 μs), regardless of the chosen buffer and the inter-phase delay (Figs. 2 and 4), which is in agreement with existing studies on the electroporation strength-duration curve [60]. Thus, longer pulses than 1 μs could be applied in HF-IRE treatments to increase efficiency; however, the suggested benefit of reducing pain and muscle contractions at longer pulses would need to be reevaluated.

4.3. Permeabilization in HF-EP pulse treatment

We expected the results in permeabilization experiments to be similar to the ones in survival experiments, as survival and permeability are believed to be correlated, and increased permeabilization is a prerequisite for possible cell death [11]. First, we focused on 1 μs long pulses with 1 μs and 10 ms inter-phase delay as a function of the electric field in three electroporation buffers. Unexpectedly, in the low-conductivity buffer, permeabilization was more efficient with 1 μs than with 10 ms inter-phase delay, *i.e.*, we observed a reversed cancellation effect, opposite to our expectations and the existing literature [49]. Although cells responded very similarly in the low- and the high-conductivity buffers with the 10 ms inter-phase delay, they responded oppositely with the 1 μs inter-phase delay, thus indicating that the phenomenon causing cancellation effect happens in the time-range of 1 μs to 10 ms, as already reported for ns pulses [49,50].

Translation of results from *in vitro* to *in vivo* thus seems not to be straightforward and/or is even questionable. When inferring the *in vivo* response from the *in vitro* experiments, the importance of electroporation buffer should be taken into account, and perhaps, the buffer most similar to tissues should be used. In this paper we consider this to be the high-conductivity buffer, due to the presence of NaCl.

In Fig. 4, a wider range of inter-phase delays at 1600 V/cm was tested. We wanted to determine if in the low-conductivity buffer, the reversed cancellation effect was present also with other inter-phase and pulse lengths. However, it was mostly observed for the pulse length of 1 μs. Longer pulses mostly caused complete cell membrane permeabilization, and we could not distinguish between the effects of different inter-phase delays. The cancellation effect was however observed in high-conductivity buffer and it depends on pulse length.

Electroporation can be induced also by exposure of cells to pulse modulated sine wave signals [61]. The efficiency of treatment decreased when frequency of sinewaves was increased [59]. This is comparable to the efficiency of HF-EP: biphasic pulses with longer (10 ms) inter-phase delay are more effective in permeabilizing cells than biphasic pulses with short (1 μs) inter-phase delay (Fig. 2). In HF-EP pulse treatment this happens due to cancellation effect. This may be partly explained by the fact that at longer interphase delays, relatively more power is delivered at the lower frequency range of the signal.

4.4. Effect of electroporation buffers

It was already shown that electroporation buffer has a significant effect on the cell membrane permeabilization *in vitro* [57]. Thus, we used three different electroporation buffers: the low-conductivity potassium-phosphate buffer, usually used in many laboratories, the high-conductivity buffer where sucrose was substituted with NaCl in an *iso*-osmolar manner and the buffer without chloride. Interestingly, it was previously observed that in *in vitro* permeability experiments, cells responded very differently

when electroporated in the low- or high-conductivity buffers in the time-range of minutes, in the experiments on the so-called 'cell sensitization' [57]. Here, a parallel can be drawn as we also observed very different responses in the same buffers (Fig. 4).

In permeability experiments, it was observed that the cancellation effect was more pronounced in the low-conductivity buffers than in the high-conductivity buffers with no or very short inter-pulse intervals [51]. However, our results contradict these results, as the cancellation effect was not observed in the low-conductivity buffer; moreover, the 'sensitization' effect was observed. In our experiments, the cancellation was observed in the high-conductivity and medium-conductivity buffers (*i.e.* buffer without chloride), but not in the low-conductivity buffer. It is possible that low conductivity caused the lack of cancellation effect, although it is more possible that the high sucrose concentration was responsible, similarly, as indicated in [57].

4.4.1. Contribution of chloride channels

Since the absence of muscle contraction using HF-IRE pulses was also demonstrated [31,33,34,36,59,62–66], we assumed that HF-EP pulse treatment inhibits the induction of action potentials. Fast reversal of pulse polarities causes a reversal in depolarization and hyperpolarization of the membrane. One of the hyperpolarization-activated inward currents is produced by the chloride ions [67,68]. Opening of the Cl^- channels can be activated in the presence of Cl^- ions. The influx of Cl^- ions after the first pulse would decrease the resting potential, cause hyperpolarization and make it more difficult for excitable cells to reach membrane potential required for activation of the action potential, thus, abolishing the action potential. Similarly, due to the lower resting membrane potential, a higher electric field would need to be applied to reach the same transmembrane voltage as without the influx of chloride ions, making cells less sensitive to the following pulses, *i.e.* causing the cancellation effect. The activation time constant of voltage-gated Cl^- ion channels in skeletal muscle comprises two components, a fast gate (~16 μs) and a slow gate (~1 ms) [56], which could explain the influence of the inter-phase delay T2 on the cell membrane permeabilization and cell death. Voltage-gated chloride channels are known to be present in CHO cells [69]. Thus, we tested this theory by preparing a buffer without chloride ions. Elimination of the cancellation effect was expected, yet the cancellation effect was present. Thus, our hypothesis on the contribution of the chloride channels to the cancellation effect was dismissed.

4.5. Temperature effect

Temperature has a significant effect on the efficiency of electroporation, *e.g.* it was shown that changes in temperature affect gene electrotransfer [70,71], cell membrane permeabilization [72], skin electroporation [73] and breakdown voltage of lipid bilayers [74]. Moreover, electric pulses cause Joule heating and consequently, they can cause thermal damage [75,76]. The threshold for thermal damage is 42 °C for prolonged exposure, while the temperature should not exceed 50 °C at any time [77]. In our experiments, at applications of 1000 V the temperature increased up to 45 °C from the room temperature (24 °C), *i.e.* it increased for 22 °C and thermal damage was obtained. Between different tissues, the electric conductivity varies significantly and is generally in the range of 10^{-2} to 2 S/m [78,79] which is a similar range as the conductivities of the buffers in our study (0.1–2 S/m). Considering that in tissues the initial temperature is around 37 °C, we can expect thermal damage predominantly around the electrodes due to high current density and at very high electric fields, longer pulse lengths and shorter inter-phase delays.

When 600 V was delivered to buffers, the temperature changed for approx. 7 °C at all pulse lengths (T1 = 1, 5 and 10 μs) (Table S1). Survival experiments performed at the same condition showed different cancellation effect (Fig. 4c), therefore we can assume cancellation effect is not effected by the temperature but by the pulse parameters.

4.6. Effect of the assisted discharge

Several hypotheses were put forth to explain the cancellation effect, but so far none could completely explain the phenomenon. We focused on the assisted discharge, as the three buffers we used varied vastly in their electric conductivity. In survival assays, cells electroporated in the high-conductivity buffer and buffer without chloride were more sensitive than those in the low-conductivity buffer when the inter-phase delay was 1 μs (Fig. 2). Incomplete membrane charging and assisted membrane discharge [49] lend themselves as a plausible explanation for such behavior. In permeabilization assays, cells were generally the most sensitive to electroporation in the low-conductivity buffer (Fig. 3). As in the high-conductivity buffer and the buffer without chloride the charging was faster than in the low-conductivity buffer (Fig. 6), we cannot explain less permeabilization in the high-conductivity buffer and in the buffer without chloride than in low-conductivity buffer with the difference in membrane charging. The assisted discharge influences results up to 1.5 μs for the high-conductivity buffer and the buffer without calcium, and 2.5 μs in the low-conductivity buffer after voltage is turned on (Fig. 6). This means that permeabilization should be more efficient with the 10 ms inter-phase delay than with the 1 μs in all buffers, which is true for cell survival but not for cell membrane permeabilization. We can thus conclude that the time constant of membrane charging, together with the assisted discharge, could only partially explain the discrepancies in the experimental data.

4.7. Drawbacks of our study

We applied bursts of biphasic pulses, while in studies on cancellation effects single biphasic pulses or single trains were applied. Thus, it is possible that because our pulse application lasted for 8 s, additional effects were present [80].

It is possible that by applying different voltages in Fig. 4, or even adapting the voltages to separate electroporation buffers, the presence of a cancellation effect would be more clear. However, we decided to fix the voltage to be able to compare the results at the same electric field.

In the theoretical calculation of the assisted discharge as a function of extracellular conductivity, we assumed that all cells in the suspension are of the same size, which is a simplification [81]. The size of the radii namely follows the Gaussian distribution which means that also time constants of the membrane are statistically distributed vary through the population.

In temperature measurements, we aimed to measure the temperature always at the same spot in the cell suspension. However, due to the limited precision of positioning the probe, its position could slightly vary between the treatments. Also, we measured the macroscopic increase in temperature. It was previously shown that even when the macroscopic increase is negligible, at the cell level, there could still be some thermal damage in intermediate vicinity of the electrodes [82,83].

5. Conclusion

In our study, we focused on the previously reported cancellation effects in *in vitro* electroporation with bursts of short biphasic

pulses. The following main conclusions can be drawn from our work. (1) Cancellation effect is present in HF-EP treatments looking at survival and could be responsible for the need to apply higher electric fields in HF-IRE treatments than in IRE treatments. (2) Cancellation effect is present in a wide range of pulse parameters and depends on the inter-phase delay as well as on pulse duration, *i.e.* cancellation is less pronounced with longer pulses and longer interphase delays. (3) Cancellation effect is electroporation-buffer dependent. (4) Cancellation effect in survival experiments can be only partially explained by the assisted discharge. (5) Cancellation effect is not caused by the hyperpolarization.

Author contribution

- Tamara Polajžer: acquisition of the data, analysis and interpretation of the data, drafting the paper, final approval of the paper.
- Janja Dermol-Černe: analysis and interpretation of the data, drafting the paper, final approval of the paper.
- Matej Reberšek: design of the ns-µs pulse generator, used in the study, final approval of the paper.
- Rodney O'Connor: conception and design of the study, final approval of the paper.
- Damijan Miklavčič: conception and design of the study, analysis and interpretation of the data, drafting the paper, final approval of the paper.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

[1] T. Kotnik, L. Rems, M. Tarek, D. Miklavčič, Membrane electroporation and electropermeabilization: mechanisms and models, Annu. Rev. Biophys. (2019), https://doi.org/10.1146/annurev-biophys-052118-115451.
[2] D. Miklavčič et al., Electrochemotherapy: technological advancements for efficient electroporation-based treatment of internal tumors, Med. Biol. Eng. Comput. 50 (12) (2012) 1213–1225, https://doi.org/10.1007/s11517-012-0991-8.
[3] D. Miklavčič, B. Mali, B. Kos, R. Heller, G. Serša, Electrochemotherapy: from the drawing board into medical practice Electrochemotherapy for skin and superficial tumors, Electrochemotherapy for visceral and deep-seated tumors, Biomed. Eng. 13 (2014) 1–20, https://doi.org/10.1186/1475-925X-13-29.
[4] J. Gehl et al., Updated standard operating procedures for electrochemotherapy of cutaneous tumours and skin metastases, Acta Oncol. 57 (7) (2018) 874–882, https://doi.org/10.1080/0284186X.2018.1454602.
[5] L.G. Campana et al., Electrochemotherapy of superficial tumors – Current status: Basic principles, operating procedures, shared indications, and emerging applications, Semin. Oncol. (2019), https://doi.org/10.1053/j.seminoncol.2019.04.002.
[6] L.C. Heller, R. Heller, Electroporation gene therapy preclinical and clinical trials for melanoma, Curr. Gene Ther. 10 (4) (2010) 312–317.
[7] L. Lambricht, A. Lopes, S. Kos, G. Sersa, V. Préat, G. Vandermeulen, Clinical potential of electroporation for gene therapy and DNA vaccine delivery, Expert Opin. Drug Deliv. 13 (2) (2016) 295–310, https://doi.org/10.1517/17425247.2016.1121990.
[8] C.L. Trimble et al., Safety, efficacy, and immunogenicity of VGX-3100, a therapeutic synthetic DNA vaccine targeting human papillomavirus 16 and 18 E6 and E7 proteins for cervical intraepithelial neoplasia 2/3: a randomised, double-blind, placebo-controlled phase 2b trial, Lancet (London, England) 386 (10008) (2015) 2078–2088, https://doi.org/10.1016/S0140-6736(15)00239-1.
[9] K. Kwak et al., Multivalent human papillomavirus L1 DNA vaccination utilizing electroporation, PLoS One (2013), https://doi.org/10.1371/journal.pone.0060507.
[10] H.J. Scheffer et al., Irreversible electroporation for nonthermal tumor ablation in the clinical setting: a systematic review of safety and efficacy, J. Vasc. Interv. Radiol. 25 (7) (2014) 997–1011, https://doi.org/10.1016/j.jvir.2014.01.028.
[11] C. Jiang, R.V. Davalos, J.C. Bischof, A review of basic to clinical studies of irreversible electroporation therapy, IEEE Trans. Biomed. Eng. 62 (1) (2015) 4–20, https://doi.org/10.1109/TBME.2014.2367543.
[12] A. Wojtaszczyk, G. Caluori, M. Pešl, K. Melajova, Z. Stárek, Irreversible electroporation ablation for atrial fibrillation, J. Cardiovasc. Electrophysiol. 29 (4) (2018) 643–651, https://doi.org/10.1111/jce.13454.
[13] V.Y. Reddy et al., Pulsed field ablation for pulmonary vein isolation in atrial fibrillation, J. Am. Coll. Cardiol. (2019), https://doi.org/10.1016/j.jacc.2019.04.021.
[14] F.H.M. Wittkampf, R. van Es, K. Neven, Electroporation and its relevance for cardiac catheter ablation, JACC. Clin. Electrophysiol. 4 (8) (2018) 977–986, https://doi.org/10.1016/j.jacep.2018.06.005.
[15] M.T. Stewart et al., Intracardiac pulsed field ablation: proof of feasibility in a chronic porcine model, Hear. Rhythm (2019), https://doi.org/10.1016/j.hrthm.2018.10.030.
[16] S. Mahnič-Kalamiza, E. Vorobiev, D. Miklavčič, Electroporation in food processing and biorefinery, J. Membr. Biol. 247 (12) (2014) 1279–1304, https://doi.org/10.1007/s00232-014-9737-x.
[17] T. Kotnik, W. Frey, M. Sack, S. Haberl Meglič, M. Peterka, D. Miklavčič, Electroporation-based applications in biotechnology, Trends Biotechnol. 33 (8) (2015) 480–488, https://doi.org/10.1016/j.tibtech.2015.06.002.
[18] A. Golberg et al., Energy-efficient biomass processing with pulsed electric fields for bioeconomy and sustainable development, Biotechnol. Biofuels (2016), https://doi.org/10.1186/s13068-016-0508-z.
[19] S. Toepfl, C. Siemer, G. Saldaña-Navarro, V. Heinz, Chapter 6 – Overview of pulsed electric fields processing for food, Emerg. Technol. Food Process. (2014), https://doi.org/10.1016/B978-0-12-411479-1.00006-1.
[20] M. Marty et al., Electrochemotherapy - An easy, highly effective and safe treatment of cutaneous and subcutaneous metastases: results of ESOPE (European Standard Operating Procedures of Electrochemotherapy) study, Eur. J. Cancer, Suppl. (2006), https://doi.org/10.1016/j.ejcsup.2006.08.002.
[21] D. Miklavcic, S. Corovic, G. Pucihar, N. Pavselj, Importance of tumour coverage by sufficiently high local electric field for effective electrochemotherapy, Eur. J. Cancer Suppl. 4 (11) (2006) 45–51, https://doi.org/10.1016/J.EJCSUP.2006.08.006.
[22] C.B. Arena, R.V. Davalos, Advances in therapeutic electroporation to mitigate muscle contractions, J. Membr. Sci. Technol. 02 (01) (2012) 1–3, https://doi.org/10.4172/2155-9589.1000e102.
[23] A. Zupanic, S. Ribaric, D. Miklavcic, Increasing the repetition frequency of electric pulse delivery reduces unpleasant sensations that occur in electrochemotherapy, Neoplasma 54 (3) (2007) 246–250.
[24] R.C. Martin, E. Schwartz, J. Adams, I. Farah, B.M. Derhake, Intra-operative anesthesia management in patients undergoing surgical irreversible electroporation of the pancreas, liver, kidney, and retroperitoneal tumors, Anesthesiol. Pain Med. (2015), https://doi.org/10.5812/aapm.22786.
[25] B. Mali et al., The effect of electroporation pulses on functioning of the heart, Med. Biol. Eng. Comput. (2008), https://doi.org/10.1007/s11517-008-0346-7.
[26] B. Mali et al., Electrochemotherapy of colorectal liver metastases–an observational study of its effects on the electrocardiogram, Biomed. Eng. 14 (Suppl. 3) (2015) S5, https://doi.org/10.1186/1475-925X-14-S3-S5.
[27] C. Ball, K.R. Thomson, H. Kavnoudias, Irreversible electroporation, Anesth. Analg. 110 (5) (2010) 1305–1309, https://doi.org/10.1213/ANE.0b013e3181d27b30.
[28] D. Miklavčič et al., The effect of high frequency electric pulses on muscle contractions and antitumor efficiency in vivo for a potential use in clinical electrochemotherapy, Bioelectrochemistry 65 (2) (2005) 121–128.
[29] G. Pucihar, L.M. Mir, D. Miklavcic, The effect of pulse repetition frequency on the uptake into electropermeabilized cells in vitro with possible applications in electrochemotherapy, Bioelectrochemistry 57 (2) (2002) 167–172.
[30] G. Sersa, S. Kranjc, J. Scancar, M. Krzan, M. Cemazar, Electrochemotherapy of mouse sarcoma tumors using electric pulse trains with repetition frequencies of 1 Hz and 5 kHz, J. Membr. Biol. (2010), https://doi.org/10.1007/s00232-010-9268-z.
[31] C. Yao et al., Bipolar microsecond pulses and insulated needle electrodes for reducing muscle contractions during irreversible electroporation, IEEE Trans. Biomed. Eng. 64 (12) (2017) 2924–2937, https://doi.org/10.1109/TBME.2017.2690624.
[32] A. Golberg, B. Rubinsky, Towards electroporation based treatment planning considering electric field induced muscle contractions, Technol. Cancer Res. Treat. (2013), https://doi.org/10.7785/tcrt.2012.500249.
[33] C.B. Arena et al., High-frequency irreversible electroporation (H-FIRE) for non-thermal ablation without muscle contraction, Biomed. Eng. 10 (1) (2011) 102, https://doi.org/10.1186/1475-925X-10-102.
[34] Y. Zhao et al., Characterization of conductivity changes during high-frequency irreversible electroporation for treatment planning, IEEE Trans. Biomed. Eng. (2018), https://doi.org/10.1109/TBME.2017.2778101.

[35] M.B. Sano et al., Bursts of bipolar microsecond pulses inhibit tumor growth, Sci. Rep. 5 (2015) 14999, https://doi.org/10.1038/srep14999.
[36] S. Dong, H. Wang, Y. Zhao, Y. Sun, C. Yao, First human trial of high-frequency irreversible electroporation therapy for prostate cancer, Technol. Cancer Res. Treat. (2018), https://doi.org/10.1177/1533033818789692.
[37] D.C. Sweeney, M. Reberšek, J. Dermol, L. Rems, D. Miklavčič, R.V. Davalos, Quantification of cell membrane permeability induced by monopolar and high-frequency bipolar bursts of electrical pulses, Biochim. Biophys. Acta - Biomembr. 1858 (11) (2016) 2689–2698, https://doi.org/10.1016/J.BBAMEM.2016.06.024.
[38] M. Scuderi, M. Rebersek, D. Miklavcic, J. Dermol-Cerne, The use of high-frequency short bipolar pulses in cisplatin electrochemotherapy in vitro, Radiol. Oncol. 53 (2) (2019) 194–205, https://doi.org/10.2478/raon-2019-0025.
[39] E. Tekle, R.D. Astumiant, P. Boon Chock, Electroporation by using bipolar oscillating electric field: an improved method for DNA transfection of NIH 3T3 cells, Biochemistry 88 (1991) 4230–4234.
[40] T. Kotnik, L.M. Mir, K. Flisar, M. Puc, D. Miklavčič, Cell membrane electropermeabilization by symmetrical bipolar rectangular pulses: part I. Increased efficiency of permeabilization, Bioelectrochemistry 54 (1) (2001) 83–90, https://doi.org/10.1016/S1567-5394(01)00114-1.
[41] T. Kotnik, D. Miklavčič, L.M. Mir, Cell membrane electropermeabilization by symmetrical bipolar rectangular pulses: part II. Reduced electrolytic contamination, Bioelectrochemistry 54 (1) (2001) 91–95, https://doi.org/10.1016/S1567-5394(01)00115-3.
[42] I. Daskalov, N. Mudrov, E. Peycheva, Exploring new instrumentation parameters for electrochemotherapy. Attacking tumors with bursts of biphasic pulses instead of single pulses, IEEE Eng. Med. Biol. Mag. 18 (1), 62–6.
[43] S. Jaichandran, S.T.B. Yap, A.B.M. Khoo, L.P. Ho, S.L. Tien, O.L. Kon, In vivo liver electroporation: optimization and demonstration of therapeutic efficacy, Hum. Gene Ther. 17 (3) (2006) 362–375, https://doi.org/10.1089/hum.2006.17.362.
[44] L. Pasquet et al., Safe and efficient novel approach for non-invasive gene electrotransfer to skin, Sci. Rep. (2018), https://doi.org/10.1038/s41598-018-34968-6.
[45] M. Reberšek, D. Miklavčič, C. Bertacchini, M. Sack, Cell membrane electroporation-Part 3: the equipment, IEEE Electr. Insul. Mag. (2014), https://doi.org/10.1109/MEI.2014.6804737.
[46] B.L. Ibey et al., Bipolar nanosecond electric pulses are less efficient at electropermeabilization and killing cells than monopolar pulses, Biochem. Biophys. Res. Commun. 443 (2) (2014) 568–573, https://doi.org/10.1016/j.bbrc.2013.12.004.
[47] A.G. Pakhomov, S. Grigoryev, I. Semenov, M. Casciola, C. Jiang, S. Xiao, The second phase of bipolar, nanosecond-range electric pulses determines the electroporation efficiency, Bioelectrochemistry 122 (2018) 123–133, https://doi.org/10.1016/j.bioelechem.2018.03.014.
[48] C.M. Valdez, R.A. Barnes, C.C. Roth, E.K. Moen, G.A. Throckmorton, B.L. Ibey, Asymmetrical bipolar nanosecond electric pulse widths modify bipolar cancellation, Sci. Rep. (2017), https://doi.org/10.1038/s41598-017-16142-6.
[49] A.G. Pakhomov et al., Cancellation of cellular responses to nanoelectroporation by reversing the stimulus polarity, Cell. Mol. Life Sci. 71 (22) (2014) 4431–4441, https://doi.org/10.1007/s00018-014-1626-z.
[50] C.M. Valdez, R. Barnes, C.C. Roth, E. Moen, B. Ibey, The interphase interval within a bipolar nanosecond electric pulse modulates bipolar cancellation, Bioelectromagnetics 39 (6) (2018) 441–450, https://doi.org/10.1002/bem.22134.
[51] E.C. Gianulis, M. Casciola, S. Xiao, O.N. Pakhomova, A.G. Pakhomov, Electropermeabilization by uni- or bipolar nanosecond electric pulses: the impact of extracellular conductivity, Bioelectrochemistry 119 (2018) 10–19, https://doi.org/10.1016/j.bioelechem.2017.08.005.
[52] T.R. Gowrishankar, J.V. Stern, K.C. Smith, J.C. Weaver, Nanopore occlusion: a biophysical mechanism for bipolar cancellation in cell membranes, Biochem. Biophys. Res. Commun. (2018), https://doi.org/10.1016/j.bbrc.2018.07.024.
[53] C. Merla, A.G. Pakhomov, I. Semenov, P.T. Vernier, Frequency spectrum of induced transmembrane potential and permeabilization efficacy of bipolar electric pulses, Biochim. Biophys. Acta Biomembr. 1859 (7) (2017) 1282–1290, https://doi.org/10.1016/j.bbamem.2017.04.014.
[54] D. Shamoon et al., Assessing the electro-deformation and electro-poration of biological cells using a three-dimensional finite element model, Appl. Phys. Lett. (2019), https://doi.org/10.1063/1.5079292.
[55] T.J. Jentsch, V. Stein, F. Weinreich, A.A. Zdebik, Molecular structure and physiological function of chloride channels, Physiol. Rev. 82 (2) (2002) 503–568, https://doi.org/10.1152/physrev.00029.2001.
[56] A. Accardi, M. Pusch, Fast and slow gating relaxations in the muscle chloride channel CLC-1, J. Gen. Physiol. (2000).
[57] J. Dermol, O.N. Pakhomova, A.G. Pakhomov, D. Miklavčič, Cell electrosensitization exists only in certain electroporation buffers, 10.1371/journal.pone.0159434.
[58] T. Kotnik, D. Miklavčič, T. Slivnik, Time course of transmembrane voltage induced by time-varying electric fields - A method for theoretical analysis and its application, Bioelectrochem. Bioenerg. 45 (1) (1998) 3–16, https://doi.org/10.1016/S0302-4598(97)00093-7.
[59] S. Dong, C. Yao, Y. Zhao, Y. Lv, H. Liu, Parameters optimization of bipolar high frequency pulses on tissue ablation and inhibiting muscle contraction, IEEE Trans. Dielectr. Electr. Insul. (2018), https://doi.org/10.1109/TDEI.2018.006303.
[60] G. Pucihar, J. Krmelj, M. Reberšek, T.B. Napotnik, D. Miklavčič, Equivalent pulse parameters for electroporation, IEEE Trans. Biomed. Eng. 58 (11) (2011) 3279–3288, https://doi.org/10.1109/TBME.2011.2167232.
[61] T. Kotnik, G. Pucihar, M. Reberšek, D. Miklavčič, L.M. Mir, Role of pulse shape in cell membrane electropermeabilization, Biochim. Biophys. Acta - Biomembr. 1614 (2) (2003) 193–200, https://doi.org/10.1016/S0005-2736(03)00173-1.
[62] C.B. Arena et al., Focal blood-brain-barrier disruption with high-frequency pulsed electric fields, Technology (2014), https://doi.org/10.1142/s2339547814500186.
[63] I.A. Siddiqui et al., Induction of rapid, reproducible hepatic ablations using next-generation, high frequency irreversible electroporation (H-FIRE) in vivo, HPB (Oxford) 18 (9) (2016) 726–734, https://doi.org/10.1016/j.hpb.2016.06.015.
[64] M.B. Sano et al., Reduction of muscle contractions during irreversible electroporation therapy using high-frequency bursts of alternating polarity pulses: a laboratory investigation in an ex vivo swine model, J. Vasc. Interv. Radiol. 29 (6) (2018) 893–898, https://doi.org/10.1016/j.jvir.2017.12.019.
[65] B. Mercadal, C.B. Arena, R.V. Davalos, A. Ivorra, Avoiding nerve stimulation in irreversible electroporation: a numerical modeling study, Phys. Med. Biol. 62 (20) (2017) 8060–8079, https://doi.org/10.1088/1361-6560/aa8c53.
[66] V.M. Ringel-Scaia et al., High-frequency irreversible electroporation is an effective tumor ablation strategy that induces immunologic cell death and promotes systemic anti-tumor immunity, EBioMedicine 44 (Jun. 2019) 112–125, https://doi.org/10.1016/j.ebiom.2019.05.036.
[67] S. Clark, S.E. Jordt, T.J. Jentsch, A. Mathie, Characterization of the hyperpolarization-activated chloride current in dissociated rat sympathetic neurons, J. Physiol. 506 (Pt 3) (1998) 665–678, https://doi.org/10.1111/J.1469-7793.1998.665BV.X.
[68] M. Pusch, S.E. Jordt, V. Stein, T.J. Jentsch, Chloride dependence of hyperpolarization-activated chloride channel gates, J. Physiol. 515 (Pt 2) (1999) 341–353, https://doi.org/10.1111/J.1469-7793.1999.341AC.X.
[69] X. Li, K. Shimada, L.A. Showalter, S.A. Weinman, Biophysical properties of ClC-3 differentiate it from swelling-activated chloride channels in Chinese hamster ovary-K1 cells, J. Biol. Chem. (2000), https://doi.org/10.1074/jbc.M002712200.
[70] M. Pierre Rols, C. Delteil, G. Serin, J. Teissié, Temperature effects on electrotransfection of mammalian cells, Nucleic Acids Res. (1994), https://doi.org/10.1093/nar/22.3.540.
[71] A. Bulysheva et al., Coalesced thermal and electrotransfer mediated delivery of plasmid DNA to the skin, Bioelectrochemistry (2019), https://doi.org/10.1016/j.bioelechem.2018.10.004.
[72] M. Kandušer, M. Šentjurc, D. Miklavčič, The temperature effect during pulse application on cell membrane fluidity and permeabilization, Bioelectrochemistry (2008), https://doi.org/10.1016/j.bioelechem.2008.04.012.
[73] U.F. Pliquett, G.T. Martin, J.C. Weaver, Kinetics of the temperature rise within human stratum corneum during electroporation and pulsed high-voltage iontophoresis, Bioelectrochemistry 57 (1) (2002) 65–72.
[74] A. Velikonja, P. Kramar, D. Miklavčič, A. Maček Lebar, Specific electrical capacitance and voltage breakdown as a function of temperature for different planar lipid bilayers, Bioelectrochemistry 112 (2016) 132–137, https://doi.org/10.1016/j.bioelechem.2016.02.009.
[75] M. Faroja et al., Irreversible electroporation ablation: is all the damage nonthermal?, Radiology (2012), https://doi.org/10.1148/radiol.12120609.
[76] Long, Histological and finite element analysis of cell death due to irreversible electroporation, TCRT Exp. (2013), https://doi.org/10.7785/tcrtexpress.2013.600253.
[77] R.V. Davalos, B. Rubinsky, L.M. Mir, Theoretical analysis of the thermal effects during in vivo tissue electroporation, Bioelectrochemistry 61 (1–2) (2003) 99–107.
[78] S. Gabriel, R.W. Lau, C. Gabriel, The dielectric properties of biological tissues: II. Measurements in the frequency range 10 Hz to 20 GHz, Phys. Med. Biol. (1996), https://doi.org/10.1088/0031-9155/41/11/002.
[79] IT'IS Foundation, Tissue Properties Database V4.0. IT'IS Foundation, 2018, 10.13099/VIP21000-04-0.
[80] O.N. Pakhomova, B.W. Gregory, V.A. Khorokhorina, A.M. Bowman, S. Xiao, A.G. Pakhomov, Electroporation-induced electrosensitization, PLoS One 6 (2) (2011), https://doi.org/10.1371/journal.pone.0017100 e17100.
[81] M. Puc, T. Kotnik, L.M. Mir, D. Miklavcic, Quantitative model of small molecules uptake after in vitro cell electropermeabilization, Bioelectrochemistry 60 (1–2) (2003) 1–10.
[82] T. Kotnik, D. Miklavcic, Theoretical evaluation of the distributed power dissipation in biological cells exposed to electric fields, Bioelectromagnetics 21 (5) (2000) 385–394.
[83] P.A. Garcia, R.V. Davalos, D. Miklavcic, A numerical investigation of the electric and thermal cell kill distributions in electroporation-based therapies in tissue, PLoS One (2014), https://doi.org/10.1371/journal.pone.0103083.

2.1.2 Razvoj spremenjene občutljivosti celične linije CHO ob večkratni izpostavljenosti elektroporacijskim pulzom *in vitro*

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Terapije, ki temeljijo na elektroporaciji (elektrokemoterapija, genska elektrotransfekcija, netermično uničenje tkiva z ireverzibilno elektroporacijo, ablacija srčnega tkiva) so predvsem za enkratne terapije. Včasih pa se zgodi, da zdravljenje ni bilo popolnoma uspešno, zato je potrebno terapijo ponoviti, a se o učinkovitosti ponovnega zdravljenja ne ve veliko. V naši študiji smo preverili možnost razvoja povečane odpornosti celic na elektroporacijske pulze. CHO celice smo dolgoročno (30 zaporednih generacij) izpostavljali elektroporacijskim pulzom. Razvoj povečane odpornosti celic smo preverili pri vsaki peti generaciji, preko izdelave krivulji, ki prikazujejo povečano prepustnost membrane in preživetje celic po elektroporaciji. V nobeni od teh generacij nismo zaznali statistične razlike med kontrolo skupino ter celicam izpostavljenim elektroporacijskim pulzom. S to študijo ponujamo dokaz, da elektroporacija ne vpliva na celice na način, da bi te postale manj (ali bolj) občutljive na elektroporacijske pulze. Naši rezultati kažejo, da lahko zdravljenje z elektroporacijskimi metodami uporabimo večkrat, pri tem pa lahko pričakujemo, da bo učinek vsakega nadaljnjega zdravljenja enakovreden prvotnemu zdravljenju.

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Development of adaptive resistance to electric pulsed field treatment in CHO cell line *in vitro*

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Pulsed electric field treatment has increased over the last few decades with successful translation from *in vitro* studies into different medical treatments like electrochemotherapy, irreversible electroporation for tumor and cardiac tissue ablation and gene electrotransfer for gene therapy and DNA vaccination. Pulsed electric field treatments are efficient but localized often requiring repeated applications to obtain results due to partial response and recurrence of disease. While these treatment times are several orders of magnitude lower than conventional biochemical treatment, it has been recently suggested that cells may become resistant to electroporation in repetitive treatments. In our study, we evaluate this possibility of developing adaptive resistance in cells exposed to pulsed electric field treatment over successive lifetimes. Mammalian cells were exposed to electroporation pulses for 30 generations. Every 5th generation was analyzed by determining permeabilization and survival curve. No statistical difference between cells in control and cells exposed to pulsed electric field treatment was observed. We offer evidence that electroporation does not affect cells in a way that they would become less susceptible to pulsed electric field treatment. Our findings indicate pulsed electric field treatment can be used in repeated treatments with each treatment having equal efficiency to the initial treatment.

Electroporation or pulsed electric field (PEF) treatment can cause increase in membrane permeability and allows molecules, for which the membrane is mostly impermeable, to cross. Two distinct outcomes of electroporation can be observed and used: reversible and irreversible electroporation.

In reversible electroporation cells manage to repair the damage caused by electric pulses, *i.e.* cells survive. This has been widely used in different scientific fields for inserting molecules of interest into the cell. One of the more widely used applications is gene electro-transfer, which allows genetic manipulation. From a simple increased transfection this was developed into gene therapy[1,2] and DNA vaccination[3,4]. While with gene therapy new genes and products are expressed within the host cell, DNA vaccination induces immune response of host organism. Combination of DNA vaccine injection and electroporation is most potent DNA delivery for subsequent immune response[5]. Increased cell membrane permeability is also being exploited in electrochemotherapy (ECT). ECT is a combination of electric pulses and chemotherapeutic drug. Due to increased cell membrane permeability chemotherapeutic drug enter the cell which potentiates the cytotoxicity of the drug[6,7]. ECT has been proved as a safe and efficient procedure for skin malignancies and now studies have been initiated to treat deep-seated tumors[8,9].

The other possible outcome of electroporation is irreversible electroporation (IRE), where the damage to the cells is too extensive and leads to cell death. The use of irreversible electroporation as tissue ablation technique in recent years is increasing. IRE is one of the rising technologies as minimally invasive ablation treatment in interventional electrophysiology as it has several benefits over other types of ablation (*i.e.* radiofrequency or cryoablation) such as short treatment time, reduced thermal injury and selectivity or sparing of surrounding tissue. IRE can also be used for tumor and tissue ablation not amenable to surgical treatment[10,11]. In IRE treatment targeted tissue is efficiently destroyed, however, the integrity of nearby tissue structures like vessels and nerves are preserved as well as extracellular matrix[12,13]. This is also why IRE is on its way to become the leading therapy for cardiac ablation[14–16]. Other myocardial ablation therapies like radiofrequency or cryo-ablation may result in damaging untargeted tissue (e.g. phrenic nerve and esophagus)[17]. In cardiac ablation IRE, also known as pulsed field ablation (PFA), pulses are being delivered intracardially (*i.e.* inside of the heart) via specially designed catheters as minimally invasive procedure or epicardially (e.g. during heart surgery)[18]. PFA pulses use trains of mono- or biphasic pulses. Preclinical experiments show successful lesion formation in myocardium, while

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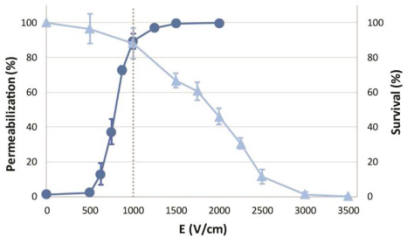

Figure 1. Preliminary experiments for voltage determination. Permeabilization (●) and survival (▲) curve are presented. The intersection point of the curves was the selected voltage/electric field for the adaptive resistance experiment.

preserving the integrity and function of nearby structures, such as lungs, coronary arteries, phrenic nerve and esophagus[17,19–22]. First human studies showed successful and immediate electrical isolation of pulmonary vein after PFA application[23,24].

PEF treatment including reversible (DNA vaccination, electrochemotherapy) and irreversible electroporation also stimulates sensory nerves, thus the procedure can be painful for patients[25,26]. Therefore, general anesthesia[27], synchronization with electrocardiogram[28–30] and administration of muscle relaxants are needed during the treatment to prevent muscle contraction, discomfort and pain due to electric pulse delivery. Electrochemotherapy and IRE are efficient and cause complete or partial response of the treated tumors/tissue which leads to prolonged survival and significant pain reduction[12,31,32].

The outcome of the electroporation based treatment depends on pulse parameters, which may need to be adjusted to specific application and targeted tissue. While effect of different pulse parameters on cells and tissues are being addressed in *in vitro* and *in vivo* experiments, not much is known about the impact of repeated treatment, *i.e.* if cells can adapt to PEF treatment in a way that they would become less affected by the PEF treatment, *i.e.* if the treatment would become less efficient when repeated. In a recent study on human cancer cells authors suggested a development of adaptive resistance to IRE[33]. Similar question was addressed by designing experiments in bacteria for 30 generations which however showed no development of adaptive resistance to PEF treatment[34]. In our study elements from both previous studies were used for designing and conducting an experiment on developing adaptive resistance in cell exposed to PEF treatment. Mammalian cells were exposed to electroporation pulses for 30 generations over the course of four months. No development of adaptive resistance to PEF treatment was observed. We were unable to develop an electroporation resistant cell line resistant either to permeabilization (reversible electroporation) or viability (irreversible electroporation). Based on results obtained we can confirm repetitive electroporation treatment in electroporation-based therapies, like electrochemotherapy, gene electrotransfer for gene therapy or DNA vaccination and IRE as an ablation method is being as efficient as when the first time used.

Results

In preliminary experiments we determined pulse amplitude to which cells will be exposed in each generation in adaptive resistance experiment. The voltage (electric field) amplitude for the adaptive resistance experiment was selected at the intersection point of permeabilization and survival curve of 8 × 100 µs with repetition rate 1 Hz, *i.e.* 200 V (1000 V/cm) (Fig. 1). At this pulse amplitude almost all cells were permeabilized, while the survival was only mildly affected and yet enough by the standard procedure to obtain a "resistant" cell line[35].

Graphical description of the experiment and forming of experimental groups is presented in the methods (Fig. 5). Permeabilization and survival curves (8 × 100 µs, 1 Hz, 0–600 V) were determined for 1st, 5th, 10th, 15th, 20th, 25th and 30th generation in three experimental groups: CTRL- never exposed to pulse treatment, CTRL + EP - exposed to PEF treatment only in 1st generation and EP- repeatedly exposed to pulse treatment (for more detailed explanation see Materials and methods section).

Over the 15/30 generation period we obtained a large amount of permeabilization and survival curves. For easier presentation we separated permeabilization and survival curves. Changes in curves at different electrical field are presented in five graphs, one for each tested point of electrical field of permeabilization and survival experiment. Rise of permeabilization curve and fall of survival curve as a function of the pulse amplitude still remains evident. This presentation allows easier comparison between CTRL and EP and between CTRL − EP and EP group at the same electric field and additionally we can compare different generations.

Every time cells were transferred to a new growing flask, they were exposed to 1000 V/cm, which caused around 80% of cells in preliminary experiment (Fig. 1) to be permeabilized. Cells were periodically exposed to this stressor, which could influence the sensitivity of cells to electroporation pulses. Looking overall at the permeabilization results obtained (Fig. 2), only one (15th generation at 1250 V cm) out of 15 experimental points show statistical difference between CTRL and EP group in experiment 1. At this experimental point the slope of permeabilization curve is high and the variability between generations is large. If resistance would develop, we would expect the whole permeabilization curve (at every experimental point) to shift towards lower % values and be statistically different than control. To investigate, if this one statistically significant differences between CTRL

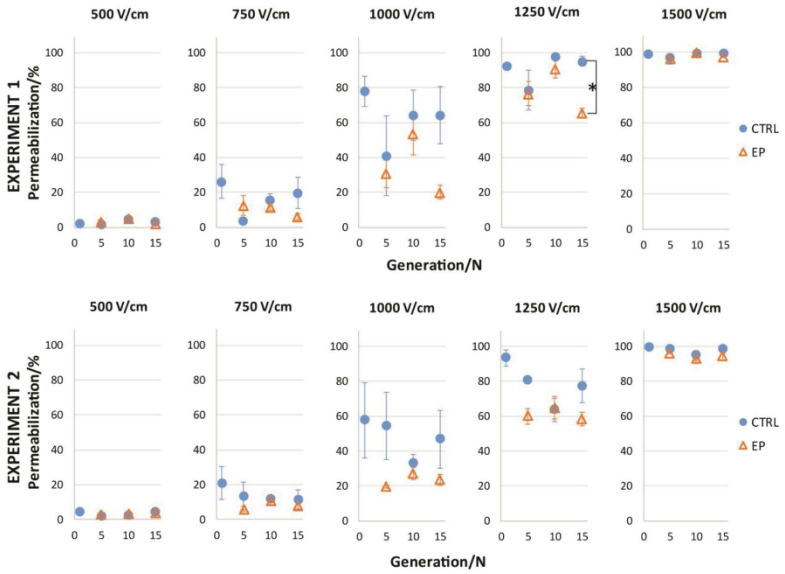

Figure 2. Permeabilization at different electric fields for 15 generation. Two experiments (upper and lower panels) both for 15 generations, are shown. Each experiment is presented at 5 different electric field values: 500, 750, 1000, 1250 and 1500 V/cm. Results are show an average ± SD of three technical repetitions. In each experiment generation efficiency of permeabilization of control samples (CTRL, ●) was compared to samples exposed to PEF treatment/electric pulses (EP, Δ) in the same experiment. Statistically significant difference, with $p < 0.05$, is observed only at 1250 V/cm between CTRL and EP group of 15th generation in experiment 1.

and EP group is repeatable and not an experimental error we repeated the experiment. This time no statistical difference between CTRL and EP group (Fig. 2) was detected. After two repetitions of the experiment, no statistical differences were reproduced. Similar observations were observed between CTRL + EP and EP group. Data on CTRL + EP (cells that were only once exposed to pulse treatment) is shown in supplementary file, Fig. S1. Electroporation pulses alone do not cause/influence cells to develop adaptive resistance to cell permeabilization caused by electroporation.

In survival experiments cells were repeatedly exposed to 1000 V/cm, which in the preliminary experiment (Fig. 1) allowed around 80% of cells to survive (together 80% of permeabilization, Fig. 2). Cells were exposed to EP in every generation, which would influence the sensitivity of cells for PEF treatment, making cells more resistant to electroporation pulses, *i.e*, shifting the survival curve towards higher % values. Similar observation as in permeabilization experiment were found in viability experiments. Variation between generations is present at the slope of survival curve (Fig. 3). In the two experiments no statistically significant difference between CTRL and EP group was observed. Similar observations were observed between CTRL + EP and EP group. Data on CTRL + EP is shown in supplementary file, Fig. S2. Electroporation pulses alone do not cause cells to develop adaptive resistance to IRE.

In 15 generations of cell line, spread over the course of 2 months, we did not succeed in developing electroporation resistant cells line. On account of "short" time, we expanded the second experiment for another 15 generations, making it a total of 30 generations and prolonging the experiment to 4 months.

Only one statistically significant difference was observed in permeabilization and survival experiments (Fig. 4), however this was not present in all of the experimental points of both curves and it disappeared with a next generation. Similar results were obtained also between CTRL + EP and EP group. Data on CTRL + EP is shown in supplementary file, Fig. S3. Electroporation pulses do not cause a development of adaptive resistance in cells to PEF treatment.

Discussion

The use of Pulsed Electric Field (PEF) treatment has increased over the last few decades. It has been successfully translated from *in vitro* studies into clinic. Electrochemotherapy is becoming an established method for treating skin metastasis and is now moving to a deep-seated tumor[36,37]. Irreversible electroporation is on its way to become the leading therapy for hard to reach tumors[38] and cardiac tissue ablation[39]. A promising medical field is DNA vaccination with the help of electric pulse treatment. Both reversible and irreversible electroporation are

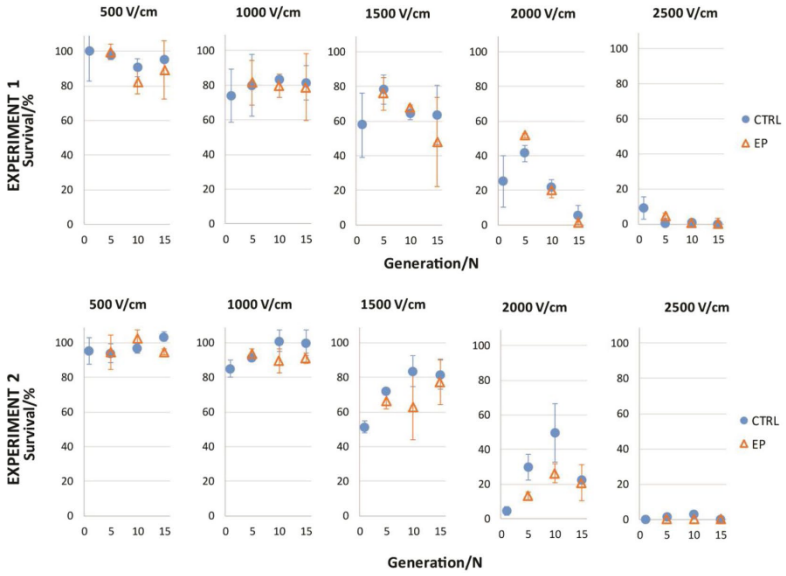

Figure 3. Survival at different electric fields for 15 generation. Two experiments (upper and lower panels) both for 15 generations, are shown. Each experiment is presented at 5 different electric field values: 500, 1000, 1500, 2000 and 2500 V/cm. Results are show an average + SD of three technical repetitions. In each experiment generation efficiency of permeabilization of control samples (CTRL, ●) was compared to samples exposed to PEF treatment/electric pulses (EP, Δ) in the same experiment. No statistically significant difference with $p < 0.05$ was observed between CTRL and EP group.

very efficient but may result in partial response or recurrent disease and thus the treatment needs to be repeated. Also in gene therapy and DNA vaccination repeatable treatment may be needed. The efficiency and impact of repeated PEF treatments is however not known. Hitherto it has not been explored if repeated PEF treatment could lead to a development of resistance to PEF treatment.

In our study, we evaluated the possibility of developing adaptive resistance to electroporation pulses. According to the available literature this was investigated only twice with different outcome *in vitro*. One experiment was performed on bacteria for 30 generations and the second experiment was on mammalian cells for 2 generations. The standard procedure to obtain a "resistant" cell line takes a few months, with low administrated doses or treatment and cells are allowed to recover in drug-free media. Produced cell line should display between two to eight-fold resistance compared to their parental cell line. In this test the sensitive parental cells are compared to the surviving daughter resistant cells by viability assays[35]. In our study we exposed mammalian cells to PEF treatment for 30 generations which lasted app. 4 months, to see if we can induce development of adaptive resistance in cells to PEF treatment. The results of our study can help to understand if PEF treatment can be used in repeated treatments in clinics.

Cells were exposed to well established and most often used electroporation pulses; 100 µs long monopolar pulses with repetition frequency 1 Hz[38,40]. Two controls were used. First control (CTRL group) represents intact parental cell line, cells that were never exposed to pulse treatment. Every time cells were just harvested and transferred to a new growing flask. This control allowed us to evaluate if pulse treatment has influenced cells to develop adaptive resistance over time. At the same time, it showed that some variably over generations is present also in untreated group. Second control (CTRL + EP) was introduced to investigate if only one exposure of cells to PEF treatment has any effect on their behavior. This happens when PEF treatment is successful and only one application is needed to achieve desired results. However, if PEF treatment results in only partial response, the treatment needs to be repeated. The baseline for this control are cells that were once exposed to PEF treatment. This control (CTRL + EP group) was first exposed to pulse treatment at the beginning of the experiment and then it was just continually transfer to a new growing flask in an absence of pulse treatment. Two controls in experiment allowed us to assess, if repetitive exposure to PEF treatment leads to any changes in cell behavior compared to cells that were never exposed to PEF treatment and cells with only one exposure to PEF treatment (cells that had only partial response to the first treatment).

In our experiment 1st generation followed by every 5th generation was being analyzed by determining permeabilization and survival curve. We would expect a shift, *i.e.* higher electrical field would be needed for

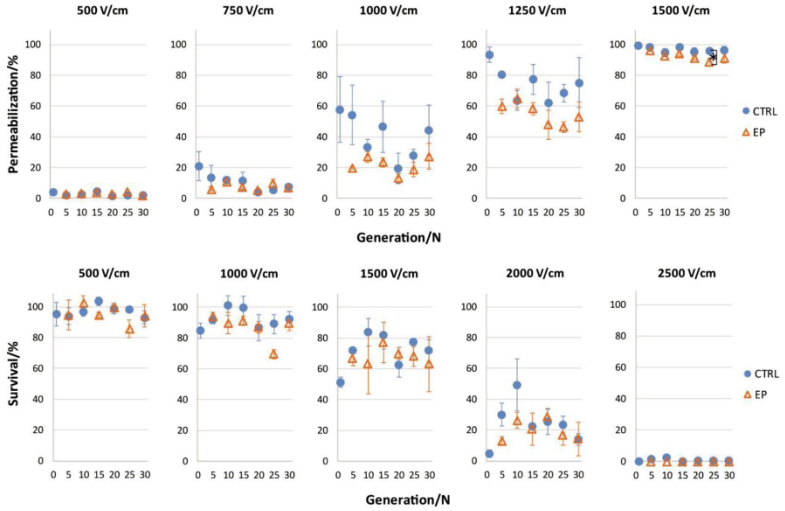

Figure 4. Permeabilization and survival in 30 generation. 30 generations of CTRL and EP group are shown at different electric fields. In each generation efficiency of survival of CTRL (●) group was compared to samples exposed to electric pulses (EP group, Δ). Statistically significant difference, with $p < 0.05$, is observed only at 1500 V/cm between CTRL and EP group of 25th generation (note that first 15 generations are the same as those in experiment 2 from Figs. 2 and 3).

permeabilization and survival curve of EP group, in comparison to control groups, if cells exposed to pulsed electric field treatment develop adaptive resistance (become resistant) to the electroporation pulses. Furthermore, we would expect the shift between EP and control groups to become bigger with each generation.

Looking overall at our result only in two data points statistical difference between CTRL and EP group and one between CTRL + EP and EP group were detected, which, however, disappeared with the next generations and with the repetition of the experiment. We thus assumed these differences are most probably a result of experimental errors and can as such be considered random. No repeatable statistical difference between EP and control groups and no visible shift in permeabilization or survival curves were observed. Electric field for no (0%) and maximum/full permeabilization (100%) and cell death stays the same even after 30 generations. Nevertheless, some variations between generations within the same experimental group (within CTRL group, within CTRL + EP group and within EP group) can be observed at intermediate electric fields/experimental points which however do not provide evidence of cells developing adaptive resistance. According to our results PEF treatment can thus be used for repeated treatments with similar efficiency as the initial treatment.

Our findings can hardly be compared to the results of previously reported study[33] where a small number of generations (only 2: 1st and 2nd generation) was used. A small number of generations used invalidates the conclusion of authors regarding adaptive resistance to PEF treatment being developed. Namely, according to standard procedure obtaining a "resistant" cell line takes a few months, while the study in[33] on adaptive resistance development due to electrical pulses based on 2 generation took only 5 days. Such a short time is not sufficient to develop a resistant cell line. In our experiment we observed a statistical difference between CTRL and EP group at 5th generation, but with the next generation this difference disappeared, and the difference was attributed to experimental error. Also, experiments in these two studies were performed on different cell lines; in[33] experiments were performed using human pancreas cancer cells, whereas in our experiment "healthy" cells from hamster ovaries were used so direct comparison is not necessarily adequate.

Even though our result show that PEF treatment can be used as safe and efficient in repeated treatments, we need to recognize some limitations of our study. Since the standard procedure to obtain a resistant cell line is conducted with low administrated doses of chemicals or treatment, we believe affecting 80% of cell viability (*i.e.* 20% cell kill) should be sufficient enough to cause a development of resistant cell line. At the same time, chosen threshold caused 80% in permeabilization assay, which from permeabilization perspective was actually a high administrated dose. Nevertheless, no development of adaptive resistance to PEF was detected in permeabilization. We believe that same cell behavior would we observed in development of adaptive resistance to PEF in survival assay, even if the used electric field would be higher. However, it would be interesting to test, if adaptive resistance to PEF in survival assay would change, if experiment was performed with PEF treatment causing higher, e.g. 80% cell kill. The study was performed only on one cell line, *i.e.* CHO cell line, which are genetically stable and are considered as non-cancer cells. For more reliable results, different cancer and non-cancer cell should be used. However, when working with cancer cell line, number of passages (generation) is limited between

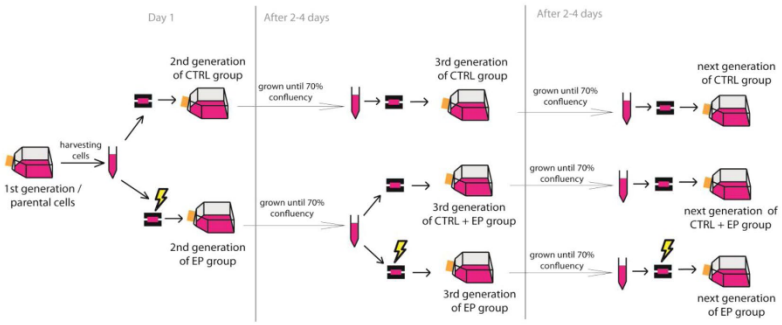

Figure 5. Scheme of the resistance experiment. Each generation cells were electroporated in transferred to a new growing flask. Experiment was repeated for 15 or 30 generations (N). 1st generation followed by every 5th generation of CTRL, CTRL + EP and EP group were analyzed for a development of adaptive resistance by preforming permeabilization and survival curve.

10–20, depending on cell line, as low passage reflects the characteristics of the primary tumor more closely and the malignancy kept[41,42]. Such experiments would be shorter than the one performed in this study, but sufficient enough to see, if cancer cell behavior is different form non-cancer cell. If the adaptive resistance would be obtained *in vitro* further *in vivo* experiments would be necessary.

Conclusion

In our study the efficiency and the effect of repeated PEF treatment was investigated *in vitro* on CHO cells. Our results show that electroporation does not affect cells in a way that they would become less susceptible to PEF treatment, *i.e.* that cells would develop adaptive resistance for reversible or irreversible electroporation. Our findings indicate PEF treatment can be used for repeated treatments, if the initial treatment resulted in only partial response or recurrence of disease.

Methods

Pulses. Eight 100 µs long monopolar pulses with repetition frequency 1 Hz of 100–600 V (0.5–3 kV/cm) were applied by the laboratory prototype pulse generator (University of Ljubljana), based on H-bridge digital amplifier with 1 kV MOSFETs (DE275-102N06A, IXYS, USA)[43]. Delivered current and voltage were measured by the oscilloscope Wavesurfer 422, 200 MHz, the current probe CP030 and the differential probe ADP305 (all from LeCroy, USA) (recording not shown). Cells in suspension were electroporated between stainless steel 304 plate electrodes with 2 mm interelectrode distance (voltage-to-distance ratio: kV/cm).

Cells. Chinese hamster ovary (CHO-K1, European Collection of Authenticated Cell Cultures) (negative for mycroplasma) were grown in HAM F-12 growth medium (PAA, Austria) in culture flasks (TPP, Switzerland) in an incubator (Kambič, Slovenia) at 37 °C with a humidified 5% CO_2. The growth medium was supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, Germany), L-glutamine (StemCell, Canada), antibiotics penicillin/streptomycin (PAA, Austria) and gentamycin (Sigma-Aldrich, Germany) (*i.e.*, complete HAM-F12). When 70% confluency was reached, cells were detached by 10x trypsin-EDTA (PAA, Austria), diluted 1:9 in Hank's basal salt solution (StemCell, Canada) and incubated at 37 °C for 2 minutes. Trypsin was inactivated by addition of fresh complete HAM F-12. Sample was centrifuged at 180 g and 22 °C for 5 minutes. Old media was removed, and the cell pellet was re-suspended in growth medium (complete HAM-F12) at the cell density 2×10^6 cells/ml.

Permeabilization assay. Electropermeabilization of cells can be quantified by penetration of a membrane-impermeable fluorescent agent like propidium iodide (PI)[44]. Binding of PI to nucleic acid enhances its fluorescence, so it can easily be detected. Before electric pulses were applied, cells suspension was mixed with propidium iodide (PI, Life Technologies) to a final concentration of 100 µg/ml. 60 µl of the sample was transferred between electrodes, and electric pulses were applied. Afterwards the sample was transferred to a 1.5 ml tube and incubated at room temperature for three minutes. 150 µl of growth medium was added to obtain a high-enough volume for measurement. The uptake of PI was detected with the flow cytometer (Attune NxT; Life Technologies, Carlsbad, CA, USA). Samples were excited with a blue laser at 488 nm and emitted fluorescence was detected through a 574/26 nm band-pass filter. 10,000 events were obtained, and data were analyzed using the Attune Nxt software. In analysis single cells were separated from debris and clusters and the percentage of PI permeabilized cells was obtained from PI fluorescence intensity histogram by gating permeabilized cells from non-permeabilized.

Viability assay. The MTS Assay is a colorimetric method for determining the number of viable cells[45]. 60 µl of the sample was transferred between electrodes, and electric pulses were applied. After pulse application, 40 µl

cell suspension was diluted in full HAM-F12 growth media to obtain cell density 2×10^4 cells/100 µl. 100 µl of cell suspension was then transferred (in three technical repetitions) to wells in the 96-well plate (TPP, Switzerland) and incubated at 37 °C and humidified 5% CO_2 atmosphere. MTS assay (CellTiter 96 AQueous One Solution Cell Proliferation Assay, Promega, USA) was used to assess cell viability 24 hours after electric pulses were applied. According to manufacturer's instructions 20 µl of MTS tetrazolium compound was added to the samples, and the 96-well plate was returned to the incubator for 2 hours. The absorbance of formazan (reduced MTS tetrazolium compound) was measured with a spectrofluorometer (Tecan Infinite M200, Tecan, Austria) at 490 nm. Percentage of viable cells was calculated by subtracting the background and normalizing the absorbance of the samples to the absorbance of the sham control (0 V/cm).

Development of adaptive resistance experiment. In preliminary experiments we determined amplitude of voltage/electric field at which high membrane permeabilization and cell survival at the same time is achieved.

Parental cells (1st generation) were detached and resuspended in complete HAM F-12 growth media at the density of 2×10^6 cells/ml. Parental cells were split to two groups: one was used as a control in the absence of any pulse treatment – control or parental cells, never exposed to pulse treatment (CTRL group), and the other was repeatedly exposed to pulse treatment to become "resistant" cell line by exposing cells to pulse treatment (EP group). 60 µl samples were placed between plate electrodes and pulses were delivered (8×100 µs, 1 Hz, 200 V). Control cells were subjected to the same procedure as the exposed sample in absence of pulses, *i.e.* 0 V/cm amplitude. Afterwards cells were transferred in new culture growing flask and placed in an incubator until a new generation of cells grew to obtain 70% of confluency was reached (2–4 days).

Cells from the second generation (CTRL and EP group) cells were harvested and resuspended incomplete HAM F-12 growth medium to the density of 2×10^6 cells/ml. CTRL group was again transferred between the electrodes (in the absence of pulse treatment) and then transferred to the new growing flask. EP group was split in two parts. One part was exposed to pulse treatment and then transferred to the new growing flask. The other part became a control, which was once exposed to electric pulses (CTRL + EP). Cells were transferred between the electrodes (in the absence of pulse treatment) and then transferred to the new growing flask. Cell were incubated until a new generation of cell grew.

Cells from the third generation (CTRL, CTRL + EP and EP group) were harvested and resuspended in complete HAM F-12 growth medium to the density of 2×10^6 cells/ml. Cells from control groups (CTRL and CTRL + EP group) were transferred between the electrodes (in the absence of pulse treatment) and then transferred to the new growing flask. Cells from EP group were exposed to pulse treatment) and then transferred to the new growing flask. Cell were incubated until a new generation of cells grew. This procedure was repeated until a wanted generation for all of the groups was obtained.

Graphical description of the experiment is presented in Fig. 5. This process/experiment was repeated twice for 15 generations. Second experiment was prolonged to 30 generations. Three technical replications were made for each group in each experiment.

The first, followed by every fifth, generation in EP and control groups (5th, 10th, 15th, 20th, 25th and 30th) were analyzed. In every fifth generation permeabilization and survival curves (typical for electroporation assays) were determined (8×100 µs, 1 Hz, 0–600 V). If cells exposed to electroporation pulses (EP group) would develop adaptive resistance to the PEF treatment, we would expect a shift in permeabilization and/or survival curve in comparison to control groups.

Statistical analysis. Statistical analysis was performed using SigmaPlot 11.0 (Systat Software, USA). The results are shown as mean ± SD. Statistically significant differences ($^*p < 0.05$) were determined by repeated measures ANOVA test, followed by multiple comparison by Hold-Sidak method.

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References

1. Heller, L. C. & Heller, R. Electroporation gene therapy preclinical and clinical trials for melanoma. *Curr. Gene Ther.* **10**, 312–7 (2010).
2. Lambricht, L. *et al.* Clinical potential of electroporation for gene therapy and DNA vaccine delivery. *Expert Opin. Drug Deliv.* **13**, 295–310 (2016).
3. Trimble, C. L. *et al.* Safety, efficacy, and immunogenicity of VGX-3100, a therapeutic synthetic DNA vaccine targeting human papillomavirus 16 and 18 E6 and E7 proteins for cervical intraepithelial neoplasia 2/3: a randomised, double-blind, placebo-controlled phase 2b trial. *Lancet (London, England)* **386**, 2078–2088 (2015).
4. Kwak, K. *et al.* Multivalent Human Papillomavirus L1 DNA Vaccination Utilizing Electroporation. *Plos One* https://doi.org/10.1371/journal.pone.0060507 (2013).
5. Lee, S. H., Danishmalik, S. N. & Sin, J. I. DNA vaccines, electroporation and their applications in cancer treatment. *Hum. Vaccines Immunother.* **11**, 1889–1900 (2015).
6. Orlowski, S., Belehradek, J., Paoletti, C. & Mir, L. M. Transient electropermeabilization of cells in culture. Increase of the cytotoxicity of anticancer drugs. *Biochem. Pharmacol.* **37**, 4727–4733 (1988).
7. Mir, L. M. Bases and rationale of the electrochemotherapy. *Eur. J. Cancer, Suppl.* **4**, 38–44 (2006).
8. Kos, B. *et al.* Robustness of treatment planning for electrochemotherapy of deep-seated tumors. *J. Membr. Biol.* **236**, 147–153 (2010).
9. Groselj, A. *et al.* Efficiency of electrochemotherapy with reduced bleomycin dose in the treatment of nonmelanoma head and neck skin cancer: Preliminary results. *Head Neck* **40**, 120–125 (2018).
10. Davalos, R. V., Mir, L. M. & Rubinsky, B. Tissue ablation with irreversible electroporation. *Ann. Biomed. Eng.* **33**, 223–231 (2005).
11. Chen, X. *et al.* Electric Ablation with Irreversible Electroporation (IRE) in Vital Hepatic Structures and Follow-up Investigation. *Sci. Rep.* **5**, 16233 (2015).

12. Scheffer, H. J. *et al.* Irreversible Electroporation for Nonthermal Tumor Ablation in the Clinical Setting: A Systematic Review of Safety and Efficacy. *J. Vasc. Interv. Radiol.* **25**, 997–1011 (2014).
13. Phillips, M., Maor, E. & Rubinsky, B. Nonthermal irreversible electroporation for tissue decellularization. *J. Biomech. Eng.* **132**, 091003 (2010).
14. Stewart, M. T. *et al.* Intracardiac pulsed field ablation: Proof of feasibility in a chronic porcine model. *Hear. Rhythm* https://doi.org/10.1016/j.hrthm.2018.10.030 (2019).
15. Wojtaszczyk, A., Caluori, G., Pešl, M., Melajova, K. & Stárek, Z. Irreversible electroporation ablation for atrial fibrillation. *J. Cardiovasc. Electrophysiol.* **29**, 643–651 (2018).
16. Wittkampf, F. H. M., van Es, R. & Neven, K. Electroporation and its Relevance for Cardiac Catheter Ablation. *JACC. Clin. Electrophysiol.* **4**, 977–986 (2018).
17. Van Driel, V. J. H. M. *et al.* Pulmonary vein stenosis after catheter ablation electroporation versus radiofrequency. *Circ. Arrhythmia Electrophysiol.* **7**, 734–738 (2014).
18. Sugrue, A. *et al.* Irreversible electroporation for catheter-based cardiac ablation: a systematic review of the preclinical experience. *J. Interv. Card. Electrophysiol.* **55**, 251–265 (2019).
19. Neven, K. *et al.* Acute and Long-Term Effects of Full-Power Electroporation Ablation Directly on the Porcine Esophagus. *Circ. Arrhythm. Electrophysiol.* **10** (2017).
20. Van Driel, V. J. H. M. *et al.* Low vulnerability of the right phrenic nerve to electroporation ablation. *Hear. Rhythm* **12**, 1838–1844 (2015).
21. Kuck, K.-H. *et al.* Cryoballoon or Radiofrequency Ablation for Paroxysmal Atrial Fibrillation. *N. Engl. J. Med.* **374**, 2235–2245 (2016).
22. Koruth, J. *et al.* Preclinical Evaluation of Pulsed Field Ablation: Electrophysiological and Histological Assessment of Thoracic Vein Isolation. *Circ. Arrhythmia Electrophysiol.* **12** (2019).
23. Reddy, V. Y. *et al.* Ablation of Atrial Fibrillation With Pulsed Electric Fields: An Ultra-Rapid, Tissue-Selective Modality for Cardiac Ablation. *JACC Clin. Electrophysiol.* **4**, 987–995 (2018).
24. Reddy, V. Y. *et al.* Pulsed Field Ablation for Pulmonary Vein Isolation in Atrial Fibrillation. *J. Am. Coll. Cardiol.* **74**, 315–326 (2019).
25. B. Arena, C. & V. Davalos, R. Advances in Therapeutic Electroporation to Mitigate Muscle Contractions. *J. Membr. Sci. Technol.* **02**, 1–3 (2012).
26. Zupanic, A., Ribaric, S. & Miklavcic, D. Increasing the repetition frequency of electric pulse delivery reduces unpleasant sensations that occur in electrochemotherapy. *Neoplasma* **54**, 246–50 (2007).
27. Martin, R. C., Schwartz, E., Adams, J., Farah, I. & Derhake, B. M. Intra-operative Anesthesia Management in Patients Undergoing Surgical Irreversible Electroporation of the Pancreas, Liver, Kidney, and Retroperitoneal Tumors. *Anesthesiol. Pain Med.* https://doi.org/10.5812/aapm.22786 (2015).
28. Mali, B. *et al.* The effect of electroporation pulses on functioning of the heart. *Med. Biol. Eng. Comput.* https://doi.org/10.1007/s11517-008-0346-7 (2008).
29. Mali, B. *et al.* Electrochemotherapy of colorectal liver metastases–an observational study of its effects on the electrocardiogram. *Biomed. Eng. Online* **14**(Suppl 3), S5 (2015).
30. Ball, C., Thomson, K. R. & Kavnoudias, H. Irreversible Electroporation. *Anesth. Analg.* **110**, 1305–1309 (2010).
31. Gasbarrini, A., Campos, W. K., Campanacci, L. & Boriani, S. Electrochemotherapy to Metastatic Spinal Melanoma. *Spine (Phila. Pa. 1976).* **40**, E1340–E1346 (2015).
32. Cornelis, F. H. *et al.* Percutaneous Image-Guided Electrochemotherapy of Spine Metastases: Initial Experience. *Cardiovasc. Intervent. Radiol.* **42**, 1806–1809 (2019).
33. Shao, Q. *et al.* Physical and Chemical Enhancement of and Adaptive Resistance to Irreversible Electroporation of Pancreatic Cancer. *Ann. Biomed. Eng.* **46**, 25–36 (2017).
34. Gusbeth, C., Frey, W., Volkmann, H., Schwartz, T. & Bluhm, H. Pulsed electric field treatment for bacteria reduction and its impact on hospital wastewater. *Chemosphere* **75**, 228–233 (2009).
35. McDermott, M. *et al. In vitro* development of chemotherapy and targeted therapy drug-resistant cancer cell lines: A practical guide with case studies. *Front. Oncol.* **4** MAR (2014).
36. Campana, L. G. *et al.* Electrochemotherapy of superficial tumors – Current status:: Basic principles, operating procedures, shared indications, and emerging applications. *Seminars in Oncology* **46**, 173–191 (2019).
37. Campana, L. G. *et al.* Electrochemotherapy – Emerging applications technical advances, new indications, combined approaches, and multi-institutional collaboration. *European Journal of Surgical Oncology* **45**, 92–102 (2019).
38. Aycock, K. N. & Davalos, R. V. Irreversible Electroporation: Background, Theory, and Review of Recent Developments in. *Clinical Oncology. Bioelectricity* **1**, 214–234 (2019).
39. Maor, E. *et al.* Pulsed electric fields for cardiac ablation and beyond: A state-of-the-art review. *Hear. Rhythm* **16**, 1112–1120 (2019).
40. Gehl, J. *et al.* Updated standard operating procedures for electrochemotherapy of cutaneous tumours and skin metastases. *Acta Oncol.* **57**, 874–882 (2018).
41. Arul, M., Roslani, A. C., Ng, C. L. L. & Cheah, S. H. Culture of low passage colorectal cancer cells and demonstration of variation in selected tumour marker expression. *Cytotechnology* **66**, 481–491 (2014).
42. Ossowski, L. & Reich, E. Loss of malignancy during serial passage of human carcinoma in culture and discordance between malignancy and transformation parameters. *Cancer Res.* **40**, 2310–2315 (1980).
43. Sweeney, D. C. *et al.* Quantification of cell membrane permeability induced by monopolar and high-frequency bipolar bursts of electrical pulses. *Biochim. Biophys. Acta - Biomembr.* **1858**, 2689–2698 (2016).
44. Batista Napotnik, T. & Miklavčič, D. *In vitro* electroporation detection methods – An overview. *Bioelectrochemistry* **120**, 166–182 (2018).
45. Šatkauskas, S., Jakštys, B., Ruzgys, P. & Jakutavičiūtė, M. Different Cell Viability Assays Following Electroporation *In Vitro*. In *Handbook of Electroporation* 1–14, https://doi.org/10.1007/978-3-319-26779-1_140-1 (Springer International Publishing, 2016).

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Author contributions
T.P.: acquisition of the data, analysis and interpretation of the data, drafting the paper, final approval of the paper. D.M.: conception and design of the study, interpretation of the data, drafting the paper, final approval of the paper.

Competing interests
The authors declare no competing interests.

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2.1.3 *In vitro* analiza sproščenih molekulskih vzorcev povezanih s poškodbo iz celic po elektroporaciji

Polajžer T., Jarm T., Miklavčič D. 2020. Analysis of damage-associated molecular pattern molecules due to electroporation of cells *in vitro*. Radiology and Oncology, 54: 317-328

Celice lahko umrejo po poti imunogene celične smrti, pri kateri se iz celic sproščajo molekulski vzorci povezani s poškodbo (DAMP). DAMP molekule se vežejo na celice imunskega sistema. Ob tem se sproži kaskada dogodkov aktivacije imunskega sistema, od sproščanja vnetnih mediatorjev do priklica celic imunskega sistema, kar povzroči uničenje preostalih tumorskih celic. Aktivacija imunskega sistema je pomemben sestavni del pri zdravljenju tumorja z elektrokemoterapijo (ECT) in ireverzibilno elektroporacijo (IRE). V tej študiji smo suspenzijo celic, pridobljenih iz ovarijev kitajskega hrčka, izpostavili 100 µs dolgim pulzom. Sproščanje DAMP molekul – natančneje: adenozintrifosfata (ATP), kalretikulina, nukleinskih kislin in sečne kisline smo preučevali v različnih časih po izpostavitvi elektroporacijskim pulzom različnih amplitud. Ocenili smo statistično korelacijo med sproščanjem DAMP in povečano prepustnostjo membrane ter preživetjem celic, oziroma med sproščanjem DAMP in reverzibilno ter ireverzibilno elektroporacijo. Na splošno se sproščanje DAMP molekul povečuje z naraščajočo amplitudo pulza. Koncentracija DAMP molekul je odvisna od časovnega intervala med izpostavljenostjo celic elektroporaciji in analizo. Stabilnost nekaterih DAMP molekul je časovno odvisna, kar je potrebno upoštevati pri načrtovanju poskusov detekcije DAMP molekul po elektroporaciji. Koncentracija večine DAMP molekul močno korelira s celično smrtjo. V analiziranih vzorcih pa nismo zaznali sečne kisline. Sproščanje DAMP molekul bi lahko služilo kot marker za predvidevanje tipa celične smrti. Razumevanje sproščanja DAMP molekul ima velik vpliv na zdravljenje z elektroporacijo, saj bi lahko tako bolje nadzorovali prisotnost DAMP molekul, ki je glede na cilj terapije različno zaželena. Pri elektrokemoterapiji je aktivacija imunskega sistema zaželena, saj pripomore k uničenju tumorskih celic, medtem ko sproščanje DAMP pri ablaciji srčnega tkiva lahko povzroči močnejše vnetje in fibrozo tkiva.

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research article

Analysis of damage-associated molecular pattern molecules due to electroporation of cells in vitro

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Background. Tumor cells can die via immunogenic cell death pathway, in which damage-associated molecular pattern molecules (DAMPs) are released from the cells. These molecules activate cells involved in the immune response. Both innate and adaptive immune response can be activated, causing a destruction of the remaining infected cells. Activation of immune response is also an important component of tumor treatment with electrochemotherapy (ECT) and irreversible electroporation (IRE). We thus explored, if and when specific DAMPs are released as a consequence of electroporation *in vitro*.

Materials and methods. In this *in vitro* study, 100 µs long electric pulses were applied to a suspension of Chinese hamster ovary cells. The release of DAMPs – specifically: adenosine triphosphate (ATP), calreticulin, nucleic acids and uric acid was investigated at different time points after exposing the cells to electric pulses of different amplitudes. The release of DAMPs was statistically correlated with cell permeabilization and cell survival, e.g. reversible and irreversible electroporation.

Results. In general, the release of DAMPs increases with increasing pulse amplitude. Concentration of DAMPs depend on the time interval between exposure of the cells to pulses and the analysis. Concentrations of most DAMPs correlate strongly with cell death. However, we detected no uric acid in the investigated samples.

Conclusions. Release of DAMPs can serve as a marker for prediction of cell death. Since the stability of certain DAMPs is time dependent, this should be considered when designing protocols for detecting DAMPs after electric pulse treatment.

Key words: electroporation; pulsed electric field treatment; damage-associated molecular pattern molecules; immunogenic cell death; electrochemotherapy

Introduction

Electroporation or pulsed electric field (PEF) treatment can cause changes in membrane permeability, which allows molecules, that are otherwise membrane impermeable, to cross the plasma membrane. In reversible electroporation the damage to cell membrane is repaired, enabling the cell to reestablish its metabolism and survive. This type of electroporation is used in multiple therapies. Electrochemotherapy (ECT) is one of such widely used therapies in which the increased cell membrane permeability enables chemotherapeutic drug to enter the cell and thus potentiates the cytotoxicity of the drug.[1,2] In irreversible electroporation (IRE) the damage to the cells however is too severe for the cells to recover which leads to cell death. While the cells are destroyed, the integrity of tissue like vessels, nerves and extracellular matrix remains preserved[3,4], making this therapy very appealing for ablation of tumor and other tissues, otherwise unsuitable for surgical removal or thermal ablation such as radiofrequency ablation or cryo-ablation.[5,6]

In ECT eight square 100 µs electrical pulses, with an amplitude of 100-1000 V are usually used to induce a reversible membrane permeabilization. For IRE, more pulses (80-100 pulses) at higher amplitude (up to 3000 V) are required, to overwhelm the reparative capacity of the cells which leads to cell death.[7] From morphological, biochemical, and functional perspectives, different cell death pathways/types can be activated.[8] Historically, based on morphological changes, three different forms of cell death were defined: apoptosis (cell shrinkage, chromatin condensation, formation of apoptotic bodies); autophagy (cytoplasmic vacuolization); and necrosis (loss of plasma membrane integrity).[8,9] Such classification is still employed, but in newer classification based on genetic, biochemical, pharmacological and functional differences, cell death is either accidental (uncontrollable death caused by disassembly of the plasma membrane) or regulated (activation of signal transduction). Depending on signaling pathways different types of regulated cell death are being characterized, e.g. intrinsic and extrinsic apoptosis, necroptosis, ferroptosis, pyroptosis, immunogenic cell death, lysosome-dependent cell death, mitochondrial permeably transition driven necrosis and many others gathered and described by Galluzi *et al*..[10] In electroporation studies, cell death has been most extensively explored in the range of nanosecond pulse treatment, where the majority of studies confirmed cell death by apoptosis (intrinsic and extrinsic) and only few studies indicated necrosis.[11,12] Both pathways were confirmed also in microsecond pulse treatment.[13-9] Nevertheless, in recent studies new cell death types were also detected like pyroptosis[20], necroptosis[20,21] and immunogenic cell death.[22-29]

In IRE [18,30-34] and ECT with either bleomycin or cisplatin[26,35-37] used for cancer treatment, involvement and importance of host immune response was demonstrated, counteracting tumor escape mechanisms.[29,38,39] After these therapies, dying tumor cells can release specific molecules, which are being recognized by the cells of immune system. These molecules can activate the innate and adaptive immune response, leading to the destruction of the remaining tumor cells in the body[40] and inducing long-lasting protective antitumor immunity.[41] Some studies even suggest that immunogenic effect of IRE is more pronounced than in other ablation therapies like radiofrequency ablation[31] and cryoablation.[32] Evidence suggests that administration of immune-stimulating molecules can even enhance the local effectiveness of ECT[35] and IRE[29,42-44] allowing simultaneous treatment of distant tumors.

Our immune system consists of two complementary and closely collaborative systems, an innate (non-specific) and an adaptive (antigen-specific) system. Activation of immune system is essential for our survival, as it distinguishes and eliminates potentially harmful molecules, even the ones that derive from the host/our own tissues. Well known are the pathogen-associated molecules (PAMPs), which are present on microbes and are being recognized by cells of the innate immune system when they bind to pattern recognition receptors (PRRs). The same pathways are activated by the host's damage-associated molecular pattern molecules (DAMPs), which act as endogenous damage signal in case of cell death or response to stress, leading to inflammatory response.[45-47] Release of DAMPs characterizes immunogenic cell death (ICD). Most of DAMPs are normally located intracellularly[48], where under normal physiological conditions have an important intracellular role. When a cell is damaged or dies, DAMPs are actively or passively exposed or released to extracellular space.[49-51] The release of DAMPs is often accompanied by cytokines, chemokines and other inflammatory mediators.[52] In extracellular space DAMPs have a completely different function, as they are being recognized by pattern recognition receptors (PRRs), such as TRLs, NOD-like, PRLs and RAGE receptors on immune cells.[50,53] Binding of DAMPs to these receptors stimulates innate immune response through promoting the release of pro-inflammatory mediators and recruiting immune cells (dendritic cells, macrophages, T cells and neutrophils). Usually, the exposure of different DAMPs depends on endoplasmic reticulum stress, followed by reactive oxygen species (ROS) production.[41] Release of DAMPs correlates with the degree of trauma.[54] Some DAMPs can even be involved in tissue repair pathway.[55-57] It depends on DAMPs and their triggered pathways, together with cytokines and growth factor to determine, if mild acute inflammation and wound healing [56,58] or severe inflammation and fibrosis will follow.[59]

Electroporation causes an increase in membrane permeability and allows molecules, for which the membrane is usually impermeable, including DAMPs, to cross it. ATP, one of the main DAMPs, was even used as an indicator of cell membrane permeabilization in the first electroporation studies.[60] In recent years reports on electroporation studies have started to emerge investigating the immunogenic cell death caused by electroporation. Studies detected DAMPs, like ATP, high-mobility group box 1 protein (HMGB1) release and calreti-

culin externalization, as they are the gold standard for predicting the ICD in cancer cells.[41] So far mostly single (or a small subset of) DAMPs were studied. Most studies involved nanosecond pulse treatment[22-25], whereas studies using microsecond[26,29], millisecond[28] and H-FIRE pulse treatments[27] are even more scarce. For now, different DAMPs were investigated at different intervals after electroporation ranging from 30 min to 72 hours, using different types of cancer cells.

Because in both ECT and IRE the immune system response is essential for successful and complete tumor eradication, we decided to explore if and when specific DAMPs are released in response to electroporation *in vitro*. The experiments were performed using 100 µs long pulses, as they are most commonly used in ECT treatment and in IRE for soft tissue ablation.

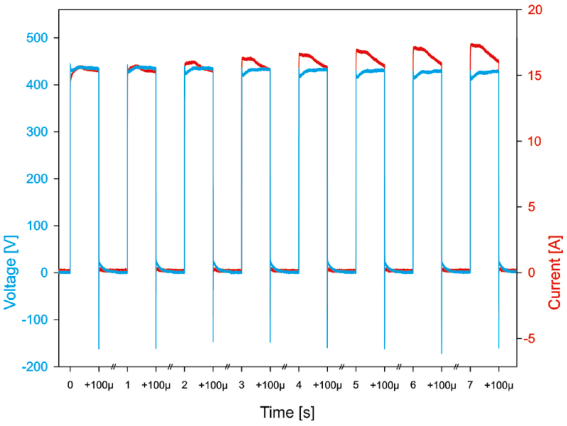

FIGURE 1. Application of 500 V pulses. Blue line shows voltage and red line shows current. Due to sequencing, all eight pulses are in one picture, separated by //.

Materials and methods

Cell preparation

Chinese hamster ovary (CHO-K1) from European Collection of Authenticated Cell Cultures were grown in culture flasks (TPP, Switzerland) filled with HAM F-12 growth medium (PAA, Austria) at 37°C with a humidified 5% CO_2. The growth medium was enriched with 10% fetal bovine serum (FBS) (Sigma-Aldrich, Germany), L-glutamine (StemCell, Canada) and antibiotics penicillin/streptomycin (PAA, Austria) and gentamycin (Sigma-Aldrich, Germany). At 70% confluency, cells were detached with trypsin solution (10x trypsin-EDTA (PAA, Austria) 1:9 diluted in Hank's basal salt solution (StemCell, Canada), which was inactivated after 3 minutes by the growth medium. After 5 minutes of centrifugation at 180 g and 22°C supernatant was removed. Cell were mixed with the growth medium to obtain cell density at $2x10^6$ cells/ml.

Electric pulse generation

Laboratory prototype pulse generator (University of Ljubljana), based on H-bridge digital amplifier with 1 kV MOSFETs (DE275-102N06A, IXYS, USA), described in [61] was used. Eight 100 µs long monopolar electric pulses with repetition frequency 1 Hz and amplitude of 0–600 V (0-3 kV/cm; voltage to distance ratio) with increments of 100 V (and additional increments in the permeabilization assay) were applied between stainless steel 304 plate electrodes (d = 2 mm). Oscilloscope HDO6104A-MS, differential probe HVD3206A and the current probe CP031A, all from LeCroy, USA, were used to monitor the delivered pulses, i.e. voltage and current. The delivered voltage was approximately 10-15% lower than the value set on the pulse generator and the current was in the range of 3-21 A. When pulses with high amplitudes were applied (Figure 1), current decreased slightly during the pulse, presumingly due to electrochemistry at electrode-electrolyte interface reducing the available interface area for ion exchange between the metal electrode and the electrolyte and possibly also due to ion depletion at the said interface.

Results

First, the permeabilization and the survival curves were obtained to determine experimental points for the studies on release of DAMPs. Permeabilization and survival curves are presented in all figures showing the concentration of various DAMPs to visualize how the presence of DAMPs is related to changes in permeabilization and cell viability. In figures the permeabilization and survival curves are shown only at pulse amplitudes tested for the presence of DAMPs; in steps of 50 V in the range of pulses where changes in permeabilization occur and in steps of 100 V above 200 V.

The concentration of ATP in supernatant was first measured with fluorescent method 30 minutes and

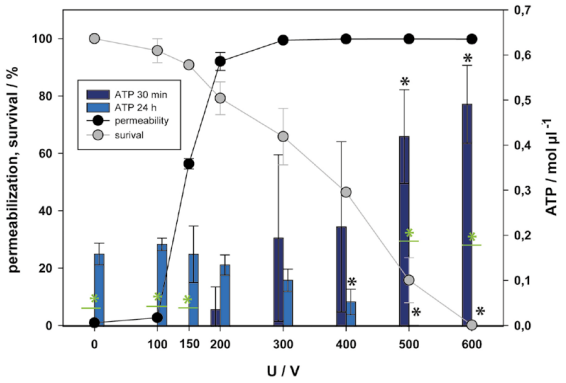

FIGURE 2. Release of adenosine triphosphate (ATP) as a function of electric pulse amplitude determined by fluorescent assay. Two-time points after electroporation were assessed. Permeabilization and survival curves are also presented. Black and green asterisks (*) indicate statistically significant differences between the samples at different voltages and the corresponding control at 0 V (one-way analysis of variance [ANOVA] followed by Holm-Sidak post-hoc test, ($p < 0.05$) and within the pair of samples at different voltages (t-test, $p < 0.05$), respectively).

24 hours after electroporation (Figure 2). At 30 minutes the concentration of ATP in supernatant was detected at 200 V. However, statistical difference between the control and the treatment groups (obtained by one-way analysis of variance [ANOVA] followed by the post-hoc test) was only detected at 500 V and above. The concentration of ATP in supernatant grew with increasing pulse amplitude, which after 24 hours led to decreased cell viability; e.g. correlation between the cell survival and ATP concentration in supernatant detected after 30 min is quite strong and negative; R = -0.864. Also, weak correlation between cell permeabilization and ATP concentration in supernatant (R = 0.594) confirms, that ATP presence in supernatant is more strongly correlated with the irreversible than the reversible electroporation. It may indicate that strong ATP release from cells leads to cell death.

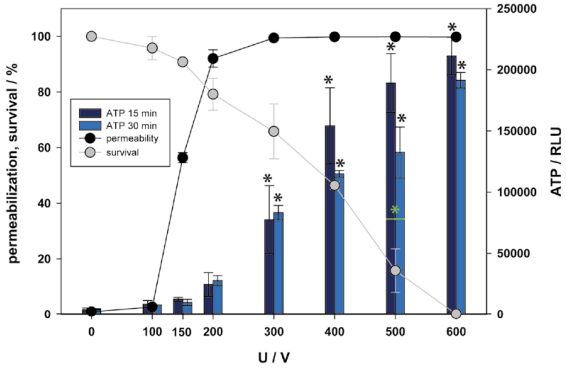

FIGURE 3. Release of adenosine triphosphate (ATP), as a function of electric pulse amplitude determined by luminescence assay. Two-time points after electroporation were assessed. Permeabilization and survival curves are also presented. Black and green asterisks (*) indicate statistically significant differences between the samples at different voltages and the corresponding control at 0 V (one-way analysis of variance [ANOVA] followed by Holm-Sidak post-hoc test, ($p < 0.05$) and within the pair of samples at different voltages (t-test, $p < 0.05$), respectively).

After 24 hours (Figure 2) the lowest ATP concentration was achieved at the pulse amplitude resulting in death of most cells (500, 600 V). The concentration of ATP at these points is statistically different to the results obtained 30 minutes after pulse treatment. After 24 hours the concentration of ATP had decreased with the lower viability, but statistical differences between the control and the treatment groups were present from 400 to 600 V. At 24 hours there is a positive statistical correlation between the cell survival and concentration of ATP in supernatant (R = 0.888), which is stronger than the correlation to permeabilization (R = -0.695).

Since no ATP was detected in supernatant within the range of reversible electroporation after 30 minutes using the fluorescent method (Figure 2), we also used a more sensitive luminescent method (Figure 3). Furthermore, since ATP analysis showed that 24 h after treatment ATP is not detected in all samples, we were also interested in how fast ATP was degraded. Scuderi *et al.* showed complete resealing of plasma membrane 10 minutes after pulse treatment using 8 x 100 µs pulses.[63] Thus, another time point for ATP measurement was chosen, *i.e.* 15 minutes after (Figure 3). With more sensitive luminescent detection assay, ATP was detected in supernatant already at 100 V, however statistically significant difference to control was only detected at 300 V and higher. These results are more reliable due to higher assay sensitivity however even with this method statistically significant amount of ATP in supernatant is detected in the range of irreversible electroporation, as increased electric field/voltage kills more cells more ATP is present in the extracellular space. This is also confirmed by a strong correlation between the survival and the amount of ATP in supernatant, R = -0.947 for 15 min and R = -0.964 for 30 min, and much weaker correlation between the permeabilization and the amount of ATP (R = 0.704 for 15 min and R = 0.728 for 30 min). In our results, only one significant difference was found in detected ATP amount between 15 and 30 minutes after pulse treatment at 500 V. Since this

difference was not detected for all the experimental points (voltages), we believe that ATP in extracellular space is not degraded in this first 30 minutes after pulse treatment.

Calreticulin (CRT) is an endoplasmic reticulum protein which needs to be transferred to the outer leaflet of the plasma membrane in order to act as a DAMP. Externalization of calreticulin to outer membrane in an active process involving also its transport across the cell. Due to this active and time demanding process the externalization of calreticulin was investigated 4 and 24 hours (also used in previous studies[23-25,28]) after pulse treatment (Figure 4) on viable cells (determined by propidium iodide [PI] staining). Calreticulin was first detected at 300 V and its fluorescence increased with increasing voltage of pulses. Furthermore, the lowest viability at 600 V with < 5% of viable cell has the strongest signal of calreticulin after 4 and 24 hours. This could indicate the amount of externalized calreticulin per viable cell increases with the level of stress (amplitude of applied electric pulses). Furthermore, analysis shows a strong correlation between survival determined by MTS test and externalization of calreticulin, as survival decreased, the detection of calreticulin increased (R = -0.801 for 4h and R = -0.946 for 24h) and weak correlation between permeabilization and externalization of calreticulin was observed (R = 0.535 for 4h and R = 0.556 for 24h). Since calreticulin was detected only in viable cells (determined by PI) additional information on viability was obtained, and results were normalized to control (0 V) for each investigated time point separately. Except for the results at 300 V, no statistically significant difference between 4 and 24 hours was detected at any other experimental point, suggesting that calreticulin can be detected 4 hours after pulse treatment and that expression of the protein remains stable for the next 20 hours.

Until now, nucleic acids (in the role of DAMPs) have not been investigated in relation to electroporation. Most of RNA (except fresh transcripted mRNA) is located in cytoplasm, while DNA is located in the cell nucleus. The concentration of RNA and DNA in supernatant has been detected 15, 30 minutes and 24 hours after electroporation like in ATP assay (Figure 5 and 6). Concentration of DNA/RNA started to rise from 400 V up (Figure 5 and 6). This happened at the same pulse amplitudes where after 24 hours cell viability was affected, indicating the amount of nucleic acid occurs in the range of cell death, *i.e.* irreversible electroporation. Exposure of cells to higher pulse amplitudes caused higher

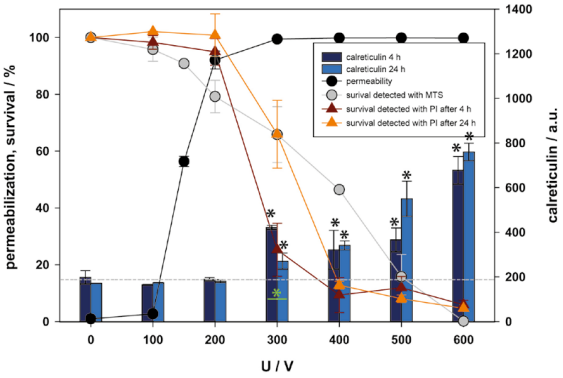

FIGURE 4. Externalization of calreticulin as a function of electric pulse amplitude. Two-time points after electroporation were assessed. Permeabilization and survival (MTS) curves are also presented. Survival detected by propidium iodide (PI) protocol is normalized to control and presented with red (4 hours after pulse treatment) and orange (24 hours after pulse treatment) line. Approximate baseline of calreticulin is presented with -----. Black and green asterisks (*) indicate statistically significant differences between the samples at different voltages and the corresponding control at 0 V (one-way analysis of variance [ANOVA] followed by Holm-Sidak post-hoc test, ($p < 0.05$) and within the pair of samples at different voltages (t-test, $p < 0.05$), respectively).

release of nucleic acids, which after 24 h resulted in lower cell viability. This is confirmed also by a strong negative correlation between survival and

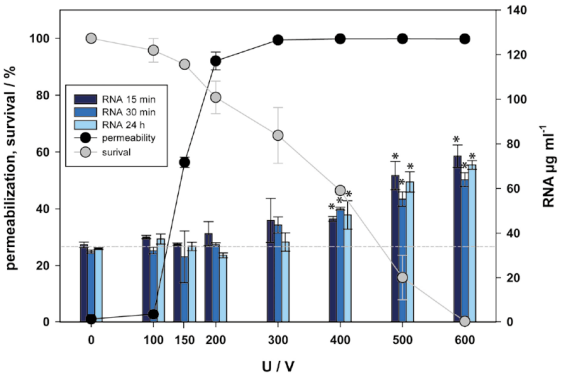

FIGURE 5. Release of RNA as a function of electric pulse amplitude. Three-time points after electroporation were assessed. Permeabilization and survival curves are also presented. Approximate baseline of RNA is presented with -----. Black and green asterisks (*) indicate statistically significant differences between the samples at different voltages and the corresponding control at 0 V (one-way analysis of variance [ANOVA] followed by Holm-Sidak post-hoc test, ($p < 0.05$) and within the pair of samples at different voltages (t-test, $p < 0.05$), respectively).

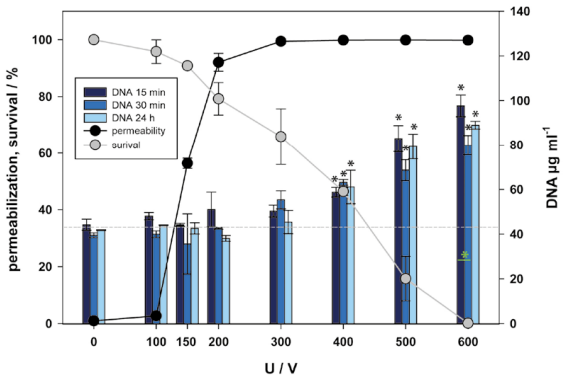

FIGURE 6. Release of DNA as a function of electric pulse amplitude. Three-time points after electroporation were assessed. Permeabilization and survival curves are also presented. Approximate baseline of DNA is presented with -----. Black and green asterisks (*) indicate statistically significant differences between the samples at different voltages and the corresponding control at 0 V (one-way analysis of variance [ANOVA] followed by Holm-Sidak post-hoc test, ($p < 0.05$) and within the pair of samples at different voltages (t-test, $p < 0.05$) respectively.

release of RNA (R = -0.909 for 15 min, R = -0.909 for 30 min, R = -0.919 for 24 h) and weak correlation between permeabilization and release of RNA (R = 0.584 for 15 min, R = 0.696 for 30 min, R is not significant for 24 h), respectively. Similarly, for DNA, a strong correlation between survival and release of DNA (R = -0.935 for 15 min, R = -0.919 for 30 min, R = -0.928 for 24 h) and a weak correlation between permeabilization and release of DNA (R = 0.571 for 15 min, R = 0.689 for 30 min, R is not significant for 24 h) was found. This correlation may indicate that loss of nucleic acids results in cell death.

Comparison of the concentration of RNA detected in supernatant 15, 30 minutes and 24 hours after pulse treatment did not show any significant differences. Comparison of the concentration of DNA detected in supernatant 15, 30 minutes and 24 hours after pulse treatment showed only significant differences between 15 and 30 minutes at 600 V, yet interestingly this was not the case between 15/30 minutes and 24 hours after pulse treatment. According to our results, the released nucleic acids *in vitro* are stable and are not degraded within 24 hours after pulse treatment.

We also tried to detect uric acid, another well know DAMP molecule. The release of uric acid in supernatant was analyzed 24 hours after pulse treatment (Figure 7). In our experiments we were however unable to detect any uric acid in supernatant after pulse treatment. Initial experiments were also performed after 30 minutes, but results were the same (data not shown).

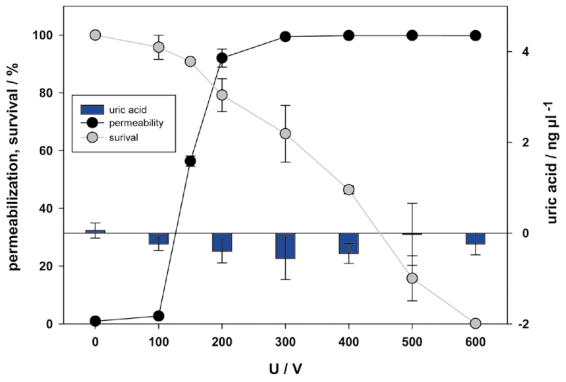

FIGURE 7. Release of uric acid as a function of electric pulse amplitude. Amount of uric acid was analysed 24 hours after pulse treatment in supernatant. Permeabilization and survival curves are also presented. No statistical difference was detected.

Discussion

Besides the induced membrane permeabilization, followed by cell death, activation of the immune response seems to be an important component in effectiveness of ECT[26,35-37] and IRE[18,30-34] treatment *in vivo*. Activation of the immune system can be triggered by a special type of cell death, called immunogenic cell death (ICD) in which DAMPs are the key mediators.[64] Presence of DAMPs after pulse treatment has been detected in different types of cancer cells and normal tissues.[22-29] However, only one study was performed with 100 us pulses, which are predominantly used in ECT and IRE.[26] The authors investigated release of ATP, calreticulin and HMGB1 due to electroporation pulses alone, bleomycin alone and combination of electroporation pulses and bleomycin. Their study demonstrated the release of ATP and calreticulin after pulse treatment, but how this correlates to reversible and/or irreversible electroporation remained elusive. This question is addressed in our study, were release/detection of different DAMPs was correlated with permeabilization and survival curve *in vitro*.

Detected amounts of DAMPs were correlated to cell membrane permeabilization determined by PI assay immediately after pulse treatment and cell survival, analyzed 24 hours after treatment by

MTS test, *i.e.* to reversible and irreversible electroporation, respectively. It is generally believed that membrane permeabilization and cell survival after pulse treatment are causally related, *i.e.* in IRE cell death occurs due to membrane permeabilization and loss of cell homeostasis (in this study correlation coefficient between the two is -0.680) and in ECT increased accumulation of drug leads to increased cell cytotoxicity.

In ECT and IRE it was also demonstrated that the immune response plays an important role in achieving therapeutic effect.[33-36] We have therefore determined different DAMPs at different times after exposing the cells to electric pulses and determined whether the concentrations of extracellular DAMPs were better correlated to cell death or to membrane permeabilization. While activation inflammatory response and activation of immune system is desired in cancer therapies, on the other hand it can be a wanted or an unwanted effect in gene therapy.[68,69] In DNA vaccination therapies, changed permeability of cell membrane enhances the introduction of DNA vaccine inside of cell and the presence of DAMPs additionally activates inflammatory response, which leads to enhanced production of antibodies, thus enhancing the efficiency of vaccination.[65-67] Nevertheless, in most cases of gene therapy, the immune response in unwanted, as it may destroy the transfected cells and prevent transgenic protein expression.[68,69] By now many DAMPs have been identified and the number is still increasing. Most known are the HMGB1, nucleic acids, proteins like heat-shock proteins, S100 and calreticulin, purine metabolites like ATP and uric acid and saccharides. A list of know DAMPs and their receptors is given by Roh and Sohn.[48]

ATP is a well-known molecule in biology and biochemistry for being a universal energy source in the cell and necessary for multiple cell processes and cell metabolism. Interestingly, first studies of electroporation and increase in plasma membrane permeability involved adenosine triphosphate ATP detection in electroporation buffer.[70] The released ATP is also considered a DAMP. In our study two types of ATP detection methods with different sensitivities were used.[71,72] In a previous study performed by Calvet *et al.*[26] using the same pulses as in our study, the release of ATP was detected 30 minutes after the treatment. We were able to confirm their observations with the fluorescence and the luminescence method (Figures 2 and 3 respectively). Furthermore, the investigation of the effects at different pulse amplitudes showed that the release of ATP increases with increasing amplitude. This was expected, as ATP release was previously used as a permeability marker after electroporation.[70] However, in our results statistical differences between the control and the treatment groups were not detected in the range where the permeabilization curve is ascending, but was detected only in the range of pulse amplitudes at which all cells were already permeabilized and many were dead. Since ANOVA analysis is less sensitive when large amount of samples with big differences between them are analyzed, additional ANOVA was performed, taking into account only the results from 0 to 300 V (e.g. where permeabilization changes from 0 to 100%). Now additional analysis showed that statistical differences between control and the treatment groups are present at 200 V and above in luminescent method, suggesting ATP release as possible membrane permeabilization detection method. In the fluorescence method, this statistical difference was obtained only at 300 V. Such difference in analysis and also a bigger ratio of ATP between the control and the treatment groups indicates that the luminescence method is more sensitive method than the fluorescence method. Taken into consideration ATP release at all investigated voltages (also the one leading to cell death after 24 hours) the release of ATP is more strongly correlated to cell death/irreversible electroporation (R = 0.888) than permeability/reversible electroporation (R = -0.695).

24 hours after pulse treatment (Figure 2) the highest amount of ATP was detected in the supernatant of control sample and the amount of ATP decreased with increasing pulse amplitude. This can be explained by homeostasis of ATP in living cells. In a living homeostatic cell most of the ATP is located intracellularly, however in considerably lower concentration ATP is also present in extracellular space.[73] When cells are damaged, considerable release of ATP molecule affects ATP pumps, causing depletion of intracellular K^+ and accumulation of intracellular NA^+ and Ca^{2+} and leading to cell death.[74] A previous study showed that electroporation pulses cause ATP depletion, which in 24 results in lower viability, presumingly by affecting Ca^{2+}-ATPase.[75] In our study the effect on survival was also confirmed by very strong positive correlation between survival/irreversible electroporation and amount of ATP detected in supernatant (R = 0.888). Nevertheless, we need to consider, that some of the ATP detected in supernatant could be from the cells damaged due to cell handling during experiment. In extracellular space ATP is degraded by nucleotides like CD39 and CD37, which convert

ATP through ADP and AMP to adenosine[73], which explains why ATP was detected 30 minutes at very high voltages (500, 600 V), but was no longer detected after 24 hours (Figure 2). This can also explain why Calvet *et al.*[26], was unable to detect ATP 30 hours after pulse treatment alone, however it does not explain, why ATP was still detected when bleomycin alone or in combination with electroporation pulses was used. How fast ATP degrades in extracellular space, remains unknown. Our results do not indicate that ATP degrades within the first 30 minutes after pulse treatment, since no difference between 15 and 30 minutes after pulse treatment was detected. In a different study[76] the results for ATP 4 hours after pulse treatment was lower in samples exposed to pulse treatment than in the control. If this is taken into consideration together with our results, then ATP degradation *in vitro* occurs somewhere between 30 minutes and 4 hours after pulse treatment.

Calreticulin was another molecule of interest in our study. This highly conserved protein has major functions in lumen of the endoplasmic reticulum (ER). It is involved in correct folding of proteins that are produced in endoplasmic reticulum[77] and in regulation of calcium metabolism, as it affects Ca^{2+} capacity of the ER stores.[78] In the early phase of cell death, activated ER stress leads to translocation of calreticulin to cells surface trough ER-Golgi pathway or lysosome exocytosis.[79,80] Calreticulin, as DAMP, was investigated previously in electroporation studies.[22-26,28] In Calvets's study[26], which used the same pulses as in our study (eight 100 µs pulses), calreticulin was determined 30 hours after treatment using different treatments. Calreticulin was detected on the plasma membrane after electroporation pulses alone or in combination with bleomycin (ECT), yet no externalization was detected in cells threated with bleomycin alone. Since only calreticulin, exposed on the cell surface acts as a DAMP, only viable cells (determined by PI) were taken into analysis. The presence of calreticulin on the cell surface was previously detected already 4 hours after electroporation with millisecond pulses[28], thus we assumed 4 hours is sufficient time for calreticulin to transfer to cells surface. Additionally, calreticulin was detected also 24 after pulse treatment. In our study calreticulin was investigated 4 and 24 hours after treatment (Figure 4), which is after the resealing of cell membrane.[63] Even though calreticulin was detected on the surface of live cells, it was detected only in the range of irreversible electroporation. Additionally, calreticulin detection increased with decreasing cell viability (less viable cells), implicating that bigger stress or in this case pulse amplitude causes more calreticulin molecules to be externalized to cell surface. Nevertheless, since it is believed that externalization of calreticulin occurs in early phase of cell death[79,80], it is possible that cell determined as viable would die within next hours.

In comparison to ATP, calreticulin is more stable. Only at 300 V the difference between 4 and 24 hours was statistically significant. Stability of externalized calreticulin was previously also confirmed in another in vitro study.[24] Nevertheless, *in vivo* study shows expression is the strongest between four and six hours, and diminishes 24 h after the treatment.[28]

So far studies investigating DAMPs, released by the electroporation treatment, included ATP, calreticulin and HMGB1. In addition to ATP and calreticulin we also included other known DAMPs in our study, namely nucleic acids and uric acid, which so far have not been investigated as DAMPs after electroporation. Inside the cells nucleic acids are the source of genetic information. As DAMPs in extracellular space nucleic acids bind to TLR receptors. Bound DNA can even attract HMGB1 (a non-histone nuclear protein, which can be actively or passively released into extracellular space, where it acts as a DAMP[81]) and together they form complexes stimulating dendritic cells to produce type 1 interferon (non-specific immune response), which can lead to anti-DNA autoantibody production (specific immune response).[82] Nucleic acids (RNA and DNA) can be detected in supernatant already within minutes after pulse treatment (Figure 5,6). Nevertheless, we need to consider – based on the control, 0 V in Figures 5 and 6, that some of the nucleic acids detected in supernatant could be from the cells damaged due to cell handling during experiment. Since RNA is more abundantly present in cells than DNA[83,84], the same was expected to be the case in the supernatant after pulse treatment. However, the amount of detected DNA in our samples was bigger than that of RNA. Since RNA is more prone to degradation than DNA[84], it is possible that some of the RNA was destroyed during the process of analysis. Nevertheless, the amount of released nucleic acids increases with increasing voltage of electric pulses to which the cells were exposed. Our results indicate that the release of nucleic acids (RNA and DNA) occurs in the range of irreversible electroporation; *i.e.* pulse amplitudes that lead to cell death as determined by MTS test at 24 h post treatment. This was confirmed also by very strong negative

correlation between the cell survival and the release for RNA and DNA.

Uric acid is a product of purine metabolism within the cell, like degradation of nucleic acids, and is released from injured and dying cells.[85] A molecule that is soluble inside the cell, accumulates in extracellular space, where it is transformed in insoluble crystal of monosodium urate, stimulating the maturation of dendritic cells and T-cell response.[85,86] Here, the presence of uric acid after electroporation was investigated for the first time. Presence of uric acid in supernatant was investigated 24 hours after electroporation treatment (Figure 7). We expected uric acid to show a similar behavior in pulse parameter dependency as other DAMPs. However, we did not detect uric acid in supernatant after pulse treatment. Standard curve was obtained, therefore Uric Acid Assay Kit worked. Maybe uric acid production did not happen or uric acid was still inside of cells and not yet in supernatant as predicted. Furthermore, we found no existing data on CHO cells and uric acid in the literature, so maybe formation of uric acid in ovarian cells does not occur.

With respect to the results obtained, detection of DAMPs and its correlation to cell membrane permeabilization and cell survival seems to be more complex than initially thought. Even a DAMP like ATP, which can be released due to electroporation alone is better correlated to cell survival than membrane permeabilization (Tables 1, 2). A recent study performed by Ringel-Scaia *et al.*[27], in which multiple signaling pathways were analyzed, showed that the cell and cell population is a dynamic system which changes with time. Two hours after pulse treatment RNA analyses showed activation of immunosuppressive pathway, cell injury and apoptosis. With time these genes became less pronounces and after 24 hours change in gene expression indicated proinflammatory response, cell repair and necrosis/pyroptosis. This explains changes in DAMPs detection hours after pulse treatment, including the presence and absence of different DAMPs and its correlation with cell survival. Since statistical correlations between DAMP release and cell survival is much stronger than with membrane permeabilization, involvement of immune system in IRE can be explained. However, activation of immune system was demonstrated also in ECT treatments, were reversible electroporation is used. How can that be, if correlation between membrane permeabilization and released DAMPs is weak or does not even exist? Modeling[87] and *in vivo* experiments[88] show that application of nominally reversible electroporation pulses such as those used for ECT of tumors still causes some cell death by means of irreversible electroporation in tissue close to the electrodes, due to inhomogeneous electric field distribution, which can thus lead to the release of DAMPs and activation of the immune system.

The aim of this study was to explore, if and when specific DAMPs are released as a consequence of electroporation and if the release of DAMPs can be correlated to reversible and/or irreversible electroporation. Even though detection of certain DAMPs remains uncertain, others show strong correlation to cell survival/irreversible elec-

TABLE 1. Correlation (R) between survival and release of damage-associated molecular pattern molecules (DAMPs) after pulse treatment. Investigated time points for each molecule are presented in the bottom row. Correlation was evaluated with Pearson correlation coefficient and survival was analyzed via MTS assay 24 hours after pulse treatment

	R vs. survival (MTS)				
PI	-0.680				
ATP		-0.947 (L)	-0.964 (L)/-0.864 (F)		0.888 (F)
DNA		-0.935	-0.919		-0.928
RNA		-0.909	-0.909		-0.919
CRT				-0.801	-0.946
uric acid					NS
time points after EP	*3 min*	*15 min*	*30 min*	*4 h*	*24 h*

ATP = adenosine triphosphate; CRT = calreticulin; (F) = fluorescence assay; (L) = luminescence assay; NS = no statistical significance; PI = propidium iodide

TABLE 2. Correlation (R) between permeabilization and release of damage-associated molecular pattern molecules (DAMPs) after pulse treatment. Investigated time points for each molecule are present in the bottom row. Correlation was evaluated with Pearson correlation coefficient and permeabilization was analyzed by propidium iodide (PI) assay 3 minutes after pulse treatment

	R vs permeabilization (PI)				
MTS					-0.680
ATP		0.704	0.728 (L)/0.594 (F)		-0.695 (F)
DNA		0.571	0.689		NS
RNA		0.584	0.696		NS
CRT				0.535	0.556
uric acid					NS
time points after EP	*3 min*	*15 min*	*30 min*	*4 h*	*24 h*

ATP = adenosine triphosphate; CRT = calreticulin; (F) = fluorescence assay; (L) = luminescence assay; NS = no statistical significance

troporation and much weaker correlation to membrane permeabilization/reversible electroporation. Release of DAMPs could perhaps serve as a predictor of cell death. In addition, it may indicates that the stability of certain DAMPs is questionable and thus their presence and detectability is time dependent. This needs to be taken into consideration when designing protocols to detect DAMPs after electroporation treatment. Finally, to obtain a better insight of DAMP release with respect to electroporation treatment other cell types including also cancer cell types should be investigated.

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References

1. Orlowski S, Belehradek J, Paoletti C, Mir LM. Transient electropermeabilization of cells in culture. Increase of the cytotoxicity of anticancer drugs. *J Biochem Pharmacol Res* 1988; **3:** 4727-33. doi: 10.1016/0006-2952(88)90344-9
2. Mir LM. Bases and rationale of the electrochemotherapy. *EJC Suppl* 2006; **4:** 38-44. doi: 0.1016/j.ejcsup.2006.08.005
3. Scheffer HJ, Nielsen K, De Jong MC, Van Tilborg AAJM, Vieveen JM, Bouwman A, et al. Irreversible electroporation for nonthermal tumor ablation in the clinical setting: a systematic review of safety and efficacy. *J Vasc Interv Radiol* 2014; **25:** 997-1011. doi: 10.1016/j.jvir.2014.01.028
4. Phillips M, Maor E, Rubinsky B. Nonthermal irreversible electroporation for tissue decellularization. *J Biomech Eng* 2010; **132:** 091003. doi: 10.1115/1.4001882
5. Davalos RV, Mir LM, Rubinsky B. Tissue ablation with irreversible electroporation. *Ann Biomed Eng* 2005; **3:** 223-31. doi: 10.1007/s10439-005-8981-8
6. Chen X, Ren Z, Zhu T, Zhang X, Peng Z, Xie H, et al. Electric ablation with irreversible electroporation (IRE) in vital hepatic structures and follow-up investigation. *Sci Rep* 2015; **5:** 16233. doi: 10.1038/srep16233
7. Jiang C, Davalos RV, Bischof JC. A review of basic to clinical studies of irreversible electroporation therapy. *IEEE Trans Biomed Eng* 2015; **62:** 4-20. doi: 10.1109/TBME.2014.2367543
8. Galluzzi L, Maiuri MC, Vitale I, Zischka H, Castedo M, Zitvogel L, et al. Cell death modalities: classification and pathophysiological implications. *Cell Death Differ* 2007; **14:** 1237-43. doi: 10.1038/sj.cdd.4402148
9. Schweichel JU, Merker HJ. The morphology of various types of cell death in prenatal tissues. *Exp Teratol* 1973; **7:** 253-66. doi: 10.1002/tera.1420070306
10. Galluzzi L, Vitale I, Aaronson SA, Abrams JM, Adam D, Agostinis P, et al. Molecular mechanisms of cell death: recommendations of the nomenclature committee on cell death 2018. *Cell Death Differ* 2018; **25:** 486-541. doi: 10.1038/s41418-017-0012-4
11. Batista Napotnik T, Rebersek M, Vernier PT, Mali B, Miklavcic D. Effects of high voltage nanosecond electric pulses on eukaryotic cells (in vitro): a systematic review. *Bioelectrochemistry* 2016; **110:** 1-12. doi: 10.1016/j.bioelechem.2016.02.011
12. Beebe SJ. Regulated and apoptotic cell death after nanosecond electroporation. In: Miklavčič D, editor. *Handbook of electroporation*. Heidelberg: Springer International Publishing; 2017. p. 511-28. doi: 10.1007/978-3-319-32886-7_146
13. Chai W, Zhang W, Wei Z, Xu Y, Shi J, Luo X, et al. Irreversible electroporation of the uterine cervix in a rabbit model. *Biomed Microdevices* 2017; **19:** 103. doi: 10.1007/s10544-017-0248-2
14. Kim HB, Sung CK, Baik KY, Moon KW, Kim HS, Yi JH, et al. Changes of apoptosis in tumor tissues with time after irreversible electroporation. *Biochem Biophys Res Commun* 2013; **435:** 651-6. doi: 10.1016/j.bbrc.2013.05.039
15. Lee EW, Loh CT, Kee ST. Imaging guided percutaneous irreversible electroporation: Ultrasound and immunohistological correlation. *Technol Cancer Res Treat* 2007; **6:** 287-93. doi: 10.1177/153303460700600404
16. Lee EW, Wong D, Tafti BA, Prieto V, Totonchy M, Hilton J, et al. Irreversible electroporation in eradication of rabbit VX2 liver tumor. *J Vasc Interv Radiol* 2012; **23:** 833-40. doi: 10.1016/j.jvir.2012.02.017
17. Zhang Z, Li W, Procissi D, Tyler P, Omary RA, Larson AC. Rapid dramatic alterations to the tumor microstructure in pancreatic cancer following irreversible electroporation ablation. *Nanomedicine* 2014; **9:** 1181-92. doi: 10.2217/nnm.13.72
18. José A, Sobrevals L, Ivorra A, Fillat C. Irreversible electroporation shows efficacy against pancreatic carcinoma without systemic toxicity in mouse models. *Cancer Lett* 2012; **317:** 16-23. doi: 10.1016/j.canlet.2011.11.004
19. Al-Sakere B, André F, Bernat C, Connault E, Opolon P, Davalos RV, et al. Tumor ablation with irreversible electroporation. *PLoS One* 2007; **2:** e1135. doi: 10.1371/journal.pone.0001135
20. Zhang Y, Lyu C, Liu Y, Lv Y, Chang TT, Rubinsky B. Molecular and histological study on the effects of non-thermal irreversible electroporation on the liver. *Biochem Biophys Res Commun* 2018; **500:** 665-70. doi: 10.1016/j.bbrc.2018.04.132
21. López-Alonso B, Hernáez A, Sarnago H, Naval A, Güemes A, Junquera C, et al. Histopathological and ultrastructural changes after electroporation in pig liver using parallel-plate electrodes and high-performance generator. *Sci Rep* 2019; **9:** 2467. doi: 10.1038/s41598-019-39433-6
22. Nuccitelli R, Berridge JC, Mallon Z, Kreis M, Athos B, Nuccitelli P. Nanoelectroablation of murine tumors triggers a cd8-dependent inhibition of secondary tumor growth. *PLoS One* 2015; **10:** e0134364. doi: 10.1371/journal.pone.0134364
23. Nuccitelli R, McDaniel A, Anand S, Cha J, Mallon Z, Berridge J, et al. Nano-pulse stimulation is a physical modality that can trigger immunogenic tumor cell death. *J Immunother Cancer* 2017; **5:** 32. doi: 10.1186/s40425-017-0234-5
24. Guo S, Jing Y, Burcus NI, Lassiter BP, Tanaz R, Heller R, et al. Nano-pulse stimulation induces potent immune responses, eradicating local breast cancer while reducing distant metastases. *Int J Cancer* 2018; **142:** 629-40. doi: 10.1002/ijc.31071
25. Rossi A, Pakhomova ON, Mollica PA, Casciola M, Mangalanathan U, Pakhomov AG, et al. Nanosecond pulsed electric fields induce endoplasmic reticulum stress accompanied by immunogenic cell death in murine models of lymphoma and colorectal cancer. *Cancers* 2019; **11:** 2034. doi: 10.3390/cancers11122034
26. Calvet CY, Famin D, André FM, Mir LM. Electrochemotherapy with bleomycin induces hallmarks of immunogenic cell death in murine colon cancer cells. *Oncoimmunology* 2014; **3:** e28131. doi: 10.4161/onci.28131
27. Ringel-Scaia VM, Beitel-White N, Lorenzo MF, Brock RM, Huie KE, Coutermarsh-Ott S. High-frequency irreversible electroporation is an effective tumor ablation strategy that induces immunologic cell death and promotes systemic anti-tumor immunity. *EBioMedicine* 2019; **44:** 112-25. doi: 10.1016/j.ebiom.2019.05.036
28. Schultheis K, Smith, TRF, Kiosses WB, Kraynyak KA, Wong A, Oh J, et al. Delineating the cellular mechanisms associated with skin electroporation. *Hum Gene Ther Methods* 2018; **29:** 177-88. doi: 10.1089/hgtb.2017.105
29. Zhao J, Wen X, Tian L, Li T, Xu C, Wen X, et al. Irreversible electroporation reverses resistance to immune checkpoint blockade in pancreatic cancer. *Nat Commun* 2019; **10:** 1-14. doi: 10.1038/s41467-019-08782-1

30. Vogl TJ, Wissniowski TT, Naguib NNN, Hammerstingl RM, Mack MG, Münch S, et al. Activation of tumor-specific T lymphocytes after laser-induced thermotherapy in patients with colorectal liver metastases. *Cancer Immunol Immunother* 2019; **58:** 1557-63. doi: 10.1007/s00262-009-0663-1

31. Bulvik BE, Rozenblum N, Gourevich S, Ahmed M, Andriyanov AV, Galun E, et al. Irreversible electroporation versus radiofrequency ablation: a comparison of local and systemic effects in a small-animal model. *Radiology* 2016; **280:** 413-24. doi: 10.1148/radiol.2015151166

32. White SB, Zhang Z, Chen J, Gogineni VR, Larson AC. Early immunologic response of irreversible electroporation versus cryoablation in a rodent model of pancreatic cancer. *J Vasc Interv Radiol* 2018; **29:** 1764-9. doi: 10.1016/j.jvir.2018.07.009

33. Scheffer HJ, Stam AGM, Geboers B, Vroomen LGPH, Ruarus A, de Bruijn B, et al. Irreversible electroporation of locally advanced pancreatic cancer transiently alleviates immune suppression and creates a window for antitumor T cell activation. *Oncoimmunology* 2019; **8:** 1652532. doi: 10.1080/2162402X.2019.1652532

34. Pandit H, Hong YK, Li Y, Rostas J, Pulliam Z, Li P, et al. Evaluating the regulatory immunomodulation effect of irreversible electroporation (ire) in pancreatic adenocarcinoma. *Ann Surg Oncol* 2019; **26:** 800-6. doi: 10.1245/s10434-018-07144-3

35. Calvet CY, Mir LM. The promising alliance of anti-cancer electrochemotherapy with immunotherapy. *Cancer Metastasis Rev* 2016; **35:** 165-77. doi: 10.1007/s10555-016-9615-3

36. Sersa G, Teissie J, Cemazar M, Signori E, Kamensek U, Marshall G, et al. Electrochemotherapy of tumors as in situ vaccination boosted by immunogene electrotransfer. *Cancer Immunol Immunother* 2015; **64:** 1315-27. doi: 10.1007/s00262-015-1724-2

37. Serša G, Miklavcic D, Cemazar M, Belehradek J, Jarm T, Mir LM. Electrochemotherapy with CDDP on LPB sarcoma: comparison of the anti- tumor effectiveness in immunocompetent and immunodeficient mice. *Bioelectrochem Bioenerg* 1997; **43:** 279-83. doi: 10.1016/S0302-4598(96)05194-X

38. Gerlini G, Tun-Kyi A, Dudli C, Burg G, Pimpinelli N, Nestle FO. Metastatic melanoma secreted IL-10 down-regulates CD1 molecules on dendritic cells in metastatic tumor lesions. *Am J Pathol* 2004; **165:** 1853-63. doi: 10.1016/S0002-9440(10)63238-5

39. Gerlini G, Di Gennaro P, Mariotti G, Urso C, Chiarugi A, Pimpinelli N, et al. Indoleamine 2,3-dioxygenase cells correspond to the BDCA2 plasmacytoid dendritic cells in human melanoma sentinel nodes. *J Investig Dermatol* 2010; **130:** 898-901. doi: 10.1038/jid.2009.307

40. Geboers B, Scheffer HJ, Graybill PM, Ruarus AH, Nieuwenhuizen S, Puijk RS, et al. High-voltage electrical pulses in oncology: irreversible electroporation, electrochemotherapy, gene electrotransfer, electrofusion, and electroimmunotherapy. *Radiology* 2020; **295:** 192190. doi: 10.1148/radiol.2020192190

41. Zhou J, Wang G, Chen Y, Wang H, Hua Y, Cai Z. Immunogenic cell death in cancer therapy: Present and emerging inducers. *J Cell Mol Med* 2019; **23:** 4854-65. doi: 10.1111/jcmm.14356

42. Alnaggar M, Lin M, Mesmar A, Liang S, Qaid A, Xu K, et al. Allogenic natural killer cell immunotherapy combined with irreversible electroporation for stage iv hepatocellular carcinoma: survival outcome. *Cell Physiol Biochem* 2018; **48:** 1882-93. doi: 10.1159/000492509

43. Yang Y, Qin Z, Du D, Wu Y, Qiu S, Mu F, et al. Safety and short-term efficacy of irreversible electroporation and allogenic natural killer cell immunotherapy combination in the treatment of patients with unresectable primary liver cancer. *Cardiovasc Intervent Radiol* 2019; **42:** 48-59. doi: 10.1007/s00270-018-2069-y

44. Lin M, Liang S, Wang X, Liang Y, Zhang M, Chen J. Percutaneous irreversible electroporation combined with allogeneic natural killer cell immunotherapy for patients with unresectable (Stage III/IV) pancreatic cancer: a promising treatment. *J Cancer Res Clin Oncol* 2017; **143:** 2607-18. doi: 10.1007/s00432-017-2513-4

45. Diercks GFH, Kluin PM. Basic principles of the immune system and autoimmunity. In: Jonkman FM, editor. *Autoimmune bullous diseases*. Heidelberg: Springer International Publishing; 2016. p. 3-12. doi: 10.1007/978-3-319-23754-1_1

46. Kellie S, Al-Mansour Z. Overview of the immune system. In: Skwarczynski M, Toth I, editors. *Micro- and nanotechnology in vaccine development*. Elsevier Inc; 2017. p. 63-81. doi: 10.1016/B978-0-323-39981-4.00004-X

47. Chaplin DD. Overview of the immune response. *J Allergy Clin Immunol* 2010; **125:** S3. doi: 10.1016/j.jaci.2009.12.980

48. Roh JS, Sohn DH. Damage-associated molecular patterns in inflammatory diseases. *Immune Netw* 2018; **18:** e27. doi: 10.4110/in.2018.18.e27

49. Obeid M, Tesniere A, Ghiringhelli F, Fimia GM, Apetoh L, Perfettini JL, et al. Calreticulin exposure dictates the immunogenicity of cancer cell death. *Nat Med* 2007; **13:** 54-61. doi: 10.1038/nm1523

50. Kato J, Svensson CI. Role of extracellular damage-associated molecular pattern molecules (DAMPs) as mediators of persistent pain. *Prog Mol Biol Transl Sci* 2015; **131:** 251-79. doi: 10.1016/bs.pmbts.2014.11.014

51. Bianchi ME. DAMPs, PAMPs and alarmins: all we need to know about danger. *J Leukoc Biol* 2007; **81:**1-5. doi: 10.1189/jlb.0306164

52. Chan JK, Roth J, Oppenheim JJ, Tracey KJ, Vogl T, Feldmann M. Science in medicine Alarmins : awaiting a clinical response. *J Clin Invest* 2012; **122:** 2711-9. doi: 10.1172/JCI62423.tification

53. Rock KL, Lai JJ, Kono H. Innate and adaptive immune responses to cell death. *Immunol Rev* 2011; **243:** 191-205. doi: 10.1111/j.1600-065X.2011.01040.x

54. Stoecklein VM, Osuka A, Lederer JA. Trauma equals danger - damage control by the immune system. *J Leukoc Biol* 2012; **92:** 539-51. doi: 10.1189/jlb.0212072

55. Wynn TA, Ramalingam TR. Mechanisms of fibrosis: therapeutic translation for fibrotic disease. *Nat Med* 2012; **18:** 1028-40. doi: 10.1038/nm.2807

56. Straino S, Di Carlo A, Mangoni A, De Mori R, Guerra L, Maurelli R. High-mobility group box 1 protein in human and murine skin: involvement in wound healing. *J Invest Dermatol* 2008; **128:** 1545-53. doi: 10.1038/sj.jid.5701212

57. Yang S, Xu L, Yang T, Wang F. High-mobility group box-1 and its role in angiogenesis. *J Leukoc Biol* 2014; **95:** 563-74. doi: 10.1189/jlb.0713412

58. Zampell JC, Yan A, Avraham T, Andrade V, Malliaris S, Aschen S, et al. Temporal and spatial patterns of endogenous danger signal expression after wound healing and in response to lymphedema. *Am J Physiol Cell Physiol* 2011; **300:** 1107-21. doi: 10.1152/ajpcell.00378.2010

59. Duffield JS, Lupher M, Thannickal VJ, Wynn TA. Host responses in tissue repair and fibrosis. *Annu Rev Pathol* 2013; **8:** 241-76. doi: 10.1146/annurev-pathol-020712-163930

60. Rols MP, Teissié J. Electropermeabilization of mammalian cells. Quantitative analysis of the phenomenon. *Biophys J* 1990; **58:** 1089-98. doi: 10.1016/S0006-3495(90)82451-6

61. Sweeney DC, Reberšek M, Dermol J, Rems L, Miklavčič D, Davalos RV. Quantification of cell membrane permeability induced by monopolar and high-frequency bipolar bursts of electrical pulses. *Biochim Biophys Acta Biomembr* 2016; **1858:** 2689-98. doi: 10.1016/j.bbamem.2016.06.024

62. Batista Napotnik T, Miklavčič D. In vitro electroporation detection methods – an overview. *Bioelectrochemistry* 2018; **120:** 166-82. doi: 10.1016/j.bioelechem.2017.12.005

63. Scuderi M, Rebersek M, Miklavcic D, Dermol-Cerne J. The use of high-frequency short bipolar pulses in cisplatin electrochemotherapy in vitro. *Radiol Oncol* 2019; **53:** 194-205. doi: 10.2478/raon-2019-0025

64. O'Brien MA, Power DG, Clover AJP, Bird B, Soden DM, Forde PF. Local tumour ablative therapies: opportunities for maximising immune engagement and activation. *Biochim Biophys Acta* 2014; **1846:** 510-23. doi: 10.1016/j.bbcan.2014.09.005

65. Babiuk S, Baca-Estrada ME, Foldvari M, Middleton DM, Rabussay D, Widera G, et al. Increased gene expression and inflammatory cell infiltration caused by electroporation are both important for improving the efficacy of DNA vaccines. *J Biotechnol* 2004; **110:** 1-10. doi: 10.1016/j.jbiotec.2004.01.015

66. Roos AK, Moreno S, Leder C, Pavlenko M, King A, Pisa P. Enhancement of cellular immune response to a prostate cancer DNA vaccine by intradermal electroporation. *Mol Ther* 2006; **13:** 320-7. doi: 10.1016/j.ymthe.2005.08.005

67. Chiarella P, Massi E, De Robertis M, Sibilio A, Parrella P, Fazio VM, et al. Electroporation of skeletal muscle induces danger signal release and antigen-presenting cell recruitment independently of DNA vaccine administration. *Expert Opin Biol Ther* 2008; **8:** 1645-57. doi: 10.1517/14712598.8.11.1645

68. Bessis N, Garcia Cozar FJ, Boissier MC. Immune responses to gene therapy vectors: influence on vector function and effector mechanisms. *Gene Ther* 2004; **11(Suppl 1):** S10-7.. doi: 10.1038/sj.gt.3302364

69. Shirley JL, de Jong YP, Terhorst C, Herzog RW. Immune responses to viral gene therapy vectors. *Mol Ther* 2020; **28:** 709-22. doi: 10.1016/j.ymthe.2020.01.001

70. Rols MP, Teissié J. Electropermeabilization of mammalian cells. Quantitative analysis of the phenomenon. *Biophys J* 1990; **58:** 1089-98. doi: 10.1016/S0006-3495(90)82451-6

71. Fan F, Wood KV. Bioluminescent assays for high-throughput screening. *Assay Drug Dev Technol* 2007; **5:** 127-36. doi: 10.1089/adt.2006.053

72. Wood KV. The bioluminescence advantage. [cited 2020 May 12]. Available at: https://worldwide.promega.com/resources/pubhub/enotes/the-bioluminescence-advantage/

73. Falzoni S, Donvito G, Di Virgilio F. Detecting adenosine triphosphate in the pericellular space. *Interface Focus* 2013; **3:** 2012. doi: 10.1089/adt.2006.053

74. Wang XQ, Xiao AY, Sheline C, Hyrc K, Yang A, Goldberg MP, et al. Apoptotic insults impair Na+, K+-ATPase activity as a mechanism of neuronal death mediated by concurrent ATP deficiency and oxidant stress. *J Cell Sci* 2003; **116:** 2099-110. doi: 10.1242/jcs.00420

75. Hansen EL, Sozer EB, Romeo S, Frandsen SK, Vernier PT, Gehl J. Dose-dependent ATP depletion and cancer cell death following calcium electroporation, relative effect of calcium concentration and electriamount strength. *PLoS One* 2015; **10:** e0122973. doi: 10.1371/journal.pone.0122973

76. Ashdown CP, Johns SC, Aminov E, Unanian M, Connacher W, Friend J, et al. Pulsed low-frequency magnetic fields induce tumor membrane disruption and altered cell viability. *Biophys J* 2020; **118:** 1552-63. doi: 10.1016/j.bpj.2020.02.013

77. Krause KH, Michalak M. Calreticulin. *Cell* 1997; **88:** 439-43. doi: 10.1016/s0092-8674(00)81884-x

78. Gelebart P, Opas M, Michalak M. Calreticulin, a Ca2+-binding chaperone of the endoplasmic reticulum. *Int J Biochem Cell Biol* 2005; **37:** 260-6. doi: 10.1016/j.biocel.2004.02.030

79. Panaretakis T, Kepp O, Brockmeier U, Tesniere A, Bjorklund AC, Chapman DC, et al. Mechanisms of pre-apoptotic calreticulin exposure in immunogenic cell death. *Embo J* 2009; **28:** 578-90. doi: 10.1038/emboj.2009.1

80. Kranz P, Neumann F, Wolf A, Classen F, Pompsch M, Ocklenburg T, et al. PDI is an essential redox-sensitive activator of PERK during the unfolded protein response (UPR). *Cell Death Dis* 2017; **8:** e2986. doi: 10.1038/cddis.2017.369

81. Hou W, Zhang Q, Yan Z, Chen R, Zeh HJ, Kang R, et al. Strange attractors: DAMPs and autophagy link tumor cell death and immunity. *Cell Death Dis* 2013; **4:** e966. doi: 10.1038/cddis.2013.493

82. Pisetsky DS. The origin and properties of extracellular DNA: from PAMP to DAMP. *Clin Immunolog* 2012; **144:** 32-40. doi: 10.1016/j.clim.2012.04.006

83. Shinohara K, Toné S, Ejima T, Ohigashi T, Ito A. Quantitative distribution of DNA, RNA, histone and proteins other than histone in mammalian cells, nuclei and a chromosome at high resolution observed by scanning transmission soft x-ray microscopy (stxm). *Cells* 2019; **8:** 164. doi: 10.3390/cells8020164

84. Mackenzie RJ. DNA vs. RNA – 5 key differences and comparison. Technology Networks. [cited 2020 Jun 9]. Available at: https://www.technologynetworks.com/genomics/lists/what-are-the-key-differences-between-dna-and-rna-296719.

85. Shi Y, Evans JE, Rock KL. Molecular identification of a danger signal that alerts the immune system to dying cells. *Nature* 2003; **425:** 516-21. doi: 10.1038/nature01991

86. Shi Y, Galusha SA, Rock KL. Cutting Edge: elimination of an endogenous adjuvant reduces the activation of cd8 t lymphocytes to transplanted cells and in an autoimmune diabetes model. *J Immunol* 2006; **176:** 3905-8. doi: 10.4049/jimmunol.176.7.3905

87. Miklavcic D, Semrov D, Mekid H, Mir LM. A validated model of in vivo electric field distribution in tissues for electrochemotherapy and fo: DNA electrotransfer for gene therapy. *Biochim Biophys Acta Gen Subj* 2000; **1523:** 73-83. doi: 10.1016/S0304-4165(00)00101-X

88. Zmuc J, Gasljevic G, Sersa G, Edhemovic I, Boc N, Seliskar A, et al. Large liver blood vessels and bile ducts are not damaged by electrochemotherapy with bleomycin in pigs. *Sci Rep* 2019; **9:** 3649. doi: 10.1038/s41598-019-40395-y

2.1.4 Celična smrt zaradi elektroporacije

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Pri elektroporaciji celice izpostavimo kratkotrajnim električnim pulzom, kar povzroči začasno povečanje prepustnosti membrane. Reverzibilno elektroporacijo uporabljamo za vnos genov in povečan vnos zdravil v celico. Ireverzibilno elektroporacijo (katere izid je smrt celice) pa uspešno uporabljamo kot metodo netermične ablacije mehkih tkiv, kot so tumorji ali srčno tkivo. Na izid zdravljenja s terapijami, ki temeljijo na elektroporaciji, lahko vplivajo tudi mehanizmi celične smrti, na katere vlivajo tudi različni parametri električnih pulzov in pogoji pri različnih terapijah. V pregledni članek smo vključil vso literaturo, ki smo jo našli preko podatkovne zbirke Pubmed ob iskanju z gesli ‘‘Electroporation cell death apoptosis”, ‘‘Electroporation cell death necrosis”, ”Electroporation cell death necroptosis”, ‘‘Electroporation cell death pyroptosis”, ‘‘Electroporation cell death pyroptotic”, ‘‘Electroporation nanosecond apoptosis”, ‘‘Electroporation nanosecond necrosis”, ‘‘Electroporation calcium cell death”, in ‘‘Electroporation immune”. Tej literaturi smo dodali še literaturo poznanih člankov, ki se dotikajo tematike celični smrti pri elektroporaciji. Z uporabo tako zbrane literature smo naredili pregled celičnih poškodb, popravljalnih mehanizmov membrane in poti celične smrti pri elektroporaciji. Ugotovili smo, da elektroporacija ne povzroči le poškodb plazemske membrane, temveč vpliva tudi na nastanek ROS, dvig znotrajceličnega Ca^{2+}, izgubo ATP, ter poškodbe mitohondrija, DNA in membranskih proteinov. Vse to pa lahko vodi v različne poti celične smrti, med katerimi literatura omenja apoptozo, nekrozo, piroptozo in nekroptozo. Znanje o popravljalnih mehanizmih membrane, mehanizmih celične smrt po izpostavitvi električnim pulzom ter identifikacija tarčnih molekul elektroporacijskih pulzov je ključna za optimizacijo že obstoječih in novih metod, ki temeljijo na uporabi elektroporacije v medicini.

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Cell death due to electroporation – A review

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ABSTRACT

Exposure of cells to high voltage electric pulses increases transiently membrane permeability through membrane electroporation. Electroporation can be reversible and is used in gene transfer and enhanced drug delivery but can also lead to cell death. Electroporation resulting in cell death (termed as irreversible electroporation) has been successfully used as a new non-thermal ablation method of soft tissue such as tumours or arrhythmogenic heart tissue. Even though the mechanisms of cell death can influence the outcome of electroporation-based treatments due to use of different electric pulse parameters and conditions, these are not elucidated yet. We review the mechanisms of cell death after electroporation reported in literature, cell injuries that may lead to cell death after electroporation and membrane repair mechanisms involved. The knowledge of membrane repair and cell death mechanisms after cell exposure to electric pulses, targets of electric field in cells need to be identified to optimize existing and develop of new electroporation-based techniques used in medicine, biotechnology, and food technology.

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1. Introduction

Electroporation is a phenomenon that occurs when cells are exposed to electric field of sufficient amplitude: their plasma membrane permeability becomes increased [1]. On one hand, in reversible electroporation, the increased permeability is only temporary, after a certain time cells repair their plasma membrane and re-establish homeostasis. Reversible electroporation is used in biotechnology and medicine for delivery of otherwise impermeant molecules to cells such as chemotherapeutics in electrochemotherapy (ECT) [2] or nucleic acids in gene electrotransfer (GET) [3]. On the other hand, in irreversible electroporation (usually with the use of higher number of electric pulses and of higher amplitude), the cells are damaged beyond repair and they die [4]. Irreversible electroporation is already used as a focal ablative technique for treating tumours, especially those unsuitable for surgery or thermal ablation because of their specific anatomic location [4]. Irreversible electroporation (called also as pulsed field ablation) is resurging as efficient, safe and fast ablation modality [5]. This technology is expected to represent a major advance in the field of treating heart arrhythmias [6]. The mechanisms of cell death also due to expanding interest in cardiac ablation are therefore of significant interest.

Irreversible electroporation leads to cell death of different types, namely necrosis, apoptosis, and also types of immunogenic cell death such as necroptosis and pyroptosis that have gained attention in recent years. It is important to know cell death pathways and how electric pulses of different parameters influence them in order to control and optimize therapeutic protocols such as tumour ablation and pulsed field ablation in heart. Different types of cell death have also different systemic responses in terms of abscopal effects and long-term immune response which is _especially important in oncology. Moreover, in gene electrotransfer, EP itself can also stimulate immune response [7]. Therefore, there is a need for better understanding of triggering cell death and immune response by electroporation especially considering the expanding interest in applying this technology clinically. And the last but not least, with the increasing knowledge of specific repair and cell death mechanisms after electric field exposure, direct and indirect targets of electric field in cells can be identified which can lead to a development of new electroporation-based techniques used in medicine, biotechnology, and food technology [8].

1.1. Cell injury and cell death

Cells constantly adapt to physiological demands to maintain their viability and homeostasis. The term cell injury is used to describe the situation when the stimulus/insult, external or internal, is excessive or when the cell is no longer capable to adapt without suffering some form of damage. Cell injury can be reparable/reversible (non-lethal damage which can generally be corrected) or irreversible (lethal damage) resulting in cell death. The transition between reversible and irreversible damage, commonly referred to as the "point of no return" is of major interest and importance for devising therapeutic strategies to prevent or trigger cell death after therapeutic intervention [9,10].

The main mechanisms of cell injury are: 1) membrane damage, 2) DNA and protein damage, 3) increase of ROS, 4) entry of Ca^{2+}, 5) mitochondrial damage, and 6) ATP depletion. They are schematically depicted in Fig. 1. We can observe the complexity of cell injury biochemical mechanisms. They are interconnected and overlapping, sometimes one injurious agent (insult) can trigger multiple pathways, therefore it is not always possible to determine a specific target or prevention mode of injury of a particular insult [9–12].

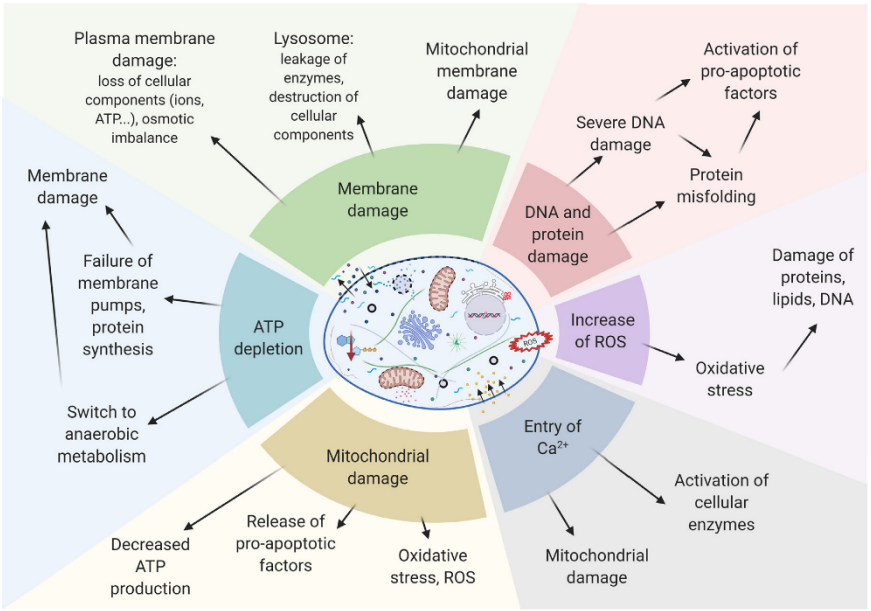

Fig. 1. Mechanisms of cell injury (derived from [9,10,12]). Created with BioRender.com.

1.2. Modes of cell death

When a cell is injured beyond repair, it dies. Historically, cell death was classified into three forms, with respect to morphological changes: type I: apoptosis (programmed cell death, cell shrinkage, caspase activation, DNA condensation, fragmentation into apoptotic bodies, absence of immune response), type II: autophagy (massive vacuolization of the cytoplasm), and type III: necrosis (accidental cell death, swelling, plasmalemmal blebs and rupture, cell lysis, immune response) [13]. This classification is still widely used, however, over the past decade new definitions of cell death emerged based on morphological, enzymological (involvement of nucleases and caspases), functional (programmed/regulated or accidental) and immunological characteristics (immunogenic or non-immunogenic) [14]. A Nomenclature Committee on Cell Death formulated guidelines for the definition and interpretation of cell death from morphological, biochemical, and functional perspectives [11]. With extensive study of cell death mechanisms, several new pathways of immunogenic cell death – a programmed/regulated cell death that can exhibit necrotic or apoptotic morphology and elicit immune response – were identified, e.g. necroptosis, ferroptosis, pyroptosis, and others [11–15].

Damaged, dying or dead cells resulting from trauma, ischemia, cancer, and other settings of tissue damage in the absence of pathogenic infection communicate a state of danger to the organism by releasing damage-associated molecular patterns (DAMPs), i.e. endogenic danger molecules. DAMPs activate the immune system by interacting with pattern recognition receptors (PRRs) on cells of innate immune system (the same PRRs that interact also with pathogen-associated molecular patterns PAMPs) and thereby trigger non-infectious inflammation and tissue repair [16–18]. DAMP molecules are passively or actively released from cytoplasm, nucleus or other cellular compartments into extracellular space (adenosine triphosphate ATP, chromatin-binding protein high mobility group B1 HMGB1, DNA etc.) or become exposed on the cell surface (calreticulin, heat shock proteins) [13,19]. DAMPs interact with PRRs (such as receptor for advanced glycation end products RAGE, Toll-like receptors TLRs, NOD-like receptors NLRs etc.) on the surface of immune cells (dendritic cells, macrophages, T cells and neutrophils) and trigger innate and adaptive immune responses via various pathways (NF-κB etc.) [17–19]. A selective interception of immunogenic cell death pathways can be a promising new tool for diagnosis, prevention and treatment of human diseases in which cell loss must be avoided (inflammatory diseases) or amplified (cancer) [13,19–22].

2. Materials and methods

2.1. Literature search strategy and selection

For the purpose of our review of electroporation-related cell death, a semi-systematic search through Pub Med database (The National Institutes of Health) was performed by employing keywords "Electroporation cell death apoptosis", "Electroporation cell death necrosis", "Electroporation cell death necroptosis", "Electroporation cell death pyroptosis", "Electroporation cell death pyroptotic", "Electroporation nanosecond apoptosis", "Electroporation nanosecond necrosis", "Electroporation calcium cell death", and "Electroporation immune". Besides that, papers from previous reviews and publications [23–25] were included to the list of papers analysed. Among them, 113 papers related to cell injury and cell death after EP with electric pulses of different parameters (ns-ms range of square monopolar or bipolar pulses) without addition of any chemical compounds (chemotherapeutics, Ca^{2+} ions, DNA) were analysed for cell injury (Tables 1 and 2).

3. Cell death and electroporation

3.1. Electroporation and cell injury

Electroporation (EP) can be considered as a membrane damage (structural and dynamical reorganization of the plasma membrane) with cell recovery as an active cellular process, which involves cellular machinery [1,26]. Membrane pore formation by itself is an injury, moreover, EP causes also lipid peroxidation [27,28] and damages membrane embedded proteins [29–31]. Since many pathways of cell injury and cell death overlap, the main reason for cell death after electroporation is still unspecified. Nevertheless, some information can be gathered from the literature. Unfortunately, it is not possible to infer if the cell death following EP is a result of multiple cell injuries or a single specific pathway following a deleterious effect acting on a specific target, or if various pathways are initiated by different electric pulses used.

An injury in plasma membrane allows Ca^{2+} ions to enter the cell from extracellular space, disrupting the intracellular calcium homeostasis [32,33]. Since Ca^{2+} is a universal carrier of biological information [34], EP can trigger numerous pathways of cell signalling including stress or cell death pathways. Pore formation causes osmotic imbalance and cell swelling which lead to necrosis and this mechanism may be calcium-dependent [35–38]. The absence of extracellular Ca^{2+} prevents cells from dying from apoptosis via endoplasmic reticulum (ER) pathway [39] or commits cells more to apoptosis than to necrosis [37]. The increase of internal Ca^{2+} can be further amplified with store-operated (capacitive) Ca^{2+} entry [40,41] or especially, Ca-induced Ca^{2+} release from internal stores [42,43].

A massive influx of Ca^{2+} after EP causes depletion of intracellular ATP due to the activation of Ca-ATPases and inhibition of ATP production in mitochondria and is linked to cell death (mostly necrosis) in calcium electroporation (Ca EP) [32,33,44–49]. Moreover, ATP and other energetically rich molecules can leak through permeabilized plasma membrane from the cell [25,50–52]. In fact, ATP leak was even one of the first assays to detect cell membrane permeabilization [53]. Also to be noted, ATP depletion switches cell death from apoptosis to necrosis [54].

Electric pulses induce production of reactive oxygen species (ROS) and oxidative damage of unsaturated lipids that are associated to cell membrane permeability, membrane resealing time, and cell damage [1,26,27,55,56] and may therefore contribute to increased permeability post-pulse [28]. Electric pulses can initiate ROS production inside the cell [57–59], mostly in mitochondria, that can damage cell molecules, trigger oxidative stress, and lead to cell death by apoptosis, necrosis, necroptosis, or pyroptosis, which appears to depend on cell type and pulse parameters [12,58,60].

Mitochondrial damage by electroporation is intricate due to complex structure and function of this cellular organelle. The effects of electric pulses on mitochondria were mainly studied using electric pulses of nanosecond duration. In contrast to conventional electroporation using μs and ms pulses it was first believed that nanosecond pulse electroporation (nsEP) can create small pores only in membranes of cell organelles like mitochondria, with negligible impact on plasma membrane [61]. Moreover, it was discovered that electroporation with nsEP induce apoptosis where mitochondria play a major role [62,63]. It was shown that nsEP cause loss of mitochondrial membrane potential (MMP) that is crucial for mitochondrial activity [35,64–70]. However, it is still not known if this is a direct or indirect (apoptosis-related) effect of nsEP. Due to Ca^{2+}-dependency of MMP dissipation it was suggested that this was not due to electroporation of the inner mitochondrial membrane [65,71]. How-

Table 1
Published papers on cell death, reporting on cell injury *in vitro*. Most commonly used electric pulse parameters (number, duration and voltage to distance ratio) for each EP-based treatment are stated in column headers.

Cell injury	ns pulses (nsEP) (mostly 10–100 pulses of 10–300 ns, 20–150 kV/cm)	μs pulses (IRE) (mostly 20–200 pulses of 70–100 μs, 1000–2000 V/cm)	ms pulses (mostly single pulses of 1–20 ms, 1000–2000 V/cm)	Bipolar (H-FIRE) (mostly 50–200 bursts containing 25–300 pulses of 1–2 μs, 500–4000 V/cm)
Membrane damage	Yes [35–38,40,62,63,65,67,69–71,71,81,104–119]	Yes [25,53,80,82,111,122–126]	Yes [62]	Yes [123]
	No [63,120,121]			No [122]
ATP depletion	Yes [51,127,128]	Yes [25,50,52,53,129]	Yes [55]	Yes [58]
Elevation of Ca^{2+}	Yes [35,37–40,65,67,69,104,105,108,109,113,117,130]			
Mitochondrial damage	Yes [35,62,65,67–71,71,79,91,112,117]			
	No [39,131]			
Increase of ROS	No [67,69]		Yes [55]	Yes [58]
DNA damage	Yes Indirect [68,71,79,81,91,132–134]	Yes Indirect [80,82]	Yes Indirect [90]	
	Direct [76,77,106]			
	Direct/indirect not clear [109]			
Protein damage	Yes [71,103]			

Table 2
Published papers on cell death, reporting on cell injury *in vivo*. Most commonly used electric pulse parameters (number, duration and voltage to distance ratio) for each EP-based treatment are stated in column headers.

Cell injury	ns pulses (nsEP) (mostly 10–100 pulses of 10–300 ns, 20–150 kV/cm)	μs pulses (IRE) (mostly 20–200 pulses of 70–100 μs, 1000–2000 V/cm)	ms pulses (mostly single pulses of 1–20 ms, 1000–2000 V/cm)	Bipolar (H-FIRE) (mostly 50–200 bursts containing 25–300 pulses of 1–2 μs, 500–4000 V/cm)
Membrane damage		Yes [78,83,89,135–145]	Yes [146]	Yes [136]
ATP depletion				
Elevation of Ca^{2+}				
Mitochondrial damage	Yes [91]	Yes [142–145]		
Increase of ROS				
DNA damage	Yes Indirect [63,91,93,105]	Yes Indirect [73,78,83–89,141,142,144,147–149]	Yes Indirect [92]	
Protein damage				No [58]

ever, the involvement of the most likely candidate for the loss of MMP, mitochondria permeability transition pore (mPTP) complex is still under debate [71]. Nevertheless, it is clear that Ca^{2+}, ATP, and ROS all influence mitochondrial physiology. Moreover, they exist in an interdependent network, with each having the ability to affect the others. It is therefore difficult to determine which effect is the cause and which is the consequence of a pathological stimulus [72].

Electric pulses can also cause DNA damage, however, it is not clear [63,73] if the effect is direct [74–77] or indirect as a consequence of apoptotic cell death [78–93].

A direct protein damage after EP was not yet extensively studied. MD and other simulations revealed that electric field exposure may result in direct detrimental effects on structure (unfolding, modifying of H-bonding, conformational changes, and/or disruption of secondary structures such as α-helix or β-sheet) of proteins,

e.g. myoglobin [94,95], tubulin [96], kinesin [97], soybean hydrophobic protein [98] or small peptide V3-loop [99], and even ion channels that exhibit pores in voltage-sensor domains after electric field exposure [31]. In experiments with purified proteins exposed to electric fields, Raman spectroscopy, dynamic light scattering and atomic force microscopy imaging, or X-ray crystallography were used to demonstrate that the intense electric fields can affect protein conformation and structure [100–102]. It thus seems that electric pulses may cause direct protein damage in biological systems [29,30,71,103].

Published papers on cell death, reporting cell injury *in vitro* and *in vivo* are listed in Tables 1 and 2. An empty cell of the table means that there was no published data on the subject found in literature search.

As evident from Tables 1 and 2, electric pulses have detrimental effects on many cellular structures and functions, directly or indirectly through different pathways of cell functions and encompass all of the mechanisms of cell injury depicted in Fig. 1. When the injuries are severe and beyond repair, cells undergo one of several types of cell death. Therefore, different therapeutic strategies have evolved over the past few decades to ablate tissues such as irreversible electroporation (IRE) that use microsecond (mostly 20–200 pulses of 70–100 μs, 1000–2000 V/cm voltage to distance ratio) or ms (mostly single pulses of 1–20 ms, 1000–2000 V/cm) electric pulses [4], high-frequency irreversible electroporation (H-FIRE) that uses bursts of bipolar microsecond electric pulses (mostly 50–200 bursts containing 25–300 pulses of 1–2 μs, 500–4000 V/cm) [58], pulsed field ablation to treat cardiac arrhythmias with a large range of pulse parameters combinations [150], nanosecond pulsed electric field (nsEP) ablation (mostly 10–100 pulses of 10–300 ns, 20–150 kV/cm) [109], as well as electrochemotherapy (ECT) that combines electric pulses of lower electric field strength with cytotoxic chemotherapeutic drugs [2] and calcium electroporation (Ca EP) that combine electric pulses (mostly 8 pulses of 100 μs, 1000 V/cm) with high doses of calcium [32] to treat cancer.

3.2. Membrane repair after electroporation

Electroporation results in an injury of plasma membrane (and also internal membranes such as in the case of nsEP) and most of detrimental effects on cells that can trigger cell death (such as Ca^{2+} influx, ATP depletion, ROS increase and mitochondrial damage) can be considered a consequence of membrane damage [151]. Therefore it is of utmost importance that cell restores its plasma membrane integrity quickly after injury to maintain cell homeostasis that depends on plasma membrane selective permeability [152].

In general, only tiny membrane injuries (lipid pores of nm range) may reseal spontaneously, for injuries larger than a few nm different active mechanisms for membrane repair have evolved in eucaryotic cells [151,153,154]. Special signalling mechanisms help cells to identify the nature, magnitude (size and number of wounds) and location of the plasma membrane injury and coordinate the appropriate repair responses [151,154]. Membrane repair response occurs in seconds to minutes (mostly within 30 s after membrane injury) therefore, all the components of membrane repair mechanisms must be ready to be activated without *de novo* protein synthesis [151,152]. Ca^{2+} influx acts as a key trigger for plasma membrane repair: membrane injury results in localized and transient increases of cytosolic free Ca^{2+} concentration which trigger repair mechanisms at the site of the injury [154].

Membrane repair mechanisms are an area of active research and are reviewed in several papers [151–157]. All of membrane repair mechanisms are Ca^{2+} dependent [155]. Cells employ multiple mechanisms simultaneously for efficient membrane repair [151]. Large wounds (several μm in diameter) are repaired by patching: cytoplasmic vesicles fuse together and form a patch to fill the wound [153,154,157]. Small or medium size holes (μm to few μm scale) are repaired by clogging with annexins and other proteins (e.g. dysferlin), followed by membrane shedding (pinching out) [154]. Small wounds (smaller than 100 nm) are repaired by two mechanisms: exocytosis followed by endocytosis and membrane shedding via ESCRT (Endosomal Sorting Complex Required for Transport) proteins [154]. In exocytosis, lysosomes migrate immediately after Ca^{2+} influx towards the injured site and fuse with plasma membrane with a help of proteins involved in membrane repair (e.g. SNARE proteins, synaptotagmins, calpains, dysferlin) [151,153,155,156,158]. Exocytosis and patching also reduce membrane tension that appear in membrane injury and allow faster spontaneous resealing of small lipid pores [153,157]. Exocytosis is however not sufficient to eliminate persistent wounds such as pore-forming proteins. Therefore, the release of lysosome content into extracellular space triggers massive endocytosis [155]. Lysosomal enzyme acidic sphingomyelinase initiates production of ceramide domains in plasma membrane near wounded site and ceramide-dependent endocytosis (through caveolar vesicles) that internalizes and later degrades the lesions/pore-forming toxins [152,154,155].

Following exposure of cells to electric pulses, the aqueous pores formed in plasma membrane (or rather increased membrane permeability) persist for several minutes or, if incubated in low temperatures, e.g. 4 °C, even hours before they reseal [1,159–161]. Closing of pores and resealing of the membrane – re-establishing its full barrier function consists of several stages: a rapid stage that lasts only microseconds after the pulse with a rapid decrease in pore size, and several slower stages that can last minutes after the pulse with slow decrease in pore size and number of pores which lead to gradual reseal [161–166]. Therefore, the resealing times obtained experimentally can be of various duration, depending on the detection method, e.g. membrane conductance relaxation (50 ms – 2 s) [162,167–169] and restoration of barrier function to ions or molecules (120 ms – 20 h) [159,161,170–173]. Moreover, the pore closure time in molecular dynamics (MD) simulations is about nine orders of magnitude shorter than typical experimentally determined membrane resealing times [1] which suggests that the pores in cell membranes are more complex (e.g. involving both membrane lipids and proteins) than those in simple bilayers in MD [31,174] or that electropermeabilization of cell membranes may involve other mechanisms than electroporation such as lipid peroxidation [1]. Considerable efforts have been made in attempt to describe resealing theoretically [166,175,176].

Depending on exposure conditions, electric pulses produce heterogeneous populations of membrane pores, with sizes ranging from 1 to 100 nm [177]. Small, nanometer scale pores that occur in lipid bilayer after EP may reseal spontaneously [153,178]. However, the resealing of EP pores is mostly an active process that requires extracellular calcium: plasma membrane integrity after EP in calcium-depleted medium requires much longer time to reestablish than in medium containing calcium ions [179–183]. The resealing is affected by pulse parameters (higher the pulse amplitude and number, longer the resealing time) [53,55,172,184], temperature [159,161], calcium concentration in extracellular medium [179], generation of reactive oxygen species [55] and medium composition (e.g. sucrose concentration, Mg^{2+}) [55,185]. Although Ca^{2+} ions are required for active repair mechanisms they also interact with lipids and may therefore have a direct effect on the dynamics of pore formation, size and resealing [182].

In a simple (spontaneous) lipid pore closure model, the fate of a pore lies in the ratio between two opposing dynamic forces: contractile line tension and expansive membrane surface tension. Electropore line tension energy is proportional to the pore radius,

therefore, the resulting total energy for a given pore (and fate: closure or expansion) is strongly dependent on the pore size [157,186–188]. The cytoskeleton, especially the actin cortex under the plasma membrane has an important effect on the stability and resealing of pores [187]. Anchoring of the actin cortex to the plasma membrane alters electropore dynamics in a manner that allows for electropore stability – opposing spontaneous closure and allowing molecule transport after pulse cessation [153,189] but also opposing pore expansion and the pores may therefore be more manageable by the cell's active wound healing mechanisms [187,189,190].

It was shown that the membrane repair kinetics after EP follows an exponential dynamics that is interrupted by abrupt recovery steps consistent with a membrane patch model [191]. The authors also suggested two other active cellular mechanisms of repair: i) a removal of leaky patches of membranes by endocytosis and ii) a calcium-induced vesicle exocytosis that reduces the plasma membrane tension and thereby enables pore repair by constriction and bilayer resealing [191]. Indeed, Huynh et al. detected a lysosomal-associated membrane protein 1 (Lamp-1) on the cell surface indicating a lysosomal exocytosis in response to EP wounding [192,193] that was correlated to a degree of damage induced by different pulse parameters [192]. Moreover, a CHMP4B subunit of ESCRT-III complex, involved in repair of small membrane wounds (less than 100 nm) mainly by plasma membrane shedding [194] was localized not only at plasma membrane after EP but also at nuclear envelope after exposure to electric pulses that affect both plasma membrane and nuclear envelope [195]. The calcium binding protein ALG-2 may also contribute to membrane repair and cell survival after EP [196]. Membrane recovery and cell survival in slightly acidic medium were better than in physiological one which can be attributed to more efficient membrane repair mechanisms (possibly exocytosis) in acidic environment [197]. A less efficient membrane repair system in cancer cells may account for higher susceptibility to EP compared to normal cells [198].

Experiments with double wounding by EP [180] revealed similar results as those with double wounding by mechanical puncture [199]: the resealing after the second wounding was faster than after the first one. Authors suggested that the membrane wound was repaired by exocytosis and the second wound resealed faster due to calcium-influx-mediated activation of Golgi apparatus (GA) and its formation of new vesicles. The increasing delay (1, 2, and 3 min delay) between two pulse trains led to a decreased molecular transport [180]. In a study with similar results (the delivery of target molecules decreased with increasing delay time between pulses; high repetition rate is more efficient for permeabilization, i.e. leading to higher permeabilization than low repetition rate) the authors suggest that the second pulse re-porates the weakened cell membrane (already containing nano-sized pores or defects) [163]. Higher EP efficiency of higher pulse repetition rate is usually attributed to the temporal summation of brief subthreshold effects/lesions which can recover without consequences if the interval between pulses is sufficiently long [200]. However, some studies revealed that low pulse repetition rates are more efficient for permeabilization than high repetition rates, e.g. the study on both microsecond and nanosecond electric pulse duration [201]: applying a pulse on a permeabilized cell membrane is likely to be less effective since the existing conducting structures prevent the formation of equally high transmembrane potential [200–202]. The authors speculate that in this case, the type of damage induced by both μs and ns pulses is similar and that the resealing of such damage happens through identical pathways [201]. However, for the similar damage, much higher number and amplitude of ns pulses compared to μs pulses was applied. Nevertheless, the effect of pulse repetition rate is still puzzling (and leading to different results, for review see the Introduction of Pakhomova et al. [200]) and suggests complex permeabilization and resealing processes [163,201].

Since the application of single nanosecond electric pulses (nsEP) cause smaller pores (with a diameter $\leq$ 2 nm) than longer, micro- or millisecond electric pulses or multiple nsEP [177,203] it was always a question whether repair mechanisms that heal nsEP disruptions differ from those that restore the membrane after longer pulses (the size of pores/membrane damage affects the mode of membrane repair [153]). Lysosomes are known to contribute to membrane repair by exocytosis [158]. However, it was shown that nsEP in the presence of extracellular calcium cause inhibition of intracellular migration of lysosomes which can be a result of calcium-induced disruption of the microtubules [203,204]. Therefore, the repair mechanisms for restoring the membrane after nsEP exposure remain unknown.

3.3. Types of cell death after electroporation

Electroporation can trigger different types of cell death in treated tissues, namely apoptosis, necrosis, necroptosis, and pyroptosis. EP-based treatments lead to typical types of cell death (such as apoptosis after IRE or nsEP, necrosis after Ca EP) however, each treatment can result in many different types of cell death. The type of cell death triggered by EP depends on pulse parameters, cell and tissue type, treating conditions and other factors. *In vivo* EP-treated cells undergo a spectrum of different cell death types depending on the location in different treatment zones where they encounter specific electric field parameters. Cells close to electrodes are exposed to electric fields with the highest amplitudes and therefore die of necrosis or even coagulative necrosis with denatured proteins as a consequence of thermal damage, whereas in other treated areas cells die of other modes of cell death such as apoptosis [83,89,138]. Cells at the margins of treated area undergo reversible EP and eventually survive. However, by adding adjuvant molecules and/or chemotherapeutics that are taken up by reversibly electroporated cells at treatment margins the efficacy of tumour treatment by irreversible electroporation can be further increased (a combination of IRE and electrochemotherapy) [205–207]. Different tissues may also respond to electric fields of the same parameters with different types of cell death [140]. In the following sections different types of cell death that occur after different electroporation treatments will be presented and discussed.

3.3.1. Apoptosis

Apoptosis is a programmed, regulated, non-inflammatory cell death which is generally characterized by distinct morphological characteristics and energy-dependent biochemical mechanisms [208]. According to existing literature it is a type of cell death most commonly occurring in electroporation-based ablations such as irreversible electroporation (IRE) [83,85–89,125,135,138,141,142,144,147,148,209,210], high-frequency irreversible electroporation (H-FIRE) [58,123,136,211,212], electrochemotherapy (ECT) [205,207,213–215], electroporation combined with electrolysis [216] and nsEP ablation [63,91,93,120]. Apoptosis was confirmed also in numerous *in vitro* studies using ms [217], μs (IRE, H-FIRE) [80,82,123,125,126,218] and ns pulses [39,62,65,66,69,79,111,114,120,130,131].

Cells that undergo apoptosis after IRE with μs pulses exhibit typical morphology: nuclear condensation and fragmentation, cell shrinkage and fragmentation to apoptotic bodies [83,141,148,207]. Apoptosis in IRE treated cells and tissues was also detected by activation of executioner caspases (proteases that play essential roles in apoptosis, coordinating the destruction of cellular structures), namely caspases −3 and −7 [88,125,126,135,138,139,141], and by DNA fragmentation, typical for apoptosis, detected by Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL)

assay [73,83,85–89,141,142,144,147,148]. A clinical study of IRE in patients with colorectal liver metastases revealed that IRE induces apoptotic cell death in colorectal liver metastases and the ablation zone shows a sharp demarcation between avital and vital tissue [143]. However, apoptosis does not occur in all regions of tissue treated with IRE: at regions close to the electrodes where electric fields reach high amplitudes and even thermal damage cells die from necrosis [83,89,138]. The occurrence of apoptosis and necrosis *in vivo* is time-dependent, but the results are sometimes difficult to discern what comes first [125,135,144,148]. Sometimes, apoptosis is absent from IRE treated tissues; cells undergo other cell death pathways such as necrosis, pyroptosis, and necroptosis [73,137,219–223].

H-FIRE affects cells and tissues in a similar way as IRE (apoptosis, necrosis) [58,123,136,210–212], although with some differences in caspase-dependency in some cells [123]. H-FIRE seem to have more effect on intracellular structures, e.g. nuclei, and less on plasma membrane than IRE [122]. H-FIRE can also induce pyroptosis [58].

Apoptosis was extensively studied in treatments *in vitro* and *in vivo* with nsEP since first confirmed [63]. The fact that cells undergo cell death without the use of chemical compounds (chemotherapeutics) led to nsEP-based treatment of tumours [224]. Apart from morphology, apoptosis due to nsEP exposure was detected by activation of executioner caspases −3 and −7 [62,63,66,69,79,91,107,112,114,119,120,225,226], and DNA fragmentation [62,63,79,91,120,227]. Apoptosis detection with phosphatidylserine externalization assay using Annexin-V must be implemented and results interpreted with caution since it can occur due to pore formation in EP, not related to apoptosis [228,229]. Therefore, the assay has to be employed with a sufficient delay after nsEP application when the pores are resealed [230]. Apoptosis by nsEP is executed via different pathways (Fig. 2). Loss of mitochondrial membrane potential [65,68–70], cytochrome *c* release [62,65,69,79,91,112], caspase-9 activation [65,69,91,112,226], upregulation of pro-apoptotic factors (BAX, BAK, BAD) and downregulation of anti-apoptotic factors (Bcl-2, Bcl-xL, Mcl-1) [79,91,93,109] confirmed the involvement of mitochondria, mostly through intrinsic apoptosis pathway, however, in some studies, BID cleavage also points to the activation of type II extrinsic-like apoptosis [69]. In some cells (HCT, B16F10, E4 SCC, Jurkat), apoptosis progresses also through type I extrinsic-like pathway without or with little involvement of mitochondria [67,69,79] and with caspase-8 activation and modulation of extrinsic apoptotic regulators which influence sensitivity to nsEP [131]. This means that nsEP have a prominent effect also on plasma membrane structures, too, not only on cell interior. Different apoptotic pathways are triggered in different cells [65], sometimes even in the same cells [69]. Moreover, different conditions and severity of injury in nsEP exposure lead to different forms of cell death: necrosis following extensive swelling as a predominant cell death mode in U-937 human monocyte cell line can be switched to apoptosis if swelling is prevented by adding sucrose to electroporation medium [37,38]. Extracellular Ca^{2+} also influence the mode of cell death in nsEP in a similar way as in Ca EP [130]. The balance between apoptosis and necrosis in cells exposed to nsEP may be influenced by the ability to repair the damage (e.g. ion balance, ATP supply) or the level of intracellular damage (higher in nsEP than μs pulses) [81]. Nevertheless, similarly to longer (μs and ms) pulses, more cells undergo necrosis when they are exposed to pulses of higher amplitude or pulse number, resulting in more severe damage [93,114].

The most intriguing question in understanding nsEP cell death is what is the primary target of nsEP that triggers the cascade of the programmed cell death. Intrinsic apoptosis is initiated by a variety of microenvironmental perturbations including growth factor withdrawal, DNA damage, endoplasmic reticulum (ER) stress, reactive oxygen species (ROS) overload, replication stress, microtubular alterations or mitotic defects [11]. As known, nsEP were also reported to cause direct DNA damage and mitotic defects [76,77,231]. Apoptosis is linked to DNA damage via several pathways [232] that were not yet fully studied in the case of nsEP. One possibility is via PLK-1 protein and centrosome-mediated apoptosis [77], PUMA and NOXA were not activated [91,112]. Exposure of cells to nsEP cause ER stress that could be related to ROS formation, permeabilization of the ER, or Ca^{2+} influx, and can further trigger mitochondria-mediated intrinsic apoptosis via PERK and IRE1 [39,128]. ROS formation that occur after nsEP [57] could trigger both intrinsic and extrinsic apoptosis via several cellular targets including those in mitochondria, DNA, ER and plasma membrane [60].

In electrochemotherapy (ECT), i.e. electroporation combined with chemotherapeutic drug, pulses (typically of 100 μs duration) with lower electric field strength and lower number of pulses are used to allow tumour cells to reseal and survive, therefore, cells predominantly die due to the cytotoxic action of chemotherapeutic drugs delivered into cells with EP [233]. With the use of electric pulses that cause increased permeability, intracellular drug accumulation is increased and it results in increased drug cytotoxicity [234]. The two drugs that have been used most often in ECT are bleomycin, and cisplatin which both target DNA [233]. Bleomycin is a cytotoxic antibiotic that generates DNA double-strand breaks (DSB) and DNA single-strand breaks (SSB) [214]. Apoptosis, along with mitotic cell death, is a predominant mode of cell death after ECT [205,207,214,215,235,236], however, other forms of cell death (necrosis, necroptosis, immunogenic cell death) were also identified [50,235,237]. It seems that the internal concentration (which is related to external concentration and electric pulse parameters) and, consequently, the amount of DSB and SSB and the ratio between them determine the mode of cell death [214,215,236,238]. SSB are responsible for the induction of apoptosis which occurs at high amounts of SSB and low to moderate amounts of DSB. At moderate level of DSB and low SSB, cells undergo mitotic cell death, a caspase-dependent programmed cell death which results from the abnormal passage through mitosis of cells containing unrepaired DNA breaks, similar to cell death caused by radiation [214,238,239]. However, at high amounts of DSB (at high doses of bleomycin), irrespective of SSB amount, cells die of a rapid apoptosis-like cell death (here termed as "pseudoapoptosis") [214,215]. "Pseudoapoptosis" in this case is a very fast (a few minutes) cell death process that displays the morphological and biochemical characteristics of apoptosis, however it seems that it does not require induction of cell endonucleases involved in typical apoptosis: it is rather caused by direct effect of bleomycin than via cell endonucleases and can therefore proceed faster than regular apoptosis. The hypothesis is that bleomycin at high concentrations acts directly as an endonuclease and can be considered as an apoptosis-mimetic drug [215].

Apart from apoptosis, there are other forms of regulated cell death such as necroptosis and pyroptosis that also occur after cell exposure to electric field. However, they both elicit immune response *in vivo*, therefore, they will be discussed in Section 3.3.3: Immunogenic cell death and immune response.

3.3.2. Necrosis / accidental cell death

Accidental cell death is a rapid, uncontrollable cell death caused by extreme conditions (heat, radiation, trauma, anoxia, infection) that lead to loss in cell homeostasis and is characterized by the rupture of the cell membrane. It was previously referred to necrosis which exhibits a typical necrotic morphology: cellular changes include cell swelling (oncosis) and blebbing, swelling of mitochondria, ER and nuclear envelope dilatations, random DNA

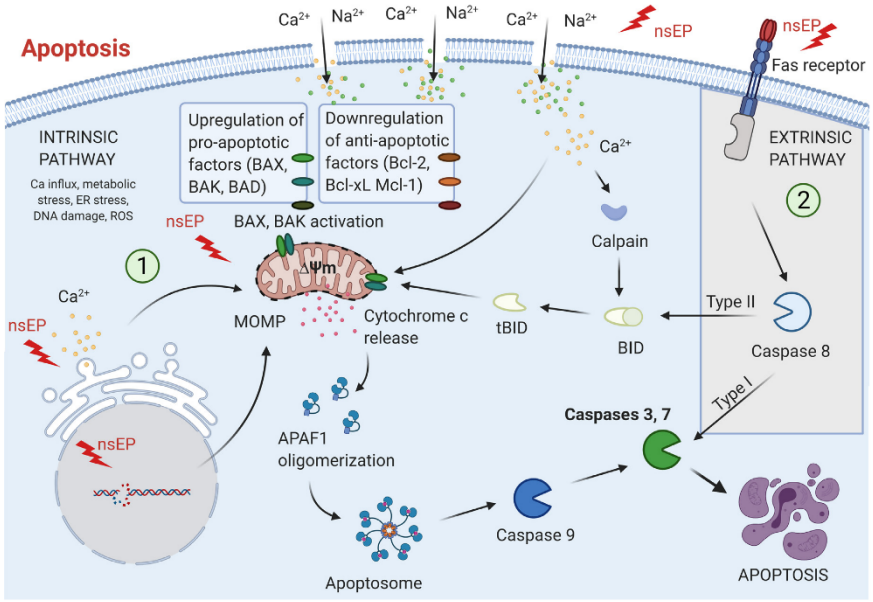

Fig. 2. Pathways of apoptosis after nanosecond pulse electroporation (nsEP). Mostly, apoptosis is executed via intrinsic pathway (1) where mitochondria play a major role. Intrinsic pathway is triggered by internal Ca^{2+} elevation, metabolic stress, ER stress, possible permeabilization of ER and mitochondrial membranes, DNA damage and ROS production that initiate upregulation of pro-apoptotic factors and downregulation of anti-apoptotic factors, dissipation of mitochondrial membrane potential, mitochondrial outer membrane permeabilization (MOMP), cytochrome c release, apoptosome formation, caspase-9 activation and subsequent activation of executioner caspases −3 and −7 which leads to apoptotic cell death. In some cells, apoptosis can progress via extrinsic pathway (2) where nsEP trigger aggregation of the Fas receptor, activation of caspase-8 and subsequent activation of executioner caspases −3 and −7 without (Type I) or with (Type II) amplification through mitochondrial pathway. Derived from [65,69,131]. Created with BioRender.com.

degradation, and finally, membrane rupture followed by spilling cell contents into its surrounding [12,240,241]. Contrary to apoptosis, necrosis was considered to be accidental, unprogrammed or unregulated, however, there are some molecular events and patterns (e.g. RIP1 and PARP activation) that typically occur during necrosis, therefore it is at least in part regulated [240–242]. Moreover, according to Nomenclature Committee on Cell Death new types of regulated cell death that also exhibit necrotic morphotype have been identified such as necroptosis and ferroptosis. Therefore, cell death types that exhibit necrotic morphotype can be accidental or regulated [11]. Most papers reporting necrosis as the form of cell death after EP were however published before the last recommendations of Nomenclature Committee on Cell Death that categorize types of cell death to accidental and regulated [11]. In most of the EP studies, researchers have identified necrosis morphologically. What the authors refer to as "necrosis" is probably, in most cases, what the Committee defines as accidental cell death however, it is impossible to distinguish the precise type of cell death that occurred after EP exposure. Hence, we refer here to necrosis as to all cell death types with necrotic morphology.

Cellular and molecular mechanisms underlying necrotic morphotype cell death are: ATP depletion, mitochondrial dysfunction, oxidative stress, protein kinase signalling, PARP activation, and plasma membrane injury [12,241]. ATP depletion is crucial for necrosis induction: in case of mitochondrial dysfunction indicative for both apoptosis and necrosis, a rapid loss of ATP can switch cell's fate from energy-dependent apoptosis to energy-independent necrosis [241,243]. The spillage of the contents of necrotic cells into the surrounding tissue activates inflammatory signalling pathways, which recruit diverse types of immune cells (neutrophils, macrophages, dendritic cells) involved in the immune response [242]. This renders necrosis also an immunogenic cell death. The cell death triggering of immune response by releasing danger signals (DAMP molecules, such as HMGB1, Heat shock proteins, calreticulin, and mRNA) will be discussed in Section 3.3.3: Immunogenic cell death and immune response.

Exposure to electric pulses caused necrosis in *in vitro* and *in vivo* studies using IRE [126,137,140,142,144,147,209,210,218–223] and H-FIRE pulses [58,136,210,212,244], Ca EP [33,44–49], ECT [235,245] and nsEP [37,38,70,81,93,114,130,134,246,247]. Necrosis was also determined as a mode of cell death after cardiac ablation with IRE for treating heart arrhythmias (for atrial fibrillations, *in vivo* experimental procedure in pigs) [248].

In Ca EP, electric pulses (mostly 100 μs long, but also with nsEP [249] and H-FIRE pulses [250]) are delivered with high concentrations of Ca^{2+} *in vitro* or *in vivo* (IC_{50} ranging from 0.4 to 5.0 mM Ca^{2+} concentration *in vitro*, and 100–500 mM Ca^{2+} with 20–80% tumour volume *in vivo*) [32]. A high Ca^{2+} uptake leads to cell death, mostly necrosis [33,44–49], however, a few studies also reported apoptosis [49,251,252] and necroptosis [250]. *In vitro*, most of the cells swell, rupture and lyse after CaEP, although some cells may exhibit apoptotic morphology and shrinkage (Fig. 3) [48,49]. Several studies reported an immediate, severe and long-lasting drop in cellular ATP level [33,46–49]. ATP depletion as a result of increased intracellular Ca^{2+} may be caused by highly increased activity of Ca-ATPases in plasma membrane (PMCA) and ER (SERCA) that try

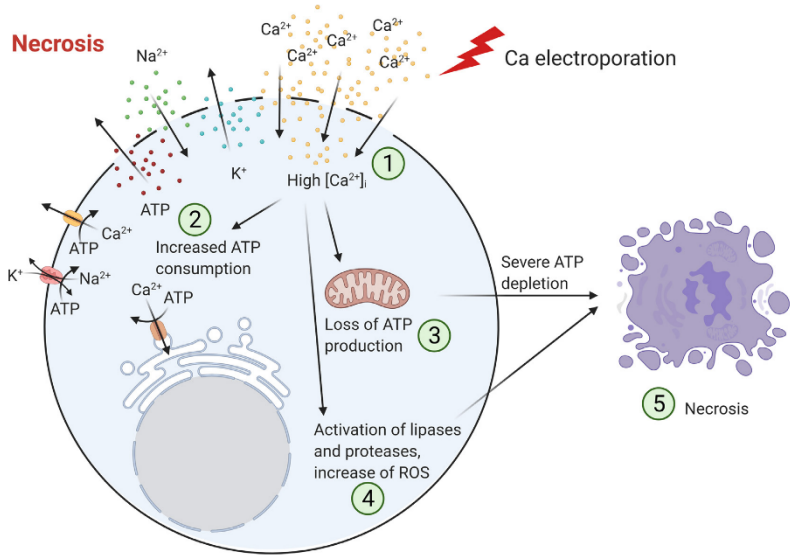

Fig. 3. Necrosis after calcium electroporation (Ca EP). Increased plasma membrane (PM) permeability causes massive Ca^{2+} influx and high intracellular Ca^{2+} concentration (1). This leads to: (2) an increased ATP consumption due to activation of Ca^{2+} pumps (PM, ER) and other pumps (such as Na^+/K^+-ATPase), and ATP loss through permeabilized PM, (3) loss of ATP production due to calcium overload in mitochondria and disruption of electrochemical gradient in mitochondria necessary for ATP production, and (4) other effects such as activation of lipases and proteases, and generation of ROS. A severe ATP depletion in cells eventually triggers necrosis (5). At the necrotic stage (5) plasma membrane is ruptured, and cell lysis occurs (this stage is symbolically depicted here on a smaller scale). Derived from [46]. Created with BioRender.com.

to restore low levels of intracellular Ca^{2+}, opening of permeability transition pores in the mitochondrial membrane, resulting in loss of ATP production, and a direct loss of ATP, i.e. leakage through permeabilized plasma membrane [32,46]. Besides the abrupt ATP depletion which is pivotal for necrosis induction, calcium overload also causes activation of lipases and proteases, and generation of ROS which may also contribute to cell death [32,46]. It has been shown that normal cells seem to be less sensitive to Ca EP than cancer cells [251–254]. Moreover, contrary to ECT, Ca EP induces cytotoxicity without any genotoxicity [33]. It was also reported to elicit immune response and long-term anti-tumour prevention mediated by DAMP molecules (HMGB1) [45].

3.3.3. Immunogenic cell death and immune response

In many studies using electric pulses of different parameters researchers report immunogenic cell death (ICD) eliciting immune response. The term immunogenic cell death is used here in a broader context of different types of cell death that can trigger immune response (necrosis, necroptosis, pyroptosis, and even apoptosis), not referring to a specific type of ICD characterized by apoptotic morphology and connected to ER stress [255].

In *in vivo* treatments of tumours with IRE and H-FIRE [58,73,73,85–88,92,129,135,137,142,144,212,221,222,244,256–260], ECT [50,235,261,262], Ca EP [44,45,253,261] and nsEP [51,127,226,263–266], immune response was observed. Besides innate immune response that recruits macrophages and natural killer cells to remove damaged and dead treated tumour cells and debris, the most important is adapted immune response: the activation of specific anti-tumour memory cells can lead to long-lasting protection against tumour that was treated and can, with an abscopal effect (*ab scopus* - away from target) prevent metastases to spread the disease, as was also reported in some cases after electroporation [45,51,58,127,226,256–258,261,264–266]. A strong positive correlation between up-regulation of cellular immunity-associated genes and decreased tumour diameter was shown [58]. Moreover, "vaccination" with ECT or nsEP-treated cancer cells protects animals against subsequent challenge with cancer cells [50,264]. Therefore, the immune response in treating cancer is advantageous. However, not all EP-based treatments were successful in eliciting a long-term anti-cancer protection [267], and in some cases incomplete eradication of tumours was reported to lead to even faster growth of recurring tumours [268].

EP is also a potent immunological adjuvant for genetic vaccination with gene electrotransfer (GET) due to a low-intensity tissue damage, which rapidly resolves, and pro-inflammatory cytokine release [7,269]. GET of cytokine genes can be used in combination with EP [270–273] or ECT [274,275] to boost the immune response after EP or ECT and enable to prevent recurrences and distant metastases. A combination of ECT with immunostimulating agents (e.g. interleukin-2) can also be an elegant and efficient way to cure both the ECT-treated nodules and distant nodules [276]. However, the immune response in gene therapy can also be unwanted since it may eliminate transfected cells or interfere with transgenic protein expression and function [277,278].

Immunogenic cell death is characterised by release of damage-associated molecular patterns (DAMPs) from dying cells [13,16,17,19,25]. Released DAMPs bind to pattern-recognition receptors (PRRs) of immune cells and elicit immune response [16–18]. The signalling of DAMP molecules is reviewed by Galluzzi [17]. Indeed, it was shown that IRE [25,129,256], H-FIRE [58], ECT [50], GET [92] and nsEP [51,127,128] cause release of DAMPs both *in vitro* and *in vivo*. In most of these studies, ATP, calreticulin and HMGB1 were detected as they represent the gold standard for predicting the ICD-inducing capacity of chemotherapeutic agents [22].

However, other DAMPs such as nucleic acids and uric acid were also investigated [25]. The release of DAMPs increases with increasing pulse amplitude, number and duration [25,51,53,128,129,256] which is consistent with the hypothesis that the release of DAMPs correlates with the degree of (membrane) injury inflicted to cells [25,279]. However, in a recent study *in vitro*, concentrations of DAMPs correlate strongly with cell death but only weakly with cell membrane permeabilization in the range of reversible EP which suggests greater complexity in DAMP signalling [25].

In necrosis, DAMPs are passively released from the cells (ATP, HMGB1, Hsp70, Hsp90, gp96) or, in the case of an ER protein calreticulin, translocated to the surface of the cells [241,242]. Historically, necrotic cells were considered as the most prominent, if not the only, source of DAMPs since apoptotic cells conserved an intact plasma membrane [280]. However, there are other forms of regulated cell death with necrotic and apoptotic-like morphology that also elicit a strong immune response such as necroptosis and pyroptosis. Since necroptosis and pyroptosis were only investigated in recent years it is possible that necrosis and apoptosis identified morphologically in some older studies may in fact be attributed to necroptosis or pyroptosis [145].

Necroptosis is a form of regulated and immunogenic cell death showing morphological features similar to necrosis [11,13,15,281]. Typical events for determining necroptosis (Fig. 4) is activation of receptor interacting serine/threonine kinase 3 (RIPK3) which subsequently activates mixed lineage kinase domain-like pseudokinase (MLKL), the effector of necroptosis: MLKL migrates to plasma membrane and causes membrane permeabilization, cell swelling and rupture which results in release of DAMPs [11,13,15,281,282]. A few studies have identified necroptosis *in vitro* or *in vivo* after IRE [73,145], electroporation combined with electrolysis [216], ECT [237], Ca EP [250] and nsEP [112]. Only two studies confirmed the activation of RIPK3 and MLKL [73,216], others determined necroptosis on the basis of morphology and time frame of cell death [145,237], or other biochemical characteristics such as sensitivity to necroptotic inhibitors [237], analysis of other cell death mechanisms [112] or activation of genes that may contribute to necroptosis signalling [250]. The lack of RIP3 expression in B16F10 melanoma cells may contribute to a weak antitumour immune response after treatment with nsEP [246].

Pyroptosis is a regulated, caspase-dependent cell death which differs from apoptosis (also regulated, caspase-dependent cell death) in morphology, biochemical pathways and immune response. Pyroptosis (Fig. 5) is driven by the activation of inflammatory caspases, most importantly, caspase −1 (but also **4**, 5, or 11) that is activated within an inflammasome, a macromolecular protein complex composed of inflammasome-initiating sensors and inflammatory caspases [283]. Inflammatory caspases activate gasdermin D, a pore-forming protein that permeabilizes plasma membrane and promote the release of DAMPs through pores and subsequent membrane ruptures. Caspase-1 also activates inflammatory cytokines, IL-1β and IL-18 which are then released through gasdermin D and membrane ruptures into the cell surrounding [11,13,281]. The release of DAMPs and, especially, the release of inflammatory cytokines promote a strong immune response [11,13,281]. Pyroptosis exhibits a distinct morphology that includes multiple bubble-like protrusions that can produce pyroptotic bodies, and a peculiar form of chromatin condensation that differs from its apoptotic counterpart. Cell lysis after membrane rupture is also typical for pyroptosis but not for apoptosis [11,284].

Activated caspase-1 and gasdermin D were found in liver tissue treated with IRE [73] or electroporation combined with electrolysis [216] and upregulation of genes associated with pyroptosis in H-FIRE treated tumours were linked to a systemic anti-tumour immune response [58]. It is likely that DAMP molecules activate

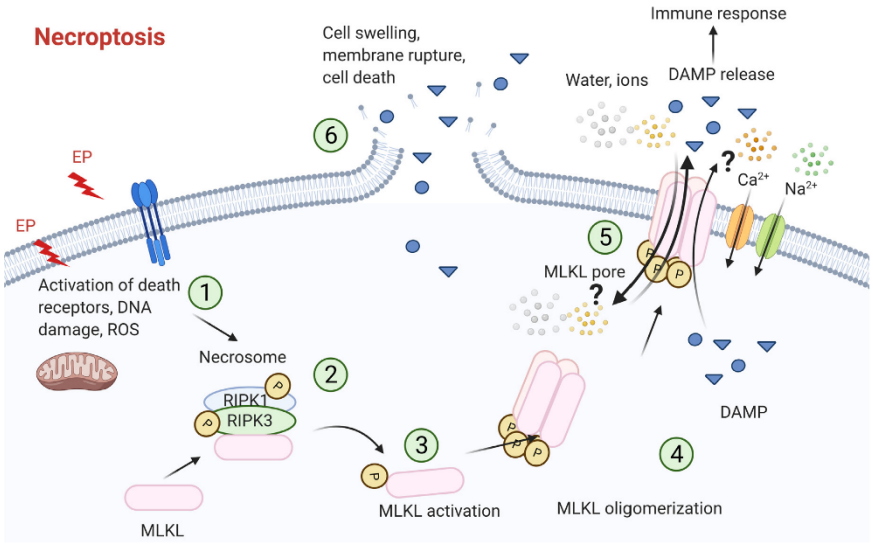

Fig. 4. Necroptosis after electroporation. Necroptosis is triggered by activation of death receptors, DNA damage or ROS production (1). The formation of necrosome enables the activation of RIPK3 kinase (2) which then activates mixed lineage kinase domain-like pseudokinase MLKL (3). Activated MLKL molecules engage in oligomerization (4) and move to plasma membrane where they possibly form pore and/or activate ion channels which allow increased transport of ions and water through plasma membrane (5). This leads to cell swelling, membrane rupture and eventually, to cell death. DAMPs released through pores and/or ruptures stimulate immune response (5, 6). Derived from [281,282]. Created with BioRender.com.

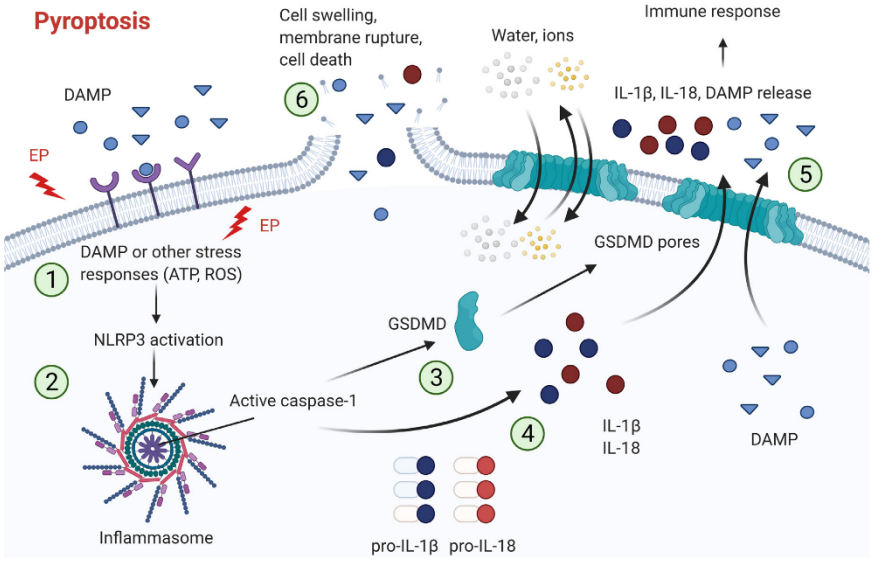

Fig. 5. Pyroptosis after electroporation. Pyroptosis is triggered by DAMPs or other stress responses (ATP, ROS) (1). Inflammatory caspases (mostly caspase-1) are activated within an inflammasome (2). Caspase-1 activates gasdermin D (GSDMD) (3), a pore-forming protein that permeabilizes plasma membrane. Caspase-1 also activates inflammatory cytokines, IL-1β and IL-18 (4). Cytokines and DAMP molecules are released through GSDMD pores out of the cell and stimulate immune response (5). Ion exchange and water influx through GSDMD pores cause cell swelling, membrane rupture, leakage of cell's constituents (including DAMPs and cytokines) and eventually, cell death (6). Derived from [281,282]. Created with BioRender.com.

NLRP3 inflammasome pathway in caspase-1-dependent pyroptosis [58].

Electroporation with a chemotherapeutic drug SN38 in the presence of free Fe^{2+} ions may lead to ferroptosis [285], a form of regulated, immunogenic cell death initiated by oxidative perturbations of the intracellular microenvironment, particularly severe lipid peroxidation, which relies on ROS generation and iron availability [11]. Since EP causes such oxidative perturbations in plasma membrane it may lead to ferroptosis [56].

Although apoptosis is considered non-immunogenic [14], nsEP and IRE treatments still elicit immune response. This can be explained by the fact that some cells in treated tumours undergo necrosis as well, especially near the electrodes, or other immunogenic forms of cell death, and it is sufficient to trigger immune response [24,112]. Moreover, some studies show that also caspase-dependent apoptotic processes can lead to immune response and exposure of DAMP molecules in pre-apoptotic stage [51,226,255,286–288], possibly through ER stress [51,128,264].

4. Conclusions

(1) There are many different forms and pathways of cell death that occur in cells and tissues. Apoptosis, necrosis, necroptosis and pyroptosis were all reported to be induced by electric pulses causing electroporation under certain conditions. The ability to trigger an immune response after electroporation-based cancer treatments is crucial for eradication of tumours on a long-term scale to prevent the recurrence. We strongly believe that with an increasing knowledge on how pulse parameters and different treatment conditions affect cell death pathways is a key to optimisation of therapies.

(2) The extent of cell death pathways needs to be evaluated in cells of different physiology to determine whether pulses are simply stimulating molecular responses or whether its effects are more specific and truly related to the electric pulse parameters. In addition, much care must be taken when comparing studies across different pulse durations and pulse shapes. Considering different types of cell death that occur after EP treatments that use a large range of pulse parameters, maybe cell death mechanisms between long and short pulses are more connected than was previously believed.

(3) Electroporated cells exhibit membrane damage, increase in intracellular Ca^{2+} concentration, mitochondrial disruption, ATP depletion, ROS production, and DNA damage, which all contribute to different forms of cell death. Nevertheless, the exact targets that may lead to different mechanisms of cell death caused by electroporation in different cells under specific conditions still need to be determined. However, this may not be an easy task, considering the cell specificity, complexity, interconnectivity and overlapping of cell injury and death pathways.

CRediT authorship contribution statement

Tina Batista Napotnik: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing - original draft, Writing - review & editing. **Tamara Polajžer:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing - original draft, Writing - review & editing. **Damijan Miklavčič:** Conceptualization, Method-

ology, Funding acquisition, Resources,Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Competing interests

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References

[1] T. Kotnik, L. Rems, M. Tarek, D. Miklavčič, Membrane Electroporation and Electropermeabilization: Mechanisms and Models, Annu. Rev. Biophys. 48 (2019) 63–91, https://doi.org/10.1146/annurev-biophys-052118-115451.

[2] L.G. Campana, I. Edhemović, D. Soden, A.M. Perrone, M. Scarpa, L. Campanacci, M. Čemažar, S. Valpione, D. Miklavčič, S. Mocellin, E. Sieni, G. Serša, Electrochemotherapy - emerging applications technical advances, new indications, combined approaches, and multi-institutional collaboration, Eur. J. Surg. Oncol. 45 (2019) 92–102, https://doi.org/10.1016/j.ejso.2018.11.023.

[3] L. Lambricht, A. Lopes, Š. Kos, G. Serša, V. Préat, G. Vandermeulen, Clinical potential of electroporation for gene therapy and DNA vaccine delivery, Expert Opinion Drug Delivery. 13 (2016) 295–310, https://doi.org/10.1517/17425247.2016.1121990.

[4] B. Geboers, H.J. Scheffer, P.M. Graybill, A.H. Ruarus, S. Nieuwenhuizen, R.S. Puijk, P.M. van den Tol, R.V. Davalos, B. Rubinsky, T.D. de Gruijl, D. Miklavčič, M.R. Meijerink, High-voltage electrical pulses in oncology: irreversible electroporation, electrochemotherapy, gene electrotransfer, electrofusion, and electroimmunotherapy, Radiology 295 (2020) 254–272, https://doi.org/10.1148/radiol.2020192190.

[5] A. Sugrue, E. Maor, A. Ivorra, V. Vaidya, C. Witt, S. Kapa, S. Asirvatham, Irreversible electroporation for the treatment of cardiac arrhythmias, Expert Rev Cardiovasc Ther. 16 (2018) 349–360, https://doi.org/10.1080/14779072.2018.1459185.

[6] C.J. Bradley, D.E. Haines, Pulsed field ablation for pulmonary vein isolation in the treatment of atrial fibrillation, J. Cardiovasc. Electrophysiol. 31 (2020) 2136–2147, https://doi.org/10.1111/jce.14414.

[7] P. Chiarella, E. Massi, M. De Robertis, A. Sibilio, P. Parrella, V.M. Fazio, E. Signori, Electroporation of skeletal muscle induces danger signal release and antigen-presenting cell recruitment independently of DNA vaccine administration, Expert Opin. Biol. Ther. 8 (2008) 1645–1657, https://doi.org/10.1517/14712598.8.11.1645.

[8] D. Miklavcic, ed., Handbook of Electroporation, Springer International Publishing, 2017. https://www.springer.com/gp/book/9783319328850 (accessed February 22, 2021).

[9] V. Kumar, A.K. Abbas, J.C. Aster, Chapter 2 - Cell Injury, Cell Death and Adaptations, in: Robbins Basic Pathology, 10 edition,., Elsevier, Philadelphia, Pennsylvania, 2017, pp. 31–56.

[10] M.A. Miller, J.F. Zachary, Mechanisms and morphology of cellular injury, adaptation, and death, Pathol. Basis Veterinary Dis. (2017) 2–43.e19, https://doi.org/10.1016/B978-0-323-35775-3.00001-1.

[11] L. Galluzzi, I. Vitale, S.A. Aaronson, J.M. Abrams, D. Adam, P. Agostinis, E.S. Alnemri, L. Altucci, I. Amelio, D.W. Andrews, M. Annicchiarico-Petruzzelli, A. V. Antonov, E. Arama, E.H. Baehrecke, N.A. Barlev, N.G. Bazan, F. Bernassola, M.J.M. Bertrand, K. Bianchi, M.V. Blagosklonny, K. Blomgren, C. Borner, P. Boya, C. Brenner, M. Campanella, E. Candi, D. Carmona-Gutierrez, F. Cecconi, F.K.-M. Chan, N.S. Chandel, E.H. Cheng, J.E. Chipuk, J.A. Cidlowski, A. Ciechanover, G.M. Cohen, M. Conrad, J.R. Cubillos-Ruiz, P.E. Czabotar, V. D'Angiolella, T.M. Dawson, V.L. Dawson, V. De Laurenzi, R. De Maria, K.-M. Debatin, R.J. DeBerardinis, M. Deshmukh, N. Di Daniele, F. Di Virgilio, V.M. Dixit, S.J. Dixon, C.S. Duckett, B.D. Dynlacht, W.S. El-Deiry, J.W. Elrod, G.M. Fimia, S. Fulda, A.J. García-Sáez, A.D. Garg, C. Garrido, E. Gavathiotis, P. Golstein, E. Gottlieb, D.R. Green, L.A. Greene, H. Gronemeyer, A. Gross, G. Hajnoczky, J.M. Hardwick, I.S. Harris, M.O. Hengartner, C. Hetz, H. Ichijo, M. Jäättelä, B. Joseph, P.J. Jost, P.P. Juin, W.J. Kaiser, M. Karin, T. Kaufmann, O. Kepp, A. Kimchi, R.N. Kitsis, D.J. Klionsky, R.A. Knight, S. Kumar, S.W. Lee, J.J. Lemasters, B. Levine, A. Linkermann, S.A. Lipton, R.A. Lockshin, C. López-Otín, S.W. Lowe, T. Luedde, E. Lugli, M. MacFarlane, F. Madeo, M. Malewicz, W. Malorni, G. Manic, J.-C. Marine, S.J. Martin, J.-C. Martinou, J.P. Medema, P. Mehlen, P. Meier, S. Melino, E.A. Miao, J.D. Molkentin, U.M. Moll, C. Muñoz-Pinedo, S. Nagata, G. Nuñez, A. Oberst, M. Oren, M. Overholtzer, M. Pagano, T. Panaretakis, M. Pasparakis, J.M. Penninger, D.M. Pereira, S. Pervaiz, M.E. Peter, M. Piacentini, P. Pinton, J.H.M. Prehn, H. Puthalakath, G.A. Rabinovich, M. Rehm, R. Rizzuto, C.M.P. Rodrigues, D.C. Rubinsztein, T. Rudel, K.M. Ryan, E. Sayan, L. Scorrano, F. Shao, Y. Shi, J. Silke, H.-U. Simon, A. Sistigu, B.R. Stockwell, A. Strasser, G. Szabadkai, S.W.G. Tait, D. Tang, N. Tavernarakis, A. Thorburn, Y. Tsujimoto, B. Turk, T. Vanden Berghe, P. Vandenabeele, M.G. Vander Heiden, A. Villunger, H.W. Virgin, K.H. Vousden, D. Vucic, E.F. Wagner, H. Walczak, D. Wallach, Y. Wang, J.A. Wells, W. Wood, J. Yuan, Z. Zakeri, B. Zhivotovsky, L. Zitvogel, G. Melino, G. Kroemer, Molecular mechanisms of cell death: recommendations of the Nomenclature Committee on Cell Death 2018, Cell Death Differ. 25 (2018) 486–541, https://doi.org/10.1038/s41418-017-0012-4.

[12] J.J. Lemasters, Chapter 1 - Molecular Mechanisms of Cell Death, in: W.B. Coleman, G.J. Tsongalis (Eds.), Molecular Pathology, 2 edition,., Academic Press, 2018, pp. 1–24, https://doi.org/10.1016/B978-0-12-802761-5.00001-8.

[13] D. Tang, R. Kang, T.V. Berghe, P. Vandenabeele, G. Kroemer, The molecular machinery of regulated cell death, Cell Res. 29 (2019) 347–364, https://doi.org/10.1038/s41422-019-0164-5.

[14] L. Galluzzi, M.C. Maiuri, I. Vitale, H. Zischka, M. Castedo, L. Zitvogel, G. Kroemer, Cell death modalities: classification and pathophysiological implications, Cell Death Differ. 14 (2007) 1237–1243, https://doi.org/10.1038/sj.cdd.4402148.

[15] M.S. D'Arcy, Cell death: a review of the major forms of apoptosis, necrosis and autophagy, Cell Biol. Int. 43 (2019) 582–592, https://doi.org/10.1002/cbin.11137.

[16] M.E. Bianchi, DAMPs, PAMPs and alarmins: all we need to know about danger, J. Leukoc. Biol. 81 (2007) 1–5, https://doi.org/10.1189/jlb.0306164.

[17] L. Galluzzi, A. Buqué, O. Kepp, L. Zitvogel, G. Kroemer, Immunogenic cell death in cancer and infectious disease, Nat. Rev. Immunol. 17 (2017) 97–111, https://doi.org/10.1038/nri.2016.107.

[18] D. Tang, R. Kang, C.B. Coyne, H.J. Zeh, M.T. Lotze, PAMPs and DAMPs: signal 0s that spur autophagy and immunity, Immunol. Rev. 249 (2012) 158–175, https://doi.org/10.1111/j.1600-065X.2012.01146.x.

[19] J.S. Roh, D.H. Sohn, Damage-associated molecular patterns in inflammatory diseases, Immune Netw. 18 (2018), https://doi.org/10.4110/in.2018.18.e27.

[20] W. Hou, Q. Zhang, Z. Yan, R. Chen, H.J. Zeh III, R. Kang, M.T. Lotze, D. Tang, Strange attractors: DAMPs and autophagy link tumor cell death and immunity, Cell Death Dis. 4 (2013), https://doi.org/10.1038/cddis.2013.493 e966.

[21] O. Krysko, T. Løve Aaes, C. Bachert, P. Vandenabeele, D.V. Krysko, Many faces of DAMPs in cancer therapy, Cell Death Dis. 4 (2013), https://doi.org/10.1038/cddis.2013.156 e631.

[22] J. Zhou, G. Wang, Y. Chen, H. Wang, Y. Hua, Z. Cai, Immunogenic cell death in cancer therapy: present and emerging inducers, J. Cell. Mol. Med. 23 (2019) 4854–4865, https://doi.org/10.1111/jcmm.14356.

[23] T. Batista Napotnik, M. Reberšek, P.T. Vernier, B. Mali, D. Miklavčič, Effects of high voltage nanosecond electric pulses on eukaryotic cells (in vitro): a systematic review, Bioelectrochemistry 110 (2016) 1–12, https://doi.org/10.1016/j.bioelechem.2016.02.011.

[24] R.M. Brock, N. Beitel-White, R.V. Davalos, I.C. Allen, Starting a fire without flame: the induction of cell death and inflammation in electroporation-based tumor ablation strategies, Front. Oncol. 10 (2020), https://doi.org/10.3389/fonc.2020.01235.

[25] T. Polajzer, T. Jarm, D. Miklavcic, Analysis of damage-associated molecular pattern molecules due to electroporation of cells in vitro, Radiol Oncol. 54 (2020) 317–328, https://doi.org/10.2478/raon-2020-0047.

[26] J. Teissie, Involvement of Reactive Oxygen Species in Membrane Electropermeabilization, in: D. Miklavcic (Ed.), Handbook of Electroporation, Springer International Publishing, Cham, 2017: pp. 1–15. https://doi.org/10.1007/978-3-319-26779-1_40-1.

[27] M. Maccarrone, M.R. Bladergroen, N. Rosato, A.F. Agro, Role of lipid peroxidation in electroporation-induced cell permeability, Biochem. Biophys. Res. Commun. 209 (1995) 417–425, https://doi.org/10.1006/bbrc.1995.1519.

[28] O. Michel, A.G. Pakhomov, M. Casciola, J. Saczko, J. Kulbacka, O.N. Pakhomova, Electropermeabilization does not correlate with plasma membrane lipid oxidation, Bioelectrochemistry 132 (2020), https://doi.org/10.1016/j.bioelechem.2019.107433 107433.

[29] W. Chen, Z. Zhongsheng, R.C. Lee, Supramembrane potential-induced electroconformational changes in sodium channel proteins: a potential mechanism involved in electric injury, Burns. 32 (2006) 52–59, https://doi.org/10.1016/j.burns.2005.08.008.

[30] W. Chen, R.C. Lee, Altered ion channel conductance and ionic selectivity induced by large imposed membrane potential pulse, Biophys. J . 67 (1994) 603–612, https://doi.org/10.1016/S0006-3495(94)80520-X.

[31] L. Rems, M.A. Kasimova, I. Testa, L. Delemotte, Pulsed electric fields can create pores in the voltage sensors of voltage-gated ion channels, Biophys. J . 119 (2020) 190–205, https://doi.org/10.1016/j.bpj.2020.05.030.

[32] S.K. Frandsen, M. Vissing, J. Gehl, A comprehensive review of calcium electroporation -a novel cancer treatment modality, Cancers (Basel). 12 (2020), https://doi.org/10.3390/cancers12020290.

[33] L. Gibot, A. Montigny, H. Baaziz, I. Fourquaux, M. Audebert, M.-P. Rols, Calcium delivery by electroporation induces in vitro cell death through mitochondrial dysfunction without DNA damages, Cancers (Basel). 12 (2020), https://doi.org/10.3390/cancers12020425.

[34] M.J. Berridge, M.D. Bootman, H.L. Roderick, Calcium signalling: dynamics, homeostasis and remodelling, Nat. Rev. Mol. Cell Biol. 4 (2003) 517–529, https://doi.org/10.1038/nrm1155.
[35] S.J. Beebe, Y.-J. Chen, N.M. Sain, K.H. Schoenbach, S. Xiao, Transient features in nanosecond pulsed electric fields differentially modulate mitochondria and viability, PLoS ONE 7 (2012), https://doi.org/10.1371/journal.pone.0051349.
[36] C. Muratori, A.G. Pakhomov, E.C. Gianulis, S.D. Jensen, O.N. Pakhomova, The cytotoxic synergy of nanosecond electric pulses and low temperature leads to apoptosis, Sci. Rep. 6 (2016) 36835, https://doi.org/10.1038/srep36835.
[37] O.N. Pakhomova, B. Gregory, I. Semenov, A.G. Pakhomov, Calcium-mediated pore expansion and cell death following nanoelectroporation, Biochim. Biophys. Acta. 2014 (1838) 2547–2554, https://doi.org/10.1016/j.bbamem.2014.06.015.
[38] O.N. Pakhomova, B.W. Gregory, I. Semenov, A.G. Pakhomov, Two modes of cell death caused by exposure to nanosecond pulsed electric field, PLoS ONE 8 (2013), https://doi.org/10.1371/journal.pone.0070278 e70278.
[39] M. Yano, M. Yano, K. Abe, S. Katsuki, H. Akiyama, Gene expression analysis of apoptosis pathway in HeLa S3 cells subjected to nanosecond pulsed electric fields, in: 2011 IEEE Pulsed Power Conference (PPC), 2011: pp. 1221–1225. https://doi.org/10.1109/PPC.2011.6191588.
[40] S.J. Beebe, P.F. Blackmore, J. White, R.P. Joshi, K.H. Schoenbach, Nanosecond pulsed electric fields modulate cell function through intracellular signal transduction mechanisms, Physiol. Meas. 25 (2004) 1077–1093, https://doi.org/10.1088/0967-3334/25/4/023.
[41] J.A. White, P.F. Blackmore, K.H. Schoenbach, S.J. Beebe, Stimulation of capacitative calcium entry in HL-60 cells by nanosecond pulsed electric fields, J. Biol. Chem. 279 (2004) 22964–22972, https://doi.org/10.1074/jbc.M311135200.
[42] I. Semenov, S. Xiao, A.G. Pakhomov, Primary pathways of intracellular Ca(2+) mobilization by nanosecond pulsed electric field, Biochim. Biophys. Acta. 2013 (1828) 981–989, https://doi.org/10.1016/j.bbamem.2012.11.032.
[43] I. Semenov, S. Xiao, O.N. Pakhomova, A.G. Pakhomov, Recruitment of the intracellular Ca2+ by ultrashort electric stimuli: the impact of pulse duration, Cell Calcium 54 (2013) 145–150, https://doi.org/10.1016/j.ceca.2013.05.008.
[44] H. Falk, L.W. Matthiessen, G. Wooler, J. Gehl, Calcium electroporation for treatment of cutaneous metastases; a randomized double-blinded phase II study, comparing the effect of calcium electroporation with electrochemotherapy, Acta Oncol. 57 (2018) 311–319, https://doi.org/10.1080/0284186X.2017.1355109.
[45] H. Falk, P.F. Forde, M.L. Bay, U.M. Mangalanathan, P. Hojman, D.M. Soden, J. Gehl, Calcium electroporation induces tumor eradication, long-lasting immunity and cytokine responses in the CT26 colon cancer mouse model, OncoImmunology. 6 (2017), https://doi.org/10.1080/2162402X.2017.1301332 e1301332.
[46] S.K. Frandsen, H. Gissel, P. Hojman, T. Tramm, J. Eriksen, J. Gehl, Direct therapeutic applications of calcium electroporation to effectively induce tumor necrosis, Cancer Res. 72 (2012) 1336–1341, https://doi.org/10.1158/0008-5472.CAN-11-3782.
[47] S.K. Frandsen, J. Gehl, Effect of calcium electroporation in combination with metformin in vivo and correlation between viability and intracellular ATP level after calcium electroporation in vitro, PLoS ONE 12 (2017), https://doi.org/10.1371/journal.pone.0181839 e0181839.
[48] E.L. Hansen, E.B. Sozer, S. Romeo, S.K. Frandsen, P.T. Vernier, J. Gehl, Dose-dependent ATP depletion and cancer cell death following calcium electroporation, relative effect of calcium concentration and electric field strength, PLoS ONE 10 (2015), https://doi.org/10.1371/journal.pone.0122973 e0122973.
[49] B. Staresinic, T. Jesenko, U. Kamensek, S. Krog Frandsen, G. Sersa, J. Gehl, M. Cemazar, Effect of calcium electroporation on tumour vasculature, Sci. Rep. 8 (2018) 9412, https://doi.org/10.1038/s41598-018-27728-z.
[50] C.Y. Calvet, D. Famin, F.M. André, L.M. Mir, Electrochemotherapy with bleomycin induces hallmarks of immunogenic cell death in murine colon cancer cells, Oncoimmunology. 3 (2014), https://doi.org/10.4161/onci.28131.
[51] R. Nuccitelli, A. McDaniel, S. Anand, J. Cha, Z. Mallon, J.C. Berridge, D. Uecker, Nano-Pulse Stimulation is a physical modality that can trigger immunogenic tumor cell death, J. ImmunoTher. Cancer 5 (2017) 32, https://doi.org/10.1186/s40425-017-0234-5.
[52] J.C. Seegers, L. Lottering, A.M. Joubert, F. Joubert, A. Koorts, C.A. Engelbrecht, D.H. van Papendorp, A pulsed DC electric field affects P2-purinergic receptor functions by altering the ATP levels in in vitro and in vivo systems, Med. Hypotheses 58 (2002) 171–176, https://doi.org/10.1054/mehy.2001.1506.
[53] M.P. Rols, J. Teissié, Electropermeabilization of mammalian cells. Quantitative analysis of the phenomenon, Biophys. J. 58 (1990) 1089–1098.
[54] P. Nicotera, M. Leist, E. Ferrando-May, Intracellular ATP, a switch in the decision between apoptosis and necrosis, Toxicol. Lett. 102–103 (1998) 139–142, https://doi.org/10.1016/S0378-4274(98)00298-7.
[55] B. Gabriel, J. Teissié, Generation of reactive-oxygen species induced by electropermeabilization of Chinese hamster ovary cells and their consequence on cell viability, Eur. J. Biochem. 223 (1994) 25–33, https://doi.org/10.1111/j.1432-1033.1994.tb18962.x.
[56] D. Wiczew, N. Szulc, M. Tarek, On the permeability of cell membranes subjected to lipid oxidation, BioRxiv. (2020) 2020.11.30.403345. https://doi.org/10.1101/2020.11.30.403345.
[57] O.N. Pakhomova, V.A. Khorokhorina, A.M. Bowman, R. Rodaitė-Riševičienė, G. Saulis, S. Xiao, A.G. Pakhomov, Oxidative effects of nanosecond pulsed electric field exposure in cells and cell-free media, Arch. Biochem. Biophys. 527 (2012) 55–64, https://doi.org/10.1016/j.abb.2012.08.004.
[58] V.M. Ringel-Scaia, N. Beitel-White, M.F. Lorenzo, R.M. Brock, K.E. Huie, S. Coutermarsh-Ott, K. Eden, D.K. McDaniel, S.S. Verbridge, J.H. Rossmeisl, K.J. Oestreich, R.V. Davalos, I.C. Allen, High-frequency irreversible electroporation is an effective tumor ablation strategy that induces immunologic cell death and promotes systemic anti-tumor immunity, EBioMedicine. 44 (2019) 112–125, https://doi.org/10.1016/j.ebiom.2019.05.036.
[59] W. Szlasa, A. Kiełbik, A. Szewczyk, N. Rembiałkowska, V. Novickij, M. Tarek, J. Saczko, J. Kulbacka, Oxidative effects during irreversible electroporation of melanoma cells—in vitro study, Molecules 26 (2021) 154, https://doi.org/10.3390/molecules26010154.
[60] M. Redza-Dutordoir, D.A. Averill-Bates, Activation of apoptosis signalling pathways by reactive oxygen species, Biochimica et Biophysica Acta (BBA) - Molecular Cell Res. 1863 (2016) 2977–2992, https://doi.org/10.1016/j.bbamcr.2016.09.012.
[61] K.H. Schoenbach, S.J. Beebe, E.S. Buescher, Intracellular effect of ultrashort electrical pulses, Bioelectromagnetics. 22 (2001) 440–448.
[62] S.J. Beebe, P.M. Fox, L.J. Rec, E.L.K. Willis, K.H. Schoenbach, Nanosecond, high-intensity pulsed electric fields induce apoptosis in human cells, FASEB J. 17 (2003) 1493–1495, https://doi.org/10.1096/fj.02-0859fje.
[63] S.J. Beebe, P.M. Fox, L.J. Rec, K. Somers, R.H. Stark, K.H. Schoenbach, Nanosecond pulsed electric field (nsPEF) effects on cells and tissues: apoptosis induction and tumor growth inhibition, IEEE Trans. Plasma Sci. 30 (2002) 286–292, https://doi.org/10.1109/TPS.2002.1003872.
[64] T.B. Batista Napotnik, Y.-H. Wu, M.A. Gundersen, D. Miklavčič, P.T. Vernier, Nanosecond electric pulses cause mitochondrial membrane permeabilization in Jurkat cells, Bioelectromagnetics. 33 (2012) 257–264, https://doi.org/10.1002/bem.20707.
[65] S. Beebe, N. Sain, W. Ren, Induction of Cell Death Mechanisms and Apoptosis by Nanosecond Pulsed Electric Fields (nsPEFs), Cells. 2 (2013) 136–162, https://doi.org/10.3390/cells2010136.
[66] S.J. Beebe, W.E. Ford, W. Ren, X. Chen, K.H. Schoenbach, Non-ionizing radiation with nanosecond pulsed electric fields as a cancer treatment: in vitro studies, in: Annual International Conference of the IEEE Engineering in Medicine and Biology Society, 2009. EMBC 2009, 2009: pp. 6509–6512. https://doi.org/10.1109/IEMBS.2009.5333139.
[67] W.E. Ford, W. Ren, P.F. Blackmore, K.H. Schoenbach, S.J. Beebe, Nanosecond pulsed electric fields stimulate apoptosis without release of pro-apoptotic factors from mitochondria in B16f10 melanoma, Arch. Biochem. Biophys. 497 (2010) 82–89, https://doi.org/10.1016/j.abb.2010.03.008.
[68] Y. Mi, C. Sun, C. Yao, C. Li, D. Mo, L. Tang, H. Liu, Effects of steep pulsed electric fields (SPEF) on mitochondrial transmembrane potential of human liver cancer cell, Conf Proc IEEE Eng. Med. Biol. Soc. 2007 (2007) 5815–5818, https://doi.org/10.1109/IEMBS.2007.4353669.
[69] W. Ren, S.J. Beebe, An apoptosis targeted stimulus with nanosecond pulsed electric fields (nsPEFs) in E4 squamous cell carcinoma, Apoptosis 16 (2011) 382–393, https://doi.org/10.1007/s10495-010-0572-y.
[70] D. Xiao, L. Tang, C. Zeng, J. Wang, X. Luo, C. Yao, C. Sun, Effect of actin cytoskeleton disruption on electric pulse-induced apoptosis and electroporation in tumour cells, Cell Biol. Int. 35 (2011) 99–104, https://doi.org/10.1042/CBI20100464.
[71] S.J. Beebe, Considering effects of nanosecond pulsed electric fields on proteins, Bioelectrochemistry 103 (2015) 52–59, https://doi.org/10.1016/j.bioelechem.2014.08.014.
[72] P.S. Brookes, Y. Yoon, J.L. Robotham, M.W. Anders, S.-S. Sheu, Calcium, ATP, and ROS: a mitochondrial love-hate triangle, Am. J. Physiol. Cell Physiol. 287 (2004) C817–C833, https://doi.org/10.1152/ajpcell.00139.2004.
[73] Y. Zhang, C. Lyu, Y. Liu, Y. Lv, T.T. Chang, B. Rubinsky, Molecular and histological study on the effects of non-thermal irreversible electroporation on the liver, Biochem. Biophys. Res. Commun. 500 (2018) 665–670, https://doi.org/10.1016/j.bbrc.2018.04.132.
[74] A. Goldberg, B. Rubinsky, The effect of electroporation type pulsed electric fields on DNA in aqueous solution, Technol. Cancer Res. Treat. 9 (2010) 423–430, https://doi.org/10.1177/153303461000900412.
[75] W.S. Meaking, J. Edgerton, C.W. Wharton, R.A. Meldrum, Electroporation-induced damage in mammalian cell DNA, Biochimica et Biophysica Acta (BBA) - Gene Structure and Expression. 1264 (1995) 357–362. https://doi.org/10.1016/0167-4781(95)00177-8.
[76] M. Stacey, J. Stickley, P. Fox, V. Statler, K. Schoenbach, S.J. Beebe, S. Buescher, Differential effects in cells exposed to ultra-short, high intensity electric fields: cell survival, DNA damage, and cell cycle analysis, Mutation Research/Genetic Toxicol. Environ. Mutagenesis 542 (2003) 65–75, https://doi.org/10.1016/j.mrgentox.2003.08.006.
[77] H. Zou, X.L. Gan, L.J. Linghu, C. Chen, L.N. Hu, Y. Zhang, Intense nanosecond pulsed electric fields promote cancer cell apoptosis through centrosome-dependent pathway involving reduced level of PLK1, Eur. Rev. Med. Pharmacol. Sci. 17 (2013) 152–160.
[78] B. Al-Sakere, F. André, C. Bernat, E. Connault, P. Opolon, R.V. Davalos, B. Rubinsky, L.M. Mir, Tumor Ablation with Irreversible Electroporation, PLoS ONE 2 (2007), https://doi.org/10.1371/journal.pone.0001135.
[79] E.H. Hall, K.H. Schoenbach, S.J. Beebe, Nanosecond pulsed electric fields induce apoptosis in p53-wildtype and p53-null HCT116 colon carcinoma cells, Apoptosis 12 (2007) 1721–1731, https://doi.org/10.1007/s10495-007-0083-7.

[80] F. Hofmann, H. Ohnimus, C. Scheller, W. Strupp, U. Zimmermann, C. Jassoy, Electric field pulses can induce apoptosis, J. Membrane Biol. 169 (1999) 103–109, https://doi.org/10.1007/s002329900522.

[81] B.L. Ibey, A.G. Pakhomov, B.W. Gregory, V.A. Khorokhorina, C.C. Roth, M.A. Rassokhin, J.A. Bernhard, G.J. Wilmink, O.N. Pakhomova, Selective cytotoxicity of intense nanosecond-duration electric pulses in mammalian cells, Biochimica et Biophysica Acta (BBA) - General Subjects. (1800 (2010)) 1210–1219, https://doi.org/10.1016/j.bbagen.2010.07.008.

[82] I. Kaminska, M. Kotulska, A. Stecka, J. Saczko, M. Drag-Zalesinska, T. Wysocka, A. Choromanska, N. Skolucka, R. Nowicki, J. Marczak, J. Kulbacka, Electroporation-induced changes in normal immature rat myoblasts (H9C2), Gen. Physiol. Biophys. 31 (2012) 19–25, https://doi.org/10.4149/gpb_2012_003.

[83] H.-B. Kim, C.-K. Sung, K.Y. Baik, K.-W. Moon, H.-S. Kim, J.-H. Yi, J.-H. Jung, M.-H. Moon, O.-K. Choi, Changes of apoptosis in tumor tissues with time after irreversible electroporation, Biochem. Biophys. Res. Commun. 435 (2013) 651–656, https://doi.org/10.1016/j.bbrc.2013.05.039.

[84] E.W. Lee, D. Wong, B.A. Tafti, V. Prieto, M. Totonchy, J. Hilton, S. Dry, S. Cho, C. T. Loh, S.T. Kee, Irreversible electroporation in eradication of rabbit VX2 liver tumor, J. Vasc. Interv. Radiol. 23 (2012) 833–840, https://doi.org/10.1016/j.jvir.2012.02.017.

[85] E.W. Lee, C. Chen, V.E. Prieto, S.M. Dry, C.T. Loh, S.T. Kee, Advanced hepatic ablation technique for creating complete cell death: irreversible electroporation, Radiology 255 (2010) 426–433, https://doi.org/10.1148/radiol.10090337.

[86] J.M. Lee, H.S. Choi, E.S. Kim, B. Keum, Y.S. Seo, Y.T. Jeen, H.S. Lee, H.J. Chun, S.H. Um, C.D. Kim, H.B. Kim, Characterization of irreversible electroporation on the stomach: a feasibility study in rats, Sci. Rep. 9 (2019) 9094, https://doi.org/10.1038/s41598-019-45659-1.

[87] K.W. Lee, J.M. Lee, H.S. Choi, E.S. Kim, B. Keum, Y.S. Seo, Y.T. Jeen, S.H. Um, H.S. Lee, H.J. Chun, C.D. Kim, C.H. Oh, H.B. Kim, Novel ablation therapy using endoscopic irreversible electroporation in the bile duct: a pilot animal study, Clin Endosc. (2020), https://doi.org/10.5946/ce.2020.126.

[88] S. Li, F. Chen, L. Shen, Q. Zeng, P. Wu, Percutaneous irreversible electroporation for breast tissue and breast cancer: safety, feasibility, skin effects and radiologic-pathologic correlation in an animal study, J. Transl. Med. 14 (2016) 238, https://doi.org/10.1186/s12967-016-0993-7.

[89] G. Long, G. Bakos, P.K. Shires, L. Gritter, J.W. Crissman, J.L. Harris, J.W. Clymer, Histological and Finite Element Analysis of Cell Death due to Irreversible Electroporation, Technol. Cancer Res. Treat. 13 (2014) 561–569, https://doi.org/10.7785/tcrtexpress.2013.600253.

[90] J. Piñero, M. López-Baena, T. Ortiz, F. Cortés, Apoptotic and necrotic cell death are both induced by electroporation in HL60 human promyeloid leukaemia cells, Apoptosis 2 (1997) 330–336, https://doi.org/10.1023/A:1026497306006.

[91] Z. Ren, X. Chen, G. Cui, S. Yin, L. Chen, J. Jiang, Z. Hu, H. Xie, S. Zheng, L. Zhou, Nanosecond pulsed electric field inhibits cancer growth followed by alteration in expressions of NF-κB and Wnt/β-catenin signaling molecules, PLoS ONE 8 (2013), https://doi.org/10.1371/journal.pone.0074322.

[92] K. Schultheis, T.R.F. Smith, W.B. Kiosses, K.A. Kraynyak, A. Wong, J. Oh, K.E. Broderick, Delineating the cellular mechanisms associated with skin electroporation, Hum Gene Ther Methods. 29 (2018) 177–188, https://doi.org/10.1089/hgtb.2017.105.

[93] D. Yin, W.G. Yang, J. Weissberg, C.B. Goff, W. Chen, Y. Kuwayama, A. Leiter, H. Xing, A. Meixel, D. Gaut, F. Kirkbir, D. Sawcer, P.T. Vernier, J.W. Said, M.A. Gundersen, H.P. Koeffler, Cutaneous papilloma and squamous cell carcinoma therapy utilizing nanosecond pulsed electric fields (nsPEF), PLoS ONE 7 (2012), https://doi.org/10.1371/journal.pone.0043891.

[94] P. Marracino, A. Paffi, R. Reale, M. Liberti, G. D'Inzeo, F. Apollonio, Technology of high-intensity electric field pulses: a way to control protein unfolding, Phys. Chem. Biophys. 3 (2013).

[95] P. Marracino, F. Apollonio, M. Liberti, G. d'Inzeo, A. Amadei, Effect of High exogenous electric pulses on protein conformation: myoglobin as a case study, J. Phys. Chem. B. 117 (2013) 2273–2279, https://doi.org/10.1021/jp309857b.

[96] P. Marracino, D. Havelka, J. Průša, M. Liberti, J. Tuszynski, A.T. Ayoub, F. Apollonio, M. Cifra, Tubulin response to intense nanosecond-scale electric field in molecular dynamics simulation, Sci. Rep. 9 (2019) 10477, https://doi.org/10.1038/s41598-019-46636-4.

[97] J. Průša, M. Cifra, Molecular dynamics simulation of the nanosecond pulsed electric field effect on kinesin nanomotor, Sci. Rep. 9 (2019) 19721, https://doi.org/10.1038/s41598-019-56052-3.

[98] A. Singh, V. Orsat, V. Raghavan, Soybean Hydrophobic Protein Response to External Electric Field: A Molecular Modeling Approach, Biomolecules. 3 (2013) 168–179, https://doi.org/10.3390/biom3010168.

[99] P. Ojeda-May, M.E. Garcia, Electric field-driven disruption of a native β-Sheet protein conformation and generation of a helix-structure, Biophys J. 99 (2010) 595–599, https://doi.org/10.1016/j.bpj.2010.04.040.

[100] D.E. Chafai, V. Sulimenko, D. Havelka, L. Kubínová, P. Dráber, M. Cifra, Reversible and irreversible modulation of tubulin self-assembly by intense nanosecond pulsed electric fields, Adv. Mater. 31 (2019) 1903636, https://doi.org/10.1002/adma.201903636.

[101] D.R. Hekstra, K.I. White, M.A. Socolich, R.W. Henning, V. Šrajer, R. Ranganathan, Electric-field-stimulated protein mechanics, Nature 540 (2016) 400–405, https://doi.org/10.1038/nature20571.

[102] Y.-Y. Liu, Y. Zhang, X.-A. Zeng, H. El-Mashad, Z.-L. Pan, Q.-J. Wang, Effect of pulsed electric field on microstructure of some amino acid group of soy protein isolates, Int. J. Food Eng. 10 (2014) 113–120, https://doi.org/10.1515/ijfe-2013-0033.

[103] M. Stacey, P. Fox, S. Buescher, J. Kolb, Nanosecond pulsed electric field induced cytoskeleton, nuclear membrane and telomere damage adversely impact cell survival, Bioelectrochemistry 82 (2011) 131–134, https://doi.org/10.1016/j.bioelechem.2011.06.002.

[104] S.J. Beebe, P.F. Blackmore, E. Hall, J.A. White, L.K. Willis, L. Fauntleroy, J.F. Kolb, K.H. Schoenbach, Dynamic effects and applications for nanosecond pulsed electric fields in cells and tissues, in: Proc. SPIE 5692, Advanced Biomedical and Clinical Diagnostic Systems III, 2005: pp. 260–269. https://doi.org/10.1117/12.604449.

[105] S.J. Beebe, J. White, P.F. Blackmore, Y. Deng, K. Somers, K.H. Schoenbach, Diverse effects of nanosecond pulsed electric fields on cells and tissues, DNA Cell Biol. 22 (2003) 785–796, https://doi.org/10.1089/104454903322624993.

[106] E.H. Hall, K.H. Schoenbach, S.J. Beebe, Nanosecond pulsed electric fields have differential effects on cells in the S-phase, DNA Cell Biol. 26 (2007) 160–171, https://doi.org/10.1089/dna.2006.0514.

[107] E.H. Hall, K.H. Schoenbach, S.J. Beebe, Nanosecond pulsed electric fields (nsPEF) induce direct electric field effects and biological effects on human colon carcinoma cells, DNA Cell Biol. 24 (2005) 283–291, https://doi.org/10.1089/dna.2005.24.283.

[108] B.L. Ibey, J.C. Ullery, O.N. Pakhomova, C.C. Roth, I. Semenov, H.T. Beier, M. Tarango, S. Xiao, K.H. Schoenbach, A.G. Pakhomov, Bipolar nanosecond electric pulses are less efficient at electropermeabilization and killing cells than monopolar pulses, Biochem. Biophys. Res. Commun. 443 (2014) 568–573, https://doi.org/10.1016/j.bbrc.2013.12.004.

[109] R. Nuccitelli, X. Chen, A.G. Pakhomov, W.H. Baldwin, S. Sheikh, J.L. Pomicter, W. Ren, C. Osgood, R.J. Swanson, J.F. Kolb, S.J. Beebe, K.H. Schoenbach, A new pulsed electric field therapy for melanoma disrupts the tumor's blood supply and causes complete remission without recurrence, Int. J. Cancer. 125 (2009) 438–445, https://doi.org/10.1002/ijc.24345.

[110] W. Qi, J. Guo, S. Wu, B. Su, L. Zhang, J. Pan, J. Zhang, Synergistic effect of nanosecond pulsed electric field combined with low-dose of pingyangmycin on salivary adenoid cystic carcinoma, Oncol. Rep. 31 (2014) 2220–2228, https://doi.org/10.3892/or.2014.3063.

[111] X. Rao, X. Chen, J. Zhou, L. Sun, J. Liu, A digital controlled pulse generator for a possible tumor therapy combining irreversible electroporation with nanosecond pulse stimulation, IEEE Trans. Biomed. Circuits Syst. 14 (2020) 595–605, https://doi.org/10.1109/TBCAS.2020.2987376.

[112] W. Ren, N.M. Sain, S.J. Beebe, Nanosecond pulsed electric fields (nsPEFs) activate intrinsic caspase-dependent and caspase-independent cell death in Jurkat cells, Biochem. Biophys. Res. Commun. 421 (2012) 808–812, https://doi.org/10.1016/j.bbrc.2012.04.094.

[113] P.T. Vernier, Y. Sun, L. Marcu, S. Salemi, C.M. Craft, M.A. Gundersen, Calcium bursts induced by nanosecond electric pulses, Biochem. Biophys. Res. Commun. 310 (2003) 286–295.

[114] P.T. Vernier, A. Li, L. Marcu, C.M. Craft, M.A. Gundersen, Ultrashort pulsed electric fields induce membrane phospholipid translocation and caspase activation: differential sensitivities of Jurkat T lymphoblasts and rat glioma C6 cells, IEEE Trans. Dielectr. Electr. Insul. 10 (2003) 795–809, https://doi.org/10.1109/TDEI.2003.1237329.

[115] J. Wang, J. Guo, S. Wu, H. Feng, S. Sun, J. Pan, J. Zhang, S.J. Beebe, Synergistic effects of nanosecond pulsed electric fields combined with low concentration of gemcitabine on human oral squamous cell carcinoma in vitro, PLoS ONE 7 (2012), https://doi.org/10.1371/journal.pone.0043213.

[116] S. Wu, J. Guo, B. Su, J. Zhang, J. Fang, Nanosecond pulsed electric fields adjuvant chemotherapy for breast cancer: an in vitro study, in: in: 2013 19th IEEE Pulsed Power Conference (PPC), 2013, pp. 1–5, https://doi.org/10.1109/PPC.2013.6627553.

[117] D. Xiao, C. Yao, H. Liu, C. Li, J. Cheng, F. Guo, L. Tang, Irreversible electroporation and apoptosis in human liver cancer cells induced by nanosecond electric pulses, Bioelectromagnetics. (2013), https://doi.org/10.1002/bem.21796.

[118] H. Xu, P.D. Nallathamby, X.-H.N. Xu, Real-time imaging and tuning subcellular structures and membrane transport kinetics of single live cells at nanosecond regime, J. Phys. Chem. B. 113 (2009) 14393–14404, https://doi.org/10.1021/jp9021739.

[119] W. Yang, Y.-H. Wu, D. Yin, H.P. Koeffler, D.E. Sawcer, P.T. Vernier, M.A. Gundersen, Differential sensitivities of malignant and normal skin cells to nanosecond pulsed electric fields, Technol. Cancer Res. Treat. 10 (2011) 281–286.

[120] S.J. Beebe, P. Fox, L. Rec, L. Willis, K. Schoenbach, Nanosecond pulsed electric field effects on human cells, in: in: Conference Record of the Twenty-Fifth International Power Modulator Symposium, 2002 and 2002 High-Voltage Workshop, 2002, pp. 652–656, https://doi.org/10.1109/MODSYM.2002.1189562.

[121] K. Morotomi-Yano, S. Oyadomari, H. Akiyama, K. Yano, Nanosecond pulsed electric fields act as a novel cellular stress that induces translational suppression accompanied by eIF2α phosphorylation and 4E-BP1 dephosphorylation, Exp. Cell Res. 318 (2012) 1733–1744, https://doi.org/10.1016/j.yexcr.2012.04.016.

[122] J.W. Ivey, E.L. Latouche, M.B. Sano, J.H. Rossmeisl, R.V. Davalos, S.S. Verbridge, Targeted cellular ablation based on the morphology of malignant cells, Sci. Rep. 5 (2015) 17157, https://doi.org/10.1038/srep17157.

[123] B. Mercadal, N. Beitel-White, K.N. Aycock, Q. Castellví, R.V. Davalos, A. Ivorra, Dynamics of Cell Death After Conventional IRE and H-FIRE Treatments, Ann. Biomed. Eng. 48 (2020) 1451–1462, https://doi.org/10.1007/s10439-020-02462-8.

[124] E. Tekle, M.D. Wolfe, H. Oubrahim, P.B. Chock, Phagocytic clearance of electric field induced 'apoptosis-mimetic' cells, Biochem. Biophys. Res. Commun. 376 (2008) 256–260, https://doi.org/10.1016/j.bbrc.2008.08.060.

[125] H. Zhang, K. Liu, Z. Xue, H. Yin, H. Dong, W. Jin, X. Shi, H. Wang, H. Wang, High-voltage pulsed electric field plus photodynamic therapy kills breast cancer cells by triggering apoptosis, Am. J. Transl. Res. 10 (2018) 334–351.

[126] W. Zhou, Z. Xiong, Y. Liu, C. Yao, C. Li, Low voltage irreversible electroporation induced apoptosis in HeLa cells, J. Cancer Res. Ther. 8 (2012) 80–85, https://doi.org/10.4103/0973-1482.95179.

[127] S. Guo, Y. Jing, N.I. Burcus, B.P. Lassiter, R. Tanaz, R. Heller, S.J. Beebe, Nano-pulse stimulation induces potent immune responses, eradicating local breast cancer while reducing distant metastases, Int. J. Cancer. 142 (2018) 629–640, https://doi.org/10.1002/ijc.31071.

[128] A. Rossi, O.N. Pakhomova, P.A. Mollica, M. Casciola, U. Mangalanathan, A.G. Pakhomov, C. Muratori, Nanosecond pulsed electric fields induce endoplasmic reticulum stress accompanied by immunogenic cell death in murine models of lymphoma and colorectal cancer, Cancers (Basel). 11 (2019), https://doi.org/10.3390/cancers11122034.

[129] J. Zhao, X. Wen, L. Tian, T. Li, C. Xu, X. Wen, M.P. Melancon, S. Gupta, B. Shen, W. Peng, C. Li, Irreversible electroporation reverses resistance to immune checkpoint blockade in pancreatic cancer, Nat. Commun. 10 (2019), https://doi.org/10.1038/s41467-019-08782-1.

[130] K. Morotomi-Yano, H. Akiyama, K. Yano, Different involvement of extracellular calcium in two modes of cell death induced by nanosecond pulsed electric fields, Arch. Biochem. Biophys. 555–556 (2014) 47–54, https://doi.org/10.1016/j.abb.2014.05.020.

[131] L.E. Estlack, C.C. Roth, G.L.T. Iii, W.A.L. Iii, B.L. Ibey, Nanosecond pulsed electric fields modulate the expression of Fas/CD95 death receptor pathway regulators in U937 and Jurkat Cells, Apoptosis 19 (2014) 1755–1768, https://doi.org/10.1007/s10495-014-1041-9.

[132] F. Guo, C. Yao, C. Li, Y. Mi, Y. Wen, J. Tang, Dependence on pulse duration and number of tumor cell apoptosis by nanosecond pulsed electric fields, IEEE Trans. Dielectr. Electr. Insul. 20 (2013), https://doi.org/10.1109/TDEI.2013.6571434.

[133] X. Miao, S. Yin, Z. Shao, Y. Zhang, X. Chen, Nanosecond pulsed electric field inhibits proliferation and induces apoptosis in human osteosarcoma, J. Orthop. Surg. Res. 10 (2015) 104, https://doi.org/10.1186/s13018-015-0247-z.

[134] K. Morotomi-Yano, H. Akiyama, K.-I. Yano, Nanosecond pulsed electric fields induce poly(ADP-ribose) formation and non-apoptotic cell death in HeLa S3 cells, Biochem. Biophys. Res. Commun. (2013), https://doi.org/10.1016/j.bbrc.2013.07.083.

[135] Y. Guo, Y. Zhang, R. Klein, G.M. Nijm, A.V. Sahakian, R.A. Omary, G.-Y. Yang, A. C. Larson, Liver-directed irreversible electroporation therapy: longitudinal efficacy studies in a rat model of hepatocellular carcinoma, Cancer Res. 70 (2010) 1555, https://doi.org/10.1158/0008-5472.CAN-09-3067.

[136] C.B. Arena, M.B. Sano, J.H. Rossmeisl, J.L. Caldwell, P.A. Garcia, M.N. Rylander, R.V. Davalos, High-frequency irreversible electroporation (H-FIRE) for non-thermal ablation without muscle contraction, Biomed. Eng. Online. 10 (2011) 102, https://doi.org/10.1186/1475-925X-10-102.

[137] A. Josè, L. Sobrevals, A. Ivorra, C. Fillat, Irreversible electroporation shows efficacy against pancreatic carcinoma without systemic toxicity in mouse models, Cancer Lett. 317 (2012) 16–23, https://doi.org/10.1016/j.canlet.2011.11.004.

[138] M. Faroja, M. Ahmed, L. Appelbaum, E. Ben-David, M. Moussa, J. Sosna, I. Nissenbaum, S.N. Goldberg, Irreversible electroporation ablation: is all the damage nonthermal?, Radiology 266 (2013) 462–470, https://doiorg/10.1148/radiol.12120609.

[139] L. Appelbaum, E. Ben-David, M. Faroja, Y. Nissenbaum, J. Sosna, S.N. Goldberg, Irreversible electroporation ablation: creation of large-volume ablation zones in in vivo porcine liver with four-electrode arrays, Radiology 270 (2013) 416–424, https://doi.org/10.1148/radiol.13130349.

[140] E. Ben-David, M. Ahmed, M. Faroja, M. Moussa, A. Wandel, J. Sosna, L. Appelbaum, I. Nissenbaum, S.N. Goldberg, Irreversible electroporation: treatment effect is susceptible to local environment and tissue properties, Radiology 269 (2013) 738–747, https://doi.org/10.1148/radiol.13122590.

[141] Z. Zhang, W. Li, D. Procissi, P. Tyler, R.A. Omary, A.C. Larson, Rapid dramatic alterations to the tumor microstructure in pancreatic cancer following irreversible electroporation ablation, Nanomedicine (Lond). 9 (2014) 1181–1192, https://doi.org/10.2217/nnm.13.72.

[142] W. Zhang, W. Wang, W. Chai, X. Luo, J. Li, J. Shi, L. Bi, L. Niu, Breast tissue ablation with irreversible electroporation in rabbits: a safety and feasibility study, PLoS ONE 12 (2017), https://doi.org/10.1371/journal.pone.0181555 e0181555.

[143] H.J. Scheffer, K. Nielsen, A. a. J.M. van Tilborg, J.M. Vieveen, R.A. Bouwman, G. Kazemier, H.W.M. Niessen, S. Meijer, C. van Kuijk, M.P. van den Tol, M.R. Meijerink, Ablation of colorectal liver metastases by irreversible electroporation: results of the COLDFIRE-I ablate-and-resect study, Eur Radiol. 24 (2014) 2467–2475. https://doi.org/10.1007/s00330-014-3259-x.

[144] W. Chai, W. Zhang, Z. Wei, Y. Xu, J. Shi, X. Luo, J. Zeng, M. Cui, J. Li, L. Niu, Irreversible electroporation of the uterine cervix in a rabbit model, Biomed. Microdevices. 19 (2017) 103, https://doi.org/10.1007/s10544-017-0248-2.

[145] B. López-Alonso, A. Hernáez, H. Sarnago, A. Naval, A. Güemes, C. Junquera, J. M. Burdío, T. Castiella, E. Monleón, J. Gracia-Llanes, F. Burdío, E. Mejía, O. Lucía, Histopathological and ultrastructural changes after electroporation in pig liver using parallel-plate electrodes and high-performance generator, Sci. Rep. 9 (2019) 2647, https://doi.org/10.1038/s41598-019-39433-6.

[146] J.F. Edd, L. Horowitz, R.V. Davalos, L.M. Mir, B. Rubinsky, In vivo results of a new focal tissue ablation technique: irreversible electroporation, IEEE Trans. Biomed. Eng. 53 (2006) 1409–1415, https://doi.org/10.1109/TBME.2006.873745.

[147] S. Hu, C. Sun, B. Wang, K. Zhou, L. Pan, J. Shangguan, J. Yang, V. Yaghmai, M. Figini, Z. Zhang, Diffusion-weighted MR imaging to evaluate immediate response to irreversible electroporation in a rabbit VX2 Liver tumor model, J. Vasc. Interv. Radiol. 30 (2019) 1863–1869, https://doi.org/10.1016/j.jvir.2019.05.030.

[148] X. Luo, X. Liang, J. Li, J. Shi, W. Zhang, W. Chai, J. Wu, S. Guo, G. Fang, X. Zhou, J. Zhang, K. Xu, J. Zeng, L. Niu, The effects of irreversible electroporation on the colon in a porcine model, PLoS ONE 11 (2016), https://doi.org/10.1371/journal.pone.0167275 e0167275.

[149] W. Zhang, W. Chai, J. Zeng, J. Chen, L. Bi, L. Niu, Irreversible electroporation for the treatment of rabbit VX2 breast cancer, Biomed. Microdevices. 19 (2017) 29, https://doi.org/10.1007/s10544-017-0173-4.

[150] A. Sugrue, V. Vaidya, C. Witt, C.V. DeSimone, O. Yasin, E. Maor, A.M. Killu, S. Kapa, C.J. McLeod, D. Miklavčič, S.J. Asirvatham, Irreversible electroporation for catheter-based cardiac ablation: a systematic review of the preclinical experience, J. Interv. Card Electrophysiol. 55 (2019) 251–265, https://doi.org/10.1007/s10840-019-00574-3.

[151] A. Horn, J.K. Jaiswal, Cellular mechanisms and signals that coordinate plasma membrane repair, Cell Mol. Life Sci. 75 (2018) 3751–3770, https://doi.org/10.1007/s00018-018-2888-7.

[152] M. Corrotte, T. Castro-Gomes, Chapter One - Lysosomes and plasma membrane repair, in: L.O. Andrade (Ed.), Current Topics in Membranes, Academic Press, 2019, pp. 1–16, https://doi.org/10.1016/bs.ctm.2019.08.001.

[153] S.T. Cooper, P.L. McNeil, Membrane Repair: Mechanisms and Pathophysiology, Physiol. Rev. 95 (2015) 1205–1240, https://doi.org/10.1152/physrev.00037.2014.

[154] A.J. Jimenez, F. Perez, Plasma membrane repair: the adaptable cell life-insurance, Curr Opin Cell Biol. 47 (2017) 99–107, https://doi.org/10.1016/j.ceb.2017.03.011.

[155] N.W. Andrews, M. Corrotte, Plasma membrane repair, Curr. Biol. 28 (2018) R392–R397, https://doi.org/10.1016/j.cub.2017.12.034.

[156] A.D. Blazek, B.J. Paleo, N. Weisleder, Plasma Membrane Repair: A Central Process for Maintaining Cellular Homeostasis, Physiol. (Bethesda). 30 (2015) 438–448, https://doi.org/10.1152/physiol.00019.2015.

[157] A.J. Jimenez, F. Perez, Physico-chemical and biological considerations for membrane wound evolution and repair in animal cells, Semin. Cell Dev. Biol. 45 (2015) 2–9, https://doi.org/10.1016/j.semcdb.2015.09.023.

[158] A. Reddy, E.V. Caler, N.W. Andrews, Plasma membrane repair is mediated by Ca2+-regulated exocytosis of lysosomes, Cell 106 (2001) 157–169, https://doi.org/10.1016/S0092-8674(01)00421-4.

[159] K. Kinosita, T.Y. Tsong, Formation and resealing of pores of controlled sizes in human erythrocyte membrane, Nature 268 (1977) 438–441, https://doi.org/10.1038/268438a0.

[160] G. Saulis, The loading of human erythrocytes with small molecules by electroporation, Cell Mol. Biol. Lett. 10 (2005) 23–35.

[161] G. Saulis, M.S. Venslauskas, J. Naktinis, Kinetics of pore resealing in cell membranes after electroporation, J. Electroanal. Chem. Interfacial Electrochem. 321 (1991) 1–13, https://doi.org/10.1016/0022-0728(91)85564-6.

[162] L.V. Chernomordik, S.I. Sukharev, S.V. Popov, V.F. Pastushenko, A.V. Sokirko, I. G. Abidor, Y.A. Chizmadzhev, The electrical breakdown of cell and lipid membranes: the similarity of phenomenologies, Biochimica et Biophysica Acta (BBA) - Biomembranes. 902 (1987) 360–373, https://doi.org/10.1016/0005-2736(87)90204-5.

[163] Y. Demiryurek, M. Nickaeen, M. Zheng, M. Yu, J.D. Zahn, D.I. Shreiber, H. Lin, J. W. Shan, Transport, resealing, and re-poration dynamics of two-pulse electroporation-mediated molecular delivery, Biochimica et Biophysica Acta (BBA) - Biomembranes. 1848 (2015) 1706–1714, https://doi.org/10.1016/j.bbamem.2015.04.007.

[164] T. Kotnik, P. Kramar, G. Pucihar, D. Miklavcic, M. Tarek, Cell membrane electroporation- Part 1: The phenomenon, IEEE Electr. Insul. Mag. 28 (2012) 14–23, https://doi.org/10.1109/MEI.2012.6268438.

[165] M. Pavlin, D. Miklavcic, Theoretical and experimental analysis of conductivity, ion diffusion and molecular transport during cell electroporation–relation between short-lived and long-lived pores, Bioelectrochemistry 74 (2008) 38–46, https://doi.org/10.1016/j.bioelechem.2008.04.016.

[166] G. Pucihar, T. Kotnik, D. Miklavčič, J. Teissié, Kinetics of transmembrane transport of small molecules into electropermeabilized cells, Biophys. J . 95 (2008) 2837–2848, https://doi.org/10.1529/biophysj.108.135541.

[167] H. He, D.C. Chang, Y.-K. Lee, Nonlinear current response of micro electroporation and resealing dynamics for human cancer cells, Bioelectrochemistry 72 (2008) 161–168, https://doi.org/10.1016/j.bioelechem.2008.01.007.

[168] M. Hibino, H. Itoh, K. Kinosita, Time courses of cell electroporation as revealed by submicrosecond imaging of transmembrane potential, Biophys J. 64 (1993) 1789–1800.

[169] K. Kinosita, I. Ashikawa, N. Saita, H. Yoshimura, H. Itoh, K. Nagayama, A. Ikegami, Electroporation of cell membrane visualized under a pulsed-laser fluorescence microscope, Biophys. J. 53 (1988) 1015–1019.
[170] G. Pucihar, T. Kotnik, J. Teissié, D. Miklavcic, Electropermeabilization of dense cell suspensions, Eur. Biophys. J. 36 (2007) 173–185, https://doi.org/10.1007/s00249-006-0115-1.
[171] M.P. Rols, J. Teissié, Electropermeabilization of mammalian cells to macromolecules: control by pulse duration, Biophys. J. 75 (1998) 1415–1423.
[172] G. Saulis, R. Saulė, Size of the pores created by an electric pulse: Microsecond vs millisecond pulses, Biochimica et Biophysica Acta (BBA) - Biomembranes. 1818 (2012) 3032–3039, https://doi.org/10.1016/j.bbamem.2012.06.018.
[173] A.E. Sowers, Fusion events and nonfusion contents mixing events induced in erythrocyte ghosts by an electric pulse, Biophys. J. 54 (1988) 619–626, https://doi.org/10.1016/S0006-3495(88)82997-7.
[174] J.C. Weaver, P.T. Vernier, Pore lifetimes in cell electroporation: Complex dark pores?, ArXiv:1708.07478 [Physics]. (2017). http://arxiv.org/abs/1708.07478 (accessed January 13, 2021).
[175] R.W. Glaser, S.L. Leikin, L.V. Chernomordik, V.F. Pastushenko, A.I. Sokirko, Reversible electrical breakdown of lipid bilayers: formation and evolution of pores, Biochimica et Biophysica Acta (BBA) - Biomembranes. 940 (1988) 275–287, https://doi.org/10.1016/0005-2736(88)90202-7.
[176] G. Saulis, Kinetics of pore disappearance in a cell after electroporation, Biomed. Sci. Instrum. 35 (1999) 409–414.
[177] A.M. Bowman, O.M. Nesin, O.N. Pakhomova, A.G. Pakhomov, Analysis of plasma membrane integrity by fluorescent detection of Tl+ uptake, J. Membrane Biol. 236 (2010) 15–26, https://doi.org/10.1007/s00232-010-9269-y.
[178] Z.A. Levine, P.T. Vernier, Life cycle of an electropore: field-dependent and field-independent steps in pore creation and annihilation, J. Membr. Biol. 236 (2010) 27–36, https://doi.org/10.1007/s00232-010-9277-y.
[179] F. Ciobanu, M. Golzio, E. Kovacs, J. Teissié, Control by low levels of calcium of mammalian cell membrane electropermeabilization, J. Membrane Biol. 251 (2018) 221–228, https://doi.org/10.1007/s00232-017-9981-y.
[180] J. Dermol, O.N. Pakhomova, A.G. Pakhomov, D. Miklavčič, Cell Electrosensitization Exists Only in Certain Electroporation Buffers, PLoS ONE 11 (2016), https://doi.org/10.1371/journal.pone.0159434.
[181] C.S. Djuzenova, U. Zimmermann, H. Frank, V.L. Sukhorukov, E. Richter, G. Fuhr, Effect of medium conductivity and composition on the uptake of propidium iodide into electropermeabilized myeloma cells, Biochim. Biophys. Acta. 1284 (1996) 143–152.
[182] D. Navickaite, P. Ruzgys, V. Novickij, M. Jakutaviciute, M. Maciulevicius, R. Sinceviciute, S. Satkauskas, Extracellular-Ca2+-induced decrease in small molecule electrotransfer efficiency: comparison between microsecond and nanosecond electric pulses, Pharmaceutics. 12 (2020), https://doi.org/10.3390/pharmaceutics12050422.
[183] R.R. Swezey, D. Epel, Stable, resealable pores formed in sea urchin eggs by electric discharge (electroporation) permit substrate loading for assay of enzymes in vivo, Cell Regul. 1 (1989) 65–74, https://doi.org/10.1091/mbc.1.1.65.
[184] B. Jakstys, M. Jakutaviciute, D. Uzdavinyte, I. Satkauskiene, S. Satkauskas, Correlation between the loss of intracellular molecules and cell viability after cell electroporation, Bioelectrochemistry 135 (2020), https://doi.org/10.1016/j.bioelechem.2020.107550 107550.
[185] M.P. Rols, J. Teissie, Modulation of electrically induced permeabilization and fusion of Chinese hamster ovary cells by osmotic pressure, Biochemistry 29 (1990) 4561–4567, https://doi.org/10.1021/bi00471a009.
[186] R.P. Joshi, K.H. Schoenbach, Electroporation dynamics in biological cells subjected to ultrafast electrical pulses: a numerical simulation study, Phys. Rev. E. 62 (2000) 1025–1033, https://doi.org/10.1103/PhysRevE.62.1025.
[187] S.M. Kennedy, Z. Ji, N.B. Rockweiler, A.R. Hahn, J.H. Booske, S.C. Hagness, The Role of Plasmalemmal-Cortical Anchoring on the Stability of Transmembrane Electropores, IEEE Trans Dielectr Electr Insul. 16 (2009) 1251–1258, https://doi.org/10.1109/TDEI.2009.5293935.
[188] D.V. Zhelev, D. Needham, Tension-stabilized pores in giant vesicles: determination of pore size and pore line tension, Biochim. Biophys. Acta. 1147 (1993) 89–104.
[189] D.L. Perrier, A. Vahid, V. Kathavi, L. Stam, L. Rems, Y. Mulla, A. Muralidharan, G.H. Koenderink, M.T. Kreutzer, P.E. Boukany, Response of an actin network in vesicles under electric pulses, Sci. Rep. 9 (2019) 8151, https://doi.org/10.1038/s41598-019-44613-5.
[190] H. Krassen, U. Pliquett, E. Neumann, Nonlinear current–voltage relationship of the plasma membrane of single CHO cells, Bioelectrochemistry 70 (2007) 71–77, https://doi.org/10.1016/j.bioelechem.2006.03.033.
[191] A. Hai, M.E. Spira, On-chip electroporation, membrane repair dynamics and transient in-cell recordings by arrays of gold mushroom-shaped microelectrodes, Lab Chip. 12 (2012) 2865–2873, https://doi.org/10.1039/C2LC40091J.
[192] C. Huynh, D. Roth, D.M. Ward, J. Kaplan, N.W. Andrews, Defective lysosomal exocytosis and plasma membrane repair in Chediak–Higashi/beige cells, Proc. Natl. Acad. Sci. U S A. 101 (2004) 16795–16800, https://doi.org/10.1073/pnas.0405905101.
[193] C. Huynh, N.W. Andrews, The small chemical vacuolin-1 alters the morphology of lysosomes without inhibiting Ca2+-regulated exocytosis, EMBO Rep. 6 (2005) 843–847, https://doi.org/10.1038/sj.embor.7400495.
[194] A.J. Jimenez, P. Maiuri, J. Lafaurie-Janvore, S. Divoux, M. Piel, F. Perez, ESCRT Machinery Is Required for Plasma Membrane Repair, Science 343 (2014), https://doi.org/10.1126/science.1247136.
[195] X. Ding, M.P. Stewart, A. Sharei, J.C. Weaver, R.S. Langer, K.F. Jensen, High-throughput nuclear delivery and rapid expression of DNA via mechanical and electrical cell–membrane disruption, Nat. Biomed. Eng. 1 (2017) 1–7, https://doi.org/10.1038/s41551-017-0039.
[196] J.M. la Cour, P. Winding Gojkovic, S.E.B. Ambjørner, J. Bagge, S.M. Jensen, S. Panina, M.W. Berchtold, ALG-2 participates in recovery of cells after plasma membrane damage by electroporation and digitonin treatment, PLoS ONE 13 (2018), https://doi.org/10.1371/journal.pone.0204520.
[197] T. Potočnik, D. Miklavčič, A. Maček Lebar, Effect of electroporation and recovery medium pH on cell membrane permeabilization, cell survival and gene transfer efficiency in vitro, Bioelectrochemistry 130 (2019), https://doi.org/10.1016/j.bioelechem.2019.107342 107342.
[198] S.K. Frandsen, A.K. McNeil, I. Novak, P.L. McNeil, J. Gehl, Difference in membrane repair capacity between cancer cell lines and a normal cell line, J. Membr. Biol. 249 (2016) 569–576, https://doi.org/10.1007/s00232-016-9910-5.
[199] T. Togo, J.M. Alderton, G.Q. Bi, R.A. Steinhardt, The mechanism of facilitated cell membrane resealing, J. Cell Sci. 112 (1999) 719–731.
[200] O.N. Pakhomova, B.W. Gregory, V.A. Khorokhorina, A.M. Bowman, S. Xiao, A. G. Pakhomov, Electroporation-induced electrosensitization, PLoS ONE 6 (2011), https://doi.org/10.1371/journal.pone.0017100.
[201] A. Silve, A. Guimerà Brunet, B. Al-Sakere, A. Ivorra, L.M. Mir, Comparison of the effects of the repetition rate between microsecond and nanosecond pulses: Electropermeabilization-induced electro-desensitization?, Biochimica et Biophysica Acta (BBA) - General Subjects. 1840 (2014) 2139–2151, https://doi.org/10.1016/j.bbagen.2014.02.011.
[202] B.L. Ibey, D.G. Mixon, J.A. Payne, A. Bowman, K. Sickendick, G.J. Wilmink, W.P. Roach, A.G. Pakhomov, Plasma membrane permeabilization by trains of ultrashort electric pulses, Bioelectrochemistry 79 (2010) 114–121, https://doi.org/10.1016/j.bioelechem.2010.01.001.
[203] G.L. Thompson, C.C. Roth, D.R. Dalzell, M. Kuipers, B.L. Ibey, Calcium influx affects intracellular transport and membrane repair following nanosecond pulsed electric field exposure, J. Biomed. Opt. 19 (2014), https://doi.org/10.1117/1.JBO.19.5.055005 055005.
[204] G.L. Thompson, H.T. Beier, B.L. Ibey, tracking lysosome migration within chinese hamster ovary (cho) cells following exposure to nanosecond pulsed electric fields, Bioengineering (Basel). 5 (2018), https://doi.org/10.3390/bioengineering5040103.
[205] N. Bhutiani, S. Agle, Y. Li, S. Li, R.C.G. Martin, Irreversible electroporation enhances delivery of gemcitabine to pancreatic adenocarcinoma, J. Surg. Oncol. 114 (2016) 181–186, https://doi.org/10.1002/jso.24288.
[206] K.N. Aycock, R.V. Davalos, Irreversible electroporation: background theory, and review of recent developments in clinical oncology, Bioelectricity. 1 (2019) 214–234, https://doi.org/10.1089/bioe.2019.0029.
[207] R.E. Neal, J.H. Rossmeisl, V. D'Alfonso, J.L. Robertson, P.A. Garcia, S. Elankumaran, R.V. Davalos, In vitro and numerical support for combinatorial irreversible electroporation and electrochemotherapy glioma treatment, Ann. Biomed. Eng. 42 (2014) 475–487, https://doi.org/10.1007/s10439-013-0923-2.
[208] S. Elmore, Apoptosis: a review of programmed cell death, Toxicol. Pathol. 35 (2007) 495–516, https://doi.org/10.1080/01926230701320337.
[209] F. Izzo, F. Ionna, V. Granata, V. Albino, R. Patrone, F. Longo, A. Guida, P. Delrio, D. Rega, D. Scala, R. Pezzuto, R. Fusco, E. Di Bernardo, V. D'Alessio, R. Grassi, D. Contartese, R. Palaia, New deployable expandable electrodes in the electroporation treatment in a pig model: a feasibility and usability preliminary study, Cancers (Basel). 12 (2020), https://doi.org/10.3390/cancers12020515.
[210] C.R. Tracy, W. Kabbani, J.A. Cadeddu, Irreversible electroporation (IRE): a novel method for renal tissue ablation, BJU Int. 107 (2011) 1982–1987, https://doi.org/10.1111/j.1464-410X.2010.09797.x.
[211] T.J. O'Brien, M. Passeri, M.F. Lorenzo, J.K. Sulzer, W.B. Lyman, J.H. Swet, D. Vrochides, E.H. Baker, D.A. Iannitti, R.V. Davalos, I.H. McKillop, Experimental high-frequency irreversible electroporation using a single-needle delivery approach for nonthermal pancreatic ablation in vivo, J. Vasc. Interv. Radiol. 30 (2019) 854–862.e7, https://doi.org/10.1016/j.jvir.2019.01.032.
[212] I.A. Siddiqui, E.L. Latouche, M.R. DeWitt, J.H. Swet, R.C. Kirks, E.H. Baker, D.A. Iannitti, D. Vrochides, R.V. Davalos, I.H. McKillop, Induction of rapid, reproducible hepatic ablations using next-generation, high frequency irreversible electroporation (H-FIRE) in vivo, HPB (Oxford). 18 (2016) 726–734, https://doi.org/10.1016/j.hpb.2016.06.015.
[213] D. Miklavčič, D. Šemrov, H. Mekid, L.M. Mir, A validated model of in vivo electric field distribution in tissues for electrochemotherapy and for DNA electrotransfer for gene therapy, Biochimica et Biophysica Acta (BBA) - General Subjects. 1523 (2000) 73–83, https://doi.org/10.1016/S0304-4165(00)00101-X.
[214] O. Tounekti, A. Kenani, N. Foray, S. Orlowski, L.M. Mir, The ratio of single- to double-strand DNA breaks and their absolute values determine cell death pathway, Br. J. Cancer 84 (2001) 1272–1279, https://doi.org/10.1054/bjoc.2001.1786.
[215] O. Tounekti, G. Pron, J. Belehradek, L.M. Mir, Bleomycin, an apoptosis-mimetic drug that induces two types of cell death depending on the number of molecules internalized, Cancer Res. 53 (1993) 5462–5469.

[216] Y. Lv, Y. Zhang, B. Rubinsky, Molecular and histological study on the effects of electrolytic electroporation on the liver, Bioelectrochemistry 125 (2019) 79–89, https://doi.org/10.1016/j.bioelechem.2018.09.007.
[217] N. Matsuki, M. Takeda, T. Ishikawa, A. Kinjo, T. Hayasaka, Y. Imai, T. Yamaguchi, Activation of caspases and apoptosis in response to low-voltage electric pulses, Oncol. Rep. 23 (2010) 1425–1433, https://doi.org/10.3892/or_00000780.
[218] D.M. Cvetković, M.N. Živanović, M.G. Milutinović, T.R. Djukić, M.D. Radović, A. M. Cvetković, N.D. Filipović, N.D. Zdravković, Real-time monitoring of cytotoxic effects of electroporation on breast and colon cancer cell lines, Bioelectrochemistry 113 (2017) 85–94, https://doi.org/10.1016/j.bioelechem.2016.10.005.
[219] K.P. Charpentier, F. Wolf, L. Noble, B. Winn, M. Resnick, D.E. Dupuy, Irreversible electroporation of the pancreas in swine: a pilot study, HPB (Oxford). 12 (2010) 348–351, https://doi.org/10.1111/j.1477-2574.2010.00174.x.
[220] X. Chen, Z. Ren, T. Zhu, X. Zhang, Z. Peng, H. Xie, L. Zhou, S. Yin, J. Sun, S. Zheng, Electric Ablation with Irreversible Electroporation (IRE) in Vital Hepatic Structures and Follow-up Investigation, Sci. Rep. 5 (2015) 16233, https://doi.org/10.1038/srep16233.
[221] A. Deodhar, S. Monette, G.W. Single, W.C. Hamilton, R.H. Thornton, C.T. Sofocleous, M. Maybody, S.B. Solomon, Percutaneous irreversible electroporation lung ablation: preliminary results in a porcine model, Cardiovasc Intervent Radiol. 34 (2011) 1278–1287, https://doi.org/10.1007/s00270-011-0143-9.
[222] C.R. Schmidt, P. Shires, M. Mootoo, Real-time ultrasound imaging of irreversible electroporation in a porcine liver model adequately characterizes the zone of cellular necrosis, HPB (Oxford). 14 (2012) 98–102, https://doi.org/10.1111/j.1477-2574.2011.00409.x.
[223] W. van den Bos, R.R. Jurhill, D.M. de Bruin, C.D. Savci-Heijink, A.W. Postema, P.G.K. Wagstaff, B.G. Muller, I.M. Varkarakis, A. Skolarikos, P.J. Zondervan, M. P. Laguna Pes, T.M. de Reijke, J.J.M.C.H. de la Rosette, Histopathological Outcomes after Irreversible Electroporation for Prostate Cancer: Results of an Ablate and Resect Study, J. Urol. 196 (2016) 552–559. https://doi.org/10.1016/j.juro.2016.02.2977.
[224] R. Nuccitelli, Tissue Ablation Using Nanosecond Electric Pulses, in: D. Miklavčič (Ed.), Handbook of Electroporation, Springer International Publishing, Cham, 2017, pp. 1787–1797, https://doi.org/10.1007/978-3-319-32886-7_93.
[225] S.J. Beebe, X. Chen, J.A. Liu, K.H. Schoenbach, Nanosecond pulsed electric field ablation of hepatocellular carcinoma, in: 2011 Annual International Conference of the IEEE Engineering in Medicine and Biology Society, EMBC, 2011: pp. 6861–6865. https://doi.org/10.1109/IEMBS.2011.6091692.
[226] R. Chen, N.M. Sain, K.T. Harlow, Y.-J. Chen, P.K. Shires, R. Heller, S.J. Beebe, A protective effect after clearance of orthotopic rat hepatocellular carcinoma by nanosecond pulsed electric fields, Eur. J. Cancer 50 (2014) 2705–2713, https://doi.org/10.1016/j.ejca.2014.07.006.
[227] Y. Mi, C. Yao, C. Li, C. Sun, L. Tang, H. Liu, Apoptosis induction effects of steep pulsed electric fields (SPEF) on human liver cancer cell SMMC-7721 in vitro, IEEE Trns. Dielectr. Electr. Insul. 16 (2009) 1302–1310.
[228] P.T. Vernier, Y. Sun, M.A. Gundersen, Nanoelectropulse-driven membrane perturbation and small molecule permeabilization, BMC Cell Biol. 7 (2006) 37, https://doi.org/10.1186/1471-2121-7-37.
[229] P.T. Vernier, Y. Sun, L. Marcu, C.M. Craft, M.A. Gundersen, Nanoelectropulse-induced phosphatidylserine translocation, Biophys. J . 86 (2004) 4040–4048, https://doi.org/10.1529/biophysj.103.037945.
[230] T. Batista Napotnik, D. Miklavčič, In vitro electroporation detection methods – an overview, Bioelectrochemistry 120 (2018) 166–182, https://doi.org/10.1016/j.bioelechem.2017.12.005.
[231] M.A. Mahlke, G. Thompson, L. Estlack, C. Navara, B.L. Ibey, Effects of nanosecond electrical pulses (nsPEFs) on cell cycle progression and susceptibility at various phases, in: G.J. Wilmink, B.L. Ibey (Eds.), Proc. SPIE 8585, Terahertz and Ultrashort Electromagnetic Pulses for Biomedical Applications, (2013) p. 858500. https://doi.org/10.1117/12.2020679.
[232] S. Nowsheen, E.S. Yang, The intersection between DNA damage response and cell death pathways, Exp. Oncol. 34 (2012) 243–254.
[233] D. Miklavčič, B. Mali, B. Kos, R. Heller, G. Serša, Electrochemotherapy: from the drawing board into medical practice, Biomed. Eng. Online. 13 (2014) 29, https://doi.org/10.1186/1475-925X-13-29.
[234] G. Sersa, M. Bosnjak, M. Cemazar, R. Heller, Preclinical Studies on Electrochemotherapy, in: D. Miklavcic (Ed.), Handbook of Electroporation, Springer International Publishing, Cham, 2016, pp. 1–15, https://doi.org/10.1007/978-3-319-26779-1_45-1.
[235] L. Bigi, G. Galdo, A.M. Cesinaro, C. Vaschieri, A. Marconi, C. Pincelli, F. Fantini, Electrochemotherapy induces apoptotic death in melanoma metastases: a histologic and immunohistochemical investigation, Clin. Cosmet. Investig. Dermatol. 9 (2016) 451–459, https://doi.org/10.2147/CCID.S115984.
[236] O. Tounekti, J. Belehradek, L.M. Mir, Relationships between DNA fragmentation, chromatin condensation, and changes in flow cytometry profiles detected during apoptosis, Exp. Cell Res. 217 (1995) 506–516, https://doi.org/10.1006/excr.1995.1116.
[237] P. Fernandes, T.R. O'Donovan, S.L. McKenna, P.F. Forde, Electrochemotherapy Causes Caspase-Independent Necrotic-Like Death in Pancreatic Cancer Cells, Cancers (Basel). 11 (2019), https://doi.org/10.3390/cancers11081177.
[238] H. Mekid, O. Tounekti, A. Spatz, M. Cemazar, F.Z. El Kebir, L.M. Mir, In vivo evolution of tumour cells after the generation of double-strand DNA breaks, Br. J. Cancer 88 (2003) 1763–1771, https://doi.org/10.1038/sj.bjc.6600959.
[239] E. Cohen-Jonathan, E.J. Bernhard, W.G. McKenna, How does radiation kill cells?, Curr. Opin. Chem. Biol. 3 (1999) 77–83, https://doi.org/10.1016/S1367-5931(99)80014-3.
[240] J.G. Nirmala, M. Lopus, Cell death mechanisms in eukaryotes, Cell Biol Toxicol. 36 (2020) 145–164, https://doi.org/10.1007/s10565-019-09496-2.
[241] S.Y. Proskuryakov, A.G. Konoplyannikov, V.L. Gabai, Necrosis: a specific form of programmed cell death?, Exp. Cell Res. 283 (2003) 1–16, https://doi.org/10.1016/s0014-4827(02)00027-7.
[242] N. Festjens, T. Vanden Berghe, P. Vandenabeele, Necrosis, a well-orchestrated form of cell demise: Signalling cascades, important mediators and concomitant immune response, Biochimica et Biophysica Acta (BBA) –, Bioenergetics. 1757 (2006) 1371–1387, https://doi.org/10.1016/j.bbabio.2006.06.014.
[243] T. Qian, B. Herman, J.J. Lemasters, The Mitochondrial Permeability Transition Mediates Both Necrotic and Apoptotic Death of Hepatocytes Exposed to Br-A23187, Toxicol. Appl. Pharmacol. 154 (1999) 117–125, https://doi.org/10.1006/taap.1998.8580.
[244] E.L. Latouche, C.B. Arena, J.W. Ivey, P.A. Garcia, T.E. Pancotto, N. Pavlisko, S.S. Verbridge, R.V. Davalos, J.H. Rossmeisl, High-Frequency Irreversible Electroporation for Intracranial Meningioma: A Feasibility Study in a Spontaneous Canine Tumor Model, Technol Cancer Res Treat. 17 (2018) 1533033818785285. https://doi.org/10.1177/1533033818785285.
[245] J. Zmuc, G. Gasljevic, G. Sersa, I. Edhemovic, N. Boc, A. Seliskar, T. Plavec, M. Brloznik, N. Milevoj, E. Brecelj, B. Kos, J. Izlakar, T. Jarm, M. Snoj, M. Stukelj, D. Miklavcic, M. Cemazar, Large Liver Blood Vessels and Bile Ducts Are Not Damaged by Electrochemotherapy with Bleomycin in Pigs, Sci. Rep. 9 (2019) 3649, https://doi.org/10.1038/s41598-019-40395-y.
[246] A. Rossi, O.N. Pakhomova, A.G. Pakhomov, S. Weygandt, A.A. Bulysheva, L.E. Murray, P.A. Mollica, C. Muratori, Mechanisms and immunogenicity of nsPEF-induced cell death in B16F10 melanoma tumors, Sci. Rep. 9 (2019) 431, https://doi.org/10.1038/s41598-018-36527-5.
[247] C. Yao, Y. Mi, X. Hu, C. Li, C. Sun, J. Tang, Xiaojuan Wu, Experiment and mechanism research of SKOV3 cancer cell apoptosis induced by nanosecond pulsed electric field, in: 30th Annual International Conference of the IEEE Engineering in Medicine and Biology Society, 2008. EMBS 2008, 2008: pp. 1044–1047. https://doi.org/10.1109/IEMBS.2008.4649338.
[248] J. Lavee, G. Onik, P. Mikus, B. Rubinsky, A novel nonthermal energy source for surgical epicardial atrial ablation: irreversible electroporation, Heart Surg. Forum. 10 (2007) E162–E167, https://doi.org/10.1532/HSF98.20061202.
[249] V. Novickij, R. Česna, E. Perminaitė, A. Zinkevičienė, D. Characiejus, J. Novickij, S. Šatkauskas, P. Ruzgys, I. Girkontaitė, Antitumor Response and Immunomodulatory Effects of Sub-Microsecond Irreversible Electroporation and Its Combination with Calcium Electroporation, Cancers (Basel). 11 (2019), https://doi.org/10.3390/cancers11111763.
[250] E.M. Wasson, N. Alinezhadbalalami, R.M. Brock, I.C. Allen, S.S. Verbridge, R.V. Davalos, Understanding the role of calcium-mediated cell death in high-frequency irreversible electroporation, Bioelectrochemistry 131 (2020), https://doi.org/10.1016/j.bioelechem.2019.107369 107369.
[251] A. Szewczyk, J. Gehl, M. Daczewska, J. Saczko, S.K. Frandsen, J. Kulbacka, Calcium electroporation for treatment of sarcoma in preclinical studies, Oncotarget. 9 (2018) 11604–11618. https://doi.org/10.18632/oncotarget.24352.
[252] A. Zielichowska, M. Daczewska, J. Saczko, O. Michel, J. Kulbacka, Applications of calcium electroporation to effective apoptosis induction in fibrosarcoma cells and stimulation of normal muscle cells, Bioelectrochemistry 109 (2016) 70–78, https://doi.org/10.1016/j.bioelechem.2016.01.005.
[253] S.K. Frandsen, M.B. Krüger, U.M. Mangalanathan, T. Tramm, F. Mahmood, I. Novak, J. Gehl, Normal and malignant cells exhibit differential responses to calcium electroporation, Cancer Res. 77 (2017) 4389–4401, https://doi.org/10.1158/0008-5472.CAN-16-1611.
[254] S.K. Frandsen, L. Gibot, M. Madi, J. Gehl, M.-P. Rols, Calcium electroporation: evidence for differential effects in normal and malignant cell lines, evaluated in a 3D spheroid model, PLoS ONE 10 (2015), https://doi.org/10.1371/journal.pone.0144028 e0144028.
[255] A.D. Garg, A.M. Dudek-Peric, E. Romano, P. Agostinis, Immunogenic cell death, Int. J. Dev. Biol. 59 (2015) 131–140, https://doi.org/10.1387/ijdb.150061pa.
[256] C. He, X. Huang, Y. Zhang, X. Lin, S. Li, T-cell activation and immune memory enhancement induced by irreversible electroporation in pancreatic cancer, Clin. Transl. Med. 10 (2020), https://doi.org/10.1002/ctm2.39.
[257] H. Pandit, Y.K. Hong, Y. Li, J. Rostas, Z. Pulliam, S.P. Li, R.C.G. Martin, Evaluating the Regulatory Immunomodulation Effect of Irreversible Electroporation (IRE) in Pancreatic Adenocarcinoma, Ann. Surg. Oncol. 26 (2019) 800–806, https://doi.org/10.1245/s10434-018-07144-3.
[258] H.J. Scheffer, A.G.M. Stam, B. Geboers, L.G.P.H. Vroomen, A. Ruarus, B. de Bruijn, M.P. van den Tol, G. Kazemier, M.R. Meijerink, T.D. de Gruijl, Irreversible electroporation of locally advanced pancreatic cancer transiently alleviates immune suppression and creates a window for antitumor T cell activation, OncoImmunology. 8 (2019) 1652532, https://doi.org/10.1080/2162402X.2019.1652532.
[259] J.A. Vogel, E. van Veldhuisen, L.K. Alles, O.R. Busch, F. Dijk, T.M. van Gulik, G. M. Huijzer, M.G. Besselink, K.P. van Lienden, J. Verheij, Time-Dependent Impact of Irreversible Electroporation on Pathology and Ablation Size in the

Porcine Liver: A 24-Hour Experimental Study, Technol. Cancer Res. Treat. 18 (2019), https://doi.org/10.1177/1533033819876899.
[260] S.B. White, Z. Zhang, J. Chen, V.R. Gogineni, A.C. Larson, Early Immunologic Response of Irreversible Electroporation versus Cryoablation in a Rodent Model of Pancreatic Cancer, J. Vasc. Interv. Radiol. 29 (2018) 1764–1769, https://doi.org/10.1016/j.jvir.2018.07.009.
[261] H. Falk, S. Lambaa, H.H. Johannesen, G. Wooler, A. Venzo, J. Gehl, Electrochemotherapy and calcium electroporation inducing a systemic immune response with local and distant remission of tumors in a patient with malignant melanoma – a case report, Acta Oncol. 56 (2017) 1126–1131, https://doi.org/10.1080/0284186X.2017.1290274.
[262] G. Serša, D. Miklavčič, M. Čemažar, J. Belehradek, T. Jarm, L.M. Mir, Electrochemotherapy with CDDP on LPB sarcoma: comparison of the anti-tumor effectiveness in immunocompotent and immunodeficient mice, Bioelectrochem. Bioenerg. 43 (1997) 279–283, https://doi.org/10.1016/S0302-4598(96)05194-X.
[263] X. Chen, S. Yin, C. Hu, X. Chen, K. Jiang, S. Ye, X. Feng, S. Fan, H. Xie, L. Zhou, S. Zheng, Comparative study of nanosecond electric fields in vitro and in vivo on hepatocellular carcinoma indicate macrophage infiltration contribute to tumor ablation In Vivo, PLoS ONE 9 (2014), https://doi.org/10.1371/journal.pone.0086421 e86421.
[264] R. Nuccitelli, J.C. Berridge, Z. Mallon, M. Kreis, B. Athos, P. Nuccitelli, Nanoelectroablation of murine tumors triggers a CD8-dependent inhibition of secondary tumor growth, PLoS ONE 10 (2015), https://doi.org/10.1371/journal.pone.0134364 e0134364.
[265] R. Nuccitelli, K. Tran, K. Lui, J. Huynh, B. Athos, M. Kreis, P. Nuccitelli, E.C. De Fabo, Non-thermal nanoelectroablation of UV-induced murine melanomas stimulates an immune response, Pigm. Cell Melanoma Res. 25 (2012) 618–629, https://doi.org/10.1111/j.1755-148X.2012.01027.x.
[266] J.G. Skeate, D.M.D. Silva, E. Chavez-Juan, S. Anand, R. Nuccitelli, W.M. Kast, Nano-Pulse Stimulation induces immunogenic cell death in human papillomavirus-transformed tumors and initiates an adaptive immune response, PLoS ONE 13 (2018), https://doi.org/10.1371/journal.pone.0191311 e0191311.
[267] S. Guo, N.I. Burcus, J. Hornef, Y. Jing, C. Jiang, R. Heller, S.J. Beebe, Nano-Pulse Stimulation for the Treatment of Pancreatic Cancer and the Changes in Immune Profile, Cancers (Basel). 10 (2018), https://doi.org/10.3390/cancers10070217.
[268] P. Philips, Y. Li, S. Li, C.R. St Hill, R.C. Martin, Efficacy of irreversible electroporation in human pancreatic adenocarcinoma: advanced murine model, Mol. Ther. Methods Clin. Dev. 2 (2015) 15001, https://doi.org/10.1038/mtm.2015.1.
[269] L. Adam, N. Tchitchek, B. Todorova, P. Rosenbaum, C. Joly, C. Poux, C. Chapon, A.-L. Spetz, M. Ustav, R. Le Grand, F. Martinon, Innate molecular and cellular signature in the skin preceding long-lasting T Cell responses after electroporated DNA vaccination, J. Immunol. 204 (2020) 3375–3388, https://doi.org/10.4049/jimmunol.1900517.
[270] A.I. Daud, R.C. DeConti, S. Andrews, P. Urbas, A.I. Riker, V.K. Sondak, P.N. Munster, D.M. Sullivan, K.E. Ugen, J.L. Messina, R. Heller, Phase I trial of interleukin-12 plasmid electroporation in patients with metastatic melanoma, J. Clin. Oncol. 26 (2008) 5896–5903, https://doi.org/10.1200/JCO.2007.15.6794.
[271] S. Li, X. Zhang, X. Xia, Regression of Tumor Growth and Induction of Long-Term Antitumor Memory by Interleukin 12 Electro-Gene Therapy, JNCI J. National Cancer Inst. 94 (2002) 762–768, https://doi.org/10.1093/jnci/94.10.762.
[272] M.L. Lucas, R. Heller, IL-12 gene therapy using an electrically mediated nonviral approach reduces metastatic growth of melanoma, DNA Cell Biol. 22 (2003) 755–763, https://doi.org/10.1089/104454903322624966.
[273] D. Pavlin, M. Cemazar, U. Kamensek, N. Tozon, A. Pogacnik, G. Sersa, Local and systemic antitumor effect of intratumoral and peritumoral IL-12 electrogene therapy on murine sarcoma, Cancer Biol. Ther. 8 (2009) 2114–2122, https://doi.org/10.4161/cbt.8.22.9734.
[274] M. Cemazar, J.A. Avgustin, D. Pavlin, G. Sersa, A. Poli, A.K. Levacic, N. Tesic, U.L. Tratar, M. Rak, N. Tozon, Efficacy and safety of electrochemotherapy combined with peritumoral IL-12 gene electrotransfer of canine mast cell tumours, Veter. Comp. Oncol. 15 (2017) 641–654, https://doi.org/10.1111/vco.12208.
[275] G. Sersa, J. Teissie, M. Cemazar, E. Signori, U. Kamensek, G. Marshall, D. Miklavcic, Electrochemotherapy of tumors as in situ vaccination boosted by immunogene electrotransfer, Cancer Immunol. Immunother. 64 (2015) 1315–1327, https://doi.org/10.1007/s00262-015-1724-2.
[276] C.Y. Calvet, L.M. Mir, The promising alliance of anti-cancer electrochemotherapy with immunotherapy, Cancer Metastasis Rev. 35 (2016) 165–177, https://doi.org/10.1007/s10555-016-9615-3.
[277] N. Bessis, F.J. GarciaCozar, M.-C. Boissier, Immune responses to gene therapy vectors: influence on vector function and effector mechanisms, Gene Ther. 11 (2004) S10–S17, https://doi.org/10.1038/sj.gt.3302364.
[278] J.L. Shirley, Y.P. de Jong, C. Terhorst, R.W. Herzog, Immune Responses to Viral Gene Therapy Vectors, Mol. Therapy. 28 (2020) 709–722, https://doi.org/10.1016/j.ymthe.2020.01.001.
[279] V.M. Stoecklein, A. Osuka, J.A. Lederer, Trauma equals danger—damage control by the immune system, J. Leukocyte Biol. 92 (2012) 539–551, https://doi.org/10.1189/jlb.0212072.
[280] C. Brenner, L. Galluzzi, O. Kepp, G. Kroemer, Decoding cell death signals in liver inflammation, J. Hepatol. 59 (2013) 583–594, https://doi.org/10.1016/j.jhep.2013.03.033.
[281] D. Frank, J.E. Vince, Pyroptosis versus necroptosis: similarities, differences, and crosstalk, Cell Death Differ. 26 (2019) 99–114, https://doi.org/10.1038/s41418-018-0212-6.
[282] H. Flores-Romero, U. Ros, A.J. Garcia-Saez, Pore formation in regulated cell death, The EMBO J. 39 (2020) e105753. https://doi.org/10.15252/embj.2020105753.
[283] S.M. Man, R. Karki, T.-D. Kanneganti, Molecular mechanisms and functions of pyroptosis, inflammatory caspases and inflammasomes in infectious diseases, Immunol. Rev. 277 (2017) 61–75, https://doi.org/10.1111/imr.12534.
[284] X. Chen, W. He, L. Hu, J. Li, Y. Fang, X. Wang, X. Xu, Z. Wang, K. Huang, J. Han, Pyroptosis is driven by non-selective gasdermin-D pore and its morphology is different from MLKL channel-mediated necroptosis, Cell Res. 26 (2016) 1007–1020, https://doi.org/10.1038/cr.2016.100.
[285] B. Nikolova, S. Semkova, I. Tsoneva, E. Stoyanova, P. Lefterov, D. Lazarova, Z. Zhelev, I. Aoki, T. Higashi, R. Bakalova, Redox-related Molecular Mechanism of Sensitizing Colon Cancer Cells to Camptothecin Analog SN38, Anticancer Res. 40 (2020) 5159–5170. https://doi.org/10.21873/anticanres.14519.
[286] M.L. Albert, S.F. Pearce, L.M. Francisco, B. Sauter, P. Roy, R.L. Silverstein, N. Bhardwaj, Immature dendritic cells phagocytose apoptotic cells via alphavbeta5 and CD36, and cross-present antigens to cytotoxic T lymphocytes, J. Exp. Med. 188 (1998) 1359–1368, https://doi.org/10.1084/jem.188.7.1359.
[287] N.E. Blachère, R.B. Darnell, M.L. Albert, Apoptotic Cells Deliver Processed Antigen to Dendritic Cells for Cross-Presentation, PLoS Biol. 3 (2005), https://doi.org/10.1371/journal.pbio.0030185 e185.
[288] J. Savill, I. Dransfield, C. Gregory, C. Haslett, A blast from the past: clearance of apoptotic cells regulates immune responses, Nat. Rev. Immunol. 2 (2002) 965–975, https://doi.org/10.1038/nri957.

3 RAZPRAVA IN SKLEPI

3.1 RAZPRAVA

Želimo si, da bi bilo zdravljenje, tako z reverzibilno kot z ireverzibilno elektroporacijo varno, učinkovito in pacientu prijazno. To pomeni, da bi zdravljenje z elektroporacijo potekalo brez spremljevalnih zdravil, ki preprečujejo nenadzorovano krčenje mišic in občutek bolečine, kar bi hkrati omogočilo tudi varnejšo in hitrejšo obravnavo pacientov. Dodana vrednost vsakega zdravljenja je njegova varnost in učinkovitost njegove ponovitve v primeru delne ozdravitve. Vse bolj postaja jasno da, je ključnega pomena razumevanje in potencialno tudi nadzorovanje reakcij imunskega sistema kot dolgoročni odziv na zdravljenje z elektroporacijo.

V doktorski disertaciji smo: 1) preučili učinek nove oblike elektroporacijskih pulzov, ki bi lahko potencialno nadomestili trenutno uveljavljene monopolarne pulze, ki povzročajo nenadzorovane mišične kontrakcije in občutek bolečine; 2) ocenili učinkovitosti ponavljajočega zdravljenja z elektroporacijo; in 3) preučili imunogeno celično smrt, ki bi lahko vodila v aktivacijo imunskega sistema, kar vpliva in lahko izboljša izid samega zdravljenja z elektroporacijo. Rezultati, pridobljeni v okviru doktorske disertacije, so skladno s tematiko razdeljeni v tri izvirne znanstvene prispevke (Polajžer in sod., 2020a; Polajžer in sod., 2020b; Polajžer in Miklavčič, 2020). Poleg izvirnih znanstvenih prispevkov, je v okviru doktorske disertacije nastal tudi pregledni članek: Celična smrt zaradi elektroporacije (Batista Napotnik in sod., 2021).

Učinkovitost monopolarnih in visokofrekvenčnih bipolarnih pulzov je primerljiva

Pred kratkim se je pojavila nova oblika ireverzibilne elektroporacije, tako imenovana visoko frekvenčna ireverzibilna elektroporacija, pri kateri naj ne bi prišlo do depolarizacije vzdražnih celic, torej nehotenega mišičnega krčenja ali občutka bolečine, kot smo tega navajeni pri trenutno uveljavljenih monopolarnih pulzih (Arena in sod., 2011; Siddiqui in sod., 2016, 2017). V času začetka te doktorske naloge so bili generatorji pulzov oziroma elektroporatorji, ki omogočajo generiranje visokofrekvenčnih bipolarnih pulzov komercialno nedostopni. Obstajalo je le nekaj prototipov na svetu, med njimi tudi v Sloveniji na Univerzi v Ljubljani, Fakulteti za elektrotehniko v Laboratoriju za biokibernetiko. Posledično so bile študije, predvsem izvedene *in vitro,* redke (Sano in sod., 2015, 2017; Sweeney in sod., 2016). Naša študija je ena prvih v kateri smo širše preučili delovanje visokofrekvenčnih bipolarnih pulzov in njihove posebnosti.

Odsotnost nehotenega mišičnega krčenja ali občutka bolečine pri zdravljenju z visokofrekvenčnimi bipolarnimi pulzi nepomembna, če njihov učinek ni primerljiv z učinkovitostjo monopolarnih pulzih. V študiji smo, preko izdelave krivulj povečane prepustnosti membrane in preživetja celic, primerjali učinek uveljavljenih monopolarnih

pulzov in novejših visokofrekvenčnih bipolarnih pulzov v območju reverzibilne in ireverzibilne elektroporacije. Pri tem smo uporabili tipične pulze za elektrokemoterapijo – osem 100 µs dolgih pulzov (skupen čas trajanja pulzov je 800 µs) in ponavljalno frekvenco 1 Hz. Učinek visokofrekvenčnih bipolarnih pulzov smo primerjali z učinkom monopolarnih pulzov. Obe obliki pulzov sta imeli enak skupen čas trajanja (800 µs). To pomeni, da smo v obeh primerih, tako z visokofrekvenčnimi bipolarnimi pulz kot monopolarnimi pulzi dovedli enako energijo. Iz vidika povečane prepustnosti membrane in preživetja celic smo z uporabo visokofrekvenčnih bipolarnih pulzov in monopolarnih pulzov lahko dosegli primerljiv učinek elektroporacije. Za doseganje primerljive učinkovitosti smo morali pri visokofrekvenčnih bipolarnih pulzih uporabiti višjo amplitudo oziroma dovesti večjo energijo kot pri monopolarnih pulzih. Potreba po višji amplitudi je prisotna neglede na uporabljen elektroporacijski pufer ali parametre visokofrekvenčnih bipolarnih pulzov. Ta opažanja so skladna z obstoječimi študijami, s čimer smo še dodatno potrdili potencialno nadomestitev monopolarnih pulzov z visokofrekvenčnimi bipolarnimi pulzi (Dong in sod., 2018; Scuderi in sod., 2019; Sweeney in sod., 2016; Zhao in sod., 2018).

Parametri visokofrekvenčnih bipolarnih pulzov vplivajo na izraženost izničevalnega učinka

Glede na to, da so visokofrekvenčni bipolarni pulzi sestavljeni iz številnih zelo kratkih bipolarnih pulzov, smo predvidevali, da bi bil vzrok za potrebovano višjo amplitudo visokofrekvenčnih bipolarnih pulzov v primerjavi z monopolarnimi pulzi, lahko posledica prisotnosti izenačevalnega učinka. Spreminjanje parametrov (Slika 1), kot sta dolžina pozitivne in negativne faze bipolarnega pulza (v nadaljevanju dolžina pulza, Tp) in časovni zamik med pozitivno in negativno fazo bipolarnega pulza (v nadaljevanju časovni zamik, d_1) visokofrekvenčnih bipolarnih pulzov nam je tako omogočilo preučevanje, do sedaj neraziskanega, vpliva na učinkovitost elektroporacije kot tudi preučevanje pojava izničevalnega učinka v odvisnosti od omenjenih parametrov. Pri tem smo dolžini pulza ($Tp = 1$, 5 ali 10 µs) glede na izbrano dolžino prilagodili število bipolarnih pulzov (N) v vlaku, tako da smo ohranili skupen čas trajanja pulzov pri 800 µs ($Tp = 1$ µs, N = 50; $Tp = 5$ µs, N= 10; $Tp = 10$ µs, N= 5) ob ponavljalni frekvenci enaki (1 Hz) vlakov visokofrekvenčnih bipolarnih pulzov in monopolarnih pulzov. Medtem pa parameter časovnega zamika ($d_1 =$ 0,5 µs – 10 ms) nima vpliva na skupen čas trajanja pulzov. Preučevanje parametrov visokofrekvenčnih bipolarnih pulzov je omogočila tudi uporaba elektroporacijskih pufrov različne sestave in posledično različne prevodnosti. KPB pufer (angl. potassium-phospfate buffer) z nizko prevodnostjo (1,76 mS/cm) se pogosto uporablja v *in vitro* poskusih zaradi tokovnih omejitev elektroporatorjev. Drugi pufer je visoko-prevodni pufer (19,12 mS/cm), kjer smo saharozo zamenjali z NaCl, s čimer so pogoji, katerimi je izpostavljena celica bolj podobni *in vivo* pogojem.

Glede na rezultate naše študije je učinkovitost *in vitro* elektroporacije z visokofrekvenčnimi bipolarnimi pulzi odvisna od parametrov pulzov in elektroporacijskega medija. Pri krajši

dolžini pulza (1 µs) je elektroporacija manj učinkovita oziroma oz. povzroči manjšo prepustnosti membrane in večje preživetje celic. Ob podaljševanju dolžine pulza (10 µs) se poveča tudi učinkovitost reverzibilne in ireverzibilne elektroporacije oz. povzroči večjo prepustnosti membrane in manjše preživetje. Vpliv dolžine pulza na prepustnost membrane/reverzibilno elektroporacijo je prisoten le v visoko-prevodnem mediju, medtem ko je vpliv na preživetje celic je prisoten v obeh pufrih. V visoko-prevodnem pufru dolžina časovnega zamika vpliva na učinkovitost elektroporacije podobno kot dolžina pulzov, saj krajši časovni zamik (0,5 µs) povzroči slabšo učinkovitost elektroporacije, podaljševanje časovnega zamika (10 ms) pa privede do učinkovitejše elektroporacije. Predvidevamo, da je to zaradi pojava izničevalnega učinka, katerega izraženost upada s podaljševanjem dolžine pulza in časovnega zamika. Ravno nasprotno pa se celice odzovejo na parameter časovnega zamika v nizko-prevodnem pufru. Učinek elektroporacije je tam največji pri najkrajšem časovnem zamiku in s podaljševanjem časovnega zamika upada, kar je v nasprotju s teorijo o izenačevalnemu učinku. Vzrok za to bi lahko bil KPB pufer, pri katerem so že dokazali drugačen odziv celic na elektroporacijo *in vitro* v primerjavi z ostalimi pufri (Dermol in sod., 2016).

Na podlagi rezultatov pridobljenih pri visoko prevodnih medijih, ki bolj realistično predstavljajo razmere *in vivo*, predvidevamo da pri uporabi visokofrekvenčnih bipolarnih pulzov lahko pride do izničevalnega učinka, ki je odvisen od dolžine pulzov in časovnega zamika. Povečevanje dolžine bipolarnega pulza in časovnega zamika bipolarnega pulza povzroči manjšo izraženost izničevalnega učinka ob tem pa se povečuje učinkovitost elektroporacije. Zato sklepamo, da neprimerna izbira parametrov visokofrekvenčnih bipolarnih pulzov lahko zmanjša učinkovitost reverzibilne in ireverzibilne elektroporacije.

Vzrok za nastanek izničevalnega učinka ostaja nepojasnjen

Natančen mehanizem izničevalnega učinka ni znan, je pa predlaganih nekaj teorij: obrnjen elektroforezni transport ionov, dvostopenjska oksidacija membranskih fosfolipidov, aktivno praznjenje membrane, lokalno polnjenje in praznjenje membrane, pa vendar nobena od teh ni potrjena (Gianulis in sod., 2018; Gowrishankar in sod., 2018; Merla in sod., 2017; Pakhomov in sod., 2014; Valdez in sod., 2017). V tej študiji smo preučevali novo teorijo pri kateri naj imeli pomembno vlogo kloridni kanalčki. Vdor kloridnih ionov v notranjost celice preko kloridnih kanalčkov naj bi zmanjšal mirovni membrani potencial oziroma povzročili hipepolarizacijo membrane. Vzdražne celice bi tako težje dosegle prag za nastanek akcijskega potenciala. Po tej teoriji kloridni ioni v visoko-prevodnem mediju omogočajo nastanek hiperpolarizacije, zamenjava kloridnih molekul za brez-kloridno molekulo pa bi morala fenomen izničevalnega učinka odpraviti. Za preučevanje vpliva kloridnih ionov smo uporabili poseben pufer, kjer smo MgCl nadomestili z magnezijevim D-glukonat hidratom in NaCl z natrijevim glukonatom. Vseeno je bil izničevalni učinek tudi v tem pufru prisoten. V primerjavi z monopolarnimi pulzi smo potrebovali višjo amplitudo, prav tako pa je

učinkovitost elektroporacije odvisna od vsaj enega parametra pulzov, in sicer časovnega zamika (d_2), kjer krajši časovni zamik (0,5 µs) povzroči manjšo učinkovitost elektroporacije, ob podaljševanju zamika pa se učinkovitost elektroporacije poveča. Presenetljivo je bil izničevalni učinek po velikosti precej podoben tistemu v visoko-prevodnem pufru, čeprav je prevodnost pufra brez kloridnih ionov precej nižja (9,57 mS/cm). Rezultati naše študije niso potrdili teorije o vpletenosti kloridnih ionov v izničevalni učinek.

Vseeno pa smo z uporabo matematičnega modeliranja, na podlagi različne prevodnosti elektroporacijskih pufrov izničevalni učinek deloma pripisali aktivnemu praznjenja membrane, ki velja za eno od teorij nastanka izničevalnega učinka (Gianulis in sod., 2018; Gowrishankar in sod., 2018; Merla in sod., 2017; Pakhomov in sod., 2014; Valdez in sod., 2017).

Večkratno zdravljenje z elektroporacijo ne pomeni zmanjšano učinkovitosti zdravljenja

Pri vsakem zdravljenju v kliniki se pojavi vprašanje njegove učinkovitosti in ponovljivosti v primeru nepopolnega odziva bolezni na zdravljenje in potrebe po njegovi ponovitvi. Pri tem se porajajo naslednja vprašanja: kako primerljiva je učinkovitost med prvim in ponovnim zdravljenjem, kolikokrat lahko ob podobni učinkovitosti zdravljenje še ponovimo, da je ob tem zdravljenje še učinkovito oziroma kdaj ponovno zdravljenje ni več uspešno in je zato potrebno način zdravljenja spremeniti. V zdravljenju raka ta pojav poznamo pod imenom semirezistenca in redko rezistenca. Ponovljivost zdravljenja z elektroporacijo je nejasna, saj so si trenutno obstoječe študije maloštevilne, njihovi rezultati pa nasprotujoči (Gusbeth in sod., 2009; Shao in sod., 2017). Naša študija je preplet eksperimentalnih elementov obeh obstoječih študij, s čimer odpravlja njune pomanjkljivosti kot so izbira celične linije in število generacij. Čeprav je realistična ponovna uporaba zdravljenja z elektroporacijo dvakratna ali modra trikratna, pa nas je zanimalo ali je spremenjena občutljivost oz. razvoj rezistence na električne pulze sploh možna, če že, kdaj do tega pride in kaj to pomeni za metode zdravljenja z elektroporacijo.

Naša študija opravljena na CHO celicah dokazuje, da je uporaba oziroma zdravljenje samo z elektroporacijskimi pulzi (in ne v kombinaciji z drugimi zdravili) za namen tako reverzibilne elektroporacije kot tudi ireverzibilne elektroporacije enako uspešna več generaciji. Omenjeno velja za v medicini trenutno uveljavljene monopolarni pulze, in sicer osem pulzov z dolžino 100 µs in ponavljano frekvenco 1 Hz (Aycock in Davalos, 2019; Gehl in sod., 2018). Pri tem smo učinkovitost elektroporacije oz. spremenjeno občutljivost celic na elektroporacijske pulze spremljali preko povečane prepustnosti membrane (območje reverzibilne elektroporacije) in preživetja celic (območje ireverzibilne elektroporacije) v prvi/starševski generaciji in nato v vsaki 5. nadaljnji generaciji.

Prva ponovitev poskusa je trajala 15 generacij oz. približno dva meseca, druga ponovitev pa dobre štiri mesece. Časovni okvir za vzpostavitev »rezistenčne« celične linije oz. celične linije s spremenjeno občutljivostjo naj bi bil nekaj mescev, s čimer smo še povečali verjetnost razvoja takih celic (McDermott in sod., 2014). Glede na standardni protokol za nastanek rezistenčne celične linije smo izpolnili tudi ostala ključna elementa: nizka doza stresorja (v tem primeru elektroporacijskih pulzov) in okrevanje brez prisotnosti stresorja (3-4 dnevni okrevanje celic v gojišču v inkubatorju) (McDermott in sod., 2014). Nizko dozo stresorja smo dosegli s premišljeno izbiro jakosti električnega polja dovedenih pulzov. V ta namen smo izbrali amplitudo pulzov, ki povzroči povečano prepustnost membrane približno 80 % celicam in hkrati umre samo okoli 20 % celic. Jakost stresorja elektroporacijskega pulza je tako hkrati iz vidika reverzibilne elektroporacije visoka, ter nizka iz vidika ireverzibilne elektroporacije.

Ob upoštevanju vseh pogojev razvoja rezistence celične linije in obstoječe študije na evkariontskih celicah smo pričakovali zamik krivulj oziroma spremenjeno občutljivost celic na električno polje in s tem razvoj rezistence celične linije oz. celične linije z zmanjšano občutljivostjo na električne pulze. Predvidevamo, da bi celice z spremenjeno občutljivostjo za elektroporacijske pulze za enako učinkovitost v primerjavi s starševsko generacijo potrebovale nižjo ali višjo amplitudo oziroma jakost stresorja (McDermott in sod., 2014). Pri tem pa bi razlika v amplitudi naraščala tudi z vsako naslednjo generacijo. V sklopu teh predvidevanj smo postavili hipotezo, da večkratna izpostavljenost električnemu polju povzroči razvoj rezistence na električne pulze in jo po poskušali potrditi.

Naši rezultati so v nasprotju s postavljeno hipotezo. Celice niso spremenile svoje občutljivosti na električne pulze niti v 15 generacijah, niti v 30 generacijah. To velja tako za reverzibilno elektroporacijo kot ireverzibilno, torej neglede na jakost stresorja, ki je bil pri reverzibilni elektroporaciji precej močnejši kot pri ireverzibilni elektroporaciji. Glede na dolgoročen nespremenjen odziv celic na izpostavljenost elektroporacijskim pulzom, morda celo nikoli ne pride do spremenjene občutljivosti celic. Iz dobljenih rezultatov lahko trdimo, da celice zaradi izpostavljenosti elektroporacijskim pulzom ne postanejo bolj ali manj občutljive ozirom oz. ne postanejo rezistentne na takšne pulze. Ugotovljeno je v nasprotju z našo postavljeno hipotezo, zaradi česar smo hipotezo zavrnili.

Naša študija je torej zavrnila možnost razvoja spremenjene občutljivosti celic v primeru ponovnega zdravljenja s samimi elektroporacijskimi pulzi tako v območju reverzibilne kot ireverzibilne elektroporacije. Na razvoj spremenjene učinkovitosti ne vpliva niti jakost stresorja, saj kljub zelo intenzivnem stresorju (ki vpliva na 80% celic) celice niso postale bolj občutljive v območju ireverzibilne elektroporacije. Glede na rezultate študije sklepamo, da zdravljenje z elektroporacijo lahko uporabimo večkrat, pri tem pa je učinkovitost ponovljenega zdravljenja enaka. Ker smo pri tem uporabili celično linijo CHO, ki velja za

netumorsko celično linijo, predvidevamo, da to velja za gensko elektrotransfekcijo pri vnosu več odmerkov cepiv in ireverzibilno elektroporacijo, kot je ablacija srčnega tkiva, v primeru nezadostne obsežnosti tkiva pri prvem zdravljenju. Predpostavljamo, da enako velja tudi za ablacijo tumorskega tkiva, kar pa je sicer v nasprotju z študijo, ki so jo izvedli Shao in sod. (2017), a je slednja zaradi zelo majhnega števila generacij celic nezanesljiva, zato je odziv tumorskih celic na večkratno izpostavitev elektroporacijskim pulzom potrebno še preveriti. Vprašanje pa je, če se to zgodi tudi pri zdravljenju raka z elektrokemoterapijo, kjer so celice poleg električnih pulzov izpostavljene tudi kemoterapevtikom, ki sami po sebi lahko vplivajo na preživetje celic. Slednji bi morda kot kemični stresor v kombinaciji z elektroporacijskim pulzi lahko vplival na spremenjeno občutljivost celic na elektrokemoterapijo. Seveda, pa bi bilo smiselno sposobnost razvoja spremenjene občutljivosti celic na elektroporacijske pulze preveriti tudi *in vivo*, saj vemo, da se rezultati *in vitro* študij, ne odražajo vedno tudi *in vivo*, kjer gre za celosten odgovor organizma.

Sproščanje DAMP molekul iz elektroporiranih celic je izrazitejše v območju ireverzibilne elektroporacije

Aktivacija imunskega sistema pacienta, naj bi izboljšala izid zdravljenja z reverzibilno (Calvet in sod., 2014; Calvet in Mir, 2016; Serša in sod., 2015; Serša in sod., 1997; Chiarella in sod., 2008) kot tudi z ireverzibilno elektroporacijo (Bulvik in sod., 2016; José in sod., 2012; Pandit in sod., 2019; Scheffer in sod., 2019; Vogl in sod., 2009; White in sod., 2018), saj poleg uničenega tarčnega tumorskega tkiva, aktiviran imunski sistem vpliva tudi na oddaljene metastaze. Eden možnih razlogov za aktivacijo imunskega sistema pri zdravljenju z elektroporacijo je aktivacija imunogene celične smrti, o kateri se na področju elektroporacije ve dokaj malo, saj gre za novejšo obliko poimenovanja poti celične smrti. Aktivacijo imunogene celične smrti je možno spremljati *in vitro* preko sproščanja specifičnih signalnih DAMP molekul iz elektroporiranih celic. Dosedanje *in vitro* študije so bile opravljene na tumorskih in netumorskih celicah ob uporabi različnih dolžin pulzov in v kombinaciji z različnimi kemoterapevtiki (Calvet in sod., 2014; Guo in sod., 2018; Nuccitelli in sod., 2015, 2017; Ringel-Scaia in sod., 2019; Rossi in sod., 2019; Schultheis in sod., 2018; Zhao in sod., 2019). Za razliko od obstoječih študij smo v naši ocenili korelacijo med sporočanjem DAMP molekul in povečano prepustnostjo membrane oziroma reverzibilno elektroporacijo ter med sporočanjem DAMP molekul in celično smrtjo oziroma ireverzibilno elektroporacijo.

Korelacija prestavlja povezanost dveh spremenljivk in jo prikazujemo s koeficientom R. Korelacija je najmočnejša v bližini vrednosti 1 in upada z nižanjem vrednoti do 0,5. Pri vrednostih manjših od 0,5 velja, da korelacija ne obstaja. Na splošno velja prepričanje, da sta povečana prepustnost membrane in preživetje celic po elektroporaciji vzročno povezana, kjer zaradi povečane prepustnosti membrane celic pride do zmanjšanega preživetja celic. Korelacijski koeficient v tej študiji je bil R = - 0,680, kar kaže da njuna povezanost ni

najmočnejša. Pri tem velja izpostaviti, da smo za analizo celične smrti uporabili metabolni test, katerega rezultati % preživelih celic niso tako natančni kot pri testu klonogenosti, vendar hkrati dovolj zanesljivi, da ocenimo približen upad preživetja celic ob povečanju amplitude električnih pulzov.

Rezultati naše študije so najbolj primerljivi s študijo, ki so jo opravili Calvet in sod. (2014), saj pri obeh študijah uporabljamo enake pulze, in sicer osem monopolarnih pulzov, dolžine 100 µs. Medtem ko so Calvet in sod. (2014) preučevali aktivacijo imunogene celične smrt v kombinaciji s samimi elektroporacisjkimi pulzi in v kombinaciji elektroporacijskih pulzov s kemoterapevtikom, smo se v naši študiji osredotočili na delovanje samih elektroporacijskih pulzov. Predvidevamo namreč, da je izpostavljenost elektroporacijskim pulzom že samo po sebi zadošča, da pride do sproščanja DAMP molekul, saj so aktivacijo imunskega sistema, dokazali pri ireverzibilni elektroporaciji le z uporabo elektroporacijskih pulzov (Bulvik in sod., 2016; José in sod., 2012; Pandit in sod., 2019; Scheffer in sod., 2019; Vogl in sod., 2009; White in sod., 2018). Tako kot Calvet in sod. (2014) smo določali vrednosti sproščenega ATP po elektroporaciji in premik proteina kalretikulina na zunanjo stran celične membrane in dobili podobne rezultate kot omenjena študija ob uporabi sami elektroporacijskih pulzov. ATP in kalretikulin sta dve od treh DAMP molekul, ki veljajo za zlati standard imunogene celične smrti tumorskih celic (Zhou in sod., 2019). Vendar je identificiranih še veliko DAMP molekul, njihov število pa se še povečuje. Med bolj znanimi so visoko mobilen protein 1 (HMGB1), nukleinske kisline, proteini toplotnega šoka (angl. heat-shock proteins), protein S100, sečna kislina ter saharidi (Roh in Sohn, 2018). V naši študiji smo poleg sproščanja ATP in kalretikulina analizirali še sproščanje nukleinskih kislin in sečne kisline. Prisotnost slednje sicer v študiji nismo uspeli potrditi, kar pripisujemo CHO celični liniji ali neustrezni v metodi.

Statistična korelacija sproščanja DAMP molekul s povečano prepustnostjo membrane je v splošnem slaba ali je celo ne moremo potrdili. Korelacija med sproščanjem ATP in povečane prepustnosti membrane je pozitivna, saj vrednost sproščenega ATP po 15-30 minutah narašča z vrednostjo povečane prepustnosti membrane ($R = 0,594$ pri fluorescenčni metodi oziroma $R = 0,704$ in 0,728 pri luminiscenčni metodi). Količina sproščenega ATP nekaj minut po izpostavitvi elektroporacijskemu pulzu dosega pozitivno korelacijo s povečano prepustnostjo membrane. Pozitivna korelacija je bila sicer pričakovana, vendar ne tako šibka ($R = 0,594$), saj so v prvih študijah elektroporacije detekcijo sproščenega ATP iz celic uporabljali za potrjevanje povečane prepustnosti membrane. 24 ur po elektroporaciji se je korelacija spremenila v negativno korelacijo ($R = - 0,695$), saj so vrednosti ATP ob višanju amplitude upadle. Zelo šibko korelacijo sproščanja DAMP molekul z reverzibilno elektroporacijo smo potrdili tudi pri analizi kalretikulina ($R (4 h) = 0,535$ in $R (24 h) = 0,556$) tako 4 in 24 ur po elektroporaciji, kot tudi pri detekciji DNA in RNA 15 - 30 min po elektroporaciji ($R = 0,57 - 0,69$), medtem pa korelacija med sproščanjem nukleinskih kislin in reverzibilno elektroporacijo 24 ur po elektroporaciji sploh ne obstaja.

V primerjavi z reverzibilno elektroporacijo je korelacija sproščanja DAMP molekul močnejša z ireverzibilno elektroporacijo oz. celično smrtjo. Korelacija sproščenega ATP analiziranega s fluorescenčno metodo (R (30 min) = - 0,865 in R (24 h) = 0,888). Dodatna analiza ATP z luminiscenčno metodo, ki velja za bolj občutljivo metodo merjenja ATP je potrdila močno korelacijo med sproščanjem ATP in ireverzibilno elektroporacijo oziroma celično smrtjo tudi v tem primeru (R (15 min) = - 0,964, R (30 min) = - 0,947). To velja tudi za sproščanje nukleinskih kislin, ki dosega močno negativno korelacijo (R > - 0,9) pri vse točkah časovne analize (15, 30 min in 24 h).

V preteklosti so elektroporacijskim pulzom že pripisovali poškodovanje DNA in zmanjšanje znotraj celičnega ATP zaradi nastanka por v membrani in s tem povečane prepustnosti membrane. Tako bi lahko dvig ATP, DNA in RNA v odvisnosti od amplitude pulza kmalu po izpostavitvi elektroporaciji pripisali stanju oz. številu poškodb, ki jih povzroči elektroporacija. Zanimivo je, da koncentracija ATP 24h po elektroporaciji upada s povečevanjem amplitude, medtem pa koncentraciji DNA in RNA s povečevanjem amplitude naraščata. Predvidevamo, da se izvencelični ATP razgradi s pomočjo ATPaz, ki ob povečani prepustnosti membrane uidejo iz celice. Drastičen iztek ATP bi lahko vplival tudi na delovanje ATP črpalk, ki sicer vzdržujejo koncentracijo ionov na obeh straneh membrane. Posledično, zaradi tega pride do porušenega ionskega ravnotežja kar vliva na celično smrt (Hansen in sod., 2015; Wang in sod., 2003) [75]. Povišana vrednost ATP pri kontroli pa bi bila lahko posledica delnega poškodovanja celic ob pipetiranju in centrifugiranju celic.

Podobno korelacijo kot sproščanje ATP in nukleinskih kislin nakazuje tudi detekcija kalretikulina na zunanji plazemski membrani. Korelacija se v 24 urah po elektroporaciji še ojača (R (4 h) = - 0,801, R (24 h) = - 0,946). Medtem ko pasivno sproščanje ATP in nukleinskih kislin lahko pripisujemo poškodbi celične membrane oz. nastanku por, ki nastanejo v membrani pri elektroporaciji, to ne velja za premik proteina kalretikulina na zunanjo stran plazemske membrane. Kalretikulin se namreč v normalno delujoči celic nahaja v notranjosti endoplazemskega retikuluma, kjer skrbi za pravilno zvitje nastajajočih proteinov in za regulacijo Ca^{2+} metabolizma (Gelebart in sod., 2005; Krause in Michalak, 1997). V zgodnji fazi celične smrti se preko veziklov endoplazemskega retikuluma in golgijevega aparata ali z lizosomskimi vezilki premakne na celično površje, kjer prevzame vlogo DAMP molekule (Kranz in sod., 2017; Panaretakis in sod., 2009). Detekcija takšnega kalretikulina s protitelesi je zato možna le na živih celicah z zaceljeno membrano, saj bi drugače označili tudi znotraj celičen kalretikulin, ki pa ni DAMP molekula. Celice smo zato pred analizo označili s propidijevim jodidom (PI), ki obarva celice s poškodovano membrano in mrtve celice. Kljub temu smo kalretikulin na živih celicah (PI negativnih) zaznali le v območju ireverzibilne elektroporacije. Njegova detekcija pa je naraščala s povečanjem amplitude, čeprav se je število živih celic pri tem zmanjševalo iz česar sklepamo da višji stres oziroma v tem primeru amplituda pulzov povzroči, da se na površje celice premakne več molekul kalretikulina. Pri tem pa velja dodati, da naj bi do premika kalretikulina na

zunanjo plazemsko membrano prišlo v zgodnji fazi celične smrti (Kranz in sod., 2017; Panaretakis in sod., 2009), kar pomeni da tistih nekaj celice ki jih po 24h prepoznamo kot žive (PI negativinih), lahko v naslednjih nekaj urah umrejo. Obarvanje celic s PI je pokazalo, da nekaj celic preživi tudi po dovajanju pulzov z najvišjo amplitudo, kar je v nasprotju z oceno preživetja z metabolnim testom. Metodi namreč določata preživetje celic preko različnega celičnega mehanizma 24 ur po elektroporaciji (Šatkauskas in sod., 2017).

Sodeč po naših rezultatih je statistična korelacija med sproščanjem DAMP in preživetjem celic veliko močnejša kot pri povečani prepustnosti membrane, kar lahko pojasni aktivacijo imunskega sistema ob uporabi ireverzibilne elektroporacije. Vendar pa je bila aktivacija imunskega sistema dokazana tudi pri zdravljenju z reverzibilno elektroporacijo (Calvet in sod., 2014; Chiarella in sod., 2008), kar pa smo v naši študiji potrdili le v primeru izločanja molekule ATP. Vzrok za aktivacijo imunskega sistema pri zdravljenju z elektrokemoterapijo bi lahko ležal v nenamerni ireverzibilni elektroporaciji, v neposredni bližini elektrod. Študije so namreč pokazale, da pri zdravljenju z elektrokemoterapijo kljub dovajanju reverzibilnih pulzov, zaradi nehomogene porazdelitve električnega polja, pride v bližini elektrod do celične smrti s pomočjo ireverzibilne elektroporacije (Miklavčič in sod., 2000; Žmuc in sod., 2019). Znanje o aktivaciji imunogene celične smrti, njenem prispevku in vpletenosti v zdravljenje je pomembno tudi za čim boljšo napoved zdravljenja ali uporabo v kombinaciji z imuno-terapijami (Maglietti in sod., 2020).

Ključ do razumevanja aktivacije imunskega sistema bi lahko ležal v mehanizmu celične smrti po elektroporaciji

Izboljšanje metod zdravljenja z elektroporacijo zahteva tudi izboljšanje razumevanje aktivacije imunogene celične smrt, kar posledično pomeni razumevanje celične smrti pri elektroporaciji. Sodeč po rezultatih naše študije je sproščanje DAMP molekul z elektroporacijo bolj zapleteno kot smo sprva mislili, saj je tudi sproščanje ATP statistično močneje povezano v ireverzibilno elektroporacijo kot reverzibilno elektroporacijo. Nedavna študija Ringel-Scaia in sod. (2019) je pokazala, da se dinamika izražanja genov po elektroporaciji s časom spreminja. Tako naj bi se kmalu po elektroporaciji aktivirale signale poti, ki se nanašajo na poškodbo celice, apoptozo in zatiranje imunskega odziva. Sčasoma naj bi njihovo izražanje upadlo, zato pride izražanja drugih genov. Po 24 urah naj bi prevladovalo izražanje genov vpletenih v vnetni odziv, popravilo celic in nekrozo/piroptozo. Spremenjena dinamika izražanje genov bi lahko pojasnila tudi spremembe v koncentraciji DAMP molekul v različnih časovnih točkah.

Vse kaže, da je za razumevanje aktivacije imunskega sistema ključno za razumevanje celične smrti, ki jo povzročijo elektroporacijski pulzi oz. ireverzibilne elektroporacija. Na splošno o celični smrti, ki jo povzročijo električni pulzi vemo dokaj malo, kar je glede na to, da prve študije segajo daleč nazaj v zgodovino (Neumann in Rosenheck, 1972) in da je bilo od takrat

na tem področju opravljenih mnogo študij, morda znak da odgovor na to ni tako preprost. S preučevanjem literature celični smrti smo ugotovili, da gre elektroporirana celica skozi tri faze in sicer: poškodba, popravljanje in smrt. Tip celične poškodbe zaradi elektroporacijskih pulzov je odvisen od parametrov elektroporacije, ki pa se razlikujejo tudi glede na eksperimentalne pogoje (*in vivo* ali *in vitro*) (Batista Napotnik in sod., 2021). Preživetje celice je seveda odvisno od popravljalnih mehanizmov celice. Kateri mehanizem je vpleten pri odstranjevanju celičnih poškodb oziroma por v membrani je zaenkrat le slabo raziskano, pri tem pa naj bi imeli pomembno vlogo tudi parametri pulzov (Batista Napotnik in sod., 2021) kot je na primer dolžina samih pulzov (Thompson in sod., 2018; Thompson in sod., 2014). Pot celične smrti po elektroporaciji, so nekoč večinoma pripisovali apoptozi in nekrozi, medtem ko novejše študije omenjajo tudi nekroptozo in piroptozo za kateri je značilno sproščanje DAMP molekul oziroma imunogena celična smrt (Brock in sod., 2020; Mercadal in sod., 2020; Ringel-Scaia in sod., 2019). Natančen mehanizem celične smrti pri elektroporaciji do sedaj še ni poznan, vse pa kaže da je odvisen od parametrov pulzov, celične linije oz. tkiva ter od eksperimentalnih pogojev (Ball in sod., 2010; Batista Napotnik in sod., 2021; Brock in sod., 2020; Pakhomova in sod., 2013; Ringel-Scaia in sod., 2019). Na podlagi zbrane literature ocenjujemo, da bi boljše razumevanje elektroporacije na nivoju celičnih poškodb, popravljalnih mehanizmov in celične smrti prinesla sistematsko zastavljena študija. Pri tem bi različne celične linije (različnega tkivnega izvora, tumorske – netumorske, diferencirane – nediferencirane) izpostavili različnim pulzom (nanosekundne, mikrosekundne milisekundne monopolarne pulze in visokofrekvečne bipolarne pulze) pri istih eksperimentalnih pogojih. Le tako bi lahko zares ocenili vpliv pulzov in celičnih linij na poškodbe, popravljalne mehanizme in celične smrti.

3.1.1 Prispevek k znanosti

Pridobljeni rezultati na področju visokofrekvenčne ireverzibilne elektroporacije so pomembno dopolnili obstoječe znanje o delovanju in vplivu parametrov visokofrekvenčnih bipolarnih električnih pulzov na učinkovitost elektroporacije. Potrdili smo, da je učinek visokofrekvenčnih bipolarnih pulzov primerljiv s monopolarnimi pulzi, ki se trenutno večinoma uporabljajo v medicini. Na učinek zdravljenja lahko močno vpliva izbor parametrov pulzov, kot je dolžina pozitivne in negativne faze bipolarnega pulza in časovni zamik med pozitivno in negativno fazo bipolarnega pulza. Ob neustrezni izbiri lahko namreč pride do izničevalnega učinka, ki se kaže v slabši učinkovitosti zdravljenja z elektroporacijo. Poleg klinične vrednost študije ima študija tudi raziskovalno vrednost, kot je zavrnitev ene teorije in delna potrditev druge teorije o nastanku izničevalnega učinka.

Aktivacija imunskega sistema lahko v odvisnosti od namena zdravljenja pripomore k uspešnejšem zdravljenju z elektroporacijo. Aktivacija imunskega sistema pri elektroporaciji je lahko posledica sproščanja DAMP molekul iz elektroporiranih celic. Dokazali smo, da je sproščanje DAMP molekul odvisno od parametrov pulzov. Sproščanje DAMP molekul in s tem aktivacija imunskega sistema je glede na naše rezultate izrazitejša v območju

ireverzibilne elektroporacije kot reverzibilne elektroporacije, ne glede na to, da nekatere DAMP molekule izstopijo iz celice že zaradi povečane prepustnosti membrane, ki jo povzročijo elektroporacijski pulzi.

Naša študija je edina v kateri smo raziskovali razvoj odpornosti evkariontskih celic oz. razvoj zmanjšanje občutljivosti celic na električne pulze. Dokazali smo, da je učinek elektroporacijskih pulzov na celicah, vsaj do 30. generacije, enak kot ob prvem zdravljenju. S tem smo potrdili, da je elektroporacija metoda, ki jo lahko po potrebi lahko večkrat ponovimo, v primeru, delnega odziva na prvotno zdravljenje ali ponovnega pojava bolezenskega tkiva.

Rezultati pridobljeni v doktorski nalogi bodo služili kot pomembno izhodišče številnim pred-kliničnim in kliničnim študijam, kot tudi metodam zdravljenja z elektroporacijo, temelječih na reverzibilni kot tudi ireverzibilni elektroporaciji.

3.2 SKLEPI

- Visokofrekvenčni bipolarni pulzi bi lahko v prihodnosti nadomestili trenutno uveljavljene monopolarni pulze, tako iz vidika reverzibilne kot tudi ireverzibilne elektroporacije. Z visokofrekvenčnimi bipolarnimi pulzi lahko namreč dosežemo enako učinkovitosti elektroporacije kot z monopolarnimi pulzi, vendar pa je pri visokofrekvenčnih bipolarnih pulzih potrebno dovesti večjo energijo oziroma uporabiti višjo amplitudo.

- Učinek visokofrekvenčnih bipolarnih pulzov je odvisen od parametrov pulzov, kot sta dolžina pozitivne in negativne faze bipolarnega pulza in dolžina časovnega zamika med pozitivno in negativno fazo bipolarnega pulza. Krajša dolžina omenjenih parametrov pomeni manj učinkovito elektroporacijo, podaljševanje njune dolžine pa privede do učinkovitejše elektroporacije. Predvidevamo, da je razlog za to izničevalni učinek, katerega vpliv pa upada s podaljševanjem dolžine pulza.

- Prisotnost izničevalnega učinka pri visokofrekvenčnih bipolarnih pulzih je odvisna tudi od elektroporacijskega pufra, kar lahko vpliva na rezultate *in vitro* študij in njihovo napovedno vrednost za *in vivo* in klinične študije.

- Vzrok za nastanek izničevalnega učinka pri bipolarnih pulzih ostaja nepojasnjen, vendar njegov obstoj lahko vsaj deloma pripišemo aktivnemu praznjenja membrane in ne teoriji o hiperpolarizaciji celice zaradi vdora kloridnih ionov.

- Zdravljenje z elektroporacijo, tako v območju reverzibilne kot ireverzibilne elektroporacije lahko uporabimo večkrat, pri tem pa je učinkovitost vsakega ponovnega zdravljenja vedno enaka in primerljiva s prvotnim zdravljenjem. Povečanje ali zmanjšanje občutljivosti namreč kljub večkratni izpostavljenosti elektroporacijskim pulzom nismo opazili.

- Sproščanje DAMP molekul pri zdravljenju z elektroporacije je v večji meri prisotno v območju ireverzibilne elektroporacije oz. ablacije tkiva, ob katerem povzročimo smrt celic in ne območju reverzibilne elektroporacije, kjer dosežemo le povečano prepustnost celic.

- Mehanizem celične smrti, ki jo povzročijo elektroporacijski pulzi je slabo raziskan. Trenutno obstaja nekaj teorij o celičnih poškodbah, ki jih pulzi povzročijo, popravljalnih mehanizmov, ki se ob tem sprožijo ter o poteh celične smrti, do katerih elektroporacijski pulzi privedejo. V prihodnosti bo potrebno izvesti sistematično zasnovano raziskavo, kjer bi ugotovili ali je učinek elektroporacije z vidika poškodbe, popravljalnih mehanizmov in celične smrti odvisen od oblike pulzov ali tkivnega izvora celice ali obojega ali še kakšnega drugega parametra.

4 POVZETEK (SUMMARY)

4.1 POVZETEK

Kadar celico izpostavimo zunanjemu električnemu polju v membrani nastanejo pore, zaradi česar se spremeni prepustnost membrane za molekule in ione, ki drugače membrano težko prehajajo. Zaradi povečane prepustnosti membrane pojav imenujemo elektropermeabilizacija ali elektroporacija, saj naj bi se ob izpostavljenosti zunanjemu električnemu polju v membrani oblikovale pore. Elektroporacija je lahko reverzibilna/povratna ali ireverzibilna/nepovratna, odvisno od parametrov električnih pulzov, kot so električna poljska jakost, dolžina, število in ponavljalna frekvenca pulzov. Če celica ponovno vzpostavi fiziološke procese in preživi govorimo o reverzibilni elektroporaciji, če celica umre pa o ireverzibilni elektroporaciji. Elektroporacija se uporablja v širokem naboru postopkov v živilski tehnologiji, biotehnologiji in medicini. V doktorski disertaciji sem se osredotočila predvsem na metode elektrokemoterapije in genske elektrotransfekcije z reverzibilno elektroporacijo ter netermičnega odstranjevanja tkiva z ireverzibilno elektroporacijo.

Tako elektrokemoterapija kot ablacija tkiva z ireverzibilno elektroporacijo za svoje delovanje uporabljata dolge monopolarne pulze, v različnem številu in amplitudi. Dovajanje teh pulzov povzroči nenadzorovano mišično krčenje in občutek bolečine, spremembo pH in nastanek zračnih mehurčkov kot posledico elektrokemijskih reakcij. Pred kratkim se je pojavila nova oblika pulzov, ki odpravlja navedene pomanjkljivosti monopolarnih pulzov, tako imenovana visokofrekvenčna ireverzibilna elektroporacija. Visokofrekvenčne bipolarne pulze sestavljajo vlaki zelo kratkih pulzov, ki si sledijo v izmenjujoči se polariteti. V nasprotju s klasično IRE, pri terapiji z visokofrekvenčnimi bipolarnimi pulzi ne potrebujemo mišičnih relaksantov in sinhronizacije dovajanja pulzov s srčnim utripom, kar močno olajša sam proces in skrajša trajanje terapije. Potencialno rabo teh pulzov so v zadnjih letih prepoznali tudi za ablacijo srčnega tkiva, kjer je odsotnost vzdraženja srčnomišičnih celice in nastanka mehurčkov v krvi srca še posebej pomembno. V primerjavi s klasičnimi monopolarnimi pulzi imajo visokofrekvenčni bipolarni pulzi kompleksnejšo strukturo in večje število parametrov, saj lahko poleg dolžine, števila, frekvence in amplitude pulzov nadzorujemo tudi časovni zamik med pozitivno in negativno fazo bipolarnega pulza, ponavljalno frekvenco, število pulzov v vlaku in zamik med vlaki pulzov. Posledično to pomeni več spremenljivk, ki vplivajo na učinek elektroporacije. Neustrezno spreminjanje parametrov lahko povzroči tudi termične poškodbe, zato je poznavanje delovanja visokofrekvenčnih bipolarnih pulzov pulzov, tako parametrov in posebnosti, ki jih prinaša ta oblika pulza, ključna za varno in uspešno uporabo v kliniki. Vseeno pa je odsotnost nehotenega mišičnega krčenja ali občutka bolečine pri zdravljenju z visokofrekvenčnimi bipolarnimi pulzi nepomembna, če njihov učinek ni primerljiv z učinkovitostjo monopolarnih pulzih. V študiji smo, preko izdelave krivulj povečane prepustnosti membrane in preživetja celic, primerjali učinek uveljavljenih monopolarnih pulzov in novejših

visokofrekvenčnih bipolarnih pulzov v območju reverzibilne in ireverzibilne elektroporacije. Tako z uporabo monopolarnih pulzov kot visokofrekvenčnih bipolarnih pulzov smo lahko dosegli primerljiv učinek elektroporacije, iz vidika povečane prepustnosti membrane in preživetja celic. Vendar pa smo morali za doseganje primerljive učinkovitosti pri visokofrekvenčnih bipolarnih pulzih uporabiti višjo amplitudo oziroma dovesti večjo energijo kot pri monopolarnih pulzih. Učinek visokofrekvenčnih bipolarnih pulzov je odvisen od parametrov pulzov, kot sta dolžina pozitivne in negativne faze bipolarnega pulza in dolžina časovnega zamika med pozitivno in negativno fazo bipolarnega pulza. Krajša dolžina parametra pomeni manj učinkovito elektroporacijo, podaljševanje dolžine pa omogoči učinkovitejšo elektroporacijo. Predvidevamo, da je to zaradi pojava izničevalnega učinka, katerega moč upada s podaljševanjem dolžine pulza. Prisotnost izničevalnega učinka visokofrekvenčnih bipolarnih pulzih *in vitro* je odvisna od elektroporacijskega pufra. Vzrok za nastanek izničevalni učinek še vedno ni v celoti pojasnjen, vseeno pa izničevalni učinek lahko vsaj deloma pripišemo aktivnemu praznjenja membrane.

Kljub temu, da gre pri elektrokemoterapiji in ireverzibilni elektroporaciji predvsem za enkratno terapijo, pa je v primeru delnega/nepopolnega odgovora potrebno (in možno) terapijo ponoviti. Pri tem se pojavi vprašanje učinkovitosti, ali le-ta ostane enaka ali je slabša. Dosedanje študije večkrat izpostavljenih celic električnim pulzov so si nasprotujoče, prekratke ali za namen zdravljenja tumorskega tkiva neustrezne. V študiji smo spremenjeno občutljivost celic na elektroporacijske pulze spremljali preko povečane prepustnosti membrane (območje reverzibilne elektroporacije) in preživetja celic (območje ireverzibilne elektroporacije) do 30. generacije. Pri tem smo izpolnili kriterije standardnega protokola za vzpostavitev »rezistenčne« oz. celične linije s spremenjeno občutljivostjo na elektroporacijske pulze. Glede na rezultate študije sklepamo, da zdravljenje z elektroporacijo lahko uporabimo večkrat, pri tem pa je učinkovitost vsakega zdravljenja primerljiva s primarnim zdravljenjem, kar velja tako za reverzibilno kot ireverzibilno elektroporacijo.

Številne študije omenjajo pomembnost aktivacije imunskega sistema organizma k dolgoročnejšemu vpliv zdravljenja z reverzibilno kot ireverzibilno elektroporacijo, saj aktiviran imunski sistem omogoči uničenje preostanka tumorskih celic oziroma zmanjšanje oddaljenih metastaz. Aktivacija imunskega sistema pri elektroporaciji bi bila lahko posledica imunogene celične smrti (ICD) elektroporiranih celic, kjer se iz celic sproščajo posebne signalne DAMP molekule. Trenutno razumevanje signalnih poti vpletenih v celično smrt/preživetje po elektroporaciji in aktivacijo/supresijo imunskega sistema je slabo. Za razliko od obstoječih študij smo sporočanje DAMP molekul (ATP, kalretikullin, nukleinske kisline, sečna kislina) analizirali v celotnem območju elektroporacije, torej tako reverzibilne kot ireverzibilne elektroporacije ter njihovo sproščanje statistično korelirali z reverzibilno in ireverzibilno elektroporacijo. Statistična korelacija s povečano prepustnostjo membrane je v splošnem slaba ali je celo ne moremo potrdili. Statistična korelacija med sproščanjem DAMP in preživetjem celic je veliko močnejša, kar lahko pojasni aktivacijo imunskega

sistema ob uporabi ireverzibilne elektroporacije. Vzrok za aktivacijo imunskega sistema pri zdravljenju z elektrokemoterapijo oziroma reverzibilno elektroporacijo bi lahko ležal v nenamerni ireverzibilni elektroporaciji, v neposredni bližini elektrod. Študije so namreč pokazale, da pri zdravljenju z elektrokemoterapijo kljub dovajanju reverzibilnih pulzov, zaradi nehomogene porazdelitve električnega polja, pride v bližini elektrod do celične smrti zaradi ireverzibilne elektroporacije ali celo termičnih poškodb.

Izboljšanje metod zdravljenja z elektroporacijo zahteva tudi izboljšanje razumevanje aktivacije imunskega sistema. Aktivacijo imunskega sistema lahko povzroči imunogena celična smrt, o kateri pri elektroporaciji vemo bolj malo. Na splošno je znanje o celični smrti, do katere pride pri elektroporaciji pomanjkljivo. Trenutno vemo, da elektroporacija povzroči prehod celice skozi tri faze - nastanek poškodbe, popravljanje in celična smrt. Mehanizem vsakega od teh ob elektroporaciji je zaenkrat slabo raziskan, v vsaki fazi pa je do sedaj potrjenih nekaj možnih tarč, mehanizmov oz poti. Med potmi celičnih smrti je najpogosteje opisana apoptoza, medtem ko novejše študije opisujejo tudi novi poti celične smrti in sicer nekroptozo (angl. necroptosis) in piroptozo (angl. pyroptosis), kjer gre zaradi sproščanje DAMP molekul v obliki imunogene celične smrti. V prihodnosti je potrebno izvesti sistematično zasnovano študijo, kjer bi ugotovili ali je učinek elektroporacije z vidika poškodbe/celične smrti odvisen od pulzov ali tkivnega izvora celice.

4.2 SUMMARY

When the cell is exposed to an external electric field, pores are formed in the membrane. Pores change the permeability of the membrane to molecules and ions that otherwise cannot pass through the membrane. Because of the change in permeability, the phenomenon is called electropermeabilization. Another name for it is electroporation because pores are formed in the membrane. The effect of electroporation depends on parameters of electric pulses, such as the strength of the electric field, the duration, number and frequency of the pulses. If the cell survives, it is called reversible electroporation, and if the cell dies, it is called irreversible electroporation. Electroporation is used in food technology, biotechnology and medicine. In my thesis, I mainly focused on the methods of electrochemotherapy and gene therapy with reversible electroporation and non-thermal tissue removal with irreversible electroporation.

Both electrochemotherapy and tissue ablation with irreversible electroporation use long monopolar pulses of varying number and amplitude. The delivery of these pulses causes uncontrolled muscle contraction and sensation of pain, as well as pH changes and the formation of air bubbles as a result of electrochemical reactions at the electrodes. Recently, a new form of pulses called high-frequency irreversible electroporation was suggested that mitigate the above-mentioned disadvantages of monopolar pulses. High-frequency bipolar pulses consist of trains of very short pulses that follow each other in alternating polarity. Unlike the classic IRE, therapy with high-frequency bipolar pulses does not require muscle relaxants or synchronization with the heartbeat, which greatly facilitates the process and reduces the duration of therapy. In recent years, the potential of these pulses has also been recognized for ablation of cardiac tissue, where the lack of excitation of cardiomyocytes and the absence of bubbles in the blood of the heart are particularly important. Compared with conventional monopolar pulses, high-frequency bipolar pulses have a more complex structure and a larger number of parameters, because in addition to the pulse duration, number, frequency, and amplitude, we can also control the time delay between positive and negative bipolar phases and the repetition frequency of the trains. Also, such pulses can be symmetrical or asymmetrical. Thus, there are several parameters that affect the electroporation effect. Inadequate pulse parameters can also cause thermal damage. Therefore, knowledge of the parameters of the high-frequency bipolar pulse and the specific properties of this form of pulse is critical for successful application in the clinic. However, the absence of involuntary muscle contractions or pain sensations during treatment with high-frequency bipolar pulses is irrelevant if their effects are not comparable to the efficacy of monopolar pulses. In this study, we compared the effects of established monopolar pulses with newer high-frequency bipolar pulses in reversible and irreversible electroporation through membrane permeability and cell survival. By using monopolar pulses and high-frequency bipolar pulses, we were able to achieve comparable electroporation efficiency. However, to achieve comparable efficiency, we had to use a higher amplitude or apply more energy for the high-frequency bipolar pulses than for the monopolar pulses. The effect of

high-frequency bipolar pulses depends on pulse parameters such as the duration of the positive and negative phases of the bipolar pulse and the duration of the time delay between the positive and negative phases of the bipolar pulse. A shorter duration of the parameters means a less efficient electroporation, and a longer duration leads to a more efficient electroporation. We hypothesized that this is due to the presence of a cancellation effect whose strength decreases with increasing pulse duration. The presence of the cancellation effect of high-frequency bipolar pulses *in vitro* depends on the electroporation buffer. The cause of the cancellation effect is not known. However, its existence can be attributed at least in part to the active discharge of the membrane.

Although electrochemotherapy and irreversible electroporation are mainly single-shot therapies, in case of partial/incomplete response it is necessary to repeat the treatment. This raises the question of efficacy, whether it remains the same or worsens. Previous studies in which cells were repeatedly exposed to electrical pulses have been inconsistent, too short, or inadequate for the purpose of treating tumor tissue. In this study, we monitored sensitivity of cells to electroporation pulses by changes in membrane permeability (reversible electroporation range) and survival (irreversible electroporation range) up to 30 generations. Thus, we met the criteria of the standard protocol for establishing "resistance" or cell lines with reduced sensitivity to electroporation pulses. Based on the results of the study, we conclude that electroporation therapy can be applied more than once and that the efficacy of each treatment is comparable to that of the initial treatment, which is true for both reversible and irreversible electroporation.

Several researchers report a contribution of activation of the body's immune system to the long-term effects of treatment with reversible as well as irreversible electroporation since an activated immune system enables the destruction or reduction of distant metastases. Activation of the immune system by electroporation may be due to immunogenic cell death (ICD) of the electroporated cells, in which specific DAMP signaling molecules are released from the cells. Current understanding of the signaling pathways involved in cell death/survival and immune activation/suppression is poor. In contrast to existing studies, we analyzed release of DAMP molecules (ATP, calreticulin, nucleic acids, uric acid) over the entire electroporation range, i.e., both reversible and irreversible electroporation, and their release was statistically correlated with reversible and irreversible electroporation. The statistical correlation with altered membrane permeability is generally poor or not-existent. The statistical correlation between the release of DAMP and cell survival is much stronger, which could explain the activation of the immune system by irreversible electroporation. The reason for the activation of the immune system during electrochemotherapy treatment could be the unintended yet unavoidable irreversible electroporation in close proximity to the electrodes. Studies have shown that during electrochemotherapy treatment, despite the provision of reversible pulses, cell death occurs near the electrodes due to irreversible

electroporation or even thermal damage in immediate vicinity of the electrodes due to inhomogeneous distribution of the electric field.

To improve electroporation treatments, we also need to better understand how immunogenic death is activated, which in turn means understanding cell death due to electroporation. Currently, we know that a cell undergoes three phases during electroporation - damage, repair, and cell death. The mechanism of each of these phases during electroporation is currently poorly understood. In each phase, some possible targets, mechanisms, or pathways have been confirmed so far. Among the pathways of cell death, apoptosis is the most commonly described, while recent studies also include new pathways of cell death, namely necroptosis and pyroptosis both types of immunogenic cell death. In the future, a systematically designed study needs to be performed to determine whether the effect of electroporation in terms of damage/cell death depends on the pulse parameters, tissue origin of the cell or both.

5 VIRI

Ahmad F. B., Anderson R. N. 2021. The Leading Causes of Death in the US for 2020. The Journal of the American Medical Association, 325, 18: 1829–1830

Alberts B., Johnson A., Lewis J., Raff M., Roberts K., Walter P. 2002. Molecular biology of the cell. 5. izd. New York, Garland Science: 608 str.

Algazi A. P., Twitty C. G., Tsai K. K., Le M., Pierce R., Browning E., Daud A. I. 2020. Phase II Trial of IL-12 Plasmid Transfection and PD-1 Blockade in Immunologically Quiescent Melanoma. Clinical cancer research : an official journal of the American Association for Cancer Research, 26, 12: 2827–2837

Alzubi J., Lock D., Rhiel M., Schmitz S., Wild S., Mussolino C., Cornu T. I. 2021. Automated generation of gene-edited CAR T cells at clinical scale. Molecular Therapy - Methods & Clinical Development, 20: 379–388

Anguela X. M., High K. A. 2019. Entering the Modern Era of Gene Therapy. Annual Rewiew of Medicine, 70: 273–288

Arena C. B., Sano M. B., Rossmeisl J. H., Caldwell J. L., Garcia P. A., Rylander M. N., Davalos R. V. 2011. High-frequency irreversible electroporation (H-FIRE) for non-thermal ablation without muscle contraction. Biomedical engineering online, 10, 1: 102, doi: 10.1186/1475-925X-10-102: 20 str.

Aycock K. N., Davalos R. V. 2019. Irreversible Electroporation: Background, Theory, and Review of Recent Developments in Clinical Oncology. Bioelectricity, 1, 4: 214–234

Azarov J. E., Semenov I., Casciola M., Pakhomov A. G. 2019. Excitation of murine cardiac myocytes by nanosecond pulsed electric field. Journal of cardiovascular electrophysiology, 30, 3: 392–401

Bagalkot T. R., Leblanc N., Craviso G. L. 2019. Stimulation or Cancellation of Ca 2+ Influx by Bipolar Nanosecond Pulsed Electric Fields in Adrenal Chromaffin Cells Can Be Achieved by Tuning Pulse Waveform. Scientific reports, 9: 11545, doi: 0.1038/s41598-019-47929-4: 13 str.

Ball C., Thomson K. R., Kavnoudias H. 2010. Irreversible Electroporation. Anesthesia & Analgesia, 110, 5: 1305–1309

Barrau C., Teissié J., Gabriel B. 2004. Osmotically induced membrane tension facilitates the triggering of living cell electropermeabilization. Bioelectrochemistry, 63, 1–2: 327–332

Batista Napotnik T., Wu Y.-H., Gundersen M. A., Miklavčič D., Vernier P. T. 2012. Nanosecond electric pulses cause mitochondrial membrane permeabilization in Jurkat cells. Bioelectromagnetics, 33, 3: 257–264

Batista Napotnik T., Polajžer T., Miklavčič D. 2021. Cell death due to electroporation - A review. Bioelectrochemistry, 141: 107871, doi: 10.1016/j.bioelechem.2021.107871: 18 str.

Beebe S. J., Fox P. M., Rec L. J., Willis E. L. K., Schoenbach K. H. 2003. Nanosecond,

high-intensity pulsed electric fields induce apoptosis in human cells. The FASEB Journal, 17, 11: 1493–1495

Beebe S. J., Sain N., Ren W. 2013. Induction of Cell Death Mechanisms and Apoptosis by Nanosecond Pulsed Electric Fields (nsPEFs). Cells, 2, 1: 136–162

Beebe S. J. 2017. Regulated and apoptotic cell death after nanosecond electroporation. V: Handbook of Electroporation. 1. izd. Miklavčič D. (ur.). New York, Springer International Publishing: 511–528

Bermúdez-Aguirre D., Dunne C. P., Barbosa-Cánovas G. V. 2012. Effect of processing parameters on inactivation of Bacillus cereus spores in milk using pulsed electric fields. International Dairy Journal, 24, 1: 13–21

Bhatia S., Longino N. V., Miller N. J., Kulikauskas R., Iyer J. G., Ibrani D., Nghiem P. 2020. Intratumoral Delivery of Plasmid IL12 Via Electroporation Leads to Regression of Injected and Noninjected Tumors in Merkel Cell Carcinoma. Clinical cancer research : an official journal of the American Association for Cancer Research, 26, 3: 598–607

Bianchi G., Campanacci L., Ronchetti M., Donati D. 2016. Electrochemotherapy in the Treatment of Bone Metastases: A Phase II Trial. World journal of surgery, 40, 12: 3088–3094

Bower M., Sherwood L., Li Y., Martin R. 2011. Irreversible electroporation of the pancreas: definitive local therapy without systemic effects. Journal of surgical oncology, 104, 1: 22–28

Bowman A. M., Nesin O. M., Pakhomova O. N., Pakhomov A. G. 2010. Analysis of plasma membrane integrity by fluorescent detection of Tl + uptake. Journal of Membrane Biology, 236, 1: 15–26

Bradley C. J., Haines D. E. 2020. Pulsed field ablation for pulmonary vein isolation in the treatment of atrial fibrillation. Journal of cardiovascular electrophysiology, 31, 8: 2136–2147

Breton M., Delemotte L., Silve A., Mir L. M., Tarek M. 2012. Transport of siRNA through lipid membranes driven by nanosecond electric pulses: An experimental and computational study. Journal of the American Chemical Society, 134, 34: 13938–13941

Brock R. M., Beitel-White N., Davalos R. V., Allen I. C. 2020. Starting a Fire Without Flame: The Induction of Cell Death and Inflammation in Electroporation-Based Tumor Ablation Strategies. Frontiers in Oncology, 10: 1235, doi: 10.3389/fonc.2020.01235: 9 str.

Brookes P. S., Yoon Y., Robotham J. L., Anders M. W., Sheu S. S. 2004. Calcium, ATP, and ROS: A mitochondrial love-hate triangle. American Journal of Physiology - Cell Physiology, 287, 4: 56-64

Brunner S., Fürtbauer E., Sauer T., Kursa M., Wagner E. 2002. Overcoming the Nuclear Barrier: Cell Cycle Independent Nonviral Gene Transfer with Linear Polyethylenimine or Electroporation. Molecular Therapy, 5, 1: 80–86

Bulvik B. E., Rozenblum N., Gourevich S., Ahmed M., Andriyanov A. V., Galun E., Nahum

Goldberg S. 2016. Irreversible electroporation versus radiofrequency ablation: A Comparison of Local and Systemic Effects in a Small-Animal Model1. Radiology, 280, 2: 413–424

Calkins H., Hindricks G., Cappato R., Kim Y. H., Saad E. B., Aguinaga L., Yamane T. 2017. 2017 HRS/EHRA/ECAS/APHRS/SOLAECE Expert consensus statement on catheter and surgical ablation of atrial fibrillation. Heart rhythm, 14, 10: e275–e444, doi: 10.1093/europace/eux274: 160 str.

Calvet C. Y., Famin D., André F. M., Mir L. M. 2014. Electrochemotherapy with bleomycin induces hallmarks of immunogenic cell death in murine colon cancer cells. Oncoimmunology, 3: e28131, doi: 10.4161/onci.28131: 10 str.

Calvet C. Y., Mir L. M. 2016. The promising alliance of anti-cancer electrochemotherapy with immunotherapy. Cancer and Metastasis Reviews, 35, 2: 165-177

Campana L. G., Edhemovic I., Soden D., Perrone A. M., Scarpa M., Campanacci L., Serša G. 2019a. Electrochemotherapy – Emerging applications technical advances, new indications, combined approaches, and multi-institutional collaboration. European Journal of Surgical Oncology, 45, 2: 92-102

Campana L. G., Miklavčič D., Bertino G., Marconato R., Valpione S., Imarisio I., Serša G. 2019b. Electrochemotherapy of superficial tumors – Current status: Basic principles, operating procedures, shared indications, and emerging applications. Seminars in Oncology, 46, 2: 173–191

Čemažar M., Miklavcic D., in Sersa G. 1998. Intrinsic sensitivity of tumor cells to bleomycin as an indicator of tumor response to electrochemotherapy. Japanese journal of cancer research : Gann, 89, 3: 328–333

Čemažar M., Miklavcic D., Mir L. M., Belehradek J., Bonnay M., Fourcault D., Sersa G. 2001. Electrochemotherapy of tumours resistant to cisplatin: a study in a murine tumour model. European journal of cancer, 37, 9: 1166–1172

Chafai D. E., Sulimenko V., Havelka D., Kubínová L., Dráber P., Cifra M., Kubínová L. 2019. Reversible and Irreversible Modulation of Tubulin Self-Assembly by Intense Nanosecond Pulsed Electric Fields. Advanced Materials, 31, 39: 1903636, doi: 10.1002/adma.201903636, 7 str.

Chai W., Zhang W., Wei Z., Xu Y., Shi J., Luo X., Niu L. 2017. Irreversible electroporation of the uterine cervix in a rabbit model. Biomedical Microdevices, 19, 4, doi: 10.1007/s10544-017-0248-2

Chaplin D. D. 2010. Overview of the immune response. Journal of Allergy and Clinical Immunology, 125, 2: 3-23

Chen X., Ren Z., Zhu T., Zhang X., Peng Z., Xie H., Zheng S. 2015. Electric Ablation with Irreversible Electroporation (IRE) in Vital Hepatic Structures and Follow-up Investigation. Scientific Reports, 5, 1: 16233, doi: 10.1038/srep16233: 9 str.

Chiarella P., Massi E., De Robertis M., Sibilio A., Parrella P., Fazio V. M., Signori E. 2008. Electroporation of skeletal muscle induces danger signal release and antigen-presenting

cell recruitment independently of DNA vaccine administration, 8, 11: 1645–1657

Cho Y. K., Rhim H., Noh S. 2011. Radiofrequency Ablation versus Surgical Resection as Primary Treatment of Hepatocellular Carcinoma Meeting the Milan Criteria: A Systematic Review. Journal of Gastroenterology and Hepatology, 26, 9: 1354-1360

Ciobanu F., Golzio M., Kovacs E., Teissié J. 2018. Control by Low Levels of Calcium of Mammalian Cell Membrane Electropermeabilization. Journal of Membrane Biology, 251, 2: 221–228

Cooper S. T., McNeil P. L. 2015. Membrane Repair: Mechanisms and Pathophysiology. Physiological reviews, 95, 4: 1205–1240

Corrotte M., Castro-Gomes T. 2019. Lysosomes and plasma membrane repair. Current topics in membranes, 84: 1–16

Dauty E., Verkman A. S. 2005. Actin Cytoskeleton as the Principal Determinant of Size-dependent DNA Mobility in Cytoplasm: a new barrier for non-viral gene delivery. Journal of Biological Chemistry, 280, 9: 7823–7828

Davalos R. V, Mir I. L. M., Rubinsky B. 2005. Tissue ablation with irreversible electroporation. Annals of biomedical engineering, 33, 2: 223–231

Dermol J., Pakhomova O. N., Pakhomov A. G., Miklavčič D. 2016. Cell electrosensitization exists only in certain electroporation buffers. PLoS ONE, 11, 7: 1–19, doi: 10.1371/journal.pone.0159434: 19 str.

Di Gennaro P., Gerlini G., Urso C., Sestini S., Brandani P., Pimpinelli N., Borgognoni L. 2016. CD4 + FOXP3 + T regulatory cells decrease and CD3 + CD8 + T cells recruitment in TILs from melanoma metastases after electrochemotherapy. Clinical & experimental metastasis, 33, 8: 787–798

Diercks G. F. H., Kluin P. M. 2016. Basic Principles of the Immune System and Autoimmunity. V: Autoimmune Bullous Diseases. Jonkman M. K. (ur.). New york, Springer International Publishing: 3–12

Ding X., Stewart M. P., Sharei A., Weaver J. C., Langer R. S., Jensen K. F. 2017. High-throughput nuclear delivery and rapid expression of DNA via mechanical and electrical cell-membrane disruption. Nature Biomedical Engineering, 1, 3: 1–7

Dong S., Yao C., Zhao Y., Lv Y., Liu H. 2018. Parameters optimization of bipolar high frequency pulses on tissue ablation and inhibiting muscle contraction. Institute of Electrical and Electronics Engineers, 25, 1: 207-216, doi: 10.1109/TDEI.2018.006303: 10 str.

Edhemovic I., Gadzijev E. M., Brecelj E., Miklavčič D., Kos B., Zupanič A., Serša G. 2011. Electrochemotherapy: A New Technological Approach in Treatment of Metastases in the Liver. Technology in Cancer Research & Treatment, 10, 5: 475–485

Edhemovic I., Brecelj E., Gasljevic G., Marolt Music M., Gorjup V., Mali B., Serša G. 2014. Intraoperative electrochemotherapy of colorectal liver metastases. Journal of Surgical Oncology, 110, 3: 320–327

Escoffre J. M., Portet T., Favard C., Teissié J., Dean D. S., Rols M. P. 2011. Electromediated formation of DNA complexes with cell membranes and its consequences for gene delivery. Biochimica et Biophysica Acta (BBA) - Biomembranes, 1808, 6: 1538–1543

Evrendilek G. A., Karatas B., Uzuner S., Tanasov I. 2019. Design and effectiveness of pulsed electric fields towards seed disinfection. Journal of the Science of Food and Agriculture, 99, 7: 3475–3480

Fajrial A. K., He Q. Q., Wirusanti N. I., Slansky J. E., Ding X. 2020. A review of emerging physical transfection methods for CRISPR/Cas9-mediated gene editing. Theranostics, 10, 12: 5532

Faroja M., Ahmed M., Appelbaum L., Ben-David E., Moussa M., Sosna J., Goldberg S. N. 2012. Irreversible Electroporation Ablation: Is All the Damage Nonthermal? Radiology, 266, 2: 462-470

Faurie C., Reberšek M., Golzio M., Kanduser M., Escoffre J.M., Pavlin M., Rols M.P. 2010. Electro-mediated gene transfer and expression are controlled by the life-time of DNA/membrane complex formation. The Journal of Gene Medicine, 12, 1: 117–125

Festjens N., Vanden Berghe T., Vandenabeele P. 2006. Necrosis, a well-orchestrated form of cell demise: Signalling cascades, important mediators and concomitant immune response. Biochimica et Biophysica Acta (BBA) - Bioenergetics, 1757, 9–10: 1371–1387

Folegatti P. M., Bittaye M., Flaxman A., Lopez F. R., Bellamy D., Kupke A., Gilbert S. 2020. Safety and immunogenicity of a candidate Middle East respiratory syndrome coronavirus viral-vectored vaccine: a dose-escalation, open-label, non-randomised, uncontrolled, phase 1 trial. The Lancet. Infectious diseases, 20, 7, doi: 10.1016/S1473-3099(20)30160-2: 11 str.

Gabriel B., Teissie J. 1994. Generation of reactive-oxygen species induced by electropermeabilization of Chinese hamster ovary cells and their consequence on cell viability, 223, 1: 25–33

Gagneten M., Leiva G., Salvatori D., Schebor C., Olaiz N. 2019. Optimization of Pulsed Electric Field Treatment for the Extraction of Bioactive Compounds from Blackcurrant. Food and Bioprocess Technology, 12, 7: 1102–1109

Galluzzi L., Vitale I., Aaronson S. A., Abrams J. M., Adam D., Agostinis P., Kroemer G. 2018. Molecular mechanisms of cell death: Recommendations of the Nomenclature Committee on Cell Death 2018. Cell Death and Differentiation. Nature Publishing Group, 25: 486-541

Gargiulo M., Papa A., Capasso P., Moio M., Cubicciotti E., Parascandolo S. 2012. Electrochemotherapy for Non-Melanoma Head and Neck Cancers. Annals of Surgery, 255, 6: 1158–1164

Gasbarrini A., Campos W. K., Campanacci L., Boriani S. 2015. Electrochemotherapy to Metastatic Spinal Melanoma. SPINE, 40: 24, doi: 10.1097/BRS.0000000000001125: 7 str.

Geboers B., Scheffer H. J., Graybill P. M., Ruarus A. H., Nieuwenhuizen S., Puijk R. S., Meijerink M. R. 2020. High-Voltage Electrical Pulses in Oncology: Irreversible Electroporation, Electrochemotherapy, Gene Electrotransfer, Electrofusion, and Electroimmunotherapy. Radiology, 295, 2: 1254-272

Gehl J., Skovsgaard T., Mir L. M. 1998. Enhancement of cytotoxicity by electropermeabilization: an improved method for screening drugs. Anti-cancer drugs, 9, 4: 319–325

Gehl J., Serša G., Matthiessen L. W., Muir T., Soden D., Occhini A., Mir L. M. 2018. Updated standard operating procedures for electrochemotherapy of cutaneous tumours and skin metastases. Acta oncologica, 57, 7: 874–882

Gelebart P., Opas M., in Michalak M. 2005, februar. Calreticulin, a Ca2+-binding chaperone of the endoplasmic reticulum. International Journal of Biochemistry and Cell Biology, 76, 5: 779-785

Gianulis E. C., Casciola M., Xiao S., Pakhomova O. N., Pakhomov A. G. 2018. Electropermeabilization by uni- or bipolar nanosecond electric pulses: The impact of extracellular conductivity. Bioelectrochemistry, 119: 10–19

Gibot L., Montigny A., Baaziz H., Fourquaux I., Audebert M., Rols M. P. 2020. Calcium Delivery by Electroporation Induces In Vitro Cell Death through Mitochondrial Dysfunction without DNA Damages. Cancers, 12, 2: 425, doi: 10.3390/cancers12020425: 17 str.

Ginn S. L., Amaya A. K., Alexander I. E., Edelstein M., Abedi M. R. 2018. Gene therapy clinical trials worldwide to 2017: An update. The Journal of Gene Medicine, 20, 5: e3015, doi: 10.1002/jgm.3015: 17 str.

Glaser R. W., Leikin S. L., Chernomordik L. V., Pastushenko V. F., Sokirko A. I. 1988. Reversible electrical breakdown of lipid bilayers: formation and evolution of pores. Biochimica et Biophysica Acta (BBA) - Biomembranes, 940, 2: 275–287

Golberg A., Sack M., Teissie J., Pataro G., Pliquett U., Saulis G., Frey W. 2016. Energy-efficient biomass processing with pulsed electric fields for bioeconomy and sustainable development. Biotechnology for Biofuels, 9, 94, doi: 10.1186/s13068-016-0508-z: 22 str.

Gómez B., Munekata P. E. S., Gavahian M., Barba F. J., Martí-Quijal F. J., Bolumar T., Lorenzo J. M. 2019. Application of pulsed electric fields in meat and fish processing industries: An overview. Food Research International, 123: 95–105

Gong T., Liu L., Jiang W., Zhou R. 2019. DAMP-sensing receptors in sterile inflammation and inflammatory diseases. Nature Reviews Immunology 20, 2: 95–112

Gowrishankar T. R., Stern J. V., Smith K. C.,Weaver J. C. 2018. Nanopore occlusion: A biophysical mechanism for bipolar cancellation in cell membranes. Biochemical and Biophysical Research Communications, 503:1194-1199

Grošelj A., Bošnjak M., Strojan P., Krzan M., čemažar M., Serša G. 2018. Efficiency of electrochemotherapy with reduced bleomycin dose in the treatment of nonmelanoma

head and neck skin cancer: Preliminary results. Head and Neck, 40, 1: 120–125

Gudvangen E., Kim V., Novickij V., Battista F., Pakhomov A. G. 2022. Electroporation and cell killing by milli- to nanosecond pulses and avoiding neuromuscular stimulation in cancer ablation. Scientific reports, 12, 1, doi: 10.1038/s41598-022-04868-x: 15 str.

Guenther E., Klein N., Zapf S., Weil S., Schlosser C., Rubinsky B., Stehling M. K. 2019. Prostate cancer treatment with Irreversible Electroporation (IRE): Safety, efficacy and clinical experience in 471 treatments. PloS one, 14, 4, doi: 10.1371/journal.pone.0215093: 15 str.

Guo S., Jing Y., Burcus N. I., Lassiter B. P., Tanaz R., Heller R., Beebe S. J. 2018. Nano-pulse stimulation induces potent immune responses, eradicating local breast cancer while reducing distant metastases. International Journal of Cancer, 142, 3: 629–640

Gusbeth C., Frey W., Volkmann H., Schwartz T., Bluhm H. 2009. Pulsed electric field treatment for bacteria reduction and its impact on hospital wastewater. Chemosphere, 75, 2: 228–233

Hai A., Spira M. E. 2012. On-chip electroporation, membrane repair dynamics and transient in-cell recordings by arrays of gold mushroom-shaped microelectrodes. Lab on a Chip, 12, 16: 2865–2873

Haines D. E. 2022. What is Different About Pulsed Field Ablation … Everything? Journal of cardiovascular electrophysiology, 33, 3: 368-370

Hansen E. L., Sozer E. B., Romeo S., Frandsen S. K., Vernier P. T., Gehl J. 2015. Dose-Dependent ATP Depletion and Cancer Cell Death following Calcium Electroporation, Relative Effect of Calcium Concentration and Electric Field Strength. PLOS ONE, 10, 4: e0122973, doi: 10.1371/journal.pone.0122973: 12 str.

Hekstra D. R., White K. I., Socolich M. A., Henning R. W., Šrajer V., Ranganathan R. 2016. Electric-field-stimulated protein mechanics. Nature, 540, 7633: 400–405

Heller L. C., Heller R. 2010. Electroporation gene therapy preclinical and clinical trials for melanoma. Current gene therapy, 10, 4: 312–317

Heller R., Heller L. C. 2015. Gene electrotransfer clinical trials. Advances in genetics, 89: 235–262

Hristov K., Mangalanathan U., Casciola M., Pakhomova O. N., Pakhomov A. G. 2018. Expression of voltage-gated calcium channels augments cell susceptibility to membrane disruption by nanosecond pulsed electric field. Biochimica et biophysica acta. Biomembranes, 1860, 11: 2175–2183

Ibey B. L., Ullery J. C., Pakhomova O. N., Roth C. C., Semenov I., Beier H. T., Pakhomov A. G. 2014. Bipolar nanosecond electric pulses are less efficient at electropermeabilization and killing cells than monopolar pulses. Biochemical and biophysical research communications, 443, 2: 568–573

Imran K. M., Nagai-Singer M. A., Brock R. M., Alinezhadbalalami N., Davalos R. V., Allen I. C. 2022. Exploration of Novel Pathways Underlying Irreversible Electroporation Induced Anti-Tumor Immunity in Pancreatic Cancer. Frontiers in Oncology, 12, doi:

10.3389/fonc.2022.853779: 9 str.

Jakstys B., Jakutaviciute M., Uzdavinyte D., Satkauskiene I., Satkauskas S. 2020. Correlation between the loss of intracellular molecules and cell viability after cell electroporation. Bioelectrochemistry, 135: 107550, doi: 10.1016/j.bioelechem.2020.107550: 11 str.

Jarm T., Čemažar M., Miklavčič D., Serša G. 2010. Antivascular effects of electrochemotherapy: implications in treatment of bleeding metastases. Expert Review of Anticancer Therapy, 10, 5: 729–746

Jaroszeski M. J., Dang V., Pottinger C., Hickey J., Gilbert R., Heller R. 2000. Toxicity of anticancer agents mediated by electroporation in vitro. Anti-cancer drugs, 11, 3: 201–208

Jiang C., Qin Z., Bischof J. 2014. Membrane-Targeting Approaches for Enhanced Cancer Cell Destruction with Irreversible Electroporation. Annals of Biomedical Engineering, 42, 1: 193–204

José A., Sobrevals L., Ivorra A., Fillat C. 2012. Irreversible electroporation shows efficacy against pancreatic carcinoma without systemic toxicity in mouse models. Cancer Letters, 317, 1: 16–23

Kaminska I., Kotulska M., Stecka A., Saczko J., Drag-Zalesinska M., Wysocka T., Kulbacka J. 2012. Electroporation-induced changes in normal immature rat myoblasts (H9C2). Gen. Physiol. Biophys, 31: 19–25

Kato J., Svensson C. I. 2015. Role of extracellular damage-associated molecular pattern molecules (DAMPs) as mediators of persistent pain v Progress in Molecular Biology and Translational Science. Amsterdam, Elsevier: 251–279

Kellie S., Al-Mansour Z. 2017. Overview of the Immune System v Micro- and Nanotechnology in Vaccine Development. Amsterdam, Elsevier: 63–81

Klein N., Guenther E., Botea F., Pautov M., Dima S., Tomescu D., Popescu I. 2019. The combination of electroporation and electrolysis (E2) employing different electrode arrays for ablation of large tissue volumes. PLOS ONE, 14, 8: e0221393, doi: 10.1371/journal.pone.0221393: 13 str.

Koruth J., Kuroki K., Iwasawa J., Enomoto Y., Viswanathan R., Brose R., Reddy V. Y. 2019. Preclinical Evaluation of Pulsed Field Ablation: Electrophysiological and Histological Assessment of Thoracic Vein Isolation. Circulation: Arrhythmia and Electrophysiology, 12, 12, doi: 10.1161/CIRCEP.119.007781

Kotnik T., Miklavčič D., Mir L. M. 2001a. Cell membrane electropermeabilization by symmetrical bipolar rectangular pulses: Part II. Reduced electrolytic contamination. Bioelectrochemistry, 54, 1: 91–95

Kotnik T., Mir L. M., Flisar K., Puc M., Miklavčič D. 2001b. Cell membrane electropermeabilization by symmetrical bipolar rectangular pulses: Part I. Increased efficiency of permeabilization. Bioelectrochemistry, 54, 1: 83–90

Kotnik T., Pucihar G., Reberšek M., Miklavčič D., Mir L. M. 2003. Role of pulse shape in

cell membrane electropermeabilization. Biochimica et Biophysica Acta (BBA) - Biomembranes, 1614, 2: 193–200

Kotnik T., Kramar P., Pucihar G., Miklavčič D., Tarek M. 2012. Cell membrane electroporation- Part 1: The phenomenon. IEEE Electrical Insulation Magazine, 28, 5: 14–23

Kotnik T., Frey W., Sack M., Haberl Meglič S., Peterka M., Miklavčič D. 2015. Electroporation-based applications in biotechnology. Trends in biotechnology, 33, 8: 480–488

Kotnik T., Rems L., Tarek M., Miklavčič D. 2019. Membrane Electroporation and Electropermeabilization: Mechanisms and Models, 48: 63–91

Kranz P., Neumann F., Wolf A., Classen F., Pompsch M., Ocklenburg T., Brockmeier U. 2017. PDI is an essential redox-sensitive activator of PERK during the unfolded protein response (UPR). Cell death & disease, 8, 8: e2986, doi: 10.1038/cddis.2017.369: 12 str.

Krause K. H., Michalak M. 1997. Calreticulin. Cell, 88, 4: 439–443

Kreiger A., Ihan A., Avčin T. 2011. Cepljenje in cepiva - dobre prakse varnega cepljenja : univerzitetni učbenik za študente medicinske in zdravstvene fakultete. Ljubljana, Sekcija za preventivno medicino SZD: Sekcija za klinično mikrobiologijo in bolnišnične okužbe SZD: Inštitut za varovanje zdravja RS: 174 str

Kumar S., Barbhaiya C. R., Balindger S., John R. M., Epstein L. M., Koplan B. A., Michaud G. F. 2015. Better Lesion Creation And Assessment During Catheter Ablation. Journal of Atrial Fibrillation, 8, 3: 1189, doi: 10.4022/jafib.1189: 12 str.

Lambricht L., Lopes A., Kos S., Serša G., Préat V., Vandermeulen G. 2016. Clinical potential of electroporation for gene therapy and DNA vaccine delivery. Expert Opinion on Drug Delivery, 13, 2: 295–310

Lee E. W., Loh C. T., Kee S. T. 2007. Imaging guided percutaneous irreversible electroporation: Ultrasound and immunohistological correlation. Technology in Cancer Research and Treatment, 6, 4: 287–293

Levine Z. A. in Vernier P. T. 2010. Life cycle of an electropore: Field-dependent and field-independent steps in pore creation and annihilation. Journal of Membrane Biology, 236, 1: 27–36

Linnert M., Iversen H. K., Gehl J. 2012. Multiple brain metastases - current management and perspectives for treatment with electrochemotherapy. Radiology and oncology, 46, 4: 271–278

Lip G. Y. H., Fauchier L., Freedman S. B., Van Gelder I., Natale A., Gianni C., Lane D. A. 2016. Atrial fibrillation. Nature Reviews Disease Primers, 2, 1: 1–26

Maglietti F., Tellado M., De Robertis M., Michinski S., Fernández J., Signori E., Marshall G. 2020. Electroporation as the Immunotherapy Strategy for Cancer in Veterinary Medicine: State of the Art in Latin America. Vaccines, 8, 3: 1–19

Mahendran R., Ramanan K. R., Barba F. J., Lorenzo J. M., López-Fernández O., Munekata

P. E. S., Tiwari B. K. 2019. Recent advances in the application of pulsed light processing for improving food safety and increasing shelf life. Trends in Food Science & Technology, 88: 67–79

Mahnič-Kalamiza S., Miklavčič D. 2020. Scratching the electrode surface: Insights into a high-voltage pulsed-field application from in vitro & in silico studies in indifferent fluid. Electrochimica Acta, 363: 137187, doi: 10.1016/j.electacta.2020.137187: 15 str.

Mali B., Jarm T., Snoj M., Serša G., Miklavčič D. 2013. Antitumor effectiveness of electrochemotherapy: A systematic review and meta-analysis. European Journal of Surgical Oncology, 39, 1: 4–16

Maor E., Ivorra A., Leor J., Rubinsky B. 2007. The effect of irreversible electroporation on blood vessels. Technology in cancer research & treatment, 6, 4: 307–312

Maor E., Sugrue A., Witt C., Vaidya V. R., DeSimone C. V., Asirvatham S. J., Kapa S. 2019. Pulsed electric fields for cardiac ablation and beyond: A state-of-the-art review. Heart Rhythm, 16, 7: 1112–1120

Marty M., Serša G., Garbay J. R., Gehl J., Collins C. G., Snoj M., O'Sullivan G. C. 2006. Electrochemotherapy – An easy, highly effective and safe treatment of cutaneous and subcutaneous metastases: Results of ESOPE (European Standard Operating Procedures of Electrochemotherapy) study. European Journal of Cancer Supplements, 4, 11: 3–13

Matsuki N., Takeda M., Ishikawa T., Kinjo A., Hayasaka T., Imai Y., Yamaguchi T. 2010. Activation of caspases and apoptosis in response to low-voltage electric pulses. Oncology reports, 23, 5: 1425–1433

Mattes W. B. 2020. In vitro to in vivo translation. Current Opinion in Toxicology, 23–24: 114–118

McDermott M., Eustace A. J., Busschots S., Breen L., Crown J., Clynes M., Stordal B. 2014. In vitro development of chemotherapy and targeted therapy drug-resistant cancer cell lines: A practical guide with case studies. Frontiers in Oncology, 6, 4: 40, doi: 10.3389/fonc.2014.00040: 16 str.

Mercadal B., Arena C. B., Davalos R. V, Ivorra A. 2017. Avoiding nerve stimulation in irreversible electroporation: a numerical modeling study. Physics in Medicine & Biology, 62, 20: 8060–8079

Mercadal B., Beitel-White N., Aycock K. N., Castellví Q., Davalos R. V., Ivorra A. 2020. Dynamics of Cell Death After Conventional IRE and H-FIRE Treatments. Annals of Biomedical Engineering, 48: 1451-1462

Merla C., Pakhomov A. G., Semenov I., Vernier P. T. 2017. Frequency spectrum of induced transmembrane potential and permeabilization efficacy of bipolar electric pulses. Biochimica et biophysica acta. Biomembranes, 1859, 7: 1282–1290

Miklavčič D, Beravs K., Semrov D., Čemažar M., Demšar F., Serša G. 1998. The importance of electric field distribution for effective in vivo electroporation of tissues. Biophysical journal, 74, 5: 2152–2158

Miklavčič D., Šemrov D., Mekid H., Mir L. M. 2000. A validated model of in vivo electric

field distribution in tissues for electrochemotherapy and for DNA electrotransfer for gene therapy. Biochimica et Biophysica Acta - General Subjects, 1523, 1: 73–83

Miklavčič D., Pucihar G., Pavlovec M., Ribarič S., Mali M., Maček-Lebar A., Serša G. 2005. The effect of high frequency electric pulses on muscle contractions and antitumor efficiency in vivo for a potential use in clinical electrochemotherapy. Bioelectrochemistry, 65, 2: 121–128

Miklavčič Damijan, Corovic S., Pucihar G., Pavšelj N. 2006. Importance of tumour coverage by sufficiently high local electric field for effective electrochemotherapy. European Journal of Cancer Supplements, 4, 11: 45–51

Miklavčič D., Mali B., Kos B., Heller R., Serša G. 2014. Electrochemotherapy: from the drawing board into medical practice. Biomedical engineering online, 13, 1: 29, doi: 10.1186/1475-925X-13-29: 20 str.

Miller M. A., Davila A., Dukkipati S. R., Koruth J. S., Viles-Gonzalez J., Napolitano C., Reddy V. Y. 2012. Acute electrical isolation is a necessary but insufficient endpoint for achieving durable PV isolation: the importance of closing the visual gap. Europace : European pacing, arrhythmias, and cardiac electrophysiology : journal of the working groups on cardiac pacing, arrhythmias, and cardiac cellular electrophysiology of the European Society of Cardiology, 14, 5: 653–660

Miller M. A., Zachary J. F. 2017. Mechanisms and Morphology of Cellular Injury, Adaptation, and Death. V: Pathologic Basis of Veterinary Disease Expert Consult. 6th ed. Zachary J. F. (ur.). Amsterdam, Elesvier: 1-80

Mir L M, Orlowski S., Belehradek J., Paoletti C. 1991. Electrochemotherapy potentiation of antitumour effect of bleomycin by local electric pulses. European journal of cancer, 27, 1: 68–72

Mir L. M, Gehl J., Sersa G., Collins C. G., Garbay J. R., Billard V., Mir L. M. 2006. Standard operating procedures of the electrochemotherapy: Instructions for the use of bleomycin or cisplatin administered either systemically or locally and electric pulses delivered by the CliniporatorTM by means of invasive or non-invasive electrodes. The European Journal of Cancer Supplements, 4, 4: 14-25

Montanari C., Tylewicz U., Tabanelli G., Berardinelli A., Rocculi P., Ragni L., Gardini F. 2019. Heat-assisted pulsed electric field treatment for the inactivation of saccharomyces cerevisiae: Effects of the presence of citral. Frontiers in Microbiology, 10: 1737, doi: 10.3389/fmicb.2019.01737: 11 str.

Morrow M. P., Kraynyak K. A., Sylvester A. J., Shen X., Amante D., Sakata L., Bagarazzi M. L. 2016. Augmentation of cellular and humoral immune responses to HPV16 and HPV18 E6 and E7 antigens by VGX-3100. Molecular Therapy Oncolytics, 3: 16025, doi: 10.1038/mto.2016.25: 11 str.

Neumann E., Rosenheck K. 1972. Permeability changes induced by electric impulses in vesicular membranes. The Journal of Membrane Biology, 10, 1: 279–290

Nuccitelli R., Berridge J. C., Mallon Z., Kreis M., Athos B., Nuccitelli P. 2015. Nanoelectroablation of Murine Tumors Triggers a CD8-Dependent Inhibition of

Secondary Tumor Growth. PloS one, 10, 7: e0134364, doi: 10.1371/journal.pone.0134364: 17 str.

Nuccitelli R., McDaniel A., Anand S., Cha J., Mallon Z., Berridge J. C., Uecker D. 2017. Nano-Pulse Stimulation is a physical modality that can trigger immunogenic tumor cell death. Journal for ImmunoTherapy of Cancer, 5, 1: 32

Obeid M., Tesniere A., Ghiringhelli F., Fimia G. M., Apetoh L., Perfettini J. L., Kroemer G. 2007. Calreticulin exposure dictates the immunogenicity of cancer cell death. Nature Medicine, 13, 1: 54–61

Okino M., Mohri H. 1987. Effects of a high-voltage electrical impulse and an anticancer drug on in vivo growing tumors. Japanese journal of cancer research : Gann, 78, 12: 1319–1321

Oladiran O., Nwosu I. 2019. Stroke risk stratification in atrial fibrillation: a review of common risk factors. Journal of community hospital internal medicine perspectives, 9, 2: 113–120

Olaiz N., Signori E., Maglietti F., Soba A., Suárez C., Turjanski P., Marshall G. 2014. Tissue damage modeling in gene electrotransfer: The role of pH. Bioelectrochemistry, 100: 105–111

Ozturk B., Anli E. 2017. Pulsed electric fields (PEF) applications on wine production: A review. 40th World Congress of Vine and Wine, 9: 02008, doi: 10.1051/bioconf/20170902008: 4 str.

Pakhomov A. G., Semenov I., Xiao S., Pakhomova O. N., Gregory B., Schoenbach K. H., Ibey B. L. 2014. Cancellation of cellular responses to nanoelectroporation by reversing the stimulus polarity. Cellular and molecular life sciences, 71, 22: 4431–4441

Pakhomov A. G., Semenov I., Casciola M., Xiao S. 2017. Neuronal excitation and permeabilization by 200-ns pulsed electric field: An optical membrane potential study with FluoVolt dye. Biochimica et biophysica acta. Biomembranes, 1859, 7: 1273–1281

Pakhomov A. G., Pakhomova O. N. 2020. The interplay of excitation and electroporation in nanosecond pulse stimulation. Bioelectrochemistry, 136: 107598, doi: 10.1016/j.bioelechem.2020.107598: 8 str.

Pakhomova O. N., Gregory B. W., Khorokhorina V. A., Bowman A. M., Xiao S., Pakhomov A. G. 2011. Electroporation-induced electrosensitization. PLoS ONE, 6, 2, doi: 10.1371/journal.pone.0017100: 10 str.

Pakhomova O. N., Gregory B. W., Pakhomov A. G. 2013. Facilitation of electroporative drug uptake and cell killing by electrosensitization. Journal of Cellular and Molecular Medicine, 17, 1: 154–159

Pakhomova O. N., Gregory B. W., Semenov I., Pakhomov A. G. 2013. Two Modes of Cell Death Caused by Exposure to Nanosecond Pulsed Electric Field. PLoS ONE, 8, 7, doi: 10.1371/journal.pone.0070278: 10 str.

Pakhomova O. N., Gregory B., Semenov I., Pakhomov A. G. 2014. Calcium-mediated pore expansion and cell death following nanoelectroporation. Biochimica et Biophysica

Acta - Biomembranes, 1838, 10: 2547–2554

Panaretakis T., Kepp O., Brockmeier U., Tesniere A., Bjorklund A.-C., Chapman D. C., Kroemer G. 2009. Mechanisms of pre-apoptotic calreticulin exposure in immunogenic cell death. The EMBO Journal, 28, 5: 578–590

Pandit H., Hong Y. K., Li Y., Rostas J., Pulliam Z., Li S. P., Martin R. C. G. 2019. Evaluating the Regulatory Immunomodulation Effect of Irreversible Electroporation (IRE) in Pancreatic Adenocarcinoma. Annals of Surgical Oncology, 26, 3: 800–806

Patel S. 2018. Danger-Associated Molecular Patterns (DAMPs): the Derivatives and Triggers of Inflammation. Current allergy and asthma reports, 18, 11: 63, doi: 10.1007/s11882-018-0817-3: 12 str.

Pech M., Janitzky A., Wendler J. J., Strang C., Blaschke S., Dudeck O., Liehr U. B. 2011. Irreversible electroporation of renal cell carcinoma: a first-in-man phase I clinical study. Cardiovascular and interventional radiology, 34, 1: 132–138

Pirc E., Miklavčič D., Uršič K., Serša G., Reberšek M. 2021. High-Frequency and High-Voltage Asymmetric Bipolar Pulse Generator for Electroporation Based Technologies and Therapies, 10, 10: 1203, doi: 10.3390/electronics10101203: 19 str.

Polajžer T., Dermol-Černe J., Reberšek M., O'Connor R., Miklavčič D. 2020a. Cancellation effect is present in high-frequency reversible and irreversible electroporation. Bioelectrochemistry, 132: 107442, doi: 10.1016/j.bioelechem.2019.107442: 11 str.

Polajžer T., Miklavčič D. 2020b. Development of adaptive resistance to electric pulsed field treatment in CHO cell line in vitro. Scientific Reports, 10: 9988, doi: 10.1038/s41598-020-66879-w: 9 str.

Polajžer T., Jarm T., Miklavcic D. 2020. Analysis of damage-associated molecular pattern molecules due to electroporation of cells in vitro. Radiology and oncology, 54, 3: 317–328

Proskuryakov S. Y., Konoplyannikov A. G., Gabai V. L. 2003. Necrosis: A specific form of programmed cell death? Experimental Cell Research, 283, 1: 1–16

Pucihar G., Kotnik T., Kanduser M., Miklavcic D. 2001. The influence of medium conductivity on electropermeabilization and survival of cells in vitro. Bioelectrochemistry, 54, 2: 107–115

Raatikainen M. J. P., Arnar D. O., Merkely B., Nielsen J. C., Hindricks G., Heidbuchel H., Camm J. 2017. A Decade of Information on the Use of Cardiac Implantable Electronic Devices and Interventional Electrophysiological Procedures in the European Society of Cardiology Countries: 2017 Report from the European Heart Rhythm Association. EP Europace, 19, 2: 1–90

Reddy V. Y., Koruth J., Jais P., Petru J., Timko F., Skalsky I., Neuzil P. 2018. Ablation of Atrial Fibrillation With Pulsed Electric Fields: An Ultra-Rapid, Tissue-Selective Modality for Cardiac Ablation. JACC: Clinical Electrophysiology, 4, 8: 987–995

Reddy V. Y., Neuzil P., Koruth J. S., Petru J., Funosako M., Cochet H., Jais P. 2019. Pulsed Field Ablation for Pulmonary Vein Isolation in Atrial Fibrillation. Journal of the

American College of Cardiology, 74, 3: 315-326

Reddy V. Y., Dukkipati S. R., Neuzil P., Anic A., Petru J., Funasako M., Jais P. 2021. Pulsed Field Ablation of Paroxysmal Atrial Fibrillation: 1-Year Outcomes of IMPULSE, PEFCAT, and PEFCAT II. JACC. Clinical Electrophysiology, 7, 5: 614–627

Reece J. B., Urry L. A., Cain M. L., Wasserman S. A., Minorsky P. V. 2014. Campbell Biology. 8th ed. Boston, Benjamin-Cummings: 1464 str.

Rems L., Ušaj M., Kandušer M., Reberšek M., Miklavčič D., Pucihar G. 2013. Cell electrofusion using nanosecond electric pulses. Scientific Reports, 3: 49–55

Rems L., Miklavčič D. 2016. Tutorial: Electroporation of cells in complex materials and tissue. Journal of Applied Physics, 119, 201101, doi: 10.1063/1.4949264: 21 str.

Ren W., Beebe S. J. 2011. An apoptosis targeted stimulus with nanosecond pulsed electric fields (nsPEFs) in E4 squamous cell carcinoma. Apoptosis, 16, 4: 382–393

Ringel-Scaia V. M., Beitel-White N., Lorenzo M. F., Brock R. M., Huie K. E., Coutermarsh-Ott S., Allen I. C. 2019. High-frequency irreversible electroporation is an effective tumor ablation strategy that induces immunologic cell death and promotes systemic anti-tumor immunity. EBioMedicine, 44: 112–125

Rock K. L., Lai J.-J., Kono H. 2011. Innate and adaptive immune responses to cell death. Immunological reviews, 243, 1: 191–205

Roh J. S., Sohn D. H. 2018. Damage-associated molecular patterns in inflammatory diseases. Immune Network, 18, 4: e27, doi: 10.4110/in.2018.18.e27: 14 str.

Rols M. P., Teissie J. 1989. Ionic-strength modulation of electrically induced permeabilization and associated fusion of mammalian cells. European journal of biochemistry, 179, 1: 109–115

Rols M. P., Teissié J. 1990a. Electropermeabilization of mammalian cells. Quantitative analysis of the phenomenon. Biophysical journal, 58, 5: 1089–1098

Rols M. P., Teissié J. 1990b. Modulation of electrically induced permeabilization and fusion of Chinese hamster ovary cells by osmotic pressure. Biochemistry, 29, 19: 4561–4567

Rols M. P., Delteil C., Golzio M., Teissié J. 1998. Control by ATP and ADP of voltage-induced mammalian-cell-membrane permeabilization, gene transfer and resulting expression. European journal of biochemistry, 254, 2: 382–388

Rossi A., Pakhomova O. N., Mollica P. A., Casciola M., Mangalanathan U., Pakhomov A. G., Muratori C. 2019. Nanosecond pulsed electric fields induce endoplasmic reticulum stress accompanied by immunogenic cell death in murine models of lymphoma and colorectal cancer. Cancers, 11, 12: 2034, doi: 10.3390/cancers11122034: 18 str.

Rubinsky B. 2007. Irreversible Electroporation in Medicine. Technology in Cancer Research & Treatment, 6, 4: 255–259

Sachdev S., Potočnik T., Rems L., Miklavčič D. 2022. Revisiting the role of pulsed electric fields in overcoming the barriers to in vivo gene electrotransfer. Bioelectrochemistry,

144: 107994, doi: 10.1016/j.bioelechem.2021.107994: 26 str.

Sano M. B., Arena C. B., Bittleman K. R., DeWitt M. R., Cho H. J., Szot C. S., Davalos R. V. 2015. Bursts of Bipolar Microsecond Pulses Inhibit Tumor Growth. Scientific Reports, 5: 14999, doi: 10.1038/srep14999: 13 str.

Sano M. B., Fan R. E., Xing L. 2017. Asymmetric Waveforms Decrease Lethal Thresholds in High Frequency Irreversible Electroporation Therapies. Scientific Reports, 7: 40747, doi: 10.1038/srep40747: 13 str.

Šatkauskas S., Jakštys B., Ruzgys P., Jakutavičiūtė M. 2017. Different Cell Viability Assays Following Electroporation In Vitro V: Handbook of Electroporation. 1. izd. Miklavčič D. (ur.). New York, Springer International Publishing, 1411–1424

Saulis G., Saule R. 2012. Size of the pores created by an electric pulse: Microsecond vs millisecond pulses. Biochimica et Biophysica Acta (BBA) - Biomembranes, 1818, 12: 3032–3039

Scheffer H. J., Nielsen K., van Tilborg A. A. J. M., Vieveen J. M., Bouwman R. A., Kazemier G., Meijerink M. R. 2014. Ablation of colorectal liver metastases by irreversible electroporation: results of the COLDFIRE-I ablate-and-resect study. European Radiology, 24, 10: 2467–2475

Scheffer H. J., Nielsen K., de Jong M. C., van Tilborg A. A. J. M., Vieveen J. M., Bouwman A., Meijerink M. R. 2014. Irreversible Electroporation for Nonthermal Tumor Ablation in the Clinical Setting: A Systematic Review of Safety and Efficacy. Journal of Vascular and Interventional Radiology, 25, 7: 997–1011

Scheffer H. J., Stam A. G. M., Geboers B., Vroomen L. G. P. H., Ruarus A., de Bruijn B., de Gruijl T. D. 2019. Irreversible electroporation of locally advanced pancreatic cancer transiently alleviates immune suppression and creates a window for antitumor T cell activation. OncoImmunology, 8, 11: 1652532, doi: 10.1080/2162402X.2019.1652532: 8 str.

Schoenbach K. H., Pakhomov A. G., Semenov I., Xiao S., Pakhomova O. N., Ibey B. L. 2015. Ion transport into cells exposed to monopolar and bipolar nanosecond pulses. Bioelectrochemistry, 103: 44–51

Schultheis K., Smith T. R. F., Kiosses W. B., Kraynyak K. A., Wong A., Oh J., Broderick K. E. 2018. Delineating the Cellular Mechanisms Associated with Skin Electroporation. Human Gene Therapy Methods, 29, 4: 177–188

Scuderi M., Reberšek M., Miklavčič D., Dermol-Černe J. 2019. The use of high-frequency short bipolar pulses in cisplatin electrochemotherapy in vitro. Radiology and oncology, 53, 2: 194–205

Serša G., Čemažar M., Miklavčič D. 1995. Antitumor Effectiveness of Electrochemotherapy with cis-Diamminedichloroplatinum(II) in Mice! Cancer research, 55: 3450–3455

Serša G., Miklavčič D., Čemažar M., Belehradek J., Jarm T., Mir L. M. 1997. Electrochemotherapy with CDDP on LPB sarcoma: Comparison of the anti- tumor effectiveness in immunocompetent and immunodeficient mice. Bioelectrochemistry

and Bioenergetics 43: 279–283

Serša G., Teissie J., Cemazar M., Signori E., Kamensek U., Marshall G., Miklavčič D. 2015. Electrochemotherapy of tumors as in situ vaccination boosted by immunogene electrotransfer. Cancer Immunology, Immunotherapy, 64, 10: 1315-1327.

Shao Q., Liu F., Chung C., Elahi-Gedwillo K., Provenzano P. P., Forsyth B., Bischof J. C. 2017. Physical and Chemical Enhancement of and Adaptive Resistance to Irreversible Electroporation of Pancreatic Cancer. Annals of Biomedical Engineering, 46, 1: 25–36

Siddiqui I. A., Latouche E. L., DeWitt M. R., Swet J. H., Kirks R. C., Baker E. H., McKillop I. H. 2016. Induction of rapid, reproducible hepatic ablations using next-generation, high frequency irreversible electroporation (H-FIRE) in vivo. HPB : the official journal of the International Hepato Pancreato Biliary Association, 18, 9: 726–734

Siddiqui I. A., Kirks R. C., Latouche E. L., DeWitt M. R., Swet J. H., Baker E. H., McKillop I. H. 2017. High-Frequency Irreversible Electroporation: Safety and Efficacy of Next-Generation Irreversible Electroporation Adjacent to Critical Hepatic Structures. Surgical Innovation, 24, 3: 276–283

Silve A., Guimerà Brunet A., Al-Sakere B., Ivorra A., Mir L. M. 2014. Comparison of the effects of the repetition rate between microsecond and nanosecond pulses: Electropermeabilization-induced electro-desensitization? Biochimica et Biophysica Acta (BBA) - General Subjects, 1840, 7: 2139–2151

Singh S. M., D'Avila A., Singh S. K., Stelzer P., Saad E. B., Skanes A., Reddy V. Y. 2013. Clinical outcomes after repair of left atrial esophageal fistulas occurring after atrial fibrillation ablation procedures. Heart rhythm, 10, 11: 1591–1597

Sixou S., Eynard N., Escoubas J. M., Werner E., Teissié J. 1991. Optimized conditions for electrotransformation of bacteria are related to the extent of electropermeabilization. Biochimica et biophysica acta, 1088, 1: 135–138

Stewart M. T., Haines D. E., Verma A., Kirchhof N., Barka N., Grassl E., Howard B. 2019. Intracardiac pulsed field ablation: Proof of feasibility in a chronic porcine model. Heart Rhythm Society, 16, 5: 754-754

Stewart M. T., Haines D. E., Miklavčič D., Kos B., Kirchhof N., Barka N., Verma A. 2021. Safety and chronic lesion characterization of pulsed field ablation in a Porcine model. Journal of cardiovascular electrophysiology, 32, 4: 958–969

Stoecklein V. M., Osuka A., Lederer J. A. 2012. Trauma equals danger-damage control by the immune system. Journal of Leukocyte Biology, 92, 3: 539–551

Štublar J., Žižek D., Jan M., Jarm T., Miklavčič D. 2021. Zdravljenje atrijske fibrilacije s katetrsko ablacijo. Slovenian Medical Journal, 90, 7–8: 410–419

Swan R. Z., Sindram D., Martinie J. B., Iannitti D. A. 2013. Operative Microwave Ablation for Hepatocellular Carcinoma: Complications, Recurrence, and Long-Term Outcomes. Journal of Gastrointestinal Surgery, 17, 4: 719–729

Sweeney D. C., Reberšek M., Dermol J., Rems L., Miklavčič D., Davalos R. V. 2016. Quantification of cell membrane permeability induced by monopolar and high-

frequency bipolar bursts of electrical pulses. Biochimica et Biophysica Acta (BBA) - Biomembranes, 1858, 11: 2689–2698

Tarantino L., Busto G., Nasto A., Fristachi R., Cacace L., Talamo M., Ambrosino P. 2017. Percutaneous electrochemotherapy in the treatment of portal vein tumor thrombosis at hepatic hilum in patients with hepatocellular carcinoma in cirrhosis: A feasibility study. World journal of gastroenterology, 23, 5: 906–918

Tarek M. 2005. Membrane Electroporation: A Molecular Dynamics Simulation. Biophysical Journal, 88, 6: 4045–4053

Teissie J. 2017. Involvement of Reactive Oxygen Species in Membrane Electropermeabilization V: Handbook of Electroporation, 1. izd. Miklavčič D. (ur.). New York, Springer International Publishing, 1-15

Thamkaew G., Gómez G. F. 2020. Influence of pulsed and moderate electric field protocols on the reversible permeabilization and drying of Thai basil leaves. Innovative Food Science & Emerging Technologies, 64: 102430, doi: 10.1016/j.ifset.2020.102430: 12 str.

Thompson G. L., Roth C. C., Dalzell D. R., Kuipers M. A., Ibey B. L. 2014. Calcium influx affects intracellular transport and membrane repair following nanosecond pulsed electric field exposure. Journal of biomedical optics, 19, 5: 055005, doi: 10.1117/1.JBO.19.5.055005: 12 str.

Thompson G. L., Beier H. T., Ibey B. L. 2018. Tracking Lysosome Migration within Chinese Hamster Ovary (CHO) Cells Following Exposure to Nanosecond Pulsed Electric Fields. Bioengineering, 5, 4: 103, doi: 10.3390/bioengineering5040103: 13 str.

Tondo C., Iacopino S., Pieragnoli P., Molon G., Verlato R., Curnis A., Padeletti L. 2018. Pulmonary vein isolation cryoablation for patients with persistent and long-standing persistent atrial fibrillation: Clinical outcomes from the real-world multicenter observational project. Heart rhythm, 15, 3: 363–368

Topol E. J. 2021. Messenger RNA vaccines against SARS-CoV-2. Cell, 184, 6: 1401, doi: 10.1016/j.cell.2020.12.039: 1 str.

Valdez C. M., Barnes R. A., Roth C. C., Moen E. K., Throckmorton G. A., Ibey B. L. 2017. Asymmetrical bipolar nanosecond electric pulse widths modify bipolar cancellation. Scientific Reports, 7, 16372, doi: 10.1038/s41598-017-16142-6: 12 str.

Vogl T. J., Wissniowski T. T., Naguib N. N. N., Hammerstingl R. M., Mack M. G., Münch S., Hänsler J. 2009. Activation of tumor-specific T lymphocytes after laser-induced thermotherapy in patients with colorectal liver metastases. Cancer Immunology, Immunotherapy, 58, 10: 1557–1563

Volker T. T., Pianet I., Labouesse J., Teissié J. 1989. Signal transduction by membrane receptors in viable electropermeabilized cells: isoproterenol-stimulated cyclic AMP synthesis in C6 glioma cells. Biochimica et biophysica acta, 984, 2: 243–251

Wang X. Q., Xiao A. Y., Sheline C., Hyrc K., Yang A., Goldberg M. P., Yu S. P. 2003. Apoptotic insults impair Na+, K+-ATPase activity as a mechanism of neuronal death

mediated by concurrent ATP deficiency and oxidant stress. Journal of Cell Science, 116, 10: 2099–2110

Wang S., Chen J., Chen M. T., Vernier P. T., Gundersen M. A., Valderrábano M. 2009. Cardiac myocyte excitation by ultrashort high-field pulses. Biophysical journal, 96, 4: 1640–1648

Weaver J. C., Chizmadzhev Y. A., Chizmadzhev Y., Prausnitz M., Bose V., Langer R., Schulten K. 1996. Theory of electroporation: A review. Bioelectrochemistry and Bioenergetics, 41, 2: 135–160

White S. B., Zhang Z., Chen J., Larson A. C. 2018. Early Immunologic Response of Irreversible Electroporation versus Cryoablation in a Rodent Model of Pancreatic Cancer. Journal of Vascular and Interventional Radiology, 29, 12: 1764–1769

Wiczew D., Szulc N., Tarek M., Tarek M. 2020. On the permeability of cell membranes subjected to lipid oxidation. Bioelectrochemistry, 141: 107869, doi: 10.1016/j.bioelechem.2021.107869: 14 str.

Yin H., Kanasty R. L., Eltoukhy A. A., Vegas A. J., Dorkin J. R., Anderson D. G. 2014. Non-viral vectors for gene-based therapy. Nature Reviews Genetics, 15, 8: 541–555

Zhao J., Wen X., Tian L., Li T., Xu C., Wen X., Li C. 2019. Irreversible electroporation reverses resistance to immune checkpoint blockade in pancreatic cancer. Nature Communications, 10, 1: 1–14

Zhao Y., Bhonsle S., Dong S., Lv Y., Liu H., Safaai-Jazi A., Yao C. 2018. Characterization of conductivity changes during high-frequency irreversible electroporation for treatment planning. IEEE Transactions on Biomedical Engineering, 65, 8, doi: 10.1109/TBME.2017.2778101, 10 str.

Zhao Z., Anselmo A. C., Mitragotri S. 2022. Viral vector-based gene therapies in the clinic. Bioengineering & Translational Medicine, 7, 1: e10258, doi: 10.1002/btm2.10258: 20 str.

Zhou J., Wang G., Chen Y., Wang H., Hua Y., Cai Z. 2019. Immunogenic cell death in cancer therapy: Present and emerging inducers. Journal of Cellular and Molecular Medicine, 23, 8: 4854-4865

Zhou W., Xiong Z., Yao C., Li C. 2012. Low voltage irreversible electroporation induced apoptosis in HeLa cells. Journal of cancer research and therapeutics, 8, 1: 80–85

Žmuc J., Gasljevic G., Sersa G., Edhemovic I., Boc N., Seliskar A., Čemažar M. 2019. Large Liver Blood Vessels and Bile Ducts Are Not Damaged by Electrochemotherapy with Bleomycin in Pigs. Scientific Reports, 9, 1, doi: 10.1038/s41598-019-40395-y: 11 str.

Zorec B., Jelenc J., Miklavčič D., Pavšelj N. 2015. Ultrasound and electric pulses for transdermal drug delivery enhancement: Ex vivo assessment of methods with in vivo oriented experimental protocols. International Journal of Pharmaceutics, 490: 65–73

Zupanič A., Ribaric S., Miklavčič D. 2007. Increasing the repetition frequency of electric pulse delivery reduces unpleasant sensations that occur in electrochemotherapy. Neoplasma, 54, 3: 246–250

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PRILOGE

PRILOGA A: Dovoljenje za uporabo člankov Polajžer in sod. (2020a) in Batista Napotnik in sod. (2021) v tiskani in elektronski obliki

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