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Improving safety of electroporation clinical use - Izboljšanje varnosti pri klinični rabi elektroporacije

DOCTORAL DISSERTATION

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Mentor: Matej Reberšek, Ph.D

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Fakulteta za elektrotehniko

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PREFACE

The presented doctoral dissertation is the result of experimental work, data and statistical analysis, research and development related to electroporation-based treatments. The work was carried out during the doctoral studies at the Laboratory of Biocybernetics, University of Ljubljana, Faculty of Electrical Engineering, Slovenia. The results are presented in three papers published in international journals.

- **Paper 1: A. Cvetkoska**, A. Maček-Lebar, P. Trdina, D. Miklavčič, and M. Reberšek, **"Muscle contractions and pain sensation accompanying high-frequency electroporation pulses"**, *Scientific Reports*, vol. 12, article no. 8019, pp. 1-15, May 2022.
- **Paper 2: A. Cvetkoska**, E. Pirc, M. Reberšek, R. Magjarević, and D. Miklavčič, **"Towards standardization of electroporation devices and protocols"**, *IEEE Instrumentation & Measurement Magazine*, vol. 23, issue 2, pp. 74-81, April 2020.
- **Paper 3: A. Cvetkoska**, J. Dermol-Černe, D. Miklavčič, S. Kranjc Brezar, B. Markelc, G. Serša, and M. Reberšek, **"Design, development, and testing of a device for gene electrotransfer to skin cells in vivo"**, *Pharmaceutics*, vol. 14, issue 9, article no. 1826, pp. 1-14, August 2022.

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Experiments were performed within the Infrastructural Program: Network of research infrastructure centers at the University of Ljubljana (MRIC UL IP-0510). Part of the work was conducted in the scope of the Slovenian-Croatian Cooperation in Science and Technology (BI-HR/16-17-039; 2016-2017).

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Abstract

When cells are exposed to high-voltage electrical pulses, pores in the plasma membrane are formed, leading to a transient increase in plasma membrane permeability. As a result, transmembrane transport of molecules that otherwise cannot pass through the membrane is enabled. This phenomenon, called membrane electroporation, is currently used in medicine, biotechnology, food processing, and some environmentally relevant applications. Electroporation based treatments and therapies have reached clinical use such as electrochemotherapy and are further being developed for DNA vaccination and gene therapy for cancer treatment, as well as ablation of soft tissue, including cardiac tissue. Electrical pulses are generated by electrical pulse generators (i.e., electroporators) and delivered to the cells via electrodes (i.e., applicators). Applying electrical pulses to the cells (tissues) generates an electric field of certain magnitude, depending on the electrode geometry and on the dielectric properties of the tissue (in vivo). However, the thresholds of the electric field required to initiate electroporation are generally higher than the thresholds that trigger action potentials in excitable cells. This implies that electroporation cannot be successfully performed without causing (unintended) electrical stimulation of excitable cells, resulting in unwanted muscle contractions and pain sensations during electroporation treatment. Furthermore, electroporators used in a clinical setting, i.e., clinical electroporators, are considered medical devices of which development due to high-voltages and currents applied is most challenging. Also patient and operator safety must be ensured under both normal and single fault conditions. Electroporators for clinical use have to follow and meet different sets of requirements and standards which are defined and assessed by local medical regulations. Currently, there are only few approved/certified clinical electroporators on the market.

The main objective of this doctoral dissertation is to address some of the safety issues relevant to the clinical application of electroporation and to propose improvements that may contribute to an even wider application of electroporation based treatments and therapies in the future. Therefore, the topics presented in the thesis relate to minimizing muscle contraction and pain sensation during pulse delivery, as well as safer and easier development and use of clinical electroporators.

To minimize neuromuscular electrical stimulation during electroporation-based treatments, it has been proposed to replace long monopolar pulses with trains of bipolar high-frequency pulses in the microsecond range to reduce muscle contraction and pain sensation during pulse delivery. While the reduction of muscle contraction has been confirmed in several in vivo studies, the reduction of pain sensation has not yet been confirmed in humans, nor has the relationship between muscle contraction and pain sensation been investigated. Therefore, we performed the first study in humans to investigate muscle contraction and pain sensation with bipolar high-frequency electroporation pulses. Twenty-five healthy volunteers were subjected to electrical stimulation of the tibialis anterior muscle with bipolar high-frequency pulses in the range of a few microseconds and both symmetric and asymmetric interphase and interpulse delays. Our results confirm that bipolar high-frequency pulses with a pulse duration of 1 or 2 µs reduce muscle contraction and pain sensation in contrast to the longer monopolar pulses currently used. Furthermore, interphase and interpulse delays play an important role in reducing muscle contraction and/or pain sensation. With this study, we have shown that the range of optimal pulse parameters can be extended depending on the prerequisites of the therapy and that different bipolar pulse protocols can be used to achieve a reduction in muscle contraction and/or pain sensation.

We then reviewed and compared the characteristics of electroporation applications and devices described in the literature and/or available on the market. Since there is no particular standard or regulation that specifically addresses the safety of medical devices for electroporation, we proposed guidelines for the design of clinical electroporators and defined the minimum requirements for their safe and efficient use that can be incorporated into the particular standards for clinical electroporators in the future. In addition, we have defined tolerances and proposed recommendations for standardization of electrochemotherapy devices based on the standard operating procedure.

Finally, considering some of the guidelines and requirements for safe design and use of a clinical electroporator based on medical safety standards and Medical Device Regulation 2017/745, we designed and developed a new electroporation device (electroporator and applicator) for gene electrotransfer to cells in the skin. The goal was to design and develop an electroporator that will have an improved safety performance. Therefore, the work within the dissertation focused on developing new safety measures for the electroporator to protect the patient from excess output voltage, current or energy. The electroporator was tested with an electrical safety analyzer and was found to be safe in terms of leakage currents, as they were within the range of allowable values according to the general safety standard for medical electrical equipment EN/IEC 60601-1. The electroporator was then tested in vivo for gene electrotransfer to cells in mouse skin. It was shown that electroporation with the developed electroporator, applicator, and proposed pulse delivery protocol resulted in higher gene expression in skin cells compared to the currently used electroporator, electrodes, and pulse delivery protocol.

Key words: electroporation, electroporation equipment, nerve stimulation, muscle contraction, clinical electroporators, standardization, medical standards.

I Uvod

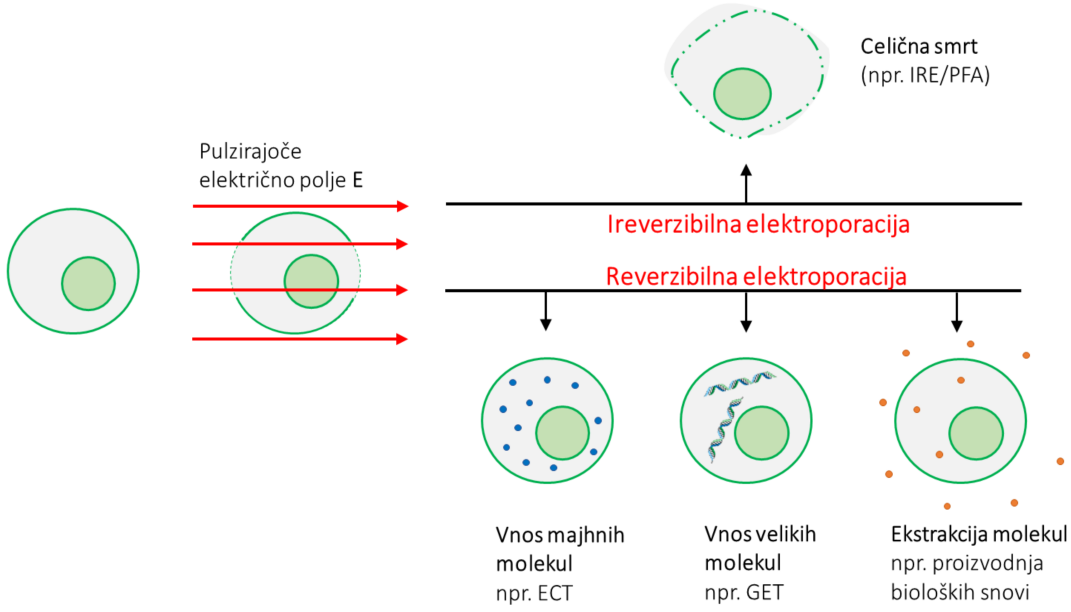
I.1 Elektroporacija

Vsaka biološka celica je obdana s celično membrano. Ta ločuje notranjost celice od zunajceličnega prostora in je sestavljena iz dvojne plasti lipidov. Celična membrana bi bila večinoma neprepustna, če ne bi bilo različnih proteinov, ki omogočajo transport le določenih molekul skozi membrano. Ko je celična membrana izpostavljena dovolj močnemu električnemu polju, pride do vsiljene transmembranske napetosti (*ang. transmembrane voltage, TMN*), npr. 500 mV, ki daleč presega transmembranske napetosti v mirovanju (običajno od -40 mV do -70 mV). Tako se začasno oblikujejo pore v dvoslojih in poveča se prepustnost celične membrane, kar omogoči transmembranski transport molekul, ki drugače membrane ne morejo prečkati [1]. Opisan pojav, imenovan elektroporacija oz. permeabilizacija membrane, je lahko reverzibilen, če si celica opomore in preživi, ali ireverzibilen, če elektroporacija povzroči celično smrt [2–4] (Slika I.1).

I.2 Elektroporacija in električna stimulacija

Elektroporacija velja za pragovni pojav, kar pomeni, da mora biti za povečanje prepustnosti celične membrane dosežen določen prag. Električna stimulacija je postopek, pri katerem se na živčna vlakna preko elektrod, pritrjenih na koži, dovaja električne pulze. Odziv človeškega telesa na električno stimulacijo je odvisen od frekvence in amplitude pulzov. Električna stimulacija vzdražnih tkiv je tako kot elektroporacija, pragovni pojav, kar pomeni, da se bo akcijski potencial sprožil, če bo dosežen določen prag. Akcijski potencial je definiran kot nenadna,

hitra, prehodna in razširljiva sprememba transmembranske napetosti. Nastane kadar zunanji dražljaj spremeni transmembransko napetost do pragovne vrednosti (odvisno od celice, okoli -55 mV za tipičen nevron). Akcijski potencial se obnaša po *principu vse ali nič*, kar pomeni, da podpragovni dražljaj ne bo sprožil akcijskega potenciala, medtem ko bo pragovni, ali nadpragovni dražljaj povzročili popoln odziv vzdražne celice [5].



Slika I.1. Simbolični prikaz različne uporabe elektroporacije. Ko je celica izpostavljena dovolj močnemu električnemu polju, se prepustnost celične membrane poveča. Elektroporacija je lahko reverzibilna, če si celica opomore in preživi; ali ireverzibilna, če elektroporacija povzroči celično smrt. V primeru reverzibilne elektroporacije, lahko vnesemo molekule v celico (npr. elektrokemoterapija (ECT), genska elektrotransfekcija (GET)) ali jih iz nje ekstrahiramo (tudi v primeru ireverzibilne elektroporacije).

Medtem ko elektroporacijo lahko dosežemo v vseh celicah, odziv na električno stimulacijo opazimo le pri vzdražnih celicah, kot so mišične in živčne. Električna mišična stimulacija je tako deležna večje pozornosti zaradi njene uporabe pri rehabilitaciji po poškodbi ali paralizi, vadbi za moč, okrevanju po vadbi, itd. Pulzi, ki jih prenašajo elektrode na mišico, posnemajo akcijski potencial, ki prihaja iz osrednjega živčnega sistema, in sprožijo mišično kontrakcijo (krčenje mišice) [6]. Vendar pa so pragovi transmembranske napetosti, potrebni za elektroporacijo (nekaj sto mV), višji od pragov, ki sprožijo akcijske potenciale v vzdražnih celi-

cah (manj kot 100 mV). To nakazuje, da elektroporacija ne bo uspešno izvedena brez sočasne (nenamerne) električne stimulacije vzdražnih celic, kar povzroča neželeno krčenje mišice in občutek bolečine med zdravljenjem z uporabo elektroporacije [7, 8].

I.2.1 Živčna vlakna

Živčno vlakno (t.j. akson) je dolg, vitek izrastek živčne celice, ki običajno prevaja akcijski potencial stran od telesa živčne celice. To omogoča, da se informacije prenesejo na različne mišice in nevrone (npr. pri krčenju mišice, zaznanju dotika ali občutka bolečine). Akcijski potencial se širi po aksonu ne da bi spremenil obliko. Hitrost širjenja akcijskega potenciala je odvisna od debeline aksona in od tega, ali je mieliniziran ali ne. V živčnem sistemu tako obstajata dve vrsti aksonov: mielinizirani - izolirani z mielinsko ovojnico, ki omogočajo hitrejše prevajanje akcijskega potenciala in počasnejši, nemielinizirani - brez mielinske ovojnice [9]. Mielinska ovojnica je prekinjena z vrzelmi (t.i. Ranvierjevimi zažemki), ki se pojavljajo na enakomernih razdaljah in omogočajo hitro prevajanje akcijskega potenciala (t.i. saltatorno prevajanje) iz enega Ranvierjevega zažemka na naslednjega. Mielinizirana živčna vlakna so razvrščena na A-alfa, A-beta in A-delta živčna vlakna. A-alfa vlakna imajo največji premer ter najvišjo prevodno hitrost in vključujejo motorične nevrone, ki prenašajo signale za krčenje mišic. Posamezen motorični nevron tako lahko aktivira več mišičnih vlaken. A-beta vlakna imajo manjši premer ter manjšo prevodno hitrost in prenašajo senzorične informacije, kot sta dotik in temperatura. A-delta vlakna so najmanjšega premera v tej skupini in prenašajo občutek ostre, zbadajoče bolečine. Lokacija vira bolečine je natančno zaznana. Na drugi strani so vlakna C, ki niso mielinizirana, odgovorna za tako imenovano bolečino druge stopnje, ki prenašajo občutek globoke, tope bolečine, ki ni dobro lokalizirana. Imajo manjši premer in manjšo prevodno hitrost kot A-delta vlakna [10–13].

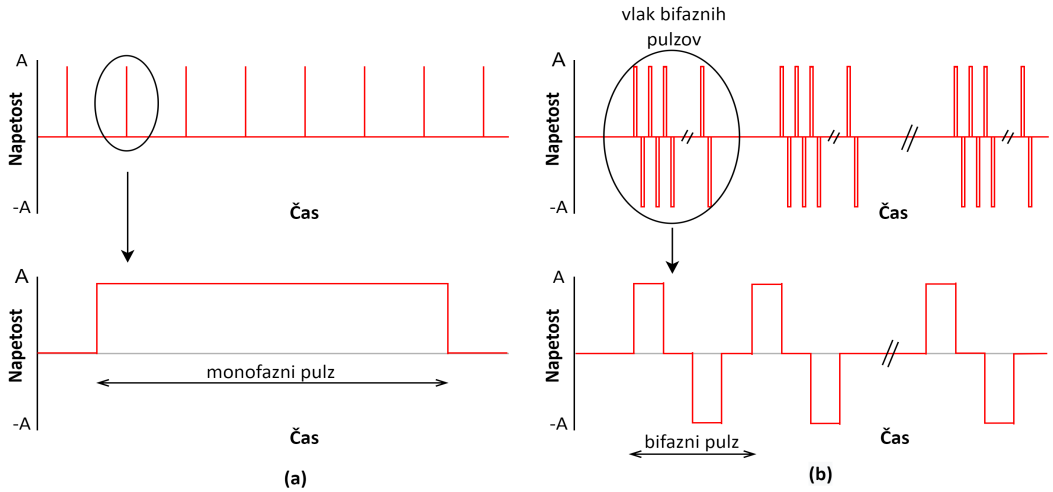
I.3 Uporaba elektroporacije

Elektroporacija je trenutno uporabljena v biotehnologiji [14], pri predelavi hrane [15] in v aplikacijah, ki so pomembne za okolje [16]. Poleg tega reverzibilna elektroporacija je uspešno uporabljena v kliničnih aplikacijah kot kombinacija visokonapetostnih električnih pulzov s kemoterapevtiki, za katere celična membrana predstavlja težko prehodno oviro - elektrokemoterapija (*ang. Electrochemotherapy, ECT*), ali z DNK - genska elektrotransfekcija (*ang. Gene Electrotransfer, GET*) [17–24] (Slika I.1). Ireverzibilna elektroporacija (IRE) se uporablja kot nova aplikacija v medicini [3] za netermično ablacijo tumorjev (ang. Nonthermal Irreversible Electroporation - NTIRE ali IRE) [25–27] in srčno ablacijo (*ang. Pulse Field Ablation – PFA*) [28–31]. Ta metoda ablacije je pokazala znatne prednosti pred trenutno uporabljenimi metodami termične ablacije, kot npr. zmanjšanje tveganja poškodbe bližnjega kritičnega tkiva. To pomeni, da srčna ablacija lahko postane prevladujoče zdravljenje v prihodnosti, zlasti v elektrofiziologiji srca [32–34].
Trenutno se v vseh zgoraj omenjenih kliničnih aplikacijah, ki temeljijo na elektroporaciji, uporabljajo relativno dolgi monopolarni pulzi (slika I.2a) z dolžino pulza 50 - 100 µs in nizko frekvenco ponavljanja pulzov (npr. 1 Hz ali 5 kHz). Za GET se najpogosteje uporabljajo pulzi, ki trajajo več milisekund (tudi do 50 ms). Amplituda pulzov se lahko giblje od nekaj deset voltov (npr. za GET) do nekaj kilovoltov (npr. za IRE). Posledično lahko dovajanje takih pulzov povzroči stimulacijo (vzdraženje) mišičnih in živčnih celic, zaradi česar je terapija lahko za pacienta neprijetna in v nekaterih primerih celo boleča. Pri zdravljenju je zato velikokrat potrebna lokalna ali splošna anestezija, skupaj z mišičnimi relaksanti, da sta zagotovljena ustrezna nevromuskularna blokada in pravilno delovanje dihal [20, 35–37]. Poleg tega mora biti dovajanje pulzov sinhronizirano s srčnim ritmom [3, 38, 39].

I.3.1 Visokofrekvenčna ireverzibilna elektroporacija

Pred kratkim je bil za ablacijo tkiva predlagan nov pulzni protokol (slika I.2b), ki ga sestavljajo kratki (0.5 - 10 µs), bipolarni pulzi, ki si sledijo z visokimi frekvencami (v območju stotih kHz) v vlakih z nizko ponavljalno frekvenco (nekoliko

Hz). Pokazano je bilo, da z njimi ne sprožimo akcijskih potencialov v živčnih vlaknih, ki bi bila sicer vzdražena z uporabo monopolarnih pulzov, kar pomeni zmanjšanega mišičnega krčenja med zdravljenjem [40, 41]. Omenjena metoda elektroporacije se je pojavila pod imenom visokofrekvenčna ireverzibilna elektroporacija (*ang. High-Frequency Irreversible Electroporation, H-FIRE*) [42–45]. Za preučevanje visokofrekvenčne ireverzibilne elektroporacije je bilo že izvedenih nekaj poskusov, ki dokazujejo zmanjšanjega mišičnega krčenja med ireverzibilno elektroporacijo [46–49]. Nadalje so pokazali z in vitro poskusi, da se lahko visokofrekvenčni pulzi uporabljajo tudi pri elektrokemoterapiji [50] in genski elektrotransfekciji [51] ter z in vivo poskusi pri ablaciji tumorjev [46] ter srčnega tkiva [30, 34, 52].

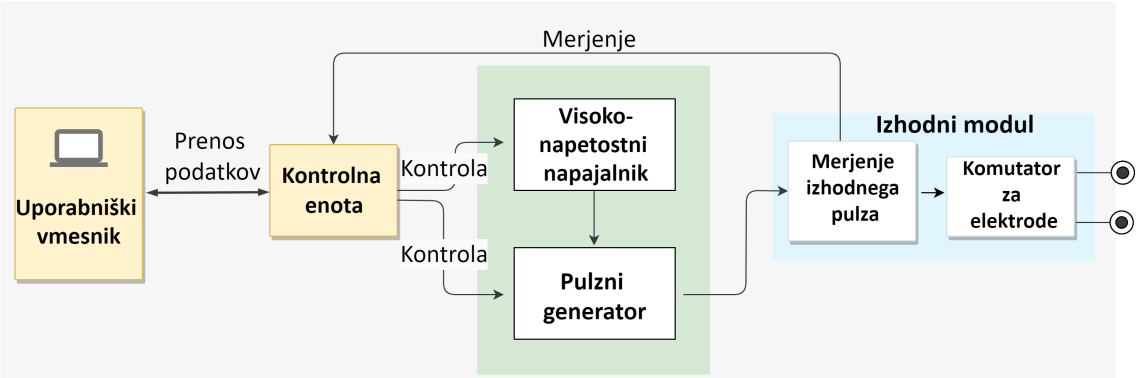


Slika I.2. (a) Najpogosteje uporabljeni dolgi monopolarni pulzi (8 pulzov x 100 µs); (b) predlagani visokofrekvenčni kratki bipolarni pulzi.

I.4 Dovajanje električnih pulzov pri elektroporaciji

Da bi dosegli uspešno elektroporacijo, morajo biti celice izpostavljene dovolj visoki električni poljski jakosti, ki jo ustvarjajo kratkotrajni električni pulzi visoke napetosti. Pulze generiramo z generatorji električnih pulzov, ti so znani tudi kot elektroporatorji, in jih preko elektrod dovedemo celicam (v tkivu). Porazdelitev električne poljske jakosti je definirana z geometrijo elektrod in dielektričnimi lastnostmi tkiva. Parametri električnih pulzov na izhodu elektropora-

torja so običajno različni po obliki, amplitudi, trajanju pulzov, številu pulzov ter frekvenci ponavljanja pulzov in vlakov, odvisno od aplikacije [53–56]. Elektroporator je večinoma sestavljen iz uporabniškega vmesnika, kontrolne enote, visokonapetostnega napajalnika in pulznega generatorja ter izhodnega modula (slika I.3). Uporabniški vmesnik omogoča nastavitev parametrov pulza, glede na določeno aplikacijo. Visokonapetostni napajalnik skupaj s pulznim generatorjem generirata in oblikujeta pulze glede na nastavljene vrednosti. Izhodni modul je sestavljen iz enote za merjenje izhodnega pulza in komutatorja za preklapljanje visokonapetostnih pulzov na različne elektrode (če se uporabljajo več elektrod) [57].



Slika I.3. Blok diagram elektroporatorja.

Elektroporatorji se glede na uporabo delijo na klinične, industrijske ali laboratorijske [55]. Nadalje je mogoče razlikovati tudi med komercialno dostopnimi in prototipnimi elektroporatorji. Elektrode so razvrščene glede na ciljno breme t.j., celice (v pogojih in vitro: enocelične komore, mikro, makro elektrode in pretočne komore) ali tkivo (ploščate, igelne, nastavljive).

I.4.1 Klinični elektroporatorji kot medicinski pripomočki

Elektroporatorji, ki se uporabljajo v medicini, t.i., klinični elektroporatorji, sodijo v skupino medicinskih pripomočkov. Razviti so bili za izvajanje protitumorskih terapij s protokoli pulzov, ki temeljijo na reverzibilni elektroporaciji (elektrokemoterapija, genska elektrotransfekcija) ter za samostojno ablacijsko terapijo, ki temelji na ireverzibilni elektroporaciji. Tumorsko tkivo mora biti pokrito z

dovolj visoko električno poljsko jakost, kar pogosto zahteva generiranje visokonapetostnih pulzov z amplitudami do 3000 V in tokovi do 50 A. Pri tem je potrebno zagotoviti varnost pacienta in operaterja tako v normalnih pogojih kot v pogojih enojne napake. Zaradi visokih napetosti in tokov je razvoj kliničnih elektroporatorjev za uporabo v medicini zelo zahteven. Klinični elektroporatorji morajo slediti in izpolnjevati medicinske varnostne standarde ter upoštevati zahteve, določene z lokalnimi zdravstvenimi predpisi, npr. Uredba o medicinskih pripomočkih 2017/745 v Evropi [58] ali 21 CFR (Kodeks zveznih predpisov) v Združenih Državah Amerike (ZDA), da bi pridobili dovoljenje za prodajo naprave na trgu, npr. certifikacijska oznaka (*ang. certification mark, CE*) v Evropi ali odobritev FDA (*ang. Food and Drug Administration*) v ZDA. Ker ima vsaka država različne regulacije, kliničnega elektroporatorja, odobrenega za uporabo v Evropi, ni mogoče uporabljati v ZDA in obratno brez dodatnega preizkusa in odobritve.

Trenutno je na trgu le nekaj certificiranih kliničnih elektroporatorjev. Najpogosteje uporabljeni klinični elektroporatorji so Cliniporator EPS02 in Cliniporator VITAE (IGEA S.p.A. Carpi (MO), Italija) [59], ki imata oznako CE za Evropo in se uporabljata tako za ECT kot GET ter NanoKnife (AngioDynamcis, Inc., Queensbury, New York, ZDA) [60], ki ima odobritev FDA-ja za kirurško ablacijo mehkih tkiv in oznako CE za Evropo. Tretji klinični elektroporator z oznako CE v Evropi je SENNEX (BionMed Technologies, Nemčija), ki se uporablja samo za ECT. Pred kratkim je bil razvit tudi nov klinični elektroporator ePORE (Mirai Medical, Galway, Irska), ki ima oznako CE, za preprosto in zanesljivo dovajanje zelo kratkih električnih pulzov, ki omogoča endoskopsko zdravljenje, brez da bi bilo potrebno pacienta hospitalizirati. Za ablacijo srčnega tkiva sta na trgu še dva klinična elektroporatorja: Farapulse (Farapulse, Inc., Boston ZDA) in CENTAURI PEF System (Galaxy Medical, San Carlos, CA, ZDA), ki imata CE oznako.

I.4.2 Varnost kliničnega elektroporatorja kot sestavni del razvoja

Razvoj kliničnih elektroporatorjev je zahteven, predvsem pri zagotavljanju varnosti pacientov in operaterjev. Da bi bila zagotovljena visoka raven varnosti za pacienta in operaterja, so vzpostavljeni standardi za preverjanje električne varnosti medicinskih pripomočkov. Uporaba načel in zahtev, opisanih v varno-

stnih standardih je pomembna in jo je treba upoštevati od začetka načrtovanja vsake medicinske električne naprave. Splošni standard za medicinsko električno opremo EN/IEC 60601-1 - 1.del: "Splošne zahteve za osnovno varnost in bistvene zmogljivosti" je splošno sprejeto merilo in skladnost s tem standardom je glavna zahteva za komercializacijo električne medicinske opreme. Zahteve določene v splošnem standardu 60601-1 so dopolnjene/preglašene s posebnimi zahtevami v spremljevalnih in partikularnih (posebnih) standardih, odvisno od vrste naprave. Spremljevalni standardi (oštevilčeni 60601-1-X) opredeljujejo splošne zahteve za varnost in delovanje določene funkcije, npr. Elektromagnetna združljivost (EN/IEC 60601-1-2). Posebni standardi (oštevilčeni 60601-2-X) določajo zahteve za posebne pripomočke, npr. srčni defibrilatorji (EN/IEC 60601-2-4). Kljub osemdesetim posebnim standardom, trenutno ne obstaja posebni standard za klinične elektroporatorje. Zato je potrebno določiti dodatna pravila za izdelavo in varno uporabo kliničnih elektroporatorjev kot relativno novih medicinskih pripomočkov poleg tistih, ki jih določajo obstoječi standardi ISO in EN/IEC za medicinske pripomočke. Ker trg kliničnih elektroporatorjev raste, bi posebni standard za klinične elektroporatorje lahko pospešil postopek certificiranja in omogočil uskladitev vseh komercialnih, certificiranih kliničnih elektroporatorjev. S tem bi izboljšali varnost, kakovost in učinkovitost teh naprav ter zagotovili varne in učinkovite terapije, ki temeljijo na elektroporaciji.

II Namen

Že od prvega uspešnega zdravljenja tumorjev z uporabo elektrokemoterapije (ECT) [61, 62] je cilj razviti učinkovitejšo, varnejšo in manj bolečo terapijo in zdravljenje z uporabo elektroporacije. Danes uporaba elektroporacije v medicini narašča še hitreje kot prej. Upoštevajoč nove tehnologije in protokole zdravljenja, pridobljeno znanje in intenzivnost raziskav, že imamo nekaj uveljavljenih protokolov, opreme ter učinkovitih in obetajočih terapij. Vendar še vedno ostajajo izzivi, ki lahko, če jih rešimo, prispevajo k še širši uporabi elektroporacije v medicini. Elektrokemoterapija je edina aplikacija elektroporacije, ki se uporablja v klinični praksi kot terapija za zdravljenje raka [63] po standardnem operativnem postopku (SOP), ki je bil posebej razvit za zdravljenje pacientov s kliničnim elektroporatorjem Cliniporator [20]. Elektroporacija ima velik potencial tudi za uporabo v medicinskih aplikacijah, kot so cepljenje z DNK in genska terapija za zdravljenje raka ter ablacija mehkega in srčnega tkiva. V doktorski disertaciji se zato osredotočam na tri različne vidike izboljšanja varnosti in uporabnosti klinične rabe elektroporacije.

V okviru **prvega prispevka k znanosti** smo preučevali mišično krčenje in občutek bolečine, ki ga povzročajo kratki bipolarni visokofrekvenčni pulzi pri zdravih prostovoljcih. Vzdraženje živcev in mišic je pogost pojav pri terapijah, ki temeljijo na elektroporaciji, saj lahko med zdravljenjem pride do premikanja elektrod in občutka nelagodja ter bolečine pri pacientih. Omenjene težave botrujejo razvoju novih metod elektroporacije, ki zmanjšujejo intenzivnost mišičnega krčenja. V znanstveni literaturi najpogosteje zasledimo uporabo vlakov zelo kratkih (v obsegu nekaj µs) bipolarnih pulzov namesto relativno dolgih monopolarnih pulzov [40, 41, 43–49]. Kljub temu, da imajo vsi podatki, pridobljeni s poskusi na celicah in živalih, modeliranjem in teoretičnimi premisleki, veliko vrednost,

ne omogočajo ocene zmanjšanja bolečine med visokofrekvenčno elektroporacijsko terapijo. Zato so poskusi na ljudeh z uporabo različnih parametrov električnih pulzov (različni protokoli bipolarnih pulzov) edini način za potrditev ali zavrnitev hipoteze, da kratki bipolarni visokofrekvenčni elektroporacijski pulzi zmanjšajo mišičnega krčenja in ne povzročajo bolečine. Poleg tega smo preučevali korelacijo med mišičnim krčenjem in občutkom bolečine, saj lahko pride do razlik v vzdraženju, ker se signali prenašajo po različnih vlaknih - mieliziranih ali nemieliziranih, pri čemer so A-delta in C glavna vlakna odgovorna za prenašanje bolečine. V nadaljevanju smo tako raziskali razmerje med mišičnim krčenjem in občutkom bolečine ob spreminjanju parametrov pulza (dolžina (trajanje) pulza, medfazna pavza – pavza med pozitivnim in negativnim pulzom ter medpulzna pavza – pavza med pulzi). Analizirali smo tudi katera bolečinska vlakna imajo večjo možnost vzdraženja (A-delta ali C vlakna) na podlagi deskriptorjev bolečine, ki so jih prostovoljci izbrali pri izpolnjevanju vprašalnika o bolečini.

Drugi prispevek k znanosti se osredotoča na pripravo izhodišč za standardizacijo kliničnih elektroporatorjev, saj varnostni standard za te naprave še ne obstaja. Odsotnost medicinskega standarda za klinične elektroporatorje je ovira za nadaljnji razvoj varnih naprav za elektroporacijo in njihovo komercializacijo. Trenutno je na trgu le nekaj certificiranih kliničnih elektroporatorjev. Ob upoštevanju standardnega operativnega postopka (SOP) za elektrokemoterapijo se v kliniki večinoma uporablja Cliniporator, ki je certificiran klinični elektroporator za zdravljenje kožnih in podkožnih tumorjev. Varnejši, učinkovitejši in komercializirani klinični elektroporatorji lahko prispevajo k bolj prepoznavnemu in učinkovitejšemu zdravljenju. Zato je potreba po pripravi koncepta standardizacije kliničnih elektroporatorjev postala zelo pomembna. Verjamemo, da bo s posebnim standardom (ali vsaj z varnostnimi priporočili) za klinične elektroporatorje razvoj teh naprav lažji in enotnejši, to pa bo prineslo varnejše in cenejše naprave. V ta namen smo preučili potrebne varnostne specifikacije, ki bi jih moral imeti klinični elektroporator na podlagi splošnih varnostnih standardov za medicinsko električno opremo EN/IEC 60601-1. Dodatno smo se osredotočili na pripravo zahtev za posebni standard za klinične elektroporatorje in opredelitev toleranc parametrov elektroporacije pri elektrokemoterapiji iz SOP-ja za lažje izvajanje terapije in vodenje operaterja. Tolerance amplitude in dolžine pulza so določene na podlagi permeabilizacijske krivulje celic [64].

Trenutno mora klinični elektroporator prestati vse varnostne teste, da je lahko sprejet za nadaljnje testiranje z ustreznimi standardi in certificiranje po Uredbi o medicinskih pripomočkih (MDR) 2017/745. To bo zagotovilo, da se klinični elektroporator lahko varno uporablja pri zdravljenju na pacientih. Ker na trgu primanjkuje ustreznih kliničnih elektroporatorjev, smo pričeli z razvojem novega kliničnega elektroporatorja, ki bo imel izboljšano varnost in bo v nadaljevanju omogočil certificiranje po novi Uredbi. Zaradi tega je namen **tretjega prispevka k znanosti** razvoj novega varnostnega ukrepa po definiranih varnostnih standardih v sklopu naprave za gensko elektrotransfekcijo v kožne celice. Osredotočili smo se na izboljšanje električne izolacije med visokonapetostnimi in nizkonapetostnimi deli naprave ob ustreznem krmiljenju visokonapetostnega napajanja. Dodatno smo se posvetili razvoju in izboljšanju varnostnih ukrepov za zaščito pacienta pred previsoko izhodno napetostjo, tokom ali energijo. S temi dodatnimi ukrepi bi zagotovili varno generiranje in dovajanje elektroporacijskih pulzov pacientu in hkrati varno uporabo za operaterja.

III Rezultati in razprava

V sklopu doktorske disertacije so predstavljeni trije izvirni prispevki k znanosti:

1. Dokaz zmanjšanja mišičnega krčenja in občutka bolečine med zdravljenjem, ki temelji na visokofrekvenčni elektroporaciji.

2. Priprava izhodišč za standardizacijo elektroporacijskih naprav za klinično uporabo.

3. Razvoj varnostnih ukrepov za elektroporacijsko napravo, ki ščitijo pacienta pred previsoko izhodno napetostjo, tokom ali energijo.

Rezultati in razprava so sestavljeni iz treh znanstevnih člankov, objavljenih v mednarodnih znanstvenih revijah, in predstavljajo delo, opravljeno v okviru te doktorske disertacije. Vsak znanstveni prispevek je obravnavan v svojem članku, kot je navedeno zgoraj. V sklopu razširjenega povzetka v slovenskem jeziku so povzeti rezultati in razprave vsakega od člankov/prispevkov:

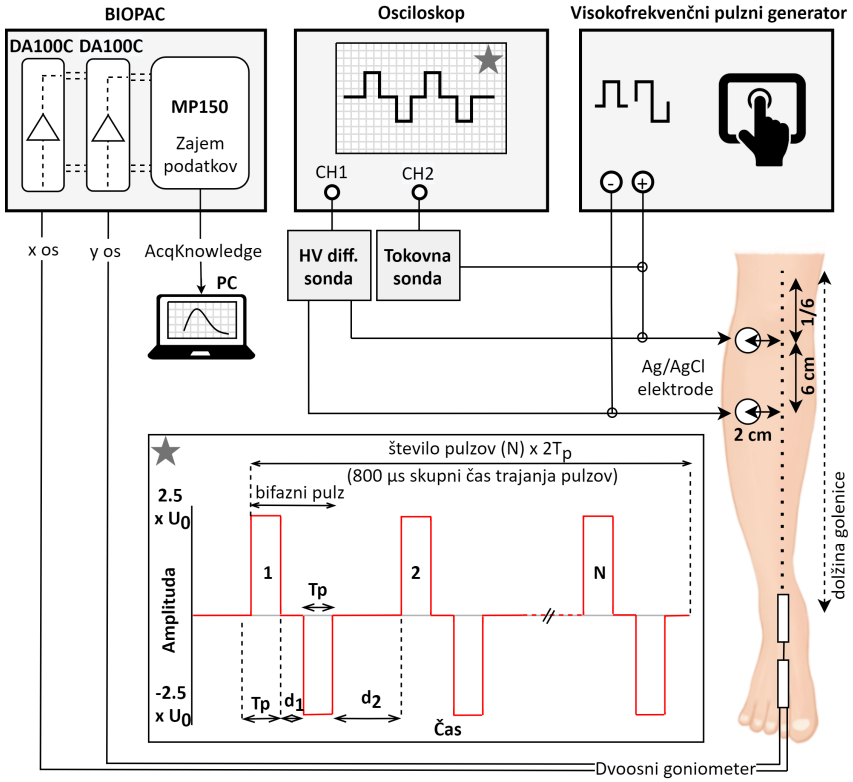
- **Članek 1** (Cvetkoska, Maček-Lebar, Trdina, Miklavčič in Reberšek) z naslovom: *"Muscle contractions and pain sensation accompanying high-frequency electroporation pulses."*
- **Članek 2** (Cvetkoska, Pirc, Reberšek, Magjarević in Miklavčič) z naslovom: *"Towards standardization of electroporation devices and protocols."*
- **Članek 3** (Cvetkoska, Dermol-Černe, Miklavčič, Kranjc-Brezar, Markelc, Serša in Reberšek) z naslovom: *"Design, development, and testing of a device for gene electrotransfer to skin cells in vivo."*

III.1 Mišično krčenje in ocena bolečine pri visokofrekvenčnih pulzih

V sklopu prvega članka/prispevka smo izvedli raziskavo v kateri je sodelovalo 25 zdravih prostovoljcev. Pri vsakem prostovoljcu smo električno stimulirali mišico tibialis anterior na desni nogi. Ker ta mišica deluje predvsem pri dorzalni fleksiji gležnja, je bil izmerjen kot dorzalne fleksije gležnja z dvoosnim goniometrom. Zaradi različnih izolacijskih lastnosti kože in podkožja, smo pri vsakem prostovoljcu določili tudi intenzivnostno - časovne krivulje [10, 65–67] za monopolarne in bipolarne pulze z različnimi dolžinami trajanja pulzov. Stimulacijsko amplitudo za bipolarne protokole smo določili glede na minimalno merljivo mišično krčenje pri stimulaciji z osmimi monopolarnimi pulzi z dolžino pulza 100 µs, dovedenih z frekvenco ponavljanja pulza 5 kHz. Stimulacijska amplituda za bipolarne protokole je bila tako 2.5-krat višja od določene amplitude za monopolarne pulze [44,50,68]. Bipolarne pulze z dolžino pulza (T_p) od 1 µs do 5 µs smo testirali med spreminjanjem medfaznih pavz (d_1, pavza med pozitivnim in negativnim pulzom, 1 µs do 100 µs) in medpulznih pavz (d_2, pavza med pulzi, 1 µs do 100 µs ter 800 µs), slika III.1. Vsak prostovoljec je bil izpostavljen 30 bipolarnim (od 51 določenih), naključno izbranim protokolom za določanje mišičnega krčenja. Da bi določili občutek bolečine ter ocenili intenzivnost in neprijetnost bolečine med stimulacijo, je vsak prostovoljec izpolnil kratke McGillove vprašalnike [69] o bolečini za naključno izbranih 15 bipolarnih protokolov (polovica od dovedenih). Skupni indeks bolečine je bil izračunan kot vsota indeksa ocene bolečine (*ang. Pain Rating Index, PRI*) in obeh vizualnih analognih lestvic (*ang. Visual Analogue Scale, VAS*) za bolečino in neprijetnost. Indeks ocene bolečine je bil izračunan iz vsote vrednosti rangov za posamezne deskriptorje (15 deskriptorjev bolečine, lestvica: 0 – 3). Za VAS analizo je bila upoštevana razdalja v centimetrih med začetkom črte na levi strani in oznako, ki jo je prostovoljec naredil (lestvica: 0 – 10). Tako je bila največja vrednost indeksa bolečine iz vprašalnikov 65 (15 x 3 + 2 x 10 = 65).

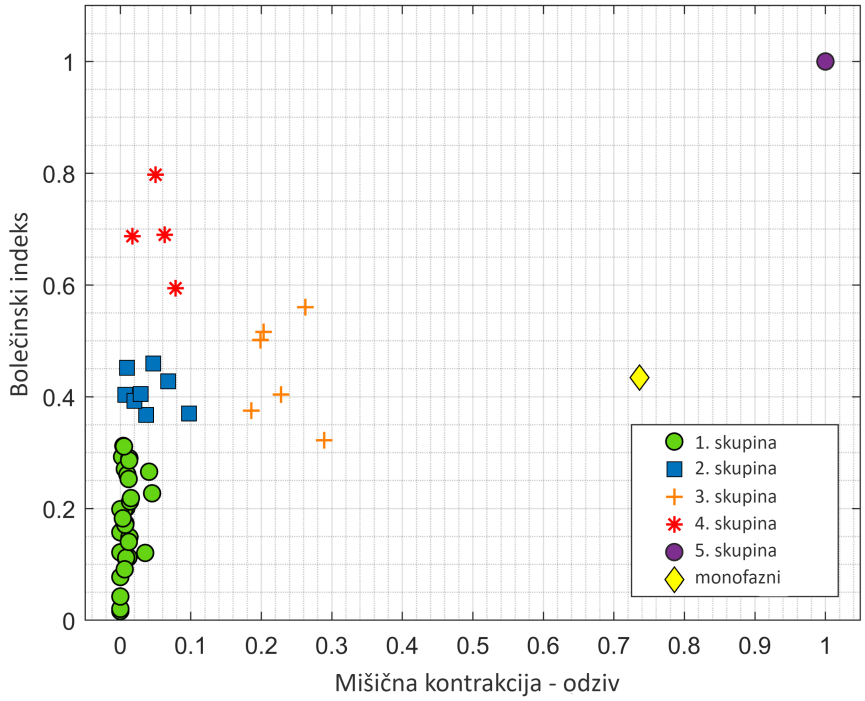
Z uporabo hierarhičnega drevesa skupin (dendrograma) s transformiranimi podatki (izvedene so bile ustrezne transformacije, da smo dobili normalno porazdelitev podatkov) smo identificirali pet skupin, ki temeljijo na podobnih odzivih

mišičnega krčenja in indeksih bolečine. Na sliki III.2 je predstavljenih vseh 51 protokolov bipolarnih pulzov, ki so označeni in pobarvani glede na skupino v katero sodijo. Vsak simbol predstavlja povprečje enega pulznega protokola v koordinatnem sistemu: x - odziv mišičnega krčenja, y - indeks bolečine. Podatki so dodatno normalizirani na podlagi protokola pulzov z največjimi vrednostmi parametrov (T_p = 5 µs, d_1 = 100 µs, d_2 = 100 µs), ki je povzročil največji odziv mišičnega krčenja (6.2° dorzalne fleksije gležnja) in najvišji indeks bolečine (13 od 65). Ta je na grafu prikazan z vijolično piko, tj., s koordinatama (1, 1).



Slika III.1. Postavitev eksperimenta. Pulzi so bili dovedeni z visokofrekvenčnim pulznim generatorjem. Elektrodi sta bili nameščeni na desni nogi: zgornja na 1/6 dolžine golenice, spodnja 6 cm nižje, obe 2 cm desno, bočno od kosti (levo na sliki). Izhodne pulze smo spremljali na osciloskopu z uporabo visokonapetostne (HV) diferencialne in tokovne sonde. Zvezdica: uporabljeni pulzi. T_p – dolžina (trajanje) pulza, d_1 – medfazna pavza, d_2 – medpulzna pavza, N – število pulzov. Odziv gležnja je bil izmerjen z dvoosnim goniometrom, povezanim z enoto Biopac. Podatke smo analizirali na osebnem računalniku (PC) s programsko opremo AcqKnowledge. DA100C – ojačevalnik, MP150 – sistem za zajem podatkov.

Na grafu je z rumenim rombom označen tudi (referenčni) protokol za določanje amplitude (8 monopolarnih pulzov x 100 µs, 5 kHz). Pomembno je omeniti, da je bila amplituda za referenčni protokol vedno 2.5-krat nižja od amplitude uporabljene za bipolarne protokole, odziv mišičnega krčenja pri referenčnem protokolu pa je bil skoraj enak za vsakega prostovoljca (minimalni merljivi odziv: 3.6°- 4° dorzalne fleksije gležnja). Na grafu lahko ločimo še štiri druge skupine (zelena, modra, oranžna in rdeča) (slika III.2).



Slika III.2. Določene skupine na podlagi hierarhičnega drevesa skupin (dendrogram). Vsaka oznaka predstavlja en protokol pulzov: x - odziv mišičnega krčenja, y - indeks bolečine. Prikazani podatki so transformirani in nato normalizirani na podlagi vijolične skupine (T_p = 5 µs, d_1 = 100 µs, d_2 = 100 µs). Rumeni romb predstavlja (referenčni) protokol za določanje amplitude (8 monopolarnih pulzov x 100 µs, 5 kHz) z 2.5-krat nižjo amplitudo.

Zelena skupina (označena z zelenimi krogi) skorajda ne povzroča mišičnega krčenja in ima nizek indeks bolečine. V tej skupini so predvsem protokoli pulzov, ki imajo kratko dolžino pulza, T_p = 1 µs in 2 µs. Modra skupina (označena z modrimi kvadratki) ima podobne odzive mišičnega krčenja, vendar nekoliko višje indekse bolečine kot zelena skupina. Protokoli pulzov v tej skupini imajo zelo kratko medfazno pavzo (d_1), vendar daljšo dolžino pulza (T_p) in daljšo medpul-

zno pavzo (d_2) kot protokoli pulzov v zeleni skupini. Oranžna skupina (označena z oranžnimi križci) ima večji odziv mišičnega krčenja kot modra pri skoraj enakem indeksu bolečine. Vsi protokoli pulzov, ki povzročijo največji odziv mišičnega krčenja (oranžna skupina), imajo $T_p = 5$ µs ter d_1 in d_2 do 10 µs. Pri podaljšanju medpulzne pavze nad 10 µs, npr. 100 µs, se odziv mišičnega krčenja zmanjša (protokoli pulzov označeni z rdečimi zvezdicami), vendar se poveča indeks bolečine. To pomeni, da bolečina ni nujno zaznana kot posledica mišičnega krčenja in obratno. V predstavljenih skupinah je oranžna skupina reprezentativna za večji odziv mišičnega krčenja, rdeča skupina pa za višji indeks bolečine. Vsi bipolarni protokoli (razen vijoličnega) so imeli nižje odzive mišičnega krčenja kot referenčni protokol (označen z rumenim rombom), tj., 8 monopolarnih pulzov x 100 µs, 5 kHz, kljub 2.5-krat višji amplitudi pulzov.

Da bi poiskali statistično značilne razlike med bipolarnimi protokoli pulza in podprli njihovo združevanje v "bipolarne protokole pulza z večjim odzivom mišičnega krčenja"in "bipolarne protokole pulza z višjim indeksom bolečine", smo izvedli N-smerno analizo variance ponovljenih meritev (*ang. N-way repeated measures analysis of variance, rmANOVA*) na transformiranih podatkih, posebej za odzive mišičnega krčenja in indekse bolečine. Protokoli, označeni z oranžnimi križci na sliki III.2, se statistično razlikujejo (višja povprečja) od protokolov v zeleni in modri skupini (minimalni odziv mišičnega krčenja). Po drugi strani, se protokoli, označeni z rdečimi zvezdicami na sliki III.2, statistično razlikujejo (višja povprečja) od protokolov v zeleni skupini (z nizkim indeksom bolečine).

Na podlagi nedavne teoretične/numerične študije [70] smo raziskali tudi, ali kratke medfazne pavze in medpulzne pavze nad 100 µs dodatno zmanjšujejo mišično krčenje. Dodatne meritve so bile opravljene na 10 prostovoljcih. Uporabili smo dodatnih 16 protokolov bipolarnih pulzov s podaljšanimi medpulznimi pavzami d_2 (200, 500, 750 in 1000 µs) in kratkimi medfaznimi pavzami d_1 (1 µs ali 5 µs). Naši rezultati potrjujejo, da podaljšanje medpulzne pavze ob kratki medfazni pavzi poveča prag mišične stimulacije, kar pomeni, da se odzivi mišičnega krčenja zmanjšajo. Nasprotno pa trendi, opaženi v naši študiji, kažejo, da podaljšane medpulzne pavze ne zmanjšajo bolečine pri prostovoljcih med stimulacijo. Daljše medpulzne pavze so se namreč izkazale za bolj boleče.

Pri vseh protokolih bipolarnih pulzov je bila medpulzna pavza (d_2) vedno

enaka ali daljša od medfazne pavze (d_1), $d_2 \geq d_1$. Izvedli smo dodatne meritve na 10 prostovoljcih, da bi ocenili odziva mišičnega krčenja in indeksa bolečine pri d_1 daljši od d_2 (obrnjene pavze, $d_1 > d_2$). Izbranih je bilo 6 izmed 51 predhodno testiranih protokolov bipolarnih pulzov, ki so imeli največjo razliko med vrednostma d_1 in d_2, saj so bila pričakovana največja odstopanja v rezultatih za te pavze. Tako je bilo dovedenih 6 novih protokolov bipolarnih pulzov (z obrnjenimi pavzami) ter 6 (izbranih), že prej uporabljenih za primerjavo. Rezultati so pokazali, da je pristop $d_2 \geq d_1$ sprejemljiv, saj ni bilo statistično značilnih razlik med primerjanimi protokoli razen pri enem paru preizkušenih protokolov bipolarnih pulzov (5-10-5-100 in 5-100-5-10). V prihodnje bi lahko na ta način zmanjšali število dodatnih poskusov.

Na podlagi izbranih deskriptorjev bolečine smo določili tudi vrsto bolečinskih vlaken, ki so bila pretežno vzdražena pri stimulaciji z visokofrekvenčnimi pulzi [12,13,71]. Na podlagi deksriptorjev bolečine, ki so bili uporabljeni v vprašalnikih, smo določili, da so med elektroporacijo pretežno vzdražena A-delta živčna vlakna. Za vsako skupino (slika III.2) je vzdraženih/stimuliranih več vlaken A-delta, kar nakazuje, da je pri kratkih bipolarnih visokofrekvenčnih pulzih večja vpletenost živčnih vlaken A-delta v zaznavanju bolečine. Edino pri oranžni skupini ni bilo statistično značilne razlike med deskriptorji, značilnimi za A-delta in C vlakna. Razlog za to je morda v tem, da so ti protokoli imeli višje odzive mišičnega krčenja. Tako so prostovoljci pogosteje izbrali deskriptor "krči" iz vprašalnikov, ki je deskriptor, ki kaže na vzdraženje C vlaken [12]. Večja vključenost A-delta vlaken je lahko posledica večje hitrosti širjenja pulza v mieliniziranih vlaknih, ki imajo tudi večji premer kot nemielinizirana C vlakna. Poleg tega imajo C vlakna daljšo kronaksijo kot A-delta, kar nakazuje, da C vlakna potrebujejo močnejši dražljaj (amplituda višjega praga) za vzdraženje pri isti dolžini pulzov [10,11,72]. Vendar se to lahko spremeni, če bi uporabili amplitude, ki so običajno višji pri terapijah, ki temeljijo na elektroporaciji.

III.2 Standardizacija elektroporacijskih naprav za klinično uporabo

Pri dovajanju električnih pulzov za elektroporacijo je zelo pomembno zagotoviti popolna poročila izmerjenih podatkov ne glede na aplikacijo. Tako omogočimo primerljivost in ponovljivost rezultatov [30, 73–75]. Potreben je tudi opis pulzov in kako so bili električni pulzi izmerjeni. Raziskovalci morajo zagotoviti tudi vse specifikacije merilne opreme. Poleg tega je treba zagotoviti parametre pulzov z ustreznim opisom uporabljenega elektroporatorja in elektrod. Za komercialno opremo je treba navesti ime podjetja in model. Če je generator pulzov laboratorijski prototip ali posebej izdelana naprava, je treba zagotoviti ustrezen opis sestavnih delov, električne konfiguracije ter sistemov za merjenje in zajemanje podatkov. Na koncu je treba izračunati električno polje, ki ga povzročijo dovedeni pulzi znotraj biološkega tkiva, in/ali navesti vse podatke, ki opisujejo obliko elektrod in njihovo postavitev glede na tarčno tkivo/vzorec.

Trenutno lahko rečemo, da je trg za klinične elektroporatorje še v razvoju in novi elektroporatorji, zasnovani za različne aplikacije šele prihajajo na trg. Odsotnost industrijskih, laboratorijskih in medicinskih standardov lahko sčasoma postane ovira za nadaljnji razvoj komercialnih naprav za elektroporacijo in pripadajočo opremo. Trenutne težave, kot so padec napetosti med dovajanjem pulza, neznani parametri pulza, nezadostno električno polje in pomanjkljiva poročila, je mogoče rešiti z navedbo novih omejitev in priporočil za napetost/tok, energijo, obremenitev, uporabljene elektrode ter izolacijo. Glede na splošni standard za medicinske pripomočke EN/IEC 60601-1, ključni varnostni dejavniki, ki jih je treba upoštevati pri načrtovanju kliničnega elektroporatorja, trenutno vključujejo: omejitev vrednosti napetosti in energije, ustrezno izolacijo, omejitev uhajavih tokov ter upoštevanje zahtev glede elektromagnetne združljivosti, kot je predstavljeno v standardu EN/IEC 60601-1-2. Dodatno je treba slediti standardom: ISO 14971 za analizo tveganja, ISO 13485 za sistem vodenja kakovosti, EN/IEC 60601-1-6 in ISO 62366 za uporabnost ter ISO 62304 in IEC 80002-1 za programsko opremo za medicinske pripomočke. Dodatna olajšava pri razvoju kliničnega elektroporatorja bi bila vzpostavitev novega posebnega standarda za klinične elektroporatorje, ki bi tako poenostavil uskladitev vseh

komercialnih, certificiranih elektroporatorjev in izboljšal varnost, kakovost in učinkovitost teh naprav.

Splošni standard za elektroporatorje bi moral definirati/vsebovati:

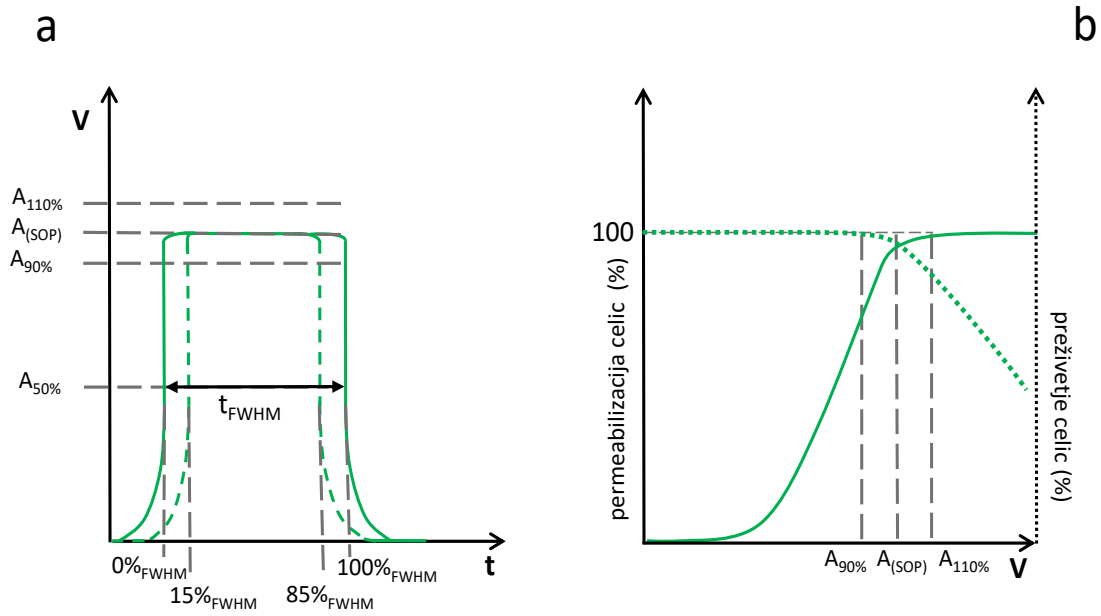
- maksimalne tolerance generiranih/dovedenih pulzov v primerjavi s pričakovanimi vrednostmi ob upoštevanju permeabilizacijske krivulje bremena (npr. celic) in tehničnih omejitev elektroporacijske naprave;
- kako določiti tehnične specifikacije naprave, tj., podati specifikacije skupaj s pogoji, pod katerimi so dosežene, npr. določiti največjo amplitudo pulzov skupaj z razponom dolžine pulza in upornostjo bremena, pri kateri je to mogoče doseči;
- opis predpulzov (amplituda, dolžina pulza in natančen čas trajanja glede na prednastavljeno zaporedje), če se uporabljajo;
- kako izvajati varnostne funkcije, kot so galvanska ločitev, omejitve napetosti, toka in energije ter opozorila, če je bilo generiranje pulza omejeno ali ustavljeno ali, če je prišlo do katerega koli drugega nepredvidljivega dogodka ali okvare;
- s kakšno obremenitvijo je treba preizkusiti elektroporatorje, da se zagotovi učinkovitost pri posameznih aplikacijah ali, da se zagotovi predvidljivo delovanje oz. delovanje znotraj predpisanih toleranc;
- pravilno izbiro in uporabo elektroporacijskih kivet in elektrod ter dovoljena odstopanja njihove geometrije glede na aplikacijo;
- maksimalne tolerance razdalj med elektrodama;
- pravilno izbiro/uporabo različnih materialov za elektrode oz. naprave.

V sklopu drugega prispevka smo določili tudi sprejemljive tolerance parametrov pulza pri elektrokemoterapiji [21] glede na standardnega operativnega postopka (SOP) [20] in predlagali priporočila za lažjo standardizacijo naprav. Predlagamo, da je galvanska ločitev izvedena v kontrolnem/napajalnem modulu in ne v izhodnem, da bi lahko zagotovili natančne parametre izhodnega signala. Izhodna napetost in tok naj se merita na izhodu pulznega generatorja. Priporočamo tudi dodatni varnostni ukrep za omejitev visokih vrednosti napetosti, toka in energije. Največji tok naprave bi moral biti omejen na 110 % ali 120 % od največjega pričakovanega toka med terapijo. Ob upoštevanju SOP za pravokotne pulze (opi-

sani z amplitudo in dolžino pulza t_{FWHM} (*ang. Full Width at Half Maximum, FWHM*) - polna dolžina (trajanje) pulza pri polovici maksimuma (maksimalne amplitude), predlagamo naslednje tolerance (slika III.3):

- amplituda pulza med 15 % in 85 % FWHM ne sme preseči 110 % ali pasti pod 90 % določene vrednosti SOP amplitude (slika III.3a);
- FWHM ne sme biti daljša ali krajša od ±8 % določene SOP FWHM;
- dovedeno število pulzov mora biti enako kot v SOP-ju in variacije tega parametra niso dovoljene;
- hitrost ponavljanja pulza, t.j. ponavljalna frekvenca pulzov lahko odstopa od hitrosti ponavljanja pulza SOP (1 Hz ali 5 kHz) za največ ±5%;
- vse elektrode morajo biti izdelane iz biokompatibilnega materiala (običajno iz nerjavečega jekla). Elektrode so lahko tudi izdelane iz materialov, ki so testirani v skladu z zahtevami, navedenimi v skupini standardov ISO 10993 za biološko vrednotenje medicinskih pripomočkov;
- elektrode so lahko namenjene tudi za večkratno uporabo; v tem primeru je treba zagotoviti jasna navodila za čiščenje in vzdrževanje elektrod po vsaki uporabi; poleg tega je treba v navodilih za uporabo navesti in zagotoviti zamenjavo elektrod v vnaprej določenih intervalih.

Za uspešno elektrokemoterapijo je pomembno, da se držimo teh toleranc, saj smo jih izračunali za določitev največjih odstopanj, kjer je še mogoče doseči želeni biološki učinek. Na primer, višje (več kot 110 %) ali nižje (manj kot 90 %) vrednosti od SOP amplitude lahko povzročijo ireverzibilno elektroporacijo oziroma nezadostno električno polje za elektrokemoterapijo (neučinkovito zdravljenje). Na podlagi permeabilizacijske krivulje (slika 2 iz reference [64]) bo tudi pri pulzih z najnižjimi ali najvišjimi definiranimi tolerancami (za amplitudo in dolžino pulza) še vedno mogoče ostati na delu permeabilizacijske krivulje, kjer bo zdravljenje z elektrokemoterapijo učinkovito (slika III.3b).



Slika III.3. a) Elektroporacijski pravokotni pulz – parametri pulza in tolerance. b) Tolerance permeabilizacijske krivulje celic (polna črta) in krivulje za preživetje celic (črtkana črta), prilagojeno iz [64]. Za amplitude med 90 % in 110% od definirane SOP amplitude bo še vedno mogoče doseči učinkovito zdravljenje. Če je vrednost amplitude višja od 110 % določene SOP amplitude, se bo preživetje celic zmanjšalo in lahko pride do ireverzibilne elektroporacije. Če pa je vrednost amplitude, nižja od 90 % določene SOP amplitude, se bo permeabilizacija celic zmanjšala, to pa lahko privede do neučinkovitega zdravljenja tumorja.

III.3 Varnostni ukrepi pri elektroporatorju za gensko elektrotransfekcijo

V okviru tretjega izvirnega prispevka k znanosti smo razvili nove varnostne ukrepe za novo napravo za gensko elektrotransfekcijo (pulzni generator in aplikator) z novim protokolom dovajanja pulzov, razvitega na podlagi numeričnega modela elektroporirane kože. Napravo smo testirali v in vivo študiji genske elektrotransfekcije kožnih celic na miših. Pokazali smo, da je genska elektrotransfekcija z razvito napravo, aplikatorjem in predlaganim protokolom za dovajanje pulzov zagotovila večjo ekspresijo genov v kožnih celicah v primerjavi s trenutno uporabljenim Cliniporatorjem, aplikatorjem z več elektrodami (*ang. Multi-Electrode Array, MEA*) in protokolom za dovajanje pulzov. Pri razvoju elektroporatorja smo sledili priporočilom, ki so predstavljeni v drugem izvirnem prispevku k zna-

nosti, in vključili nekatere varnostne zahteve za medicinsko električno napravo, predstavljene v splošnem standardu EN 60601-1:2007. Dodatno smo upoštevali smernice za razvoj naprave glede na uporabniške in tehnične zahteve in predlagali protokol zdravljenja pri genski elektrotransfekciji. Razviti elektroporator je baterijsko napajan, grafični uporabniški vmesnik je prikazan na 10.1" zaslonu na dotik, elektrode (aplikator) pa lahko namestimo na različne predele kože na telesu, ne glede na njeno "ukrivljenost". Elektroporator lahko generira pravokotne pulze od 80 V do 600 V z dolžino pulza od 10 µs do 1000 µs in frekvenco ponavljanja pulzov od 0.1 Hz do 5000 Hz.

Nova vezja so bila razvita v programskem okolju Altium Designer. Kot predlagano v poglavju III.2, smo galvansko ločitev krmilnih signalov izvedli v kontrolnem modulu s pomočjo optičnih sklopnikov. Nadgradili smo ločitveno-pretvorniško vezje z A/D in D/A pretvorniki (*ang. Analog-to-Digital and Digital-to-Analog converters*), ki zagotavlja galvansko ločitev ter omogoča nadzor in krmiljenje visokonapetostnega napajanja. Tako izvedena izolacija zagotavlja, da se visoka napetost ne prenese na nizkonapetostni del naprave v primeru okvare na visokonapetostnem delu. Pretvorniški del vezja omogoča pretvorbo digitalnega krmilnega signala v analogni signal in merjenje napetosti z A/D pretvornikom po standardiziranem protokolu SPI (*ang. Serial Peripheral Interface*). Varnostno zaščito smo razdelili na dva dela. Prvi del je vezje za meritev napetosti in toka, ki z releji preklaplja med elektroporacijskim pulzom (izhod iz pulznega generatorja) in signalom za kontakt z elektrodami (izhod iz vezja za kontakt z elektordami, ki preverja, ali so elektrode v stiku s kožo). Zaščita je izvedena tako, da ne povezuje oba signala hkrati in v primeru zaustavitve pulza izklopi oba releja. Senzorja napetosti (napetostni delilnik) in toka (senzor za meritev toka – CASR-6-NP (LEM International SA, Švica)) sta uporabljena za meritev napetosti in toka ter pretvorbo elektroporacijskega signala v signal primeren za A/D pretvornik. Drugi del je vezje, ki zazna visoke vrednosti izhodnega pulza (izhodne napetosti, izhodnega toka ali izhodne energije) in pošilja signal za zaustavitev generiranja pulzov. Visoke vrednosti napetosti in toka so zaznane z uporabo primerjalnikov, za izračun izhodne moči pa sta uporabljena analogni množilnik in integrator. Generiranje pulzov naj bi bilo ustavljeno v manj kot 1 µs po tem, ko se pojavi prevelik tok (več kot 2 A), napetost (več kot 640 V) ali energija (več kot 7 J) na izhodu. Pri testiranju vezja smo tako izmerili napetost in preklapljali

releje, vendar nam ni uspelo izmeriti toka z izbranim senzorjem. Kasneje smo zamenjali in uporabili nov tokovni senzor (ACS 70331, Allegro MicroSystems, Manchester, ZDA), a nam tudi s tem ni uspelo izmeriti toka.

Tokovni omejevalnik je bil zato razvit ločeno. Tokovni omejevalnik je sestavljen iz silicij-karbidnega JFET tranzistorja (spojni tranzistor na osnovi električnega polja, *Junction Field Effect Transistor*) UJ3N120035K3S (UnitedSiC, Princeton, USA), ki ima vlogo stikala v vezju. Ob povišanju pozitivnega toka skozi upor med vrati in izvorom, napetost na vratih JFET tranzistorja negativno narašča in JFET se začne zapirati. Izvedli smo simulacije v programskem okolju LT Spice XVII (Analog Devices, ZDA) pri različnih vrednosti upora med vrati in izvorom, da bi določili vrednost upora in tako omejili tok na 2 A (slika 3.1). Na podlagi izvedenih simulacij (slika 3.2) za vrednosti od 1 do 10 Ω smo ugotovili, da mora imeti upor vrednost 5 ali 6 Ω. Za končno izvedbo smo sicer uporabili tri zaporedno vezane upore - dva močnostna upora z upornostjo 1,5 Ω ter en upor s pozitivnim temperaturnim koeficientom z upornostjo 1 Ω, ki povečuje upornost zaradi segrevanja v primeru višjih tokov. Ta metoda se je izkazala kot uporabna pri omejevanju toka na največjo dovoljeno vrednost (2 A), vendar smo pri testiranju zaznali nekatera nihanja na začetku signala (samo v prvi 1 µs).

Simulacije so bile izvedene tudi z močnostnim silicij-karbidnim MOSFET tranzistorjem n-tipa (tranzistor s polprevodniškim efektom kovinskega oksida, *ang. Metal–Oxide–Semiconductor Field-Effect Transistor*) C2M0045170D (Wolfspeed Inc., North Carolina, ZDA) kot omejevalnik toka, za namen razvoja novega laboratorijskega viskofrekvenčnega prototipnega elektroporatorja. V tem primeru smo najprej določili vrednost upora na izvoru (R1, slika 3.3), da bi omejili tok na 120 A in nato določili vrednost upora na ponoru (R2), da bi zmanjšali oscilacije (nihanja toka), ki se pojavijo, ko je tok omejen. Za R1 smo tako izbrali upor 50 mΩ (spreminjali smo vrednosti upornosti R1 od 10 do 100 mΩ, slika 3.4). Za R2 smo nato izvedli simulacije z R1 nastavljen na 50 mΩ in spreminjali vrednosti za R2 od 100 mΩ do 2 Ω (slika 3.5). Opazili smo, da so oscilacije pri vrednostih R2 nad 700 mΩ kritično dušene. Tako smo izbrali upornost 1 Ω za R2. Dodatno je bil implementiran detektor napake desaturacije v vezju. Ta detektor zagotavlja zaščito za MOSFET v primeru, ko je napetost med ponorom in izvorom nad prednastavljeno referenčno napetostjo. Tako je doseženo hitro zaznavanje in zapiranje MOSFET-a v primeru prevelikega toka.

IV Zaključek

Da bi elektroporacijo uvedli v široko klinično uporabo, mora biti dovajanje električnih pulzov varno, učinkovito in pacientu prijazno ter povzročati minimalno mišično krčenje in ublažiti neprijetne občutke. Zelo pomembna je tudi pravilna zasnova kliničnega elektroporatorja in elektrod, saj pogojuje njihovo varno uporabo in učinkovitost terapije [56]. Poleg tega lahko certificirani/komercializirani klinični elektroporatorji prispevajo k večji uporabi elektroporacije v kliničnih okoljih z bolj prepoznavnimi, varnimi in učinkovitimi terapijami. V okviru te doktorske disertacije sem obravnavala omenjene tematike pri klinični uporabi elektroporacije in predlagane potencialne rešitve. Končne pripombe in sklepi so predstavljeni v nadaljevanju.

Študija stimulacije mišic in ocenjevanje bolečine, predstavljena v prvem prispevku k znanosti (Poglavje III.1), je potrdila hipotezo, da uporaba kratkih (1 µs, 2 µs), bipolarnih visokofrekvenčnih pulzov s kratkimi medfaznimi in medpulznimi pavzami zmanjša krčenje mišic pri zdravih prostovoljcih v primerjavi s trenutno uporabljenimi dolgimi monopolarnimi pulzi (8 pulzov z dolžino pulza 100 µs, dovedenih s frekvenco ponavljanja pulza 5 kHz). Testirani protokoli pulzov zmanjšajo tudi občutek bolečine med dovajanjem pulzov. Vendar medfazne in medpulzne pavze igrajo pomembno vlogo pri zmanjšanju mišičnega krčenja in/ali občutka bolečine, zato je kombinacija med parametri pulza bolj zapletena. Naši rezultati kažejo tudi, da so pri stimulaciji A-delta živčna vlakna vzdražena v večji meri kot C vlakna. Za vsako skupino protokolov je bilo vzdraženih/stimuliranih več vlaken A-delta, kar kaže na to, da je pri kratkih bipolarnih visokofrekvenčnih pulzih večja vpletenost A-delta živčnih vlaken pri prenosu bolečine glede na izbrane deskriptorje bolečine, ki so bili uporabljeni v študiji. S to študijo smo ugotovili tudi, da mišično krčenje in bolečina med dovajanjem pulzov nista vedno

povezana. Namreč, ni vedno nujno, da bolečina nastane kot posledica mišičnega krčenja in obratno. Do tega zaključka smo prišli, ker smo v nekaterih primerih opazili višje indekse bolečine pri parametrih pulza, ki ne povzročajo močnega mišičnega krčenja. Ena od možnih razlag bi lahko bila teorija nadzora vrat pri mehanizmih bolečine (*ang. Gate Control Theory of Pain mechanisms*) [76–78]. Ta teorija predlaga, da večja aktivnost določenih vlaken spodbuja zaviralne nevrone, kar zmanjša prenos informacij o bolečini. Če je aktiviranih več večjih vlaken (vlakna A-alfa in/ali A-beta) v primerjavi z manjšimi vlakni za bolečino (vlakna A-delta in C), ljudje občutijo manj bolečine. Na primer, neboleč dražljaj (npr. dotik/masaža na udarjenem mestu) lahko zapre živčna "vrata" za boleč dražljaj, ker se je aktivnost velikih vlaken (v tem primeru A-beta) povečala. Tako lahko zmanjšamo občutek bolečine (zmanjša se aktivnost bolečinskih vlaken), saj vsi bolečinski signali ne dosežejo centralnega živčnega sistema. V našem primeru bi to pomenilo, da s stimulacijo mišice nastane mišično krčenje, ki lahko aktivira/vzdraži velika vlakna in s tem zmanjša vzdraženost nociceptivnih (bolečinskih) vlaken, tj. vrata se zaprejo. Zato smo s to študijo tudi pokazali, da je optimalni razpon parametrov pulza mogoče povečati, saj nekatere terapije zahtevajo le specifične predpogoje (npr. le zmanjšano mišično krčenje). To pomeni, da je mogoče narediti ustrezne modifikacije parametrov pulza na podlagi posebne aplikacije elektroporacije, da lahko zmanjšamo neželene učinke in zagotovimo varno, uspešno in učinkovito terapijo. Vendar pa je za potrditev učinkovitosti novopredlagane visokofrekvenčne elektroporacije potrebna nadaljnja ocena predstavljenih protokolov bipolarnih pulzov s klinično pomembnimi visokonapetostnimi amplitudami, ki se izvajajo tudi na različnih tkivih (tumorji, srce) in lokacijah (globoko, površinsko).

Glede na nove tehnologije in povečane raziskave ter znanje, "elektroporacijska" industrija trenutno raste še hitreje kot prej. Kljub velikemu potencialu [24], je prenos aplikacij elektroporacije v kliniko (npr. transdermalna ali intradermalna genska elektrotransfekcija (GET)) počasen in zaostaja za in vitro in in vivo študijami [79, 80]. Menimo, da je neustrezna dozimetrija eden od razlogov, ker ne omogoča primerjave med različnimi raziskavami in rezultati, saj se za iste aplikacije uporabljajo različni elektroporatorji, elektrode (aplikatorji) in pulzni parametri [56,81]. Poleg tega na trgu trenutno primanjkuje certificiranih kliničnih elektroporatorjev in aplikatorjev, saj je njihova certifikacija/komercializacija za-

pletena in dolgotrajna. Klinični elektroporatorji so medicinski pripomočki in morajo izpolnjevati medicinske standarde in zahteve, določene z lokalnimi medicinskimi predpisi, npr. Uredba o medicinskih pripomočkih (MDR) 2017/745 v Evropi. Menimo, da se številni raziskovalci/razvijalci naprav za elektroporacijo ne zavedajo, kako obsežna je tehnična dokumentacija za certificiranje pripomočka po novi Uredbi in koliko varnostnih standardov ter zahtev je treba upoštevati. Postopek certificiranja je še bolj zapleten zaradi odsotnosti posebnega medicinskega varnostnega standarda za klinične elektroporatorje. Zato smo v okviru drugega izvirnega prispevka k znanosti (Poglavje III.2) določili varnostne standarde, ki jih je trenutno treba upoštevati pri razvoju kliničnih elektroporatorjev na podlagi zahtev nove Uredbe. Določili smo tudi potrebne varnostne ukrepe, ki jih je treba upoštevati pri načrtovanju kliničnih elektroporatorjev na podlagi splošnega standarda za varnost medicinske električne opreme EN/IEC 60601-1:2007: "Splošne zahteve za osnovno varnost in bistvene zmogljivosti". Poleg tega smo predlagali priporočila za zahteve, ki bi jih moral vsebovati posebni standard za klinične elektroporatorje, z namenom, da bi postal razvoj in postopek certificiranja teh naprav enostavnejši. Določili smo tudi tolerance za amplitudo in dolžino pulza na podlagi krivulj permeabilizacije celic, ki jih je možno uvesti kot dodatno izboljšavo pri trenutnem standardnem operativnem postopku za elektrokemoterapijo. S tem bi lahko elektrokemoterapijo pripeljali v širšo uporabo v klinikah, saj bi lahko imeli več ustreznih kliničnih elektroporatorjev. Da bi standardizirali protokol zdravljenja tudi pri genski elektrotransfekciji, smo v okviru tretjega izvirnega prispevka k znanosti (Poglavje III.3) predlagali smernice za standardizirani protokol zdravljenja. Ta bi lahko pomagal pri vzpostavitvi varnega in učinkovitega standardnega operativnega postopka. V nadaljevanju smo zasnovali in razvili varnostne ukrepe za nov elektroporator in aplikator za gensko elektrotransfekcijo z izboljšano varnostno učinkovitostjo v skladu z določenimi smernicami, zahtevami in varnostnimi ukrepi. Razvita naprava je bila testirana v in vivo študiji genske elektrotransfekcije kožnih celic na miših. Rezultati so pokazali, da je elektroporacija z razvito napravo, aplikatorjem in predlaganim protokolom za dovajanje pulzov, povzročila večjo ekspresijo genov v kožnih celicah v primerjavi s trenutno uporabljenim Cliniporatorjem, aplikatorjem z več elektrodami (*ang. Multi-Electrode Array, MEA*) in protokolom za dovajanje pulzov [82–84]. V okviru tretjega prispevka so bila razvita tudi nova vezja, ki omogočajo varno generiranje elektroporacijskih pulzov. Ločitveno-pretvorniško vezje z A/D in D/A pretvorniki (*ang. Analog-to-Digital*

and Digital-to-Analog converters) je bilo razvito za zagotovitev galvanske ločitve krmilnih signalov in krmiljenja visokonapetostnega napajanja. Poleg tega so bili razviti novi varnostni ukrepi za zaščito pred previsoko izhodno napetostjo, tokom ali energijo. Tokovni omejevalnik smo razvili na osnovi izvedenih simulacij v programskem okolju LT Spice. Tak varnostni ukrep bo preprečil, da pri praznjenju visokonapetostnih kondenzatorjev, tok in moč ne postaneta previsoka. Elektroporator smo dodatno testirali s certificiranim in kalibriranim analizatorjem električne varnosti Fluke ESA620 (Fluke Biomedical, Washington, ZDA) za medicinske pripomočke v skladu z medicinskim standardom EN 60601-1:2007. Poročilo o električni varnosti je pokazalo, da so uhajavi tokovi znotraj dovoljenih vrednostih po standardu (tabela 3.1), kar pomeni, da naprava ne bo škodila pacientu tudi v primeru enojne napake. Kljub temu, naprava še ni certificirana kot medicinski pripomoček. Obstajajo še nekatera odstopanja, saj nismo uspeli preveriti vseh zahtev ostalih varnostnih standardov (npr. elektromagnetna združljivost) in pripraviti celotene zahtevane tehnične dokumentacije. Prav tako nimamo vpeljanega sistema vodenja kakovosti (QMS) po standardu ISO 13485 za postopke in procese, ki so potrebni za razvoj in proizvodnjo medicinske naprave. V prihodnje je potrebno vzpostaviti QMS, načrtovati uporabnost in pripraviti načrt obvladovanja tveganja že na začetku razvojne faze. Poleg tega bo treba pripraviti tehnično dokumentacijo v skladu s prilogama II in III od MDR 2017/745. Premagovanje teh ovir nam lahko pomaga pri certificiranju kliničnega elektroporatorja, ki ga bo mogoče uporabljati s trenutnimi/predlaganimi standardnimi operativnimi postopki za nove, varne in učinkovite študije na ljudeh.

V Izvirni prispevki k znanosti

Dokaz zmanjšanja mišičnega krčenja in občutka bolečine med zdravljenjem, ki temelji na visokofrekvenčni elektroporaciji

V literaturi je vse večji poudarek na razvoju novih metod elektroporacije, ki lahko zmanjšajo intenzivnost mišičnega krčenja in občutek bolečine med zdravljenjem, ki temelji na elektroporaciji. Tako je bila predlagana uporaba vlakov kratkih ($\approx$ µs) bipolarnih pulzov namesto relativno dolgih monopolarnih pulzov. Vendar po do sedaj znanih podatkih ni moč potrditi zmanjšanja bolečine. Zato smo izvedli prvo študijo na zdravih prostovoljcih[1] z uporabo različnih vrst pulzov: monopolarnih in bipolarnih z različnimi parametri pulza (različna dolžina pulza, medfazna in medpulzna pavza). Določili smo statistično značilne razlike med protokoli in jih združili v pet različnih skupin, tj. protokoli bipolarnih pulzov z višjim/nižjim odzivom mišičnega krčenja in/ali višjim/nižjim indeksom bolečine. Izvedli smo tudi dodatne meritve s podaljšanimi medpulznimi pavzami (ob ohranjanju kratkih medfaznih pavz) ter obrnjenimi medfaznimi in medpulznimi pavzami. Na podlagi izbranih deskriptorjev bolečine smo dodatno analizirali katera bolečinska vlakna imajo večjo možnost vzdraženja (A-delta ali C vlakna). Potrdili smo hipotezo, da bipolarni, visokofrekvenčni pulzi z dolžino pulza 1 µs ali 2 µs zmanjšajo mišično krčenje in občutek bolečine v nasprotju s trenutno uporabljenimi daljšimi monopolarnimi pulzi. Vendar imajo medfazne in medpulzne pavze pomembno vlogo pri zmanjšanju mišičnega krčenja in/ali občutka bolečine, tako, da je kombinacija med optimalnimi parametri pulza bolj zapletena. Bolečina ni nujno zaznana kot posledica mišičnega krčenja in obratno. Tako se lahko razpon optimalnih parametrov poveča, odvisno od zahtev določene terapije.

[1] Študijo "Določanje neprijetnih občutkov pri terapijah z visokofrekvenčnimi elektroporacijskimi pulzi" je odobrila komisija Republike Slovenije za medicinsko etiko (št. dok. 0120-61/2020).

Priprava izhodišč za standardizacijo elektroporacijskih naprav za klinično uporabo

Odsotnost posebnega medicinskega varnostnega standarda za klinične elektroporatorje je ovira za razvoj varnih in učinkovitih naprav za elektroporacijo. Trenutno je na trgu le nekaj certificiranih kliničnih elektroporatorjev, saj proces komercializacije zahteva veliko dokumentacije in časa. Raziskovalci/razvijalci naprav za elektroporacijo se včasih ne zavedajo, kako obsežna je tehnična dokumentacija za certificiranje pripomočka po novi Uredbi o medicinskih pripomočkih (MDR) 2017/745 in koliko varnostnih standardov ter zahtev je trenutno treba upoštevati. Zato smo predlagali priporočila za lažjo standardizacijo na podlagi pregleda trenutnih aplikacij elektroporacije in pripadajoče opreme. Določili smo potrebne varnostne lastnosti in zahteve, ki jih je treba slediti pri razvoju kliničnih elektroporatorjev na podlagi splošnega varnostnega standarda za medicinsko električno opremo EN/IEC 60601-1: "Splošne zahteve za osnovno varnost in bistvene zmogljivosti". Določili smo tudi varnostne standarde, ki jih je trenutno treba upoštevati pri razvoju kliničnih elektroporatorjev na podlagi zahtev nove Uredbe. Na podlagi medicinskih varnostnih standardov smo priporočili smernice in zahteve, ki jih je treba določiti pri pripravi posebnega medicinskega standarda za klinične elektroporatorje. Poleg tega smo opredelili tolerance parametrov pulzov pri elektrokemoterapiji po standardnem operativnem postopku za lažje izvajanje terapije in vodenje operaterja ter izboljšanje/zagotavljanje kakovosti elektrokemoterapije.

Razvoj varnostnih ukrepov za elektroporacijsko napravo, ki ščitijo pacienta pred previsoko izhodno napetostjo, tokom ali energijo

Klinični elektroporator mora prestati vse varnostne teste, da je lahko sprejet za nadaljnje testiranje po ustreznih standardih in certificiranje po Uredbi o medicinskih pripomočkih 2017/745 v Evropi. Opravljanje varnostnih testov ter certifikacija kliničnega elektroporatorja bi zagotovila varno uporabo za zdravljenje v kliniki. V ta namen smo zasnovali, razvili in testirali v in vivo študiji, elektroporator z izboljšano varnostjo in aplikator za gensko elektrotransfekcijo. V prihodnosti bi ju tako lahko certificirali po novi Uredbi o medicinskih pripomočkih. Zahteve in priporočila, ki jih je treba upoštevati pri načrtovanju takšnega kliničnega elektroporatorja, smo predlagali v drugem prispevku in upoštevali med razvojem. Razvili smo električno izolacijo med visokonapetostnimi deli naprave skupaj z

ustreznim krmiljenjem visokonapetostnega napajanja. Dodatno smo se posvetili razvoju novih varnostnih ukrepov za zaščito pacienta pred previsoko izhodno napetostjo, tokom ali energijo med terapijo in tako razvili varnostni ukrep za omejevanje toka na največjo pričakovano vrednost med terapijo. Tako smo zagotovili varno generiranje in dovajanje elektroporacijskih pulzov pacientu in hkrati varno uporabo za operaterja. Elektroporator smo testirali tudi z analizatorjem električne varnosti. Glede uhajavih tokov se je izkazal za varnega, saj so bili izmerjeni uhajavi tokovi v območju dovoljenih na podlagi splošnega varnostnega standarda za medicinsko električno opremo EN/IEC 60601-1.

Improving safety of electroporation clinical use

1 Introduction

1.1 Electroporation

A biological cell is protected from its environment by the plasma membrane (cell membrane), which separates the interior of the cell from the extracellular space. The plasma membrane is composed of a two-molecule-thick layer of lipids that would make the membrane largely impenetrable barrier if there were not various proteins (and other transport mechanisms like endo- and exocytosis) that allow only specific molecules to be transported across the membrane. However, when the cell membrane is exposed to a sufficiently strong electric field, a transmembrane voltage (TMV), e.g., 500 mV is induced, which far exceeds its resting TMV (typically -40 mV to -70 mV). At this supraphysiological TMV, pores temporarily form in the bilayer and permeability of the plasma membrane increases, allowing transmembrane transport of molecules that otherwise cannot pass through the membrane [1]. This phenomenon, termed membrane electroporation/permeabilization, is called reversible, if the cell recovers and survives, or irreversible, if the exposure leads to cell death [2–4].

1.2 Electroporation vs. electrical stimulation

Electroporation is a threshold-like phenomenon. A certain threshold must be reached to increase cell membrane permeability. Electrical stimulation is a procedure in which electrical pulses are delivered to nerve fibers via electrodes attached to the skin. Electrical stimulation is also a threshold-like phenomenon, meaning that an action potential is triggered when a certain threshold is reached. The ac-

tion potential is defined as a sudden, rapid, transient, and propagating sequence of changes in the TMV. It is generated when a stimulus changes the resting TMV to the values of the threshold TMV (depending on the cell, -55 mV for a typical neuron). The action potential behaves according to the *all-or-nothing principle*, meaning that a stimulus below threshold does not elicit action potential, whereas stimulus above threshold elicits a complete response of the excitable cell [5].
While electroporation can occur in all cells, the response to electrical stimulation is only observed in excitable cells such as muscle and nerve cells. Therefore, electrical muscle stimulation has gained increasing attention due to its use in injury or paralysis rehabilitation, strength training, post-exercise recovery, etc., because the pulses delivered through the electrodes mimic the action potential that comes from the central nervous system, causing the muscles to contract [6]. However, the TMV thresholds required for electroporation are higher (several hundred mV) than those that trigger action potentials in excitable cells (less than 100 mV). This suggests that electroporation cannot be successfully performed without causing (unintended) electrical stimulation of excitable cells, resulting in unwanted muscle contractions and pain sensations during electroporation treatments [7, 8].

1.2.1 Nerve fibers

A nerve fiber (i.e., axon) is a long, slender extension of the nerve cell that typically carries the action potential away from the nerve cell body in order to transmit the action potential to various muscles and neurons (e.g., contraction of the muscle, sensation of touch or pain, etc.). The transmission does not affect the quality of the action potential in any way, nor does it reduce it. This means that the target tissue receives the same action potential no matter how far it is from the nerve cell body. There are two types of axons in the nervous system: myelinated, which are insulated by a myelin sheath and allow faster conduction of the action potential, and unmyelinated, which do not have a myelin sheath [9]. The myelin sheath has gaps (i.e., nodes of Ranvier) that occur at evenly spaced distances and enable the fast conduction of the action potential (i.e., saltatory conduction) from one node of Ranvier to the next one. Myelinated nerve fibers are further divided into A-alpha, A-beta, and A-delta nerve fibers. A-alpha fibers have the largest diameter and fastest conduction velocity and include motorneurons that transmit signals

for muscle contractions. A single motorneuron can activate multiple muscle fibers. A-beta fibers have a smaller diameter and slower conduction velocity and convey sensory information such as touch and temperature. A-delta fibers have the smallest diameter in this group and convey the sensation of sharp, prickling pain. The location of the pain source is accurately perceived. C-fibers, on the other hand, which are not myelinated, are responsible for the so-called second pain and convey the sensation of a deep, dull, aching pain that is not well localized. They have a smaller diameter and slower conduction velocity than the A-delta fibers [10–13].

1.3 Applications of electroporation

Electroporation is already widely used in various fields such as biotechnology [14], food processing [15], and environmentally relevant applications [16]. In addition, reversible electroporation has been successfully used in clinical applications as a combination of high-voltage electric fields with low-permeable chemotherapeutic drugs - electrochemotherapy (ECT) or with DNA - gene electrotransfer (GET) [17–24]. Recently, irreversible electroporation (IRE) also emerged as a new medical application [3] for non-thermal tumor [25–27] and cardiac ablation (Pulse Field Ablation - PFA) [28–31]. This ablation method offers significant advantages over currently used thermal ablation methods, such as reducing the risk of damaging nearby critical tissue. Therefore, PFA may become a dominant treatment in the future, particularly in cardiac electrophysiology [32–34].
Currently, in all of the above-mentioned electroporation-based clinical applications, relatively long monopolar pulses (50 - 100 µs) are delivered at low repetition frequencies (e.g., 1 Hz or 5 kHz). For GET, pulses in the millisecond range (up to 50 ms) are most commonly used. The amplitude of the pulses can range from a few tens of volts (e.g., for GET) to several kilovolts (e.g., for IRE). Consequently, the delivery of such pulses can lead to stimulation of nerve and muscle cells, making these treatments uncomfortable and even painful in some cases. Therefore, anesthesia is required for the treatments along with muscle relaxants to ensure adequate neuromuscular blockade and proper respiratory function [20, 35–37]. In addition, delivery of pulses should be synchronized with the cardiac rhythm [3, 38, 39].

1.3.1 High-frequency electroporation

Recently, a new waveform of electroporation pulses named high-frequency electroporation (HF-EP) has been proposed [42–45] that could replace standard monopolar pulses (50 - 100 µs) with very short (0.5 - 10 µs) bipolar pulses, which are delivered at high repetition frequencies (in the range of hundreds of kHz). The pulses are usually applied in bursts (trains) with low repetition frequencies (few Hz) and same total on-time of the pulses in each burst. It was shown that this new waveform can be potentially used for tissue ablation while avoiding the triggering of action potentials in nerve fibers that would be stimulated by monopolar pulses. Thus, the muscle contractions during the treatments could be reduced [40, 41]. Later, a series of experiments demonstrated the reduction of muscle contraction during irreversible electroporation [46–49]. In addition, it has been shown with in vitro experiments that high-frequency pulses can also be used for ECT [50] and GET [51], and with in vivo experiments for ablation of tumors [46] and cardiac tissue [30, 34, 52].

1.4 Pulse delivery in electroporation

In order to achieve successful electroporation, cells have to be exposed to a sufficiently high electric fields (related also to duration of exposure). Electroporation pulses are electrical pulses, which are generated by electrical pulse generators, also called electroporators, and delivered to the cells (in the tissue) via electrodes. Thus, the intensity of the electric field is determined by the electrode geometry and the dielectric properties of the tissue. The parameters of the electrical pulses at the output of the electroporator usually differ in shape, amplitude, pulse duration, number of pulses, pulse/burst repetition rate and pulse train, depending on the specific application [53–56].

An electroporator mainly consists of a high-voltage (HV) power supply, a pulse generator, a control unit, a user interface and an output module. The user interface enables setting of the pulse parameters as required for the specific application. For generation of electrical pulses, a HV power supply and pulse generator (for pulse shaping) are needed. The output module consists of an

output pulse measurement unit and a commutator for switching high voltage pulses to different electrodes (if multiple electrodes are used) [57]. Electroporators are generally classified as clinical, industrial, or laboratory depending on the application [55] and can be further distinguished between commercially available and prototype electroporators.

1.4.1 Clinical electroporators as medical devices

Electroporators used in a clinical setting, i.e., clinical electroporators, are considered to be medical devices. They are designed to deliver anti-tumor therapies with pulse protocols based on reversible electroporation (ECT, GET) or as a stand-alone ablation treatment based on IRE. The tumor tissue must be covered with a sufficiently high electric field, which often requires a generation of pulse amplitudes up to 3000 V and currents up to 50 A. Therefore, the clinical electroporators for medical use are challenging to develop, as patient and operator safety must be ensured under both normal and single fault conditions. They must comply with medical safety standards and meet the requirements of local medical regulations, e.g., Medical Device Regulation 2017/745 in Europe [58] or CFR (Code of Federal Regulations) Title 21 in the United States (US), in order to obtain approval to sell the device on the market, e.g. certification mark (CE) in Europe or FDA (Food and Drug Administration) approval in the US. Since each country has different regulatory regimes, a clinical electroporator approved for use in Europe cannot automatically be used in the US and vice versa.

Currently, there are only a few certified clinical electroporators on the market. The most commonly used clinical electroporators are the Cliniporator EPS02 and Cliniporator VITAE (IGEA S.p.A. Carpi (MO), Italy) [59], which are CE labeled for Europe and used for both ECT and GET, and the NanoKnife System (Angio-Dynamics Inc, New York, USA) [60], which is FDA approved for surgical ablation of soft tissue and CE labeled for Europe. Another clinical electroporator with a CE mark is SENNEX (BionMed Technologies, Germany), which is used only for ECT. Recently, a new CE approved ePORE clinical electroporation generator (Mirai Medical, Galway, Ireland) has been developed for simple and reliable delivery of ultrashort electrical pulses to enable treatment based on outpatient endoscopy. For cardiac ablation, there are two clinical electroporators available:

Farapulse (Farapulse, Inc., Boston, USA) and CENTAURI PEF System (Galaxy Medical, San Carlos, USA), which have a CE mark.

1.4.2 Safety as an integrated part of the development

The development of clinical electroporators can be challenging, especially when it comes to ensuring patient and operator safety. As a high level of patient and operator safety must be ensured, electrical safety standards have been established to verify the safety of medical devices. Applying the principles and requirements described in the safety standards is important and should be considered from the beginning of the design of every medical electrical device. The general standard EN/IEC 60601-1 is a widely accepted benchmark and compliance with this standard is the most important requirement for commercialization of electrical medical equipment. The requirements of this standard may be overridden or bypassed by specific requirements in the collateral or particular standards, depending on the type of device. Collateral standards (numbered 60601-1-X) define the general requirements for specific safety and performance aspects, such as electromagnetic compatibility (EN/IEC 60601-1-2). Particular standards (numbered 60601-2-X) define requirements for specific products, e.g., cardiac defibrillators (EN/IEC 60601-2-4). Despite the eighty particular standards, there is currently no particular standard for clinical electroporators. Therefore, in addition to the existing ISO and EN/IEC standards, it is necessary to define additional rules for manufacturing and safe use of clinical electroporators as relatively new medical devices. As the market for clinical electroporators grows, a particular standard can expedite the certification process and enable the harmonization of all commercial certified clinical electroporators to improve the safety, quality, and efficiency of these devices and provide safe and effective treatments.

2 Aim

Since the first successful tumor treatments with electrochemotherapy (ECT) [61, 62], the goal has always been to improve the therapies and treatments using the principle of electroporation to make them safer, more efficient and less painful. Nowadays, electroporation is taking an even faster rise in medicine than before. Taking into account the new technologies and treatment protocols, the increased research intensity and the growing knowledge, we already have some well-established protocols, devices, and promising treatments. However, there are still some challenges that, if solved, may contribute to a better acceptance of electroporation in clinics. ECT is the only electroporation-based application used in clinical practice as a cancer therapy [63], following the Standard Operating Procedure (SOP), which was developed specifically for the treatment of patients [20]. However, electroporation shows a great potential to be used more widely in the clinic for medical applications such as DNA vaccination and gene therapy for cancer treatment, as well as ablation of soft tissue, including cardiac tissue. Therefore, this dissertation mainly focused on three different scientific contributions to improve some of the safety aspects in the clinical application of electroporation.

In the scope of the **first scientific contribution**, we investigated muscle contraction and pain sensation elicited by short, high-frequency (HF), bipolar pulses in healthy volunteers. Stimulation of muscles and nerves is a common challenge in electroporation-based therapies during treatments, as it can lead to movement of the electrodes and cause high levels of discomfort and even pain during treatment. There is increasing emphasis in the scientific community on the development of novel electroporation techniques that can reduce the intensity or extent of muscle contraction, usually using very short (in the range of a few

µs) bipolar pulses instead of the relatively long monopolar pulses [40, 41, 43–49]. However, all the data obtained in the in vitro (cell) and in vivo (animal) experiments, modeling, and theoretical considerations, although of great value, do not allow evaluation of pain reduction during HF electroporation therapy. Therefore, experiments on healthy volunteers using different electrical pulse parameters (different pulse protocols) were the only way to confirm or reject the hypothesis that HF electroporation pulses do not cause pain. In addition, the study was extended to investigate the correlation between muscle contraction and pain sensation during the pulse treatment, because there may be differences in excitation as signals are transmitted through different fibers - myelinated or unmyelinated, with A-delta and C fibers being the main pain-conducting nerve fibers. Therefore, in our study, we further investigated the relationship between muscle contraction and pain perception while varying the pulse parameters (pulse duration, interphase delay - i.e., delay between positive and negative pulse, and interpulse delay - i.e., delay between the pulses), and analyzed which pain fibers have the higher probability of being excited (A-delta or C fibers) based on the pain descriptors selected from the pain questionnaires.

The second scientific contribution addressed the preparation of guidelines and recommendations for standardization of clinical electroporators as no safety standard concerning these devices exists. The absence of a medical-specific standard for clinical electroporators represents a barrier to further development of safe electroporation devices and their commercialization. Currently, there are only few certified clinical electroporators on the market. By following the SOP for ECT, clinicians most commonly use the Cliniporator because it is a certified and widely accepted clinical electroporator used for treatments of cutaneous and subcutaneous tumors. Safer, more efficient, and commercialized clinical electroporators can contribute to more widely recognized and effective treatment. Therefore, it is important to establish an approach for standardization of clinical electroporators. We believe that by having a particular standard (or at least safety recommendations) for clinical electroporators, the development of these devices will be easier and more uniform, resulting in safer and cheaper devices. Therefore, we studied the necessary safety features that a clinical electroporator should have, based on the general safety standards for medical electrical equipment EN/IEC 60601-1 and related safety standards. In addition, we focused

on the preparation of requirements for the particular standard for clinical electroporators and defining tolerances of electroporation parameters for ECT from the SOP for operator guidance and safer cancer treatment. Tolerances for pulse amplitude and pulse duration were defined based on the cell permeabilization curves [64].

Currently, a clinical electroporator must pass all safety tests to be eligible for further testing under relevant standards and certification under the Medical Device Regulation (MDR) 2017/745 in Europe. This would ensure that it can be used safely on patients. Since there is a lack of suitable clinical electroporators on the market, we identified the need to develop a new clinical electroporator that has improved safety performance to enable certification under the new MDR in the future. In that manner, the **third scientific contribution** focused on developing a safety measure in conjunction with the defined safety standards as part of the device for gene electrotransfer (GET) to skin cells. We focused on improving the electrical insulation between the high- and low-voltage parts of the device with an appropriate control of the high-voltage power supply. Additionally, we focused on developing a new safety measure that would detect high values of the output pulse, i.e., a high current, to limit the current to the maximum expected value during the therapy. This would ensure the safe generation and delivery of electroporation pulses to the patient while allowing the operator to safely handle the device.

3 Results and discussion

Three original scientific contributions are presented in this thesis:

1. Reduced muscle contraction and pain sensation during high-frequency electroporation treatments.
2. Preparation of a concept for standardization of electroporation devices for clinical use.
3. Development of safety measures for electroporation device to protect the patient from excess output voltage, current or energy.

The results and discussion section consists of three papers published in peer reviewed international scientific journals presenting the work done in the scope of this thesis. Each scientific contribution is addressed in a separate paper, as listed above. The results from each contribution are presented and discussed in detail in the papers. Below is a summary and additional explanation of the work done as part of this thesis. The thesis then continues with the conclusions section.

Paper 1 (Cvetkoska, Maček-Lebar, Trdina, Miklavčič, and Reberšek) entitled *Muscle contractions and pain sensation accompanying high-frequency electroporation pulses* examines muscle contraction and pain sensation caused by short bipolar high-frequency pulses in the first in-human study. Twenty-five healthy volunteers were subjected to electrical stimulation of the tibialis anterior muscle with bipolar high-frequency pulses in the range of a few microseconds (1 µs to 5 µs) and both symmetric and asymmetric interphase delays (delay between the positive and negative phase of the pulse) and interpulse delays

(delay between the pulses). To evaluate the muscle contraction, the angle of dorsiflexion of the ankle was measured with a two-axis goniometer. To examine pain sensation and to assess pain intensity and unpleasantness, each volunteer was asked to complete a short-form McGill pain questionnaire. Because of the different insulation properties of the skin and subcutaneous tissue of the volunteers, strength-duration curves were determined for monopolar and bipolar pulses with different pulse duration for each volunteer. Statistical analysis using N-way repeated measures analysis of variance (rmANOVA) and cluster analysis using a hierarchical cluster tree (dendrogram) were performed in order to find statistically significant differences between the bipolar pulse protocols and cluster them into five different clusters, i.e., bipolar pulse protocols with higher/lower muscle contraction response and/or higher/lower pain index. Based on the pain descriptors selected from the pain questionnaires, additional analysis was performed to investigate which pain fibers were more likely to be excited (A-delta or C fibers). Additional measurements with extended interpulse delays (while maintaining short interphase delays), based on modeling results from a recent paper [70], and interchanged interphase and interpulse delays were also performed. We confirmed that bipolar high-frequency pulses with a pulse duration of 1 µs or 2 µs reduce muscle contraction and pain sensation as opposed to the longer monopolar pulses currently used (8 pulses with a pulse duration of 100 µs, delivered at 5 kHz pulse repetition frequency). However, interphase and interpulse delays also play an important role in reducing muscle contraction and/or pain sensation, making the interplay of pulse parameters more complex. Pain is not necessarily elicited as a consequence of muscle contraction and vice versa. Nevertheless, our study has shown that the range of optimal pulse parameters can be extended depending on the different requirements of the therapy. However, further evaluation of the presented bipolar protocols is necessary to confirm the efficiency of the newly proposed high-frequency electroporation.

Paper 2 (Cvetkoska, Pirc, Reberšek, Magjarević, and Miklavčič) entitled *Towards standardization of electroporation devices and protocols* proposes guidelines for the safe design of clinical electroporators and defines the basic requirements for their safe and efficient use, which may be included in the particular standard in the future, as a particular standard for clinical electroporators has not yet

been established. The necessary safety features that a clinical electroporator should have are proposed based on the general safety standard for medical electrical equipment EN/IEC 60601-1 and accompanying safety standards. We also reviewed and compared the characteristics of the electroporation devices (clinical, industrial, and laboratory) found in the literature and/or on the market and emphasized the need to define electroporator performance parameters so that data/results obtained by different research groups can be compared and reproduced. In addition, we have prepared recommendations for the requirements that the particular standard should define for clinical electroporators in order to make the development and certification of such devices less demanding. Finally, we defined tolerances and prepared recommendations for standardization of electrochemotherapy devices (separately for the electroporator and electrodes) based on the standard operating procedure. Based on cell permeabilization curves, tolerances for pulse amplitude and pulse duration were determined, which may be introduced as an additional improvement to the current standard operating procedure for electrochemotherapy.

Paper 3 (Cvetkoska, Dermol-Černe, Miklavčič, Kranjc-Brezar, Markelc, Serša, and Reberšek) entitled *Design, development, and testing of a device for gene electrotransfer to skin cells in vivo* presents the development of a new electroporation device (pulse generator and applicator) and a protocol for pulse delivery for gene electrotransfer to skin cells. Based on the numerical model of the electroporated skin developed during the study, a new pulse delivery protocol was proposed, which was then used together with the device in an in vivo study of gene electrotransfer to skin cells in mice. In designing the electroporator, we followed the recommendations presented in the second paper and considered some of the safety requirements for medical electrical equipment listed in the general standard EN 60601-1:2007. We incorporated galvanic isolation so that in the event of a fault in the high-voltage part, the high-voltage would not transfer to the low-voltage part of the device. We have also developed new safety protection in the event of an overcurrent at the output of the device, which prevents the current and power from becoming too high when the high-voltage capacitors are discharged. When an overcurrent is detected, a fast limitation of the current at the output is achieved. The current limiter allows the therapy

to continue even if the current occasionally increases because it limits the current only to the maximum expected value during the therapy. To increase the likelihood of successful delivery of the pulses for safe therapy, we have also developed a circuit that checks the contact of the electrodes with the skin before the electrical pulses are delivered. This circuit is implemented as a LED light on the applicator handle. In this way, the operator is informed with a green light when the electrodes are in contact with the skin or with a red one when they are not. In addition, the developed electroporator is battery powered, the graphical user interface is displayed on a 10.1" touchscreen and the applicator can fit different skin areas on the body regardless of the curvature. It is capable of generating square wave pulses from 80 V to 600 V with a pulse duration from 10 µs to 1000 µs at a pulse repetition frequency from 0.1 Hz to 5000 Hz.
The results of the in vivo study showed that gene electrotransfer with the developed electroporator, applicator, and proposed pulse delivery protocol resulted in higher gene expression in skin cells compared to the currently used electroporator (Cliniporator), applicator (multi-electrode array - MEA electrodes), and pulse delivery protocol.

Additional results not included in paper 3

Development of the safety protection

The purpose of the third scientific contribution was to develop new safety measures for the gene electrotransfer electroporator (as described in paper 3) to protect the patient from excessive output voltage (more than 640 V), current (more than 2 A), or energy (more than 7 J). The developed protection was divided into 2 parts. The first part was the voltage and current sensor and relay (VCSR) circuit, which uses relays to switch between the electroporation pulse (output from the pulse generator) and the electrode contact signal (output from the electrode contact circuit that checks whether the electrodes are in contact with the skin or not). The protection was implemented so that it did not connect both signals simultaneously and disconnects both relays in case of a pulse termination (pulse stop signal). Voltage (voltage divider) and current sensors (fluxgate - CASR-6-NP (LEM International SA, Switzerland)) were used to measure the voltage and current of the electroporation pulse (output from

the pulse generator) and to convert this signal into a signal suitable for the A/D converter. The second part was the overvoltage, current, and energy protection (OVCEP) circuit, which detects high values of the output electroporation pulse and sends a stop signal to stop the generation of the pulses. This part of the protection was implemented with comparators (to detect high-voltage and current values) and an analog multiplier and integrator to calculate the output power. However, we were unable to measure/detect the current with either the chosen sensor or the other current sensor (ACS 70331, Allegro MicroSystems, Manchester, USA), which was implemented later. The current limiter was then developed separately, with a silicon carbide junction field effect transistor (JFET) UJ3N120035K3S (UnitedSiC, Princeton, USA) acting as a switch in the circuit. As the current through the resistor between the gate and the source increases, the voltage across the gate of the JFET negatively decreases and the JFET begins to close. Simulations were performed in LT Spice XVII (Analog Devices, Massachusetts, USA) to determine the correct value for the resistor between the gate and the source (R2, Figure 3.1), in order to limit the current to 2 A.

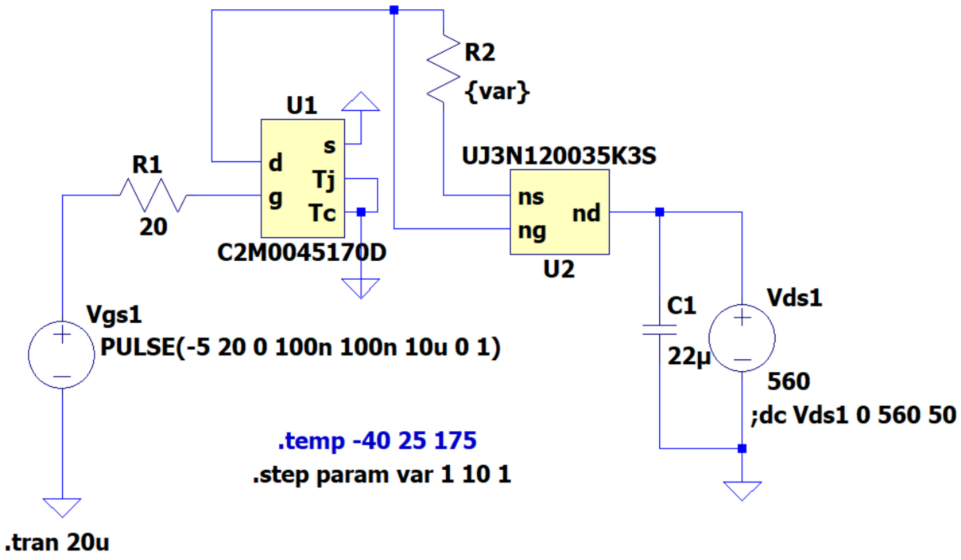


Figure 3.1. Schematic of the circuit used for the current limiting simulations. Ten different values (var: 1 Ω to 10 Ω, with 1 Ω steps) were tested for the resistance (R2) between the gate and the source of the JFET (U2). Simulations were performed for one monopolar, 10 µs pulse with an amplitude of 20 V. The high-voltage capacitor of 22 µF was charged to 560 V.

Based on the simulations performed (Figure 3.2) for values from 1 to 10 Ω (run 1-10), we determined that the resistor should have a value between 5 Ω and 6 Ω (Figure 3.2, pink and gray line, run 5 and 6). However, for the final design, we decided to connect three resistors in series - two 1.5 Ω power resistors and one 1 Ω positive temperature coefficient (PTC) resistor, which increases the resistance due to heating from the higher current flow. When the performance of the circuit was tested, this method proved useful in limiting the current to the maximum allowed value (2 A), as well as limiting the output energy. However, some oscillations were detected at the beginning of the signal (only in the first 1 µs), as it can be seen in the simulation graph in Figure 3.2.

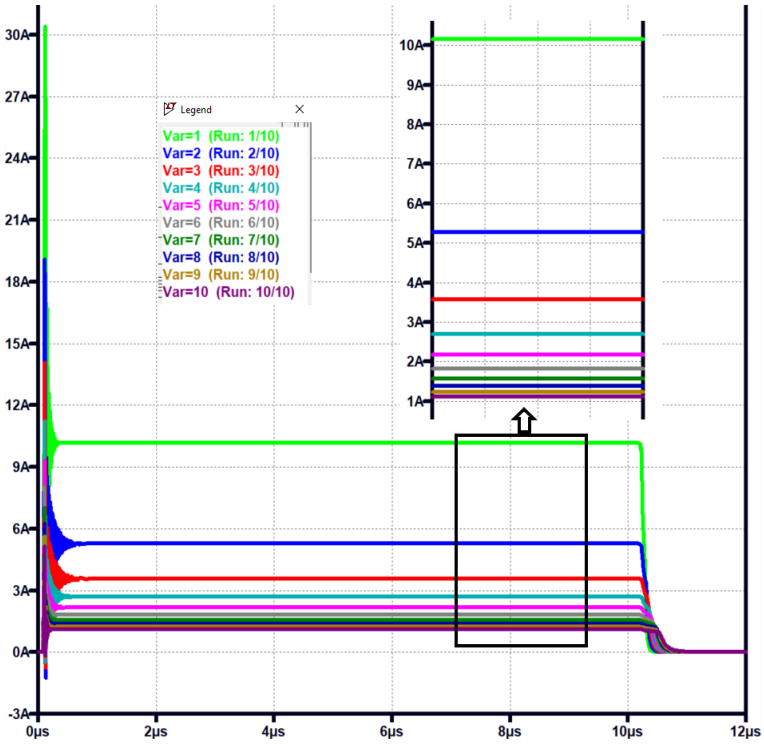


Figure 3.2. Limitation of the current to 2 A requires a resistor with a resistance of 5 Ω or 6 Ω (pink and gray line) connected between the gate and the source of the JFET. The legend shows the tested values of R2 (from top to bottom: green line - 1 Ω, purple line - 10 Ω) with respect to the limited current (y-axis).

Simulations were also performed using a silicon carbide power metal oxide semiconductor field effect transistor (MOSFET) C2M0045170D (Wolfspeed Inc., North Carolina, USA) as a current limiter. This approach was then used to

develop a safety circuit for a high-frequency laboratory prototype electroporator. In this case, we first determined the value for the resistor at the source (R1, Figure 3.3) to limit the current flow (to 120 A). Later, we determined the value for the resistance at the drain (R2, Figure 3.3) to reduce the oscillations (current fluctuations) that occur when the current is limited (simulations not shown).

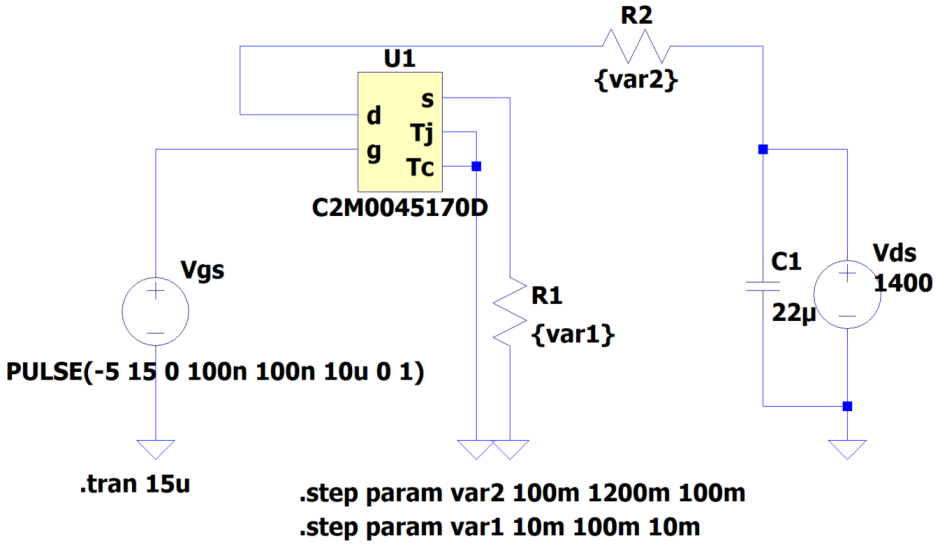


Figure 3.3. Schematic of the circuit used to perform the simulations to limit current and reduce oscillations. Ten different values (var1: 10 mΩ to 100 mΩ, with 10 mΩ steps) for resistor R1 and another twelve values (var2: 100 mΩ to 1200 mΩ, with 100 mΩ steps) for resistor R2 were tested. The simulations were performed for one monopolar, 10 µs pulse with an amplitude of 15 V. The high-voltage capacitor of 22 µF was charged to 1400 V.

The simulations in Figure 3.4 show that we need a 50 mΩ resistor for R1 (the values for var1 were changed from 10 mΩ to 100 mΩ) in order to limit the current to 120 A. This is shown in the figure with a pink line (50m, run 5). We then ran simulations for R2 where R1 was 50 mΩ and var2 (values for R2) was changed from 100 mΩ to 1.2 Ω. The simulations in Figure 3.5 show that for R2 higher than 700 mΩ the oscillations are critically damped. Therefore, we fixed the resistance of R2 to 1 Ω for the final circuit. In addition, a desaturation fault detector was added to the circuit to protect the MOSFET when the drain to source voltage is above the preset reference voltage. This provides fast detection and shutdown of the MOSFET without false triggers in the event of an overcurrent. Pulse generation will be stopped when the drain to source voltage rises above the preset reference voltage.

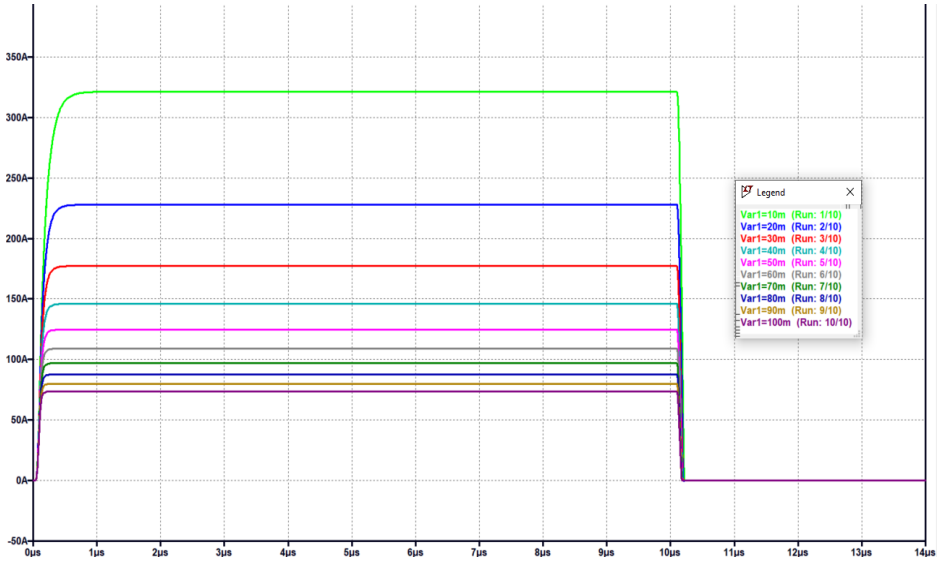


Figure 3.4. Limitation of the current will require a resistor with a resistance of 50 mΩ (pink line) connected to the source of the MOSFET. The legend shows the tested values of R1 (from top to bottom: green line - 10 mΩ, purple line - 100 mΩ) with respect to the limited current (y-axis).

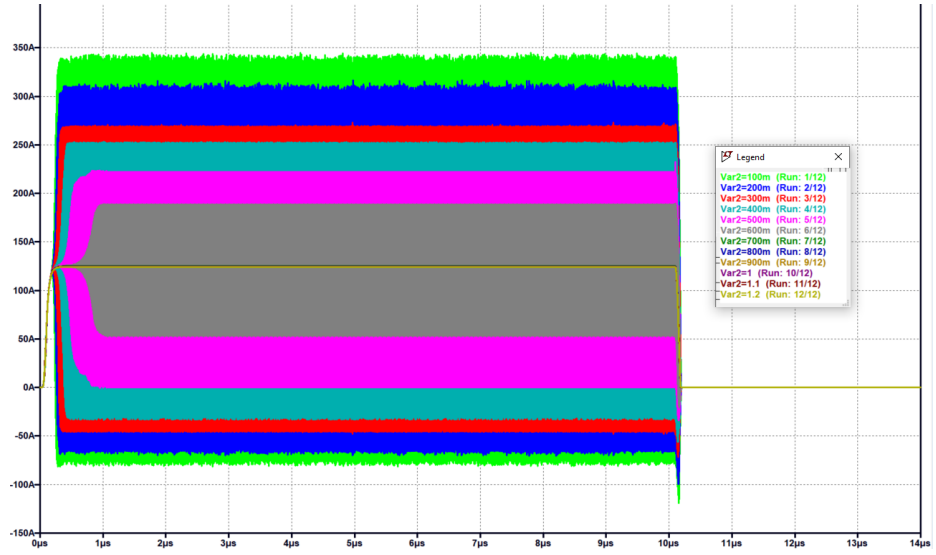


Figure 3.5. To reduce oscillations, a resistor with a resistance of at least 700 mΩ (dark green line up to light yellow line) connected to the drain of the MOSFET is required. The legend shows the tested values of R2 (from top to bottom: green line - 100 mΩ, yellow line - 1200 mΩ) with respect to the limited current (y-axis).

Results of the performed electrical safety tests

At the end, we tested/measured the leakage currents of the developed electroporator for gene electrotransfer (described in paper 3) according to the general standard EN/IEC 60601-1 for medical electrical equipment with a certified and calibrated electrical safety analyzer - Fluke ESA620 (Fluke Biomedical, Washington, USA). We tested several different types of leakage currents: earth leakage current (Figure 3.6a), touch current (Figure 3.6b), patient leakage current (Figure 3.6c), patient auxiliary leakage current (Figure 3.6d) and mains on applied parts leakage (patient leakage current on applied part, Figure 3.6e). The difference between the leakage currents depends upon how a person might come in contact with the device (Figure 3.6).

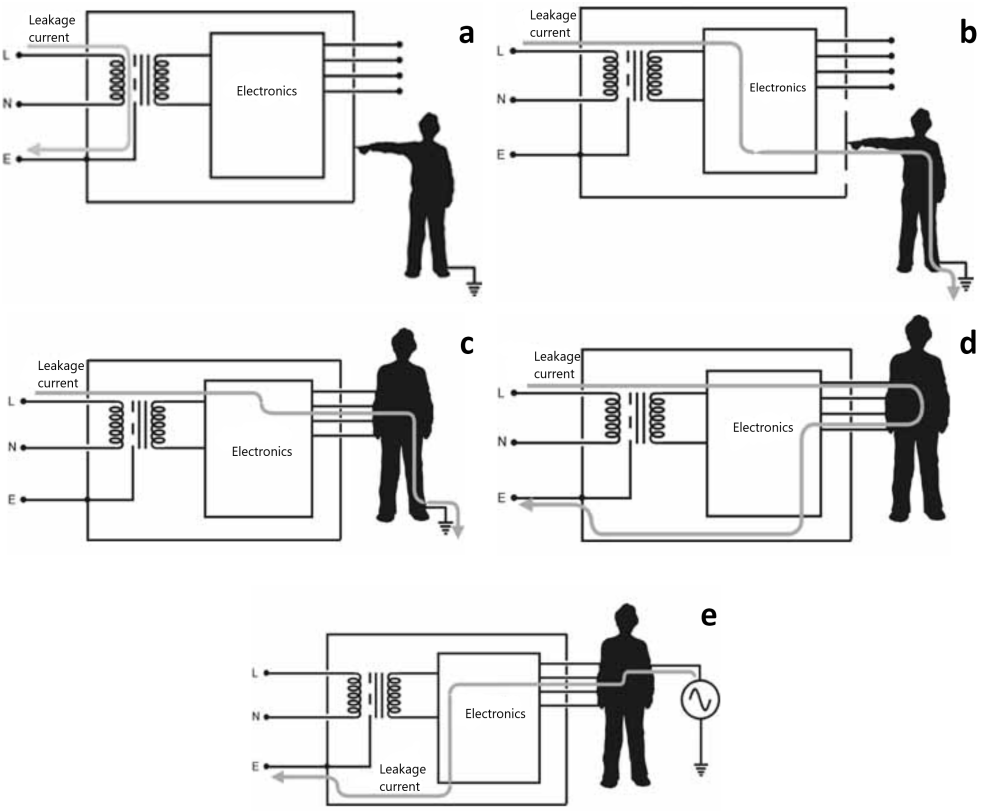


Figure 3.6. Types of leakage currents: a) earth leakage current, b) touch current, c) patient leakage current, d) patient auxiliary leakage current, e) patient leakage current on applied part.

The results from the electrical safety report are presented in Table 3.1. The allowable values from the standard for type BF (Body Floating) medical device (the device has a conductive contact with the patient) are given in the second col-

umn of Table 3.1. The results/status weather the device has passed the performed test are given in the last column of Table 3.1. The device passed all performed tests for both normal operation (e.g., normal polarity) and single fault operation (e.g., opening of the neutral connector on the mains supply), meaning that the measured values were within the allowable values according to the standard.

Table 3.1. Results of the performed electrical safety tests

Name of performed test	**Limit stated by the standard**	**Measured (actual) value**	**Result**
Earth Leakage Current (ACDC)			
normal polarity	5 mA	295 µA	Pass
reversed polarity	10 mA	286 µA	Pass
Touch Current (ACDC)			
normal polarity	100 µA	0.5 µA	Pass
normal polarity, open neutral	500 µA	0.6 µA	Pass
reversed polarity	100 µA	0.5 µA	Pass
reversed polarity, open neutral	500 µA	0.6 µA	Pass
Patient Leakage Current (AC)			
normal polarity	100 µA	0.3 µA	Pass
normal polarity, open neutral	500 µA	0.4 µA	Pass
reversed polarity	100 µA	0.3 µA	Pass
reversed polarity, open neutral	500 µA	0.4 µA	Pass
Patient Auxiliary Leakage Current (AC)			
normal polarity	100 µA	0.6 µA	Pass
normal polarity, open neutral	500 µA	0.6 µA	Pass
reversed polarity	100 µA	0.6 µA	Pass
reversed polarity, open neutral	500 µA	0.6 µA	Pass
Mains on Applied Parts Leakage (normal polarity, 110% mains voltage)			
single fault condition	5 mA	61.5 µA	Pass
single fault condition, reversed mains	5 mA	61.6 µA	Pass

3.1 Paper 1

Title: **Muscle contractions and pain sensation accompanying high-frequency electroporation pulses**

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Muscle contractions and pain sensation accompanying high-frequency electroporation pulses

Aleksandra Cvetkoska[1], Alenka Maček-Lebar[1], Peter Trdina[2], Damijan Miklavčič[1] & Matej Reberšek[1]✉

To minimize neuromuscular electrical stimulation during electroporation-based treatments, the replacement of long monophasic pulses with bursts of biphasic high-frequency pulses in the range of microseconds was suggested in order to reduce muscle contraction and pain sensation due to pulse application. This treatment modality appeared under the term high-frequency electroporation (HF-EP), which can be potentially used for some clinical applications of electroporation such as electrochemotherapy, gene electrotransfer, and tissue ablation. In cardiac tissue ablation, which utilizes irreversible electroporation, the treatment is being established as Pulsed Field Ablation. While the reduction of muscle contractions was confirmed in multiple in vivo studies, the reduction of pain sensation in humans was not confirmed yet, nor was the relationship between muscle contraction and pain sensation investigated. This is the first study in humans examining pain sensation using biphasic high-frequency electroporation pulses. Twenty-five healthy individuals were subjected to electrical stimulation of the tibialis anterior muscle with biphasic high-frequency pulses in the range of few microseconds and both, symmetric and asymmetric interphase and interpulse delays. Our results confirm that biphasic high-frequency pulses with a pulse width of 1 or 2 µs reduce muscle contraction and pain sensation as opposed to currently used longer monophasic pulses. In addition, interphase and interpulse delays play a significant role in reducing the muscle contraction and/or pain sensation. The study shows that the range of the optimal pulse parameters may be increased depending on the prerequisites of the therapy. However, further evaluation of the biphasic pulse protocols presented herein is necessary to confirm the efficiency of the newly proposed HF-EP.

Electroporation/electropermeabilization describes the phenomenon where the cell membrane is exposed to sufficiently strong electric field that is generated by short-duration, high-voltage pulses. This induces a transmembrane voltage (TMV), e.g., 500 mV, which far exceeds its resting TMV (typically -40 mV to -70 mV). Thus, plasma membrane permeability is increased and transmembrane transport of molecules is enabled which otherwise are unable to cross the membrane[1]. Electroporation can be either reversible, when the cell recovers after the treatment and survives, or irreversible when the exposure leads to cell death[2–4].

Electroporation is used in multiple clinical applications[5–7] as well as in biotechnology[8], food processing[9], and environmentally relevant applications[10]. Reversible electroporation is successfully used as combination of high-voltage pulsed electric fields with low-permeant chemotherapeutic drug or with DNA: electrochemotherapy (ECT) and gene electrotransfer (GET), respectively[11–17]. On the other hand, irreversible electroporation (IRE) appeared as a new medical application[3] for non-thermal tumor[18–20] and cardiac ablation (Pulse Field Ablation–PFA)[21–23] providing considerable benefits over existing thermal ablation methods, such as reducing the risk of damaging the nearby critical tissue. Especially in cardiac electrophysiology, PFA may represent a dominant future treatment[24–26].

Currently, in most of the electroporation-based clinical applications, relatively long monophasic pulses of 50–100 µs are delivered with low repetition rates, synchronized with the heart rhythm[3,27,28]. In gene therapy vaccination even longer pulses (in the range of milliseconds) e.g., 50 ms are applied[17]. The electric field thresholds

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required to initiate electroporation are higher than the thresholds that trigger action potentials in excitable cells, which means that electroporation is not successfully achieved without (unintended) electrical stimulation of excitable cells[29,30], i.e., muscle and nerve cells. Consequently, delivery of electroporation pulses leads to muscle contraction and sensory nerve cells (e.g., nociceptors) excitation rendering these treatments unpleasant or even painful. Muscle contraction may potentially lead to interference with the heart rhythm and/or displacement of the electrodes during the treatment, which increases the complexity of the treatments and may pose a risk to the patient. The patients need to undergo local or general anesthesia, receive muscle relaxants to ensure adequate neuromuscular blockade and proper respiratory function[31–34], and pulse delivery needs to be synchronized with the patient´s ECG[27].

Stimulation of nerves and muscles has been extensively investigated in the past, showing that short pulses and higher frequencies of alternating current (up to 10 kHz) can increase sensory, motor, and pain thresholds[35–39]. Thus, to minimize stimulation of muscle and nerves during electroporation-based treatments, the increase of the pulse repetition frequency far above the frequency of tetanic contraction was suggested. This was confirmed to be an effective treatment showing reduction of the overall muscle contractions and pain sensation[40,41]. Recently, a new waveform was suggested that could potentially replace the standard 50–100 µs monophasic pulses: a burst of short biphasic pulses (with pulse width from 1 to 10 µs) with the same total 'on-time' of the pulses (energized time)[42–44] and with pulse repetition rates ranging from 250 kHz to 1 MHz. It was shown that such pulses could be used for tissue ablation while potentially avoiding muscle contractions. This novel electroporation waveform appeared under the term high-frequency irreversible electroporation (H-FIRE)[43,44]. Moreover, a numerical modeling study[45] also suggested that by using bursts of short biphasic pulses, the same IRE efficiency for tissue ablation can be achieved while avoiding action potential triggering in the nerve fibers nearby that would be stimulated by the use of long monophasic pulses. The encouraging results obtained from the initial studies led to a series of experiments to study H-FIRE[42,46–54]. Additionally, it was demonstrated that high-frequency (HF) pulses can be efficiently used to ablate tumors[47], cardiac tissue[23,26,55] and potentially also for ECT[56] and GET[57]. However, the data obtained through cell/animal experiments, modeling, and theoretical considerations although of great value, do not allow to evaluate pain reduction during HF electroporation therapy. Moreover, the correlation between muscle contraction and pain sensation during the pulse treatment has not been examined yet. There can be differences in excitation, as the signals are transmitted via different fibers—myelinated (A-alpha, beta, gamma, delta) or unmyelinated (C-fibers), with A-delta and C-fibers being the major pain-conducting nerve fibers[58,59].

In our study, we examined pain sensation during the pulse treatment and the correlation between the elicited muscle contraction and pain sensation caused by short biphasic HF pulses in healthy individuals. Additionally, we investigated the relationship between muscle contraction and pain sensation while varying the pulse parameters (pulse width, interphase and interpulse delays). Finally, we analyzed which pain fibers have the higher possibility of being excited (A-delta or C-fibers) based on the pain descriptors selected from the pain questionnaires by the individuals.

Materials and methods

In our study, 25 healthy individuals participated. Muscle contraction was initiated by electrical stimulation of the tibialis anterior muscle on the right leg. As this muscle acts primarily in ankle dorsiflexion, the angle of ankle dorsiflexion was measured by a two-axis goniometer. Due to different insulating properties of the skin and subcutaneous tissue of the individuals, strength-duration curves were determined for monophasic and biphasic pulses with different pulse widths for each individual. Based on the amplitude of 8 monophasic pulses with a pulse width of 100 µs, delivered with 5 kHz pulse repetition rate, which results in measurable muscle contraction, the stimulus amplitude for biphasic pulse protocols was determined. Biphasic pulses with pulse width from 1 to 5 µs were tested while changing the interphase delay (time between positive and negative phase) and interpulse delay (time between the pulses) (Fig. 1). Each individual was subjected to a randomly selected group of 30 biphasic pulse protocols for muscle contraction determination. In order to examine the pain sensation and assess pain intensity and unpleasantness, each individual was requested to fill short-form McGill pain questionnaires for randomly selected half of the delivered biphasic pulse protocols after the stimulation.

Participants. The research was approved by the National Medical Ethics Committee of Slovenia (Doc. no. 0120–61/2020) and was conducted following the Declaration of Helsinki, Convention on Human Rights and Biomedicine (ETS No.164), and the Slovenian Code of Medical Ethics. Informed consent was obtained from all individuals before the start of the measurements. All of them were given the opportunity to withdraw from the study at any time, even after signing the informed consent to participate. Thirty healthy individuals volunteered to participate in the study. Five of the individuals were not included in the study due to too strong muscle contraction when the muscle was stimulated with the lowest possible amplitude of the pulse generator. The main set of measurements was thus performed on 25 individuals (12 females and 13 males) in the age range from 22 to 58 years (mean: 32.5 years, median: 27 years). Twenty individuals were younger (range: 20–32 years) and 5 elder (range: 52–58 years).

Experimental setup. For the delivery of electrical pulses, a prototype high-frequency (HF) pulse generator was used (University of Ljubljana, mPOR, Slovenia). Before measurements, the electrical safety of the pulse generator and measuring system was verified with a certified and calibrated electrical safety analyzer Fluke ESA620 (Fluke Biomedical, Washington, USA) for medical devices following the medical standard IEC 60,601–1. The available energy of the pulse generator was physically limited to 5 J with the capacitance of the integrated supply capacitor, enabling safe delivery of pulses and preventing potential damage to the skin. The lowest amplitude limit of the pulse generator was 60 V; the highest amplitude limit was 1000 V. The pulses delivered were

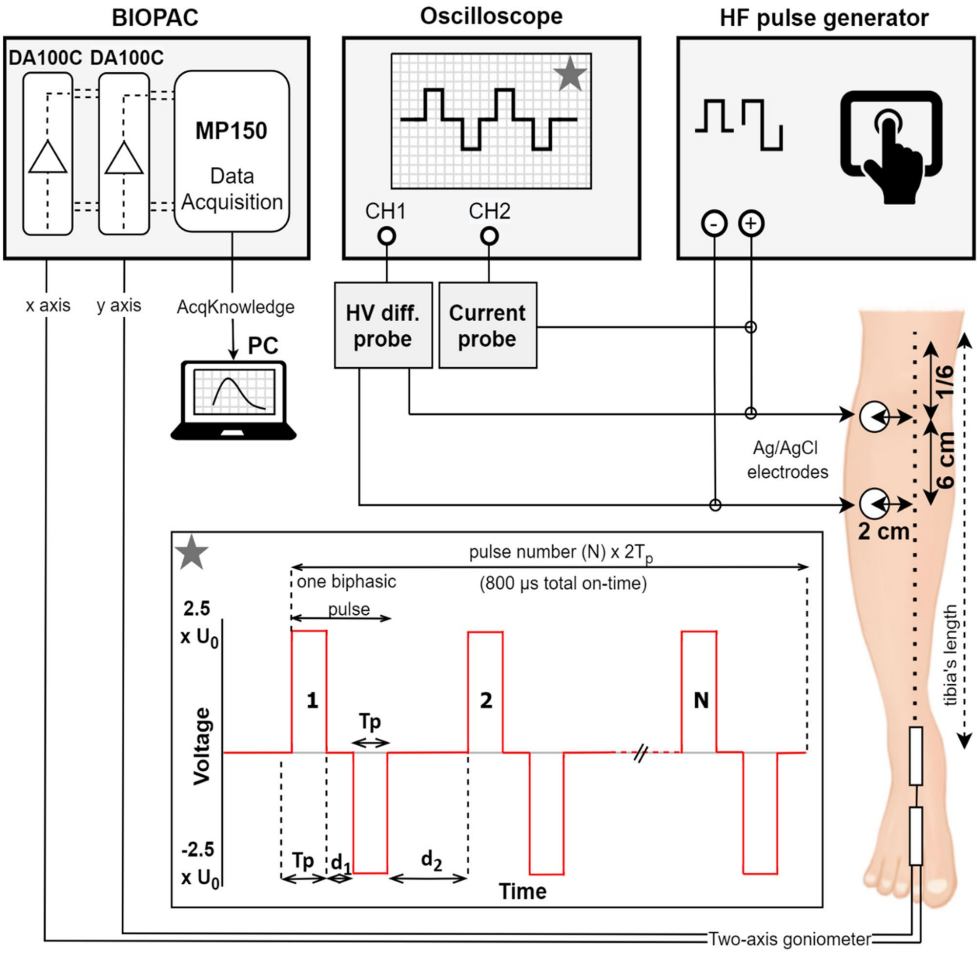

Figure 1. Experimental setup and electrodes/goniometer placement. Stimulation pulses were delivered via electrodes connected to the HF pulse generator. The electrodes (marked with circles) were placed on the right leg: the upper electrode was placed on 1/6th of the tibia's length, the lower electrode was placed 6 cm lower. Both electrodes were placed 2 cm right lateral to the bone (left in the figure). The output pulses were monitored on an oscilloscope using high-voltage (HV) differential and current probe. Asterisk: applied pulses—biphasic pulses with 800 µs total on-time. T_p-pulse width (equal for positive and negative phase), d_1-interphase delay, d_2-interpulse delay, N-number of pulses. The response from the ankle (muscle contraction) was acquired with twin-axis goniometer connected to the Biopac unit. The data was analyzed on a personal computer (PC) using the AcqKnowledge software. DA100C-amplifier, MP150-data acquisition system.

monitored by a high-voltage (HV) differential probe HVD3605A (LeCroy, USA), current probe CP031 (LeCroy, USA) and HDO6000 High-Definition oscilloscope (LeCroy, USA) via power medical isolation transformer.

Measurements were performed on the right leg in all individuals. Self-adhesive Ag/AgCl electrodes (3 M™ Red Dot™, 3 M, Minnesota, USA) for single use were connected to the pulse generator via lead wires with a clip. Before measurement, the skin was cleaned with 70% ethanol and the participant's tibia length was determined[60] in order to place the electrodes consistently for each individual. The upper electrode was placed on 1/6th of the tibia's length and the lower electrode was placed 6 cm lower. Both electrodes were placed 2 cm right, lateral to the bone (Fig. 1).

To determine muscle contraction, the angle of ankle dorsiflexion was measured with twin-axis goniometer TSD120B (Biopac Systems, Inc., USA). The upper mounting point was placed on the lower part of the right tibia and the lower mounting point was placed on the right forefoot, above the extensor tendons (Fig. 1). The goniometer was attached using double-sided tape and was additionally secured with single-sided tape. Two planes of

angular movement were simultaneously measured (foot dorsiflexion/plantarflexion and abduction/adduction). Each channel of the goniometer was connected to an DA100C amplifier as part of the MP150 data Acquisition system (Biopac Systems Inc., USA). The AcqKnowledge 4.1 software (Biopac Systems Inc., USA) was used for real-time measurements and recording of the signals (muscle contraction responses) as MATLAB data files for further analysis in MATLAB vR2018a (MathWorks Inc., Natick, MA, USA). Calibration of the goniometer was performed before each measurement using the software calibration features. The complete experimental setup is shown in Fig. 1.

In preliminary measurements, we examined how the position of the electrodes affected the muscle contraction as measured by ankle dorsiflexion. The results showed that moving the electrodes for 1–2 cm proximally/distally does not considerably affect the results while moving the electrodes for more than 4 cm laterally from the bone requires higher amplitudes (more than 20%) to be delivered to achieve the same muscle contraction response (data not shown).

Test procedure. The measurements were performed in the Laboratory for Physiological Measurements (Faculty of Electrical Engineering, University of Ljubljana, Slovenia). The duration of the measurements was approximately one hour and thirty minutes per individual. No anesthetics or nerve blockers were used during the measurements. Before the measurements, the protocol was explained to each individual. There were no serious side effects recorded, nevertheless a medical doctor was always available during the measurements. The only side effect noticed during or after measurements was slight redness at the site of the electrodes after the treatment, which disappeared within few hours. None of the individuals withdrew from the study due to pain or other reasons although they had the opportunity to withdraw from the study at any time.

Strength–duration curves. In the first part (approximately 30 min) of the measurements, we determined two strength-duration (S-D) curves per individual. These curves represent the stimulus strength (voltage) needed to produce minimal muscle contraction for certain pulse width and pulse type (monophasic/biphasic)[58,61–63]. Thus, for each individual, stimulation for one monophasic and one biphasic pulse for five pulse widths (T_p: 1, 2.5, 5, 10, or 50 µs) was performed. For the biphasic pulses, the interphase delay (delay between positive and negative phase) was randomly chosen (d_1: 1, 2.5, 5, 10, or 100 µs). Each S-D curve was measured by first applying the longest pulse width (50 µs) to the muscle and increasing the stimulus intensity (amplitude) until a lower limit of quantification (LLOQ) of muscle contraction (muscle response) was reached, defined as an angle of 3.6° to 4° ankle dorsiflexion. The LLOQ is the lowest angle that can be quantitatively determined with suitable precision and accuracy by our measurement system. Subsequently, the pulse width was decreased to 10 µs and the amplitude increased until the LLOQ of muscle contraction response was obtained. This process was repeated for pulse widths of 5, 2.5, and 1 µs. Thus, five points for each S-D curve were determined. Note that monophasic pulses were $1xT_p$ long while biphasic pulses were $2xT_p$ long; e.g., monophasic: 50 µs; biphasic: 50 µs positive + 50 µs negative = 100 µs.

Determining the stimulus amplitude for the measurements. In the same way as was the amplitude for one point on the S-D curve determined, the stimulus amplitude for 8 monophasic pulses with a pulse width of 100 µs, delivered with 5 kHz pulse repetition rate (5 pulses per one millisecond) was determined. This pulse protocol was chosen to be the amplitude determining (reference pulse protocol), as it is the most often used electroporation protocol[64] in clinical practice. The pulses were delivered with an initial amplitude of 60 V, gradually increased in small increments (5–10 V) until minimal muscle contraction was obtained. As the pulse generator was not able to deliver pulses of amplitudes lower than 60 V, five of the individuals who initially volunteered for the study were not included, due to too strong muscle contraction, i.e., above 4.6° ankle dorsiflexion when the muscle was stimulated with an amplitude of 60 V.

Biphasic pulse protocols. Twenty-five sets of biphasic pulse protocols were generated and coded with numbers from 1 to 25 at the beginning of the study, each set containing 30 randomly chosen biphasic pulse protocol numbers, within which 15 were randomly chosen for the pain questionnaires (www.random.org, RANDOM, Ireland). All of the biphasic pulse protocols were repeated nearly equal times. Before measurements, each individual drew a set number (1—25) of biphasic pulse protocols. Thus, each individual received 30 biphasic pulse protocols in addition to the reference protocol and filled out 15 pain questionnaires. There was a 2 min waiting time between each protocol. This second part of the measurements took approximately one hour.

All biphasic pulse protocols used in the study had the same total on-time as the reference pulse protocol (8 × 100 µs, 5 kHz). Therefore, in the biphasic pulse protocols the number of pulses and pulse width (duration of each phase) were changed so that the total on-time of the pulses (N x $2T_p$) was the same, i.e., 800 µs as shown in Fig. 1 (insert marked with asterisk). Additionally, for each pulse width tested (1 µs to 5 µs), the interphase d_1 (time between the end of the positive and beginning of the negative phase of the pulse) and interpulse delay d_2 (time between the end of the negative pulse and beginning of the new positive pulse) were changed. The interpulse delay d_2 was equal to or longer than the interphase delay ($d_2 \geq d_1$) in each pulse protocol. The pulse repetition rate (PRR) was calculated as $PRR = 1 / (2T_p + d_1 + d_2)$. The total number of biphasic pulse protocols examined was 51 (see Table S1 in the Supplementary files). The amplitude used for the biphasic pulse protocols was 2.5 times higher than the amplitude determined for the reference pulse protocol, since higher amplitudes are required for biphasic pulses to obtain comparable effect as with monophasic pulses[42,56,65] at the same total on-time. For example, if the amplitude determined for 8 monophasic pulses was $U_0 = 100$ V, the amplitude for the biphasic pulse protocols was 100 V x 2.5 = 250 V. Table 1 shows the values of all pulse parameters used in the study.

Polarity	Pulse width (T_p) [μs]	N	d_1 [μs]	d_2 [μs] ($d_2 \geq d_1$)	Amplitude (U)
Monophasic	100	8	/	100 (5 kHz)	U_0
Biphasic	1	400	1, 2, 5, 10, 100	1, 2, 5, 10, 100	$2.5 \times U_0$
Biphasic	2	200	1, 2, 5, 10, 100	1, 2, 5, 10, 100	$2.5 \times U_0$
Biphasic	3	133	1, 5	1, 5, 800	$2.5 \times U_0$
Biphasic	4	100	1, 5	1, 5, 800	$2.5 \times U_0$
Biphasic	5	80	1, 2, 5, 10, 100	1, 2, 5, 10, 100	$2.5 \times U_0$

Table 1. Values of the pulse parameters for all pulse protocols included in the study. All biphasic pulse protocols have equal total on-time, i.e., $N \times 2Tp = 800$ μs. Tp pulse width (equal for positive and negative phase); N number of pulses; d1 interphase delay; d2 interpulse delay.

Pain questionnaires. All individuals completed a short-form McGill Pain Questionnaire (SF-MPQ)[66] for 15 randomly chosen biphasic pulse protocols (out of the 30 examined biphasic pulse protocols for muscle contraction responses) immediately after the delivery of the specific biphasic pulse protocol, to examine the nature of pain and assess pain intensity and unpleasantness. Twenty-two individuals completed the Slovenian version, and three individuals completed the English version of the SF-MPQ, as Slovenian was not their native language. Every pain questionnaire consisted of four parts. The first part was the main component—Pain Rating Index (PRI) of the SF-MPQ, which was used to determine the sensory (pain descriptors 1–11) and affective (pain descriptors 12–15) components of pain, rated on an intensity scale as 0 = none, 1 = mild, 2 = moderate or 3 = severe. The second part of the SF-MPQ referred to two separate 10 cm horizontal Visual Analog Scales (VAS)[67] which were used to assess pain intensity and unpleasantness, respectively. Both were marked as "no pain/no unpleasantness" on one end and "most intense pain/worst possible unpleasantness" on the other end. In the third part, the SF-MPQ included the Present Pain Intensity (PPI) index, which was a five-point interval scale measuring the intensity of overall pain. The scale ranged from "mild" to "excruciating" with points from 0 to 5, respectively, and evaluated the intensity of overall pain experienced during electrical pulse delivery for each specific biphasic pulse protocol. The fourth, i.e., the last part was referred to answering three questions about the exact position of pain in the body, the duration of the pain sensation, and the pain sensation changing with time. After taking off the electrodes, each individual answered two questions regarding the sensitivity of the skin and visible signs of skin injury immediately and 6 h after the end of the measurements.

Calculation of the total pain index. The total pain index was calculated as a sum of the Pain Rating Index (PRI) and both visual analogue scales (VAS). PRI was derived from the sum of the intensity rank values of the words chosen by each individual for sensory and affective descriptors (15 pain descriptors, scale: 0–3). VAS analysis consisted of measuring the distance in centimeters by a ruler between the start of the line on the left side and the mark made by the individual (scale: 0–10). Therefore, the maximum value of the pain index from the pain questionnaires was 65 ($15 \times 3 + 2 \times 10 = 65$). The Present Pain Intensity (PPI, scale: 0–5) was not included in the calculation of the total pain index, however, it was used to estimate the overall pain intensity.

Pain descriptors. Descriptors included in the first part of the pain questionnaire were analyzed to determine which pain fibers have the higher possibility of being excited (A-delta or C-fibers). According to the literature, A-delta fibers mediate rapid nociception or first pain, typically characterized as sharp, pricking pain, while C-fibers mediate dull, aching pain, which can often be difficult to localize[68–70]. Thus, three pain descriptors for each type of fiber were chosen from the pain questionnaire: shooting, stabbing, and sharp as representative of A-delta fibers and throbbing, cramping, and aching as representative of C-fibers. Based on the intensity of the chosen descriptor from each individual, a mean intensity for each descriptor was calculated separately for each pulse protocol.

Statistical analysis. Thirty different biphasic pulse protocols per individual were delivered in order to obtain 30 muscle contraction responses (angles of ankle dorsiflexion) and 15 pain indexes, as the pain questionnaires were filled only for half of the delivered biphasic pulse protocols. The mean and median values were calculated separately for the muscle contraction responses and pain indexes for each pulse protocol. Collected data showed non-normal distribution as tested with the Sharpio-Wilk's test. Therefore, the data were transformed using inversed square root (for muscle contraction responses) and square root (for pain indexes). Both transformations were performed in Design Expert v.12 (Stat-Ease, Inc., Minneapolis, USA) and resulted in a normal distribution. To compare the transformed data with the biphasic pulse protocols[71], an N-way repeated measures analysis of variance (rmANOVA) was used. Multiple comparison test for three factors (pulse width, interphase, and interpulse delay) using the Dunn and Sidak's approach[71] was performed in order to compare intervals among the pulse protocols and find statistically different pulse protocols (separately for muscle contraction responses and pain indexes). The statistical analysis was performed in MATLAB vR2018a (MathWorks Inc., Natick, MA, USA). For all tests, the level of significance was set to 0.05.

Clustering. As there were 51 biphasic pulse protocols, clustering (of protocols) was performed using a hierarchical cluster tree (dendrogram) in MATLAB vR2018a (MathWorks Inc., Natick, MA, USA). An average was calculated from the transformed data described in the previous subsection. The average values were then

Polarity	Pulse width (T_p) [μs]	N	d_1 [μs]	d_2 [μs]	Amplitude (U)
Biphasic	1	400	1, 5	200, 500,750,1000	$2.5 \times U_0$
Biphasic	5	80	1, 5	200, 500,750,1000	$2.5 \times U_0$

Table 2. Additional biphasic pulse protocols delivered. All biphasic pulse protocols have equal total on-time, i.e., N × 2Tp = 800 μs. Tp pulse width; N number of pulses; d1 interphase delay; d2 interpulse delay.

inversely transformed and normalized. Thus, each pulse protocol was represented by a pair of two coordinates (x-muscle contraction response; y-pain index). The distance between each pair of pulse protocols was calculated using the Euclidean distance and based on the average distances, two points were linked together. According to the dendrogram, five clusters were generated. Each cluster consisted of the biphasic pulse protocols that were close to each other.

Additional measurements. *Biphasic pulse protocols with extended interpulse delay (d_2).* Based on a recent theoretical/numerical study[72], we also investigated if extending the interpulse delay (d_2) beyond 10–100 μs reduces the muscle contraction response and increases the pain. These additional measurements were performed on 10 individuals that were included in the first part of the study and volunteered again. Sixteen additional biphasic pulse protocols were derived with extended d_2 (200, 500, 750, and 1000 μs) and the interphase delay (d_1) and pulse width set to either 1 or 5 μs (Table 2). Thus, the pulse repetition rates were ranging from approximately 5 kHz (for $d_2 = 200$ μs) to 1 kHz (for $d_2 = 1000$ μs). The amplitude was determined in the same way as described previously (in the subsection: "Determining the stimulus amplitude for the measurements"). All 16 new biphasic pulse protocols were delivered on each individual in a random order. For comparison with previously used shorter interpulse delays, the pulse protocols with $d_2 = 10$ and 100 μs were also delivered. For each pulse protocol delivered, the individuals were requested to fill the short-form McGill pain questionnaire.

Interchanged interphase (d_1) and interpulse delays (d_2). The biphasic pulse protocols tested in the study were chosen so that the interpulse delay (d_2) was always equal to or longer than the interphase delay (d_1), $d_2 \geq d_1$. Additional measurements were performed on 10 individuals that were included in the first part of the study and asked to volunteer again in order to investigate the muscle contraction response and pain index when d_1 was longer than d_2 (interchanged delays, $d_1 > d_2$). Six of the 51 biphasic pulse protocols previously tested were chosen which had the highest difference between the values of d_1 and d_2, as for these delays the highest deviations in the results were expected. Three pulse protocols were chosen in order to test the effect of the pulse width ($T_p = 1$, 2, and 5 μs) when d_1 and d_2 were interchanged. The other three pulse protocols were with different delays (highest possible difference between d_1 and d_2), but all with a pulse width of 5 μs. Therefore, six additional biphasic pulse protocols with interchanged delays were generated. For comparison, the six old protocols (before the interchange) were also delivered in random order. For each pulse protocol delivered, the individuals were requested to fill the short-form McGill pain questionnaire.

Results

Strength-Duration curves. In Fig. 2, we present the mean strength-duration (S-D) curves obtained for single monophasic (each dot is a mean value of 25 measurements in 25 individuals) and biphasic pulse with various interphase delays (each dot is a mean of five measurements in five individuals). Measurements were performed at five pulse widths $T_p = 1$, 2.5, 5, 10, and 50 μs. For each pulse width, the threshold amplitude (pulse amplitude where minimal muscle contraction was observed) was recorded as a point on the graph. The thresholds rise as the pulse width and interphase delay (delay between the positive and negative pulse) are shortened. Biphasic pulses with short interphase delays ($d_1 = 1$, 2.5, and 5 μs) have higher threshold amplitudes than monophasic pulses or biphasic pulses with longer delays ($d_1 = 10$ and 100 μs) for $T_p = 1$, 2.5, 5, and 10 μs. Biphasic pulse (for all interphase delays) and monophasic pulse at all pulse widths were compared using paired t-test (with a level of significance set to 0.05). As expected for $T_p = 50$ μs, there was statistically significant difference between single monophasic pulse and biphasic pulse with $d_1 = 1$, 2.5, 5, and 10 μs (statistically lower mean values for the biphasic pulses) as for biphasic pulse the total duration was $2xT_p$ and for monophasic $1xT_p$ (Fig. 2). For all other tested pulse widths, there was a statistically significant difference between single monophasic pulse and biphasic pulse (but the mean values for the biphasic pulses were statistically higher) for the interphase delays stated in the boxes and marked with asterisks on Fig. 2. For a pulse width of 1 μs paired t-test was performed only for $d_1 = 10$ μs and 100 μs, as the threshold amplitude was higher than 1000 V (highest possible amplitude the pulse generator was able to deliver) for the rest of the interphase delays.

Clustering. Using the hierarchical cluster tree (dendrogram) with normalized data, five clusters based on similar/different muscle contraction responses and pain sensation were identified. The hierarchical cluster tree (Fig. S1) along with the table of biphasic pulse protocols and suitable coloring (Table S1) can be found in the Supplementary files. In Fig. 3, we present all 51 biphasic pulse protocols marked and colored according to the cluster they belong to. Each symbol represents the average of one pulse protocol: x-muscle contraction response, y-pain index in the coordinate system. The data is normalized based on the single-cluster pulse protocol with parameter values: $T_p = 5$ μs, $d_1 = 100$ μs, $d_2 = 100$ μs, which resulted in the highest muscle contraction response (6.2° ankle dorsiflexion) and highest pain index (13 out of 65). This is shown with a purple dot on the graph, i.e.,

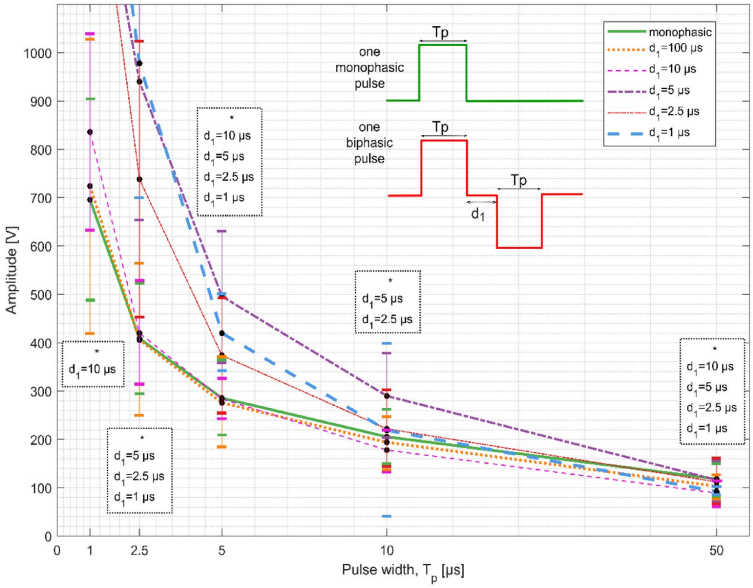

Figure 2. Threshold amplitude as a function of the pulse width for single monophasic (solid green curve) and biphasic pulses (Strength–Duration curves). Biphasic pulses are shown for each interphase delay from 1 μs to 100 μs. The results are shown as mean amplitude of the individuals (black dots) ± standard error (vertical bars). The boxes with asterisks (*) and interphase delays show statistically significant differences between the monophasic pulse and marked interphase delay (biphasic pulse) for each pulse width tested (statistically higher mean values for the biphasic pulses for all pulse widths tested except for $T_p = 50$ μs). Note that for pulse width of 1 μs, paired t-test was performed only for $d_1 = 10$ μs and 100 μs, as the threshold amplitude was higher than 1000 V for the rest of the interphase delays.

coordinates (1, 1). Additionally, the amplitude determining (reference) protocol (8 monophasic pulses × 100 μs, 5 kHz) is marked with a yellow diamond. It is important to note that the amplitude for the reference protocol was always 2.5 times lower than the amplitude used for the biphasic pulse protocols, and the muscle contraction response was almost equal for each individual (minimal muscle response: 3.6°–4° of ankle dorsiflexion).

Four other clusters (green, blue, orange, and red) can be distinguished on the graph (Fig. 3). The green cluster (marked with green circles) barely causes any muscle contraction and has low pain index. In this cluster are mainly the pulse protocols that have short pulse width, $T_p = 1$ μs and 2 μs (Table S1 in the Supplementary files). The blue cluster (marked with blue squares) has almost similar muscle contraction responses, but slightly higher pain indexes than the green one. The pulse protocols in this cluster have very short interphase delays (d_1) but longer pulse width (T_p) and interpulse delays (d_2) than the pulse protocols in the green cluster. The orange cluster (marked with orange crosses) has considerably higher muscle contraction response than the blue one at almost equal pain index. The pulse protocols that cause the highest muscle contraction response (orange cluster) all have T_p of 5 μs and d_1 and d_2 up to 10 μs. All biphasic pulse protocols except the single-cluster pulse protocol: $T_p = 5$ μs, $d_1 = 100$ μs, $d_2 = 100$ μs had lower muscle contraction responses than the reference protocol (marked with yellow diamond), i.e., 8 monophasic pulses × 100 μs, 5 kHz. When extending the interpulse delay above 10 μs, e.g., 100 μs, the muscle contraction response is reduced (pulse protocols marked with red asterisks) however, the pain index is increased. This indicates that the pain index does not necessarily correspond to the muscle contraction response and vice versa. In the clusters presented, the orange cluster is representative for higher muscle contraction response and the red cluster for higher pain index.

Statistical analysis. In order to find statistically significant differences among the biphasic pulse protocols and support their clustering into "biphasic pulse protocols with higher muscle contraction response" and "biphasic pulse protocols with higher pain index", an N-way repeated measures analysis of variance (rmANOVA) was performed on transformed data separately for muscle contraction responses and pain indexes. The complete graphs (Figs. S2 and S4) and tables (Figs. S3 and S5) are provided in the Supplementary files.

The pulse protocols marked with orange crosses in Fig. 3 are significantly different (higher means) from the pulse protocols with almost no muscle contraction response (green and blue cluster) when performing rmANOVA on the data for muscle contraction response. The pulse protocols marked with red asterisks in Fig. 3

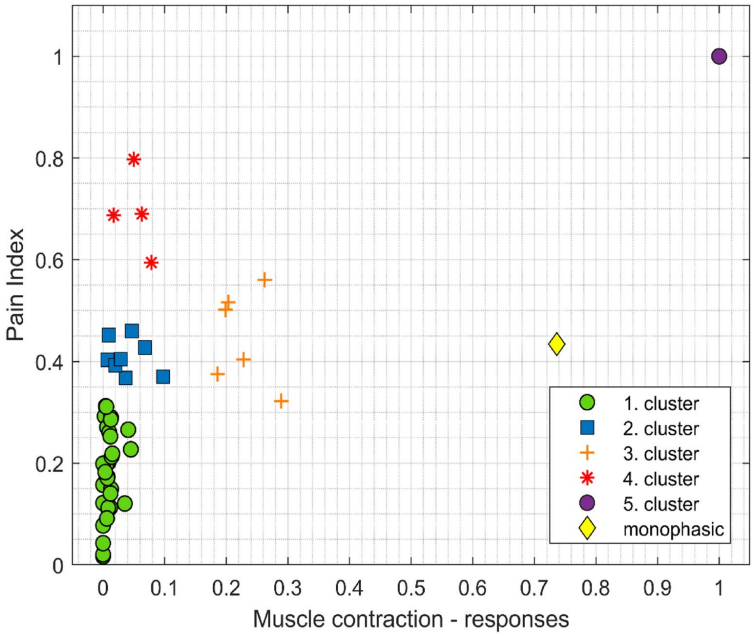

Figure 3. Clustering based on a hierarchical cluster tree (dendrogram). Each mark represents one pulse protocol: x—muscle contraction response, y—pain index. The data shown is normalized based on the purple cluster ($T_p = 5$ µs, $d_1 = 100$ µs, $d_2 = 100$ µs). Note that the yellow diamond represents the amplitude determining (reference) protocol (8 monophasic pulses × 100 µs, 5 kHz) with 2.5 lower amplitude.

are significantly different (higher means) from the pulse protocols with low pain index (green cluster) when performing rmANOVA on the data for pain indexes.

However, it is worth mentioning that when performing statistical analysis on the data for pain indexes, the statistical significance and clustering is different when the Pain Rating Indexes (PRI) and Visual Analogue Scales (VAS) are analyzed separately (data not shown).

Biphasic pulse protocols with extended interpulse delay (d_2). Twelve additional pulse protocols were tested with extended interpulse delay, $d_2 = 200$, 500, 750, and 1000 µs when the interphase delay (d_1) with pulse width were set to either 1 or 5 µs. The muscle contraction responses are shown in Fig. 4 for pulse width (T_p) of 1 µs (upper figure) and 5 µs (lower figure). The results show that as d_2 increases from 1 µs to 10 µs for $d_1 = 1$ µs, the angle of ankle dorsiflexion is increasing and reaching a peak (for $d_1 = 5$ µs the peak is at 100 µs). Beyond 10 µs for $d_1 = 1$ µs, and 100 µs for $d_1 = 5$ µs, the angle of ankle dorsiflexion is decreasing, meaning that the threshold for muscle stimulation is higher for interpulse delays above 100 µs. For interpulse delays of 5 and 10 µs, the angle is the highest, meaning that the threshold for muscle stimulation is reduced. While for a pulse width of 1 µs the muscle contraction response reaches zero for d_2 above 200 µs (upper figure), for pulse widths of 5 µs although the muscle contraction response is reduced, it does not completely disappear (lower figure). However, higher muscle contraction responses are observed only for the pulse protocols in the orange cluster (pulse protocols with a T_p of 5 µs and d_1 and d_2 up to 10 µs; see Table S1 in the Supplementary files). A difference can also be observed between d_1 of 1 and 5 µs (red and blue lines). Interestingly, for a pulse width of 1 µs, there is slightly higher muscle contraction response for d_2 of 5 µs and 10 µs. On the other hand, for a T_p of 5 µs, muscle stimulation with higher d_1 (5 µs) first reduces the muscle contraction response (angle) and as d_2 increases above 100 µs, the muscle contraction response is increasing, meaning that the muscle stimulation threshold is decreasing.

The trends observed in Fig. 4 suggest (in agreement with a recent numerical study) that for reduced muscle contraction responses, shorter interphase delays with longer interpulse delays are preferred[72]. However, as shown on the lower graph in Fig. 5 and observed in the previous sub-sections, extending the interpulse delay beyond 10 µs for longer pulse widths results in higher pain indexes (as observed in Fig. 3, red asterisks).

Interchanged interphase (d_1) and interpulse delays (d_2). In Fig. 6, the interphase (d_1) and interpulse delays (d_2) are interchanged. Six biphasic pulse protocols out of the previously tested pulse protocols were chosen (old pulse protocols ($d_2 \geq d_1$), turquoise bars in Fig. 6) for which d_1 and d_2 were interchanged (new pulse protocols ($d_1 > d_2$), purple bars in Fig. 6). Figure 6 shows the mean results with corresponding standard errors for

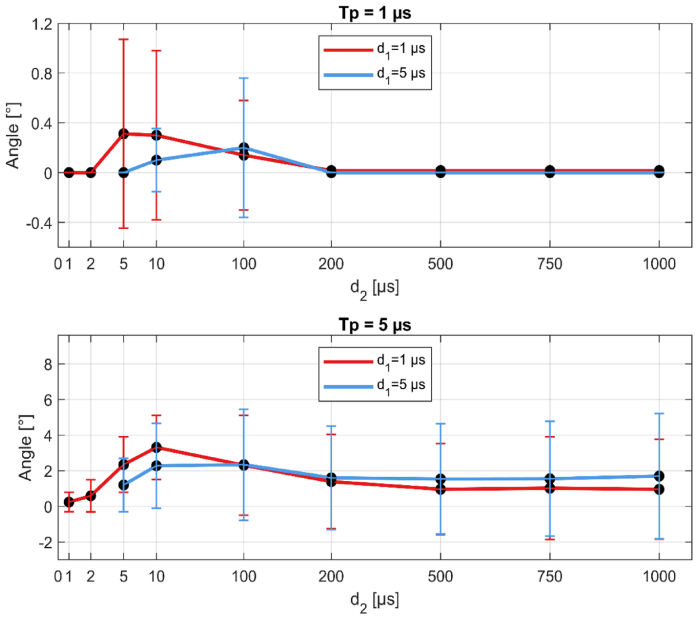

Figure 4. Longer interpulse delays reduce muscle contraction (response angle). Upper figure: 400 × 1 µs pulses, lower figure: 80 × 5 µs pulses. Note different ordinate scales (higher angles for $T_p = 5$ µs). The results are shown as the mean (black dots) ± standard error (vertical bars). T_p-pulse width, d_1-interphase delay, d_2-interpulse delay.

muscle contraction response (upper figure) and pain index (lower figure). Paired t-test was performed (with a level of significance set to 0.05) within each set for both muscle contraction response and pain index. The results show that a statistically significant difference is observed between pulse protocols 5–10-5–100 and 5–100-5–10. Interchanging d_1 from 10 to 100 and d_2 from 100 to 10 induces higher muscle contraction responses but reduces the pain (pain index).

Pain descriptors. As described in the Methods section, three descriptors were chosen for each type of nerve fiber (A-delta and C-fibers). Descriptors mean intensity from each individual was calculated for each biphasic pulse protocol. For the chosen three descriptors for each type of nerve fiber, a sum of the descriptor mean intensity was calculated separately for A-delta and C-fibers. Depending on the generated clusters (Fig. 3), an average for the biphasic pulse protocols in the same cluster was calculated from the sum of the mean intensities for each pulse protocol. Figure 7 presents a bar graph for all five clusters showing the average values of the sum of descriptors' mean intensity for each cluster with standard errors. The purple cluster (last two bars in Fig. 7) consists of a single pulse protocol, and hence the standard error is zero. Red bars show the average of the sum of descriptors mean intensity for A-delta fibers (descriptors: shooting, stabbing, and sharp), while the blue bars show the average of the sum of descriptors mean intensity for C-fibers (descriptors: throbbing, cramping, aching). A comparison between the average values of the nerve fibers within each cluster was performed using paired t-test (with a level of significance set to 0.05). The results show a statistically significant difference between the nerve fibers for the green, blue, and red clusters, indicating that more A-delta fibers are excited/stimulated by these pulse protocols.

Discussion

This study represents the first study in humans examining both muscle contraction and pain sensation during high-frequency electroporation pulses. The aim of the study was to examine high-frequency, biphasic pulse protocols, which reduce muscle contraction responses in healthy individuals. High-frequency biphasic pulses in the range of microseconds with both symmetric and asymmetric interphase and interpulse delays were tested. These pulses were recently suggested to reduce the muscle contraction and pain sensation during electroporation-based therapies, in order to enable treatments without the need of muscle relaxants and anesthesia.

Our results obtained in healthy individuals confirm that very short, biphasic high-frequency pulses significantly reduce the muscle contraction response and pain sensation. Interphase delay (between the positive and negative phase) and interpulse delay (between the pulses) however, play a significant role in reducing the muscle contraction response and pain sensation. Very short interphase and interpulse delays (1 or 2 µs) reduce

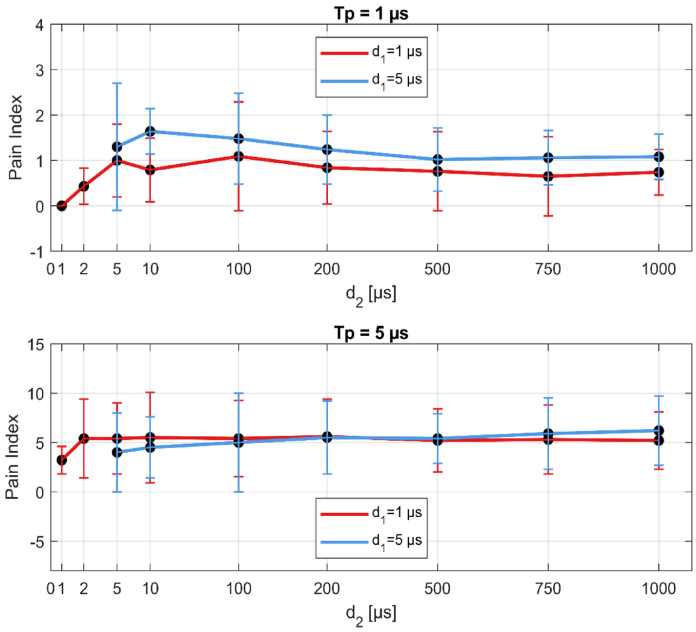

Figure 5. Longer interpulse delays slightly increase the pain index for longer pulse widths (lower figure). Upper figure: 400 × 1 µs pulses, lower figure: 80 × 5 µs pulses. Note different ordinate scales (higher pain indexes for $T_p = 5$ µs). The results are shown as the mean (black dots) ± standard error (vertical bars). T_p-pulse width, d_1-interphase delay, d_2-interpulse delay.

muscle contraction response and pain sensation even at the largest pulse width tested, i.e., 5 µs. Increasing both interphase and interpulse delays to 10 µs increases the muscle contraction response without strong pain sensation. However, in comparison to the amplitude determining protocol, i.e., reference protocol (8 monophasic pulses × 100 µs, 5 kHz with 2.5 times lower amplitude), these muscle contractions are still lower (Fig. 3).

Further increase of the interpulse delay (beyond 10 µs) additionally reduces the muscle contraction but increases the pain sensation (Fig. 4). This indicates that muscle contraction does not necessarily correlate to the pain sensation and vice versa. This may be due to different types of nerve fibers involved in the transmission of the signals-A-alpha motor fibers for muscle movement, and A-delta and C-fibers for transmitting nociception signals[58,59,73].

Reduced muscle contractions have been achieved in multiple in vivo studies with application of biphasic pulses with pulse widths from 1 to 10 µs (of each phase) but only with equal interphase and interpulse delays with a duration of 1 to 5 µs[42,46,48,49,52,74]. Modifications of the interphase and interpulse delays have not been investigated as a method to reduce excitation within the H-FIRE protocols until recently when a theoretical argument for the extended interpulse delay while minimizing the interphase delay was presented[72]. Our results confirm that extending the interpulse delay while shortening the interphase delay indeed increases the muscle stimulation thresholds, meaning that the muscle contraction responses are reduced. However, the trends observed in our study indicate that extended interpulse delay, does not reduce the pain experienced by the individuals during the delivery of such pulses. On the contrary, longer interpulse delays were reported to be more painful (Figs. 4 and 5).

The pain estimation in the study was based on patient self-reporting using a clinically validated tool—the Short-Form McGill Pain Questionnaire (SF-MPQ). With this approach, we calculated the pain index and determined the pain descriptors for each pulse protocol. Based on the chosen pain descriptors the type of pain fibers that are predominantly excited was determined. Our results indicate that the A-delta nerve fibers are predominantly excited based on the chosen pain descriptors from the pain questionnaires. For each cluster, more A-delta fibers are excited/stimulated, suggesting that with short, biphasic high-frequency pulses there is higher A-delta nerve fibers involvement in transmitting nociception. However, for the orange cluster, no statistically significant difference occurred between the fibers (Fig. 7). The reason for this may be that these biphasic pulse protocols had higher muscle contraction responses. Thus, the individuals chose the "cramping" descriptor more often, which is a descriptor indicating C-fibers involvement. In the purple cluster there were more pain descriptors indicating A-delta involvement, however, this is a single pulse protocol only and no statistical analysis could be performed. Higher involvement of A-delta fibers can be due to the higher speed of pulse propagation in myelinated fibers, which also have a larger diameter than unmyelinated C-fibers. Moreover, C-fibers have longer chronaxie than the

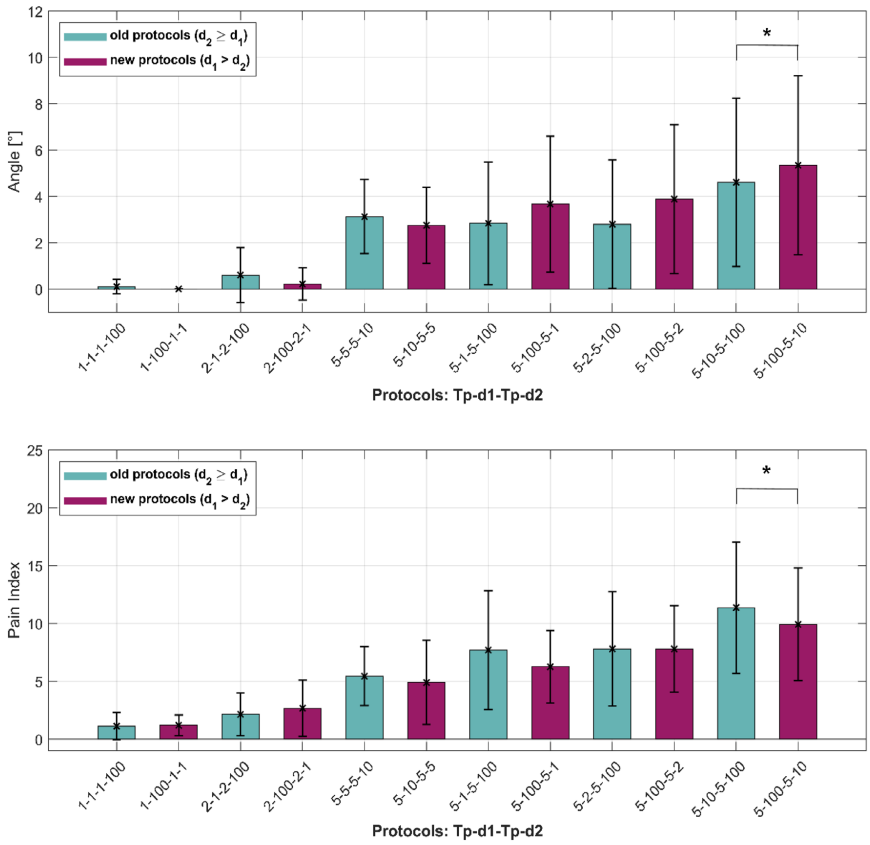

Figure 6. Interchanged interphase (d_1) and interpulse delays (d_2). Each bar represents one pulse protocol (T_p-d_1-T_p-d_2). Turquoise bars are already established biphasic pulse protocols ($d_2 \geq d_1$), purple bars are the biphasic pulse protocols generated when d_1 and d_2 were interchanged ($d_1 > d_2$). The results are shown as the mean value (bar's height) ± standard error (black vertical bars). The asterisks (*) show statistically significant differences between the pulse protocols ($P < 0.05$).

A-delta, indicating that the C-fibers require stronger stimulus (higher threshold amplitude) for excitation[58,59,73]. However, this may change if higher amplitudes would be used.

The location of the pain sensation for the short pulses with short interphase and interpulse delays was just a slight sensation right at the stimulation site, whereas for the longer interpulse delays, the individuals expressed the sensation as "spreadable" along the muscle (leg) and longer lasting.

Slight redness at the site of the electrodes was visible immediately after the measurements, which disappeared within few hours. Namely, none of the individuals reported any visible signs of injury/redness at the site of the electrodes six hours after the treatment. More importantly, with the overall present pain intensity (PPI) index (scale: 0–5) being low (average: 0.7) the treatment was reported as tolerable and none of the individuals withdrew from the study.

Our study also shows that shorter interphase delays increase the stimulation threshold (Strength-Duration (S-D) curves, Fig. 2). The addition of a secondary anodic pulse to achieve balanced charge biphasic stimuli increases the threshold amplitude. This effect becomes greater as the interphase delay approaches 1 µs. However, for a biphasic pulse with longer interphase delays, i.e., 100 µs, the S-D curve is very close to the S-D curve for a single monophasic pulse, which is in agreement with existing literature[75–77]. As expected, for longer pulse widths, i.e., $T_p = 50$ µs, a single monophasic pulse resulted in a higher threshold amplitude than a single biphasic pulse for all interphase delays tested because a single biphasic pulse consists of two pulses (positive and negative), i.e., a monophasic pulse is $1xT_p$ long and a biphasic pulse is $2xT_p$ long.

Originally, all biphasic pulse protocols had interpulse delay longer or equal to the interphase delay. Additional measurements were therefore performed with interchanged delays to confirm that the approach $d_2 \geq d_1$ is acceptable (Fig. 6). In the future, with this approach, the number of additional experiments may be reduced,

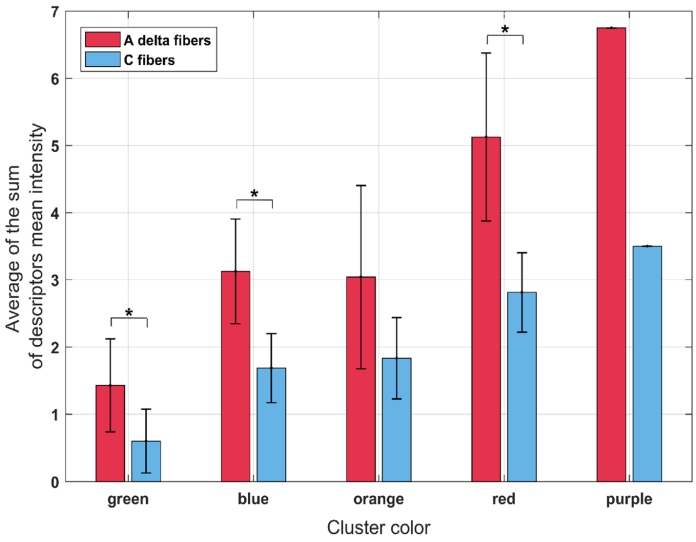

Figure 7. Sum of descriptors mean intensity for three chosen descriptors of both type of nerve fibers: A-delta (red bars) and C-fibers (blue bars). The data is shown as the average value (bar's height) ± standard error (black vertical bars) for all biphasic pulse protocols included in a particular cluster. The asterisks (*) show statistically significant difference between the nerve fibers in the cluster ($P < 0.05$). Note that the purple cluster is only one pulse protocol cluster, thus the standard error is zero.

as there were no statistically significant differences observed except for one of the tested sets of pulse protocols (5–10-5–100 and 5–100-5–10) where the muscle contraction response was higher for the interchanged delays (5–100-5–10), while the pain index was lower. Lower pain index at higher muscle contraction responses can be explained by the Gate Control Theory of Pain mechanisms. According to this theory, large fiber activity excites the inhibitory neurons, which diminishes the transmission of pain information. When there is more large fiber activity involved (A-alpha and A-beta fibers) in comparison to small fiber activity (A-delta and C-fibers), people tend to experience less pain[78–80]. This means that a non-painful input (e.g., a touch/massage on a bumped area) closes the nerve "gates" to the painful input because it increases the activity of the large fibers (A-beta fibers from the skin) and thus, prevents the pain sensation (lower activity of the pain fibers) from reaching the central nervous system. In our case, this would mean that stimulation of the muscle and the resulting muscle contraction activates/excites the large fibers and thus, reduces the excitation of the nociceptive (pain) fibers, i.e., the gates close. However, the theories and models of pain are still evolving and need further validation[81].

Limitations and drawbacks of the study. Firstly, although high-frequency electroporation protocols usually consist of more bursts of pulses delivered in succession, stimulation in our study was performed with only one burst of pulses. In addition, the total on-time of the pulses was always equal (800 µs). Second, the number of participants was limited to 25, which is enough for statistical analysis of trends, but not in-depth analysis between the pulse protocols. Moreover, the participants were in two different age groups and genders (younger-up to 32 years and elder-from 52 to 58 years; 12 male and 13 female), which also caused differences in the sensitivity. Namely, elder individuals tended to have slightly higher sensitivity (higher muscle contraction responses). This was also observed among the males compared to the females for the same biphasic pulse protocols. Therefore, relatively high standard errors and non-normal distribution of the results were observed. However, there was no statistically significant difference between the individuals' responses (obtained in Design Expert v.12), which is in agreement with existing literature[82–84]. Third, the pain questionnaire (SF-MPQ) used, although already established in practice with validated Slovenian translation, some pain descriptors were hardly understandable to some individuals. Some of the pain descriptors were also non-applicable for this kind of study and were never chosen to describe the pain sensation. Hence, the choice of only three pain descriptors for each type of nerve pain fiber for assessing selectivity (A-delta and C-fibers). Last but not least, the voltage used for the biphasic pulse protocols was established based on the reference protocol (2.5 times higher than the amplitude for the reference protocol, since higher amplitudes are required to obtain comparable effect as with monophasic pulses[42,56,65] at the same total on-time). The voltages used throughout the study were however low comparing to the voltages currently used for e.g., tissue ablations. Therefore, the VAS level (scale: 0–10) was lower (below 1) than in actual therapy[28,41,85,86]. However, we chose this approach to avoid potential damage to the underlying tissue, as we were testing 30 different biphasic pulse protocols per individual, which was almost an hour of repeated

muscle stimulation. Therefore, performing the treatment with clinically relevant high voltage pulse protocols, on different tissue (tumors or heart) or locations (deep or superficial) remains to be established.

Conclusion

In conclusion, with our study we confirmed the hypothesis that using short (1 µs, 2 µs), biphasic high-frequency pulses with short interphase and interpulse delays reduces the muscle contraction in healthy individuals. We also demonstrated that these pulse protocols reduce the pain sensation. However, the interplay between the pulse width, interphase, and interpulse delays is more complex, and modification of these parameters results in either reduced muscle contraction response or pain sensation. Pain is not necessarily induced as a consequence of the muscle contraction response and vice versa. Namely, higher pain indexes are observed for pulse parameters that do not cause high muscle contraction response. Therefore, modification of the pulse parameters should be performed for a particular application of electroporation to reduce these effects, while providing safe, effective, and successful therapy.

Data availability

The data that support the findings of this study are available in the paper and its supplementary information files. The raw data are available from the authors upon reasonable request.

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Author contributions

A.C. conducted the measurements, analyzed the results and wrote the manuscript. All authors helped with the study design. A.M.L, P.T., D.M., and M.R. corrected and revised the manuscript and gave final approval of the version to be published.

Competing interests

The authors declare no competing interests.

Additional information

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Supplementary Information

Manuscript title: Muscle contractions and pain sensation accompanying high-frequency electroporation pulses
Authors: Aleksandra Cvetkoska, Alenka Maček-Lebar, Peter Trdina, Damijan Miklavčič and Matej Reberšek

- ***Table of biphasic pulse protocols***

Table S1*. Table of tested biphasic pulse protocols with suitable coloring according to the cluster they belong to. P. No – protocol number, N – number of pulses, Tp – pulse width, d_1 – interphase delay, d_2 – interpulse delay*

P. No.	N	T_p	d_1 [µs]	d_2 [µs]
1	**400**	1	**1**	**1**
2	**400**	1	**1**	**2**
3	**400**	1	**1**	**5**
4	**400**	1	**1**	**10**
5	**400**	1	**1**	**100**
6	**400**	1	**2**	**2**
7	**400**	1	**2**	**5**
8	**400**	1	**2**	**10**
9	**400**	1	**2**	**100**
10	**400**	1	**5**	**5**
11	**400**	1	**5**	**10**
12	**400**	1	**5**	**100**
13	**400**	1	**10**	**10**
14	**400**	1	**10**	**100**
15	**400**	1	**100**	**100**
16	**200**	2	**1**	**1**
17	**200**	2	**1**	**2**
18	**200**	2	**1**	**5**
19	**200**	2	**1**	**10**
20	**200**	2	**1**	**100**
21	**200**	2	**2**	**2**
22	**200**	2	**2**	**5**
23	**200**	2	**2**	**10**
24	**200**	2	**2**	**100**
25	**200**	2	**5**	**5**
26	**200**	2	**5**	**10**
27	**200**	2	**5**	**100**
28	**200**	2	**10**	**10**
29	**200**	2	**10**	**100**
30	**200**	2	**100**	**100**

P. No.	N	T_p	d_1 [µs]	d_2 [µs]
31	**133**	3	**1**	**1**
32	**133**	3	**5**	**5**
33	**133**	3	**5**	**800**
34	**100**	4	**1**	**1**
35	**100**	4	**5**	**5**
36	**100**	4	**5**	**800**
37	**80**	5	**1**	**1**
38	**80**	5	**1**	**2**
39	**80**	5	**1**	**5**
40	**80**	5	**1**	**10**
41	**80**	5	**1**	**100**
42	**80**	5	**2**	**2**
43	**80**	5	**2**	**5**
44	**80**	5	**2**	**10**
45	**80**	5	**2**	**100**
46	**80**	5	**5**	**5**
47	**80**	5	**5**	**10**
48	**80**	5	**5**	**100**
49	**80**	5	**10**	**10**
50	**80**	5	**10**	**100**
51	**80**	5	**100**	**100**

- ***Hierarchical cluster tree (Dendrogram)***

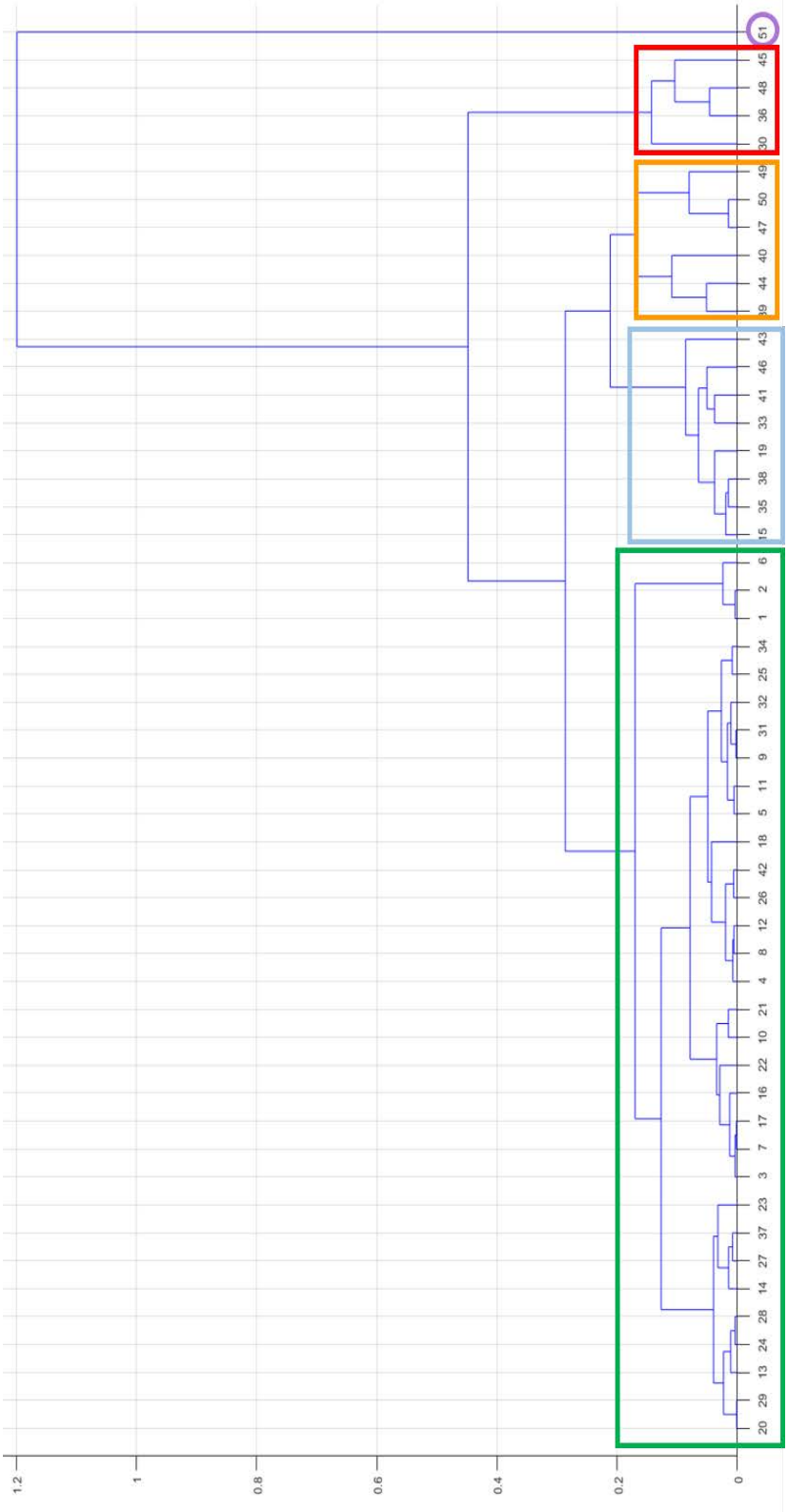

Figure S1. *Hierarchical cluster tree of the pulse protocols gathered in five clusters by color*

- **Multi-comparison analysis (N-way ANOVA)**

Multiple comparison test for three factors (pulse width, interphase, and interpulse delay) using the Dunn and Sidak's approach was performed to find the mean estimates and comparison intervals among the pulse protocols and thus, find statistically significant pulse protocols (separately for muscle contraction responses and pain indexes). Figures S2 and S4 show the performed multiple comparison test for muscle contraction responses and pain index, respectively. By selecting each pulse protocol separately (marked with blue line), all statistically significant pulse protocols were marked with red lines and the number of pulse protocols was shown below the graph.

Figure S2 provides an example for the pulse protocol 5-10-10 (T_p-d_1-d_2) marked with a blue line for which the mean estimates of the red marked pulse protocols are statistically lower. Note that the data is transformed with inverse square root transformation, thus the switched values on the graph (the red lines are shown with higher values). Figure S4 provides an example for the pulse protocol 5-100-100 (T_p-d_1-d_2) marked with a blue line for which the mean estimates of the red marked pulse protocols are statistically lower.

Figures S3 and S5 are the matrixes of the mean transformed muscle contraction responses and pain index, respectively (red numbers along the diagonal). The colored cells are marked accordingly to the pulse protocol where statistically significant difference occurs (derived from figures S2 and S4). An orange cell shows that the observed pulse protocol from the first column has a statistically higher mean value than the pulse protocol observed in the corresponding column of the cell (from the first row). A blue cell shows that the observed pulse protocol from the first row has a statistically lower mean value than the pulse protocol observed in the corresponding column of the cell (from the first row).

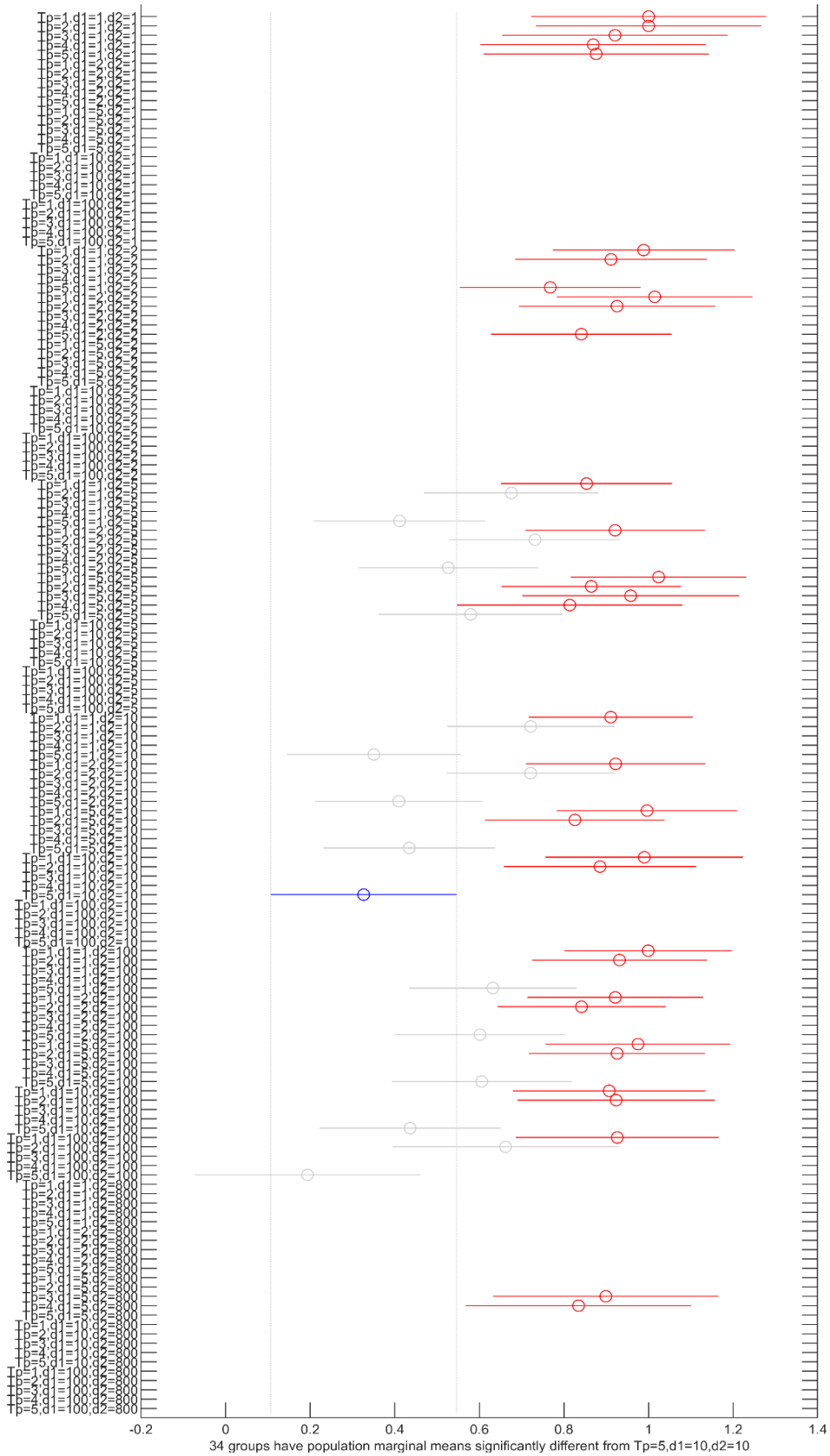

***Figure S2**. Multiple comparison test for muscle contraction responses' data (inversely transformed) between the pulse protocols showing all statistically different pulse protocols (red lines) from the observed pulse protocol (marked with blue line).*

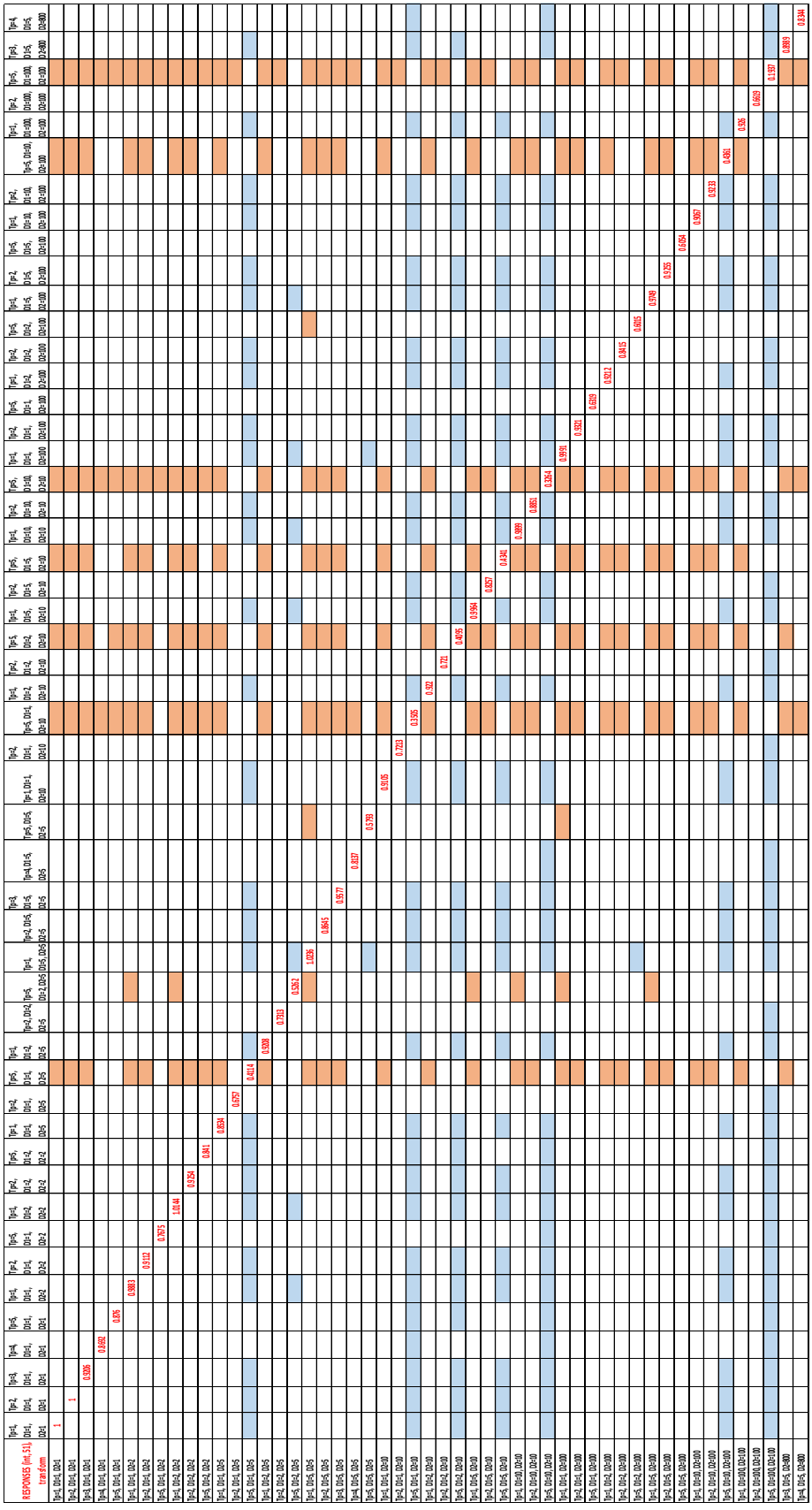

Figure S3. *Matrix of mean muscle contraction responses (transformed data) for each pulse protocol (red values along the diagonal) showing statistically different pulse protocols (orange cell – higher mean; blue cell – lower mean) as derived from figure S2.*

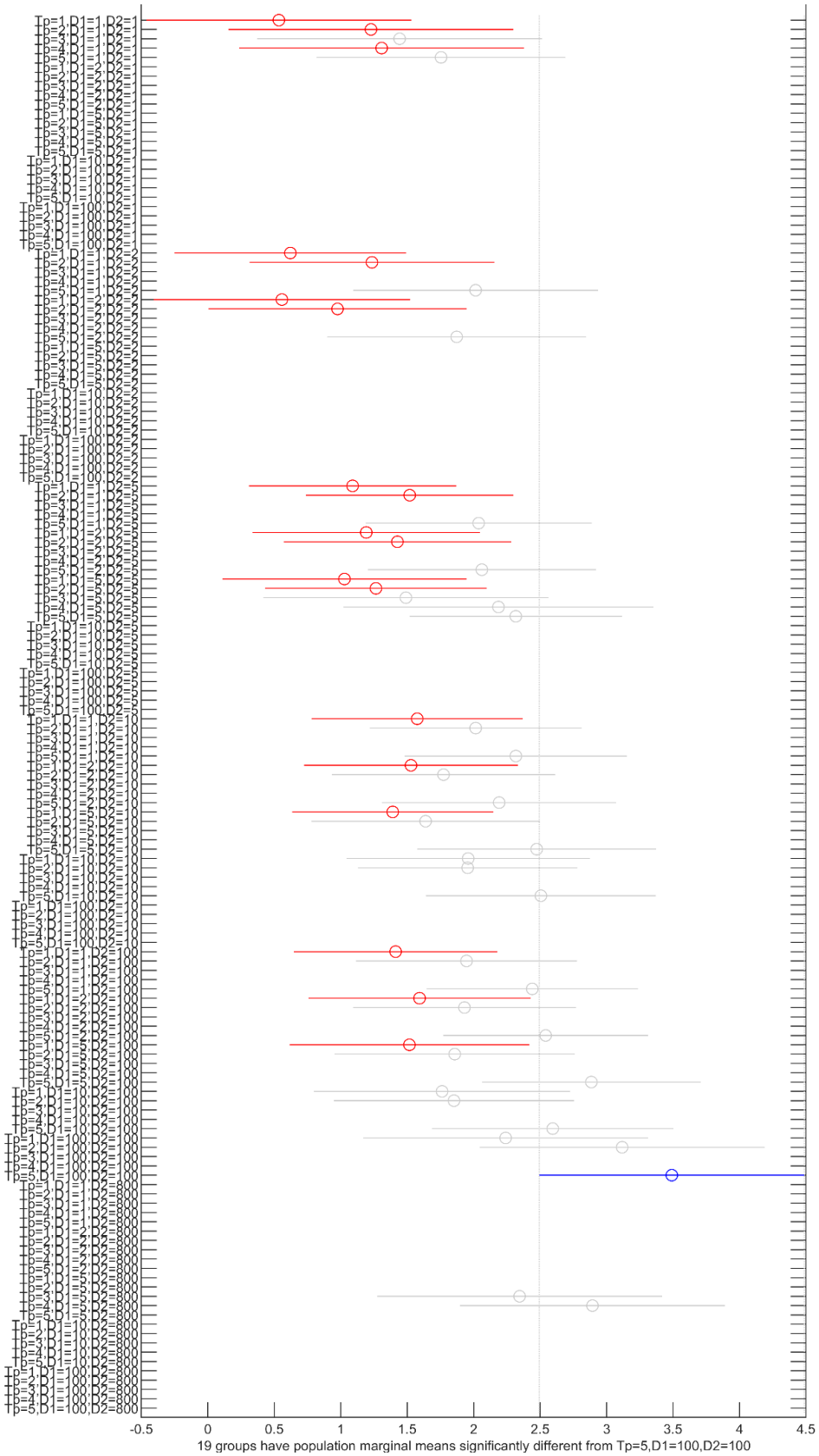

***Figure S4**. Multiple comparison test for pain indexes' data (transformed) between the protocols showing all statistically different protocols (red lines) from the observed protocol (marked with blue line).*

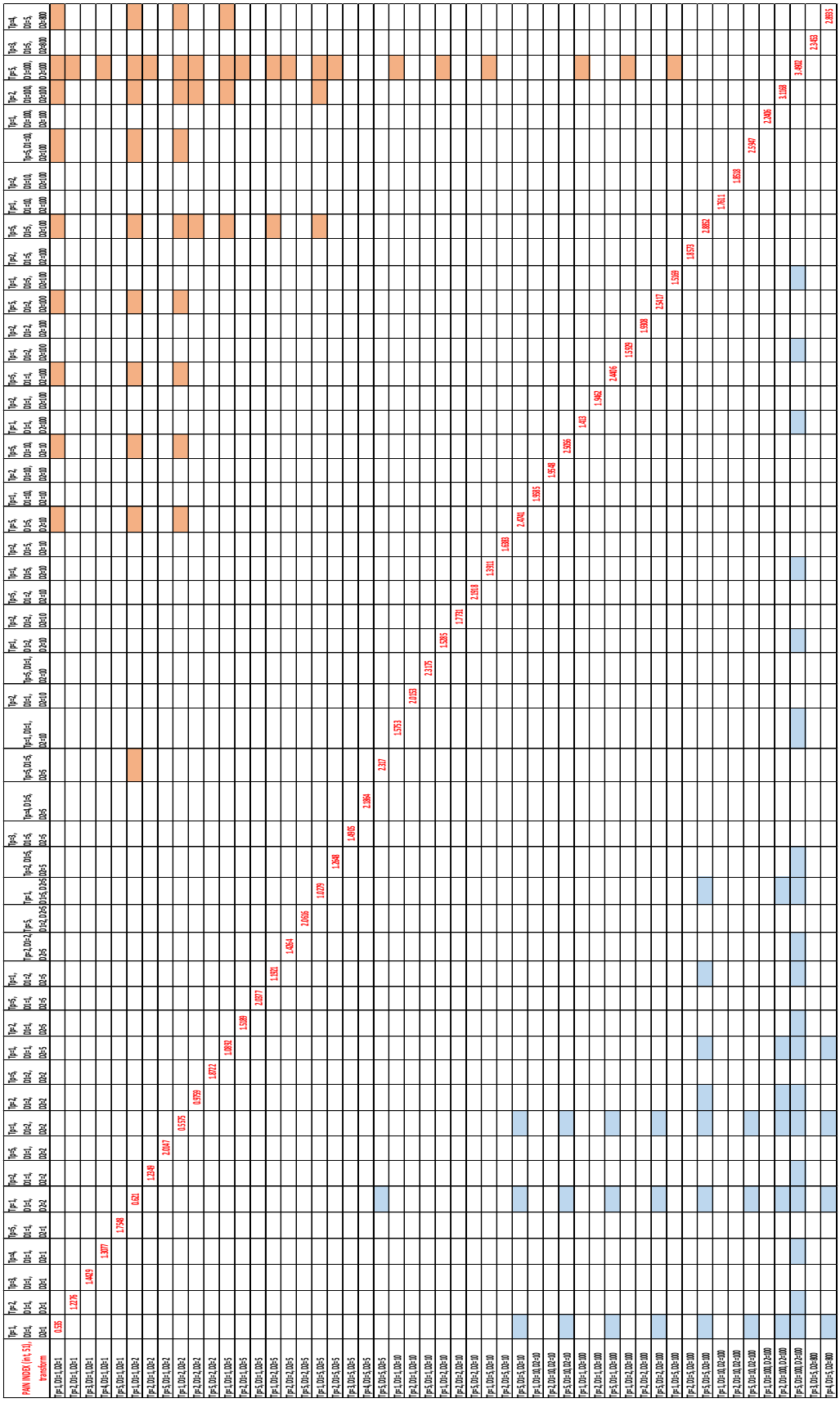

Figure S5. *Matrix of mean pain indexes (transformed data) for each pulse protocol (red values along the diagonal) showing statistically different pulse protocols (orange cell – higher mean; blue cell – lower mean) as derived from figure S4.*

3.2 Paper 2

Title: **Towards standardization of electroporation devices and protocols**

Authors: Aleksandra Cvetkoska, Eva Pirc, Matej Reberšek, Ratko Magjarević and Damijan Miklavčič

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Towards Standardization of Electroporation Devices and Protocols

Aleksandra Cvetkoska, Eva Pirc, Matej Reberšek, Ratko Magjarević, and Damijan Miklavčič

Cell exposure to high-voltage, short-duration electric pulses can lead to temporary formation of hydrophilic pores in the plasma membrane and an increase in the membrane's permeability which consequently increases the transmembrane transport of molecules that are otherwise unable to cross the membrane. This phenomenon, termed membrane electroporation, is currently an applicable technique in different areas such as biomedicine, biotechnology, food technology and environmental applications. Electroporation pulses are generated by pulse power generators known as electroporators and delivered to the cells (in tissue) via electrodes. The objective of this paper is to review and compare characteristics of electroporation applications and equipment described in the literature and/or present on the market. Since there are no specific standards or regulations that specifically refer to the safety of medical devices with intended medical uses for electroporation, we propose guidelines for the design of clinical electroporators and define minimal requirements for their safe and efficient use which can be incorporated within the particular standards in the future. In order to facilitate the comparison of data obtained by different research groups and to enable reproduction of results under the same conditions, we want to stress the necessity of defining the electroporator's output parameters and tolerances of electroporation parameters for electroporation-based therapies.

Electroporation

The Biological Phenomenon

Each biological cell is protected from its surroundings by the plasma membrane which is composed of a two-molecule thick layer of lipids. The plasma membrane would be a mostly impenetrable barrier if there were not various proteins which enable transport of specific molecules across the membrane. However, cell exposure to high-voltage, short-duration electric pulses can lead to temporary formation of hydrophilic pores in the bilayers and increase plasma membrane permeability which consequently causes increase in transmembrane transport of molecules that are otherwise unable to cross the membrane [1]. This phenomenon termed as membrane electroporation/permeabilization can be either reversible, when the cell exposure to electric field is short enough for the cells to fully recover, or irreversible, when their exposure leads to cell death.

Electroporation Applications

Electroporation has become a relevant technique in different areas such as biomedicine, biotechnology, food technology and environmental applications [2]. Reversible electroporation is already a well-established method in medicine, which combined with administration of otherwise low-permeant chemotherapeutic drugs to tumor cells, results in highly efficient local antitumor therapy called electrochemotherapy (ECT). Locally applied short, high-voltage (HV) pulses in the range of few hundreds of volts to few kilovolts increase the permeability of tumor cells membranes to facilitate cellular uptake of chemotherapeutic drugs like bleomycin and cisplatin, thus increasing their cytotoxicity [3]. Electroporation is also used for cell gene transfection, i.e., gene electrotransfer (GET), which is a non-viral gene delivery method that uses reversible electroporation for delivering DNA molecules to cells. Skin and muscle are promising targets for gene delivery, thus GET has been used in many medical applications, including vaccination, wound healing and cancer treatment. On the other hand, the potential of irreversible electroporation in medicine appeared as a nonthermal irreversible electroporation (NTIRE), also termed Pulsed Field Ablation (PFA), which has enabled the ablation of undesirable (malignant or arrhythmogenic) tissue with minimal damage to blood vessels and

This study was conducted in the scope of the Slovenian-Croatian Cooperation in Science and Technology (BI-HR/16-17-039; 2016-2017) and supported by the Slovenian Research Agency (ARRS) (MRIC UL IP-0510, P2-0249; 2015-2020). The investment is co-financed by the Republic of Slovenia and the European Regional Development Fund.

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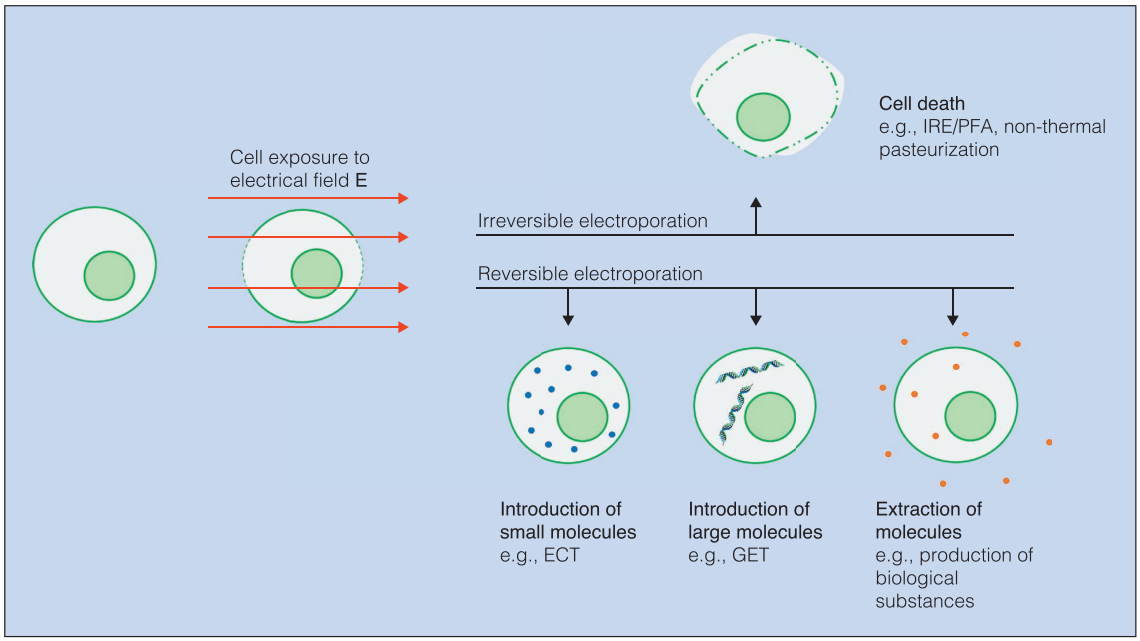

Fig. 1. Various electroporation applications. When exposure of the cell to sufficiently high electric field reaches the cell membrane threshold value, the cell gets permeabilized. Membrane electroporation/permeabilization can be either reversible, when the cell exposure to electric field is short enough for the cells to fully recover; or irreversible, when their exposure leads to cell death. In case of reversible electroporation, during the electroporation process, molecules can be introduced into the cell (electrochemotherapy (ECT), gene electrotransfer (GET)) or molecules can be extracted from the cell.

nerve conduits in the treated area [4]. Furthermore, electroporation emerged in different applications of biotechnology [5], and it is efficiently utilized for heritable genetic modification of microorganisms (electrotransformation), extraction of biomolecules, inactivation of microorganisms, and improving the mass transport in food processes. Some of the applications of electroporation are presented in Fig. 1.

Generating Electrical Pulses

To achieve successful electroporation, cells have to be exposed to sufficiently high electric fields (related also to duration of exposure). Electroporation pulses are electrical pulses, which are generated by pulse power generators known as electroporators and delivered to the cells (in tissue) via electrodes. Electrode geometry and tissue dielectric properties define the electric field intensity. Regardless of the application, electroporators have one common task: electrical pulses generation and delivery.

Electroporation success depends on the parameters of the delivered electrical pulses. The parameters of electrical pulses at the output of commercially available electroporators may vary in shape, voltage/current amplitude, pulse duration, number of pulses in a pulse train, and pulse repetition rate [6]. In electroporation, series of pulses are most commonly used for the treatment. If all pulses in a train/series of pulses are similar, the definition of the characteristics of only a single pulse and its repetition rate is sufficient for defining the treatment. The amplitude of the generated pulses may range from a few tens of volts (e.g., for GET) to a few kilovolts (e.g., for IRE) and even tens of kilovolts for microbial inactivation in liquid food pasteurization, with durations that range from nanoseconds to milliseconds for single pulse or train of pulses with predefined pulse repetition rate. Pulses may be preset for a specific application or precisely defined for a particular patient.

An electroporation pulse generator comprises an HV power supply, a pulse generator, a control unit, a user interface and an output module (Fig. 2). The user interface enables setting pulse parameters as required for a specific application. It is preferable that the device measures the parameters of delivered pulses and generates warnings in case of malfunction and/or incorrect operation.

For generation of electrical pulses, an HV power supply and pulse generator (for pulse shaping) are needed. The output module consists of an output pulse measurement unit and a commutator for switching high voltage pulses to different electrodes (if multiple electrodes are used).

Electroporators for Specific Applications

Electroporators are mainly classified as clinical, industrial or laboratory-based [7]. Electrodes together with the biological sample define the load for the output stage of the electroporator. Thus, classification of the electrodes is according to targeted load, i.e., cells (single-cell chambers, micro-electrodes, macro-electrodes and flow-through chambers) or tissue (plate, parallel needle array, hexagonal needle, finger and adjustable electrodes). Since biological loads as well as the electroporated volume intrinsically differ in different electroporation-based

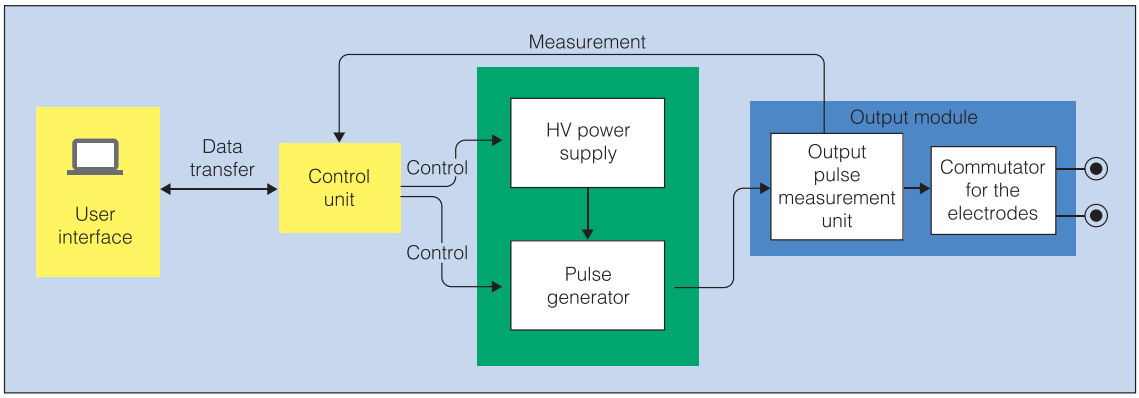

Fig. 2. Block diagram of an electroporator. An electroporator comprises a user interface, to enable setting of the pulse; a control unit; a high-voltage (HV) power supply and pulse generator for generation and forming of electrical pulses; an output module, and in case of multiple electrodes, a commutator for switching the voltage pulses to different electrodes. Measuring of the delivered pulses is preferable and warnings in case of malfunction and/or incorrect operation should be generated.

applications, pulses with specifically predefined parameters have to be used. Therefore, electroporators are often designed and developed for specific applications, where quality and efficiency have to be assured [8].

Clinical Electroporators

Electroporation-based medical/clinical applications such as ECT, GET, IRE and PFA, have so far been focused predominantly on cancer treatments. Electroporators used for medical treatments in clinics, i.e., clinical electroporators, have been developed to implement antitumor therapy using a limited number of predefined settings of electrical pulses in associations with chemotherapeutic drugs (ECT) or foreign substances such as DNA (GET) within protocols based on reversible electroporation or as stand-alone ablation treatment based on irreversible electroporation (IRE). The target tissue (e.g., tumor) should be covered with sufficiently high electric field, which should be above the threshold of reversible or irreversible electroporation 500–600 V/cm local electric field (which often requires pulse amplitudes up to 3000 V and currents up to 50 A), to achieve the desired effect. Therefore, HV pulse generators are needed which should not present potential risk for hazardous event for the patient, operator and nearby devices.

Clinical electroporators are considered to be medical devices, and thus, patient and operator safety must be ensured under both normal and single-fault conditions. Furthermore, they are obligated to meet medical device standards and follow the requirements defined by local medical regulations (e.g., Medical Device Regulation 2017/745 in Europe or Code of Federal Regulations Title 21 in the US) in order to get approval for selling the device on the market, (e.g., certification mark (CE) in Europe or Food and Drug Administration (FDA) approval in the US). In spite of agreements signed between the EU and the US, a clinical electroporator approved for use in Europe cannot be automatically used in the US and vice versa, because each country has different regulatory regimes.

Based on our review, there are only few certified clinical electroporators on the market. The most used clinical electroporators are the Cliniporator and the NanoKnife System. In Europe, the Cliniporator (two different models: Cliniporator EPS02 and Cliniporator VITAE), manufactured by IGEA S.p.A. (Carpi MO, Italy) and used for both ECT and GET, was approved by the Italian notified body and has CE mark. Another certified clinical electroporator in Europe is SENNEX (BionMed Technologies, Germany), which is used only for ECT. Recently, a new CE approved clinical electroporation generator ePORE (Mirai Medical, Galway, Ireland) was developed for simple and reliable delivery of ultra-short electrical pulses up to 250 kHz to enable treatment on an outpatient endoscopy basis. On the other hand, in the US, the NanoKnife System, manufactured by AngioDynamics, Inc. (Queensbury, NY), was approved by the FDA for surgical ablation of soft tissue [9]. Additionally, it is CE marked for Europe and has license approval for Canada for soft tissue ablation. However, it does not have FDA clearance for other treatments and therapies for specific disease or condition.

To help verify the safety of medical devices, electrical safety standards have been established in the US, Europe and other parts of the world. The first medical standard for medical electrical equipment IEC 60601 was published in 1977 by the International Electrotechnical Commission (IEC), which is a family of technical standards whose scope covers the safety, essential performance and electromagnetic compatibility of medical electrical equipment and systems. As of 2011 it consists of a general standard for medical electrical equipment: 60601-1 (EN 60601-1:2006/A1:2013 in EU or IEC 60601-1:2005/A1:2012 in the US), ten collateral standards and about 60 particular standards. Collateral standards (numbered 60601-1-X) define the general requirements for certain aspects of safety and performance, e.g., Electromagnetic Compatibility (IEC 60601-1-2). Particular standards (numbered 60601-2-X) define particular requirements for specific products or specific measurements built into products and add conditions not mentioned in

60601-1 or explain how to simplify implementation of 60601-1 to a particular device type, e.g., cardiac defibrillators (IEC 60601-2-4). In spite of the 60 particular standards, a particular standard for clinical electroporators currently does not exist. Therefore, it will be necessary to define additional rules for manufacturing and safe and efficient use of clinical electroporators as relatively new medical devices in addition to those defined by ISO and EN/IEC standards.

Considering the general standard for medical devices EN/IEC 60601-1, key safety factors that have to be considered in electroporator's design include: voltage and energy limits, adequate insulation, limitation of leakage currents, electromagnetic compatibility requirements as presented in the standard EN/IEC 60601-1-2 and consideration of fault operations while maintaining quality, efficiency and smooth operation of the device. Other standards to be considered for developing clinical electroporators are: ISO 14971 for risk analysis, ISO 13485 for quality management system, EN/IEC 60601-1-6 and ISO 62366 for usability, ISO 62304 and IEC 80002-1 for medical device software, and IEC 62311 in case of a battery powered clinical electroporator.

Industrial Electroporators

In biotechnology and even more in food processing technology, high-power and high-voltage electroporators are needed due to the requirement to electroporate large volumes of liquid. In food processing, electroporation is more frequently termed as pulsed electric field (PEF) treatment used for food structure modification or liquid food pasteurization. By PEF treatment, permeabilization of biological cells is achieved, mass transfer is improved, and therefore an efficient way for extraction of liquid and valuable substances from cells is enabled. PEF treatment systems are usually composed of a pulse power generator and a treatment chamber.

Depending on the application, a suitable pulse generator has to be chosen with adequate parameters for:

- pulse shape;
- peak voltage, which is highly dependent on the desired application;
- peak current, which is determined by the object and volume being treated;
- geometry of the treatment chamber;
- average power required, depending on the desired processing capacity (kilograms/h or liters/h).

In terms of power requirements, scale-up from several kW for laboratory to more than 100 kW for continuous-flow industrial-level processing was achieved. Therefore, regular average power of contemporary PEF devices ranges between 30 kW and 400 kW [10]. Commercial PEF treatment applications are mostly set up in potato (tuber) industry, fruit juice preservation, and vegetable processing. For juice processing, electroporation treatment systems with continuous flow have already been established with capacity of 8000 liters/h, whereas for potato processing capacity ranges up to 50–80 tons/h.

The use of new processes applied in food industry always requires appropriate process control options and set up of a systematic preventive approach to food safety – Hazard Analysis Critical Control Point (HACCP). HACCP has seven principles that need to be followed, stated in the international standard ISO 22000 FSMS 2011. In the US, the adoption of different technologies in the food processing industry is also subject to the regulation of the FDA, and in the EU it falls under the Regulation EU 2015/2283 for novel foods. Regarding safety of the device, protection against electric shock in case of insulation failure is important when using the device in wet environments. For this reason, wineries for example, are equipped with residual current devices that are responsive to a leakage current of about 30 mA. Furthermore, electromagnetic compatibility according to standards is recommended. Thus, the pulse circuit has to be shielded with metal housing, and mains and leads to the control circuity should be protected against over-voltage [11].

Laboratory Electroporators

For conducting experiments in the laboratory, users can choose between several commercially available laboratory-based electroporators. Choosing the right laboratory electroporator can be crucial for experiments and treatment protocols as some laboratory electroporators have limited range and control over pulse parameters.

An important step to be considered during electroporation is to assure pulse measuring and monitoring because only few electroporators can report and provide accurate measurements. Large variation of load characteristics is another reason to measure. The electrical properties of the sample between the electrodes might affect the current delivered (conductivity versatility). The resistance of the cuvette, for example, can vary depending on the conductivity of the media which can drastically change the required current.

However, in laboratories where experiments are done, oscilloscopes and current probes are often not readily available. Therefore, built-in measurement systems should be provided to be used with laboratory electroporators. The device should be able to perform self-tests to ensure flawless operation and detect single faults. Some use "test" pulses which should be specified and should not affect/change the sample or influence the outcome of the result. Furthermore, the device should be able to interact with the operator to ensure safe and efficient treatment and generation of output pulses, which ensure an effective experiment. The accuracy of measurements should be specified in advance, and measuring and comparison of results during experiments should be reported. Periodic calibrations of the device and equipment need to be made as well as electrode replacement based on predefined intervals. When single-use electrodes or electroporation cuvettes are used, safe disposal after the experiment should be provided due to the chemical reactions that can change the electrical properties of the electrodes in the next experiment. Recently, nanosecond electroporators were introduced and are now being used in laboratory setups. Here, measurement protocols and delivery of the pulses are more challenging. Special attention and more advanced measurement setups are required, as

those electroporators mainly do not have built-in measurement systems.

All of these problems and requirements could be solved with implementation of a specific standard for electroporators based on the application. As laboratory electroporators are not considered to be medical devices, medical device standards do not apply, and therefore, the only solution is to gather all previously mentioned requirements into one standard and agreeing on requirements that will be followed by all manufacturers to enable comparability and reproducibility of research and laboratory results. Additionally, electroporation cuvettes and electrodes should be standardized with defined tolerances and materials used.

Recommendations for Further Research and Standardization

Nowadays, the electroporation industry is growing even faster than before. Taking into consideration the new technologies, treatment protocols, increased research intensity and knowledge, we already have well-established protocols, equipment and promising treatments.

For achieving successful and efficient electroporation, it is necessary to have well defined output pulses and measure them in each treatment or experiment to make sure that the pulses are delivered as requested. Thus, suitable reproduction and comparison of results can be made if necessary. When delivering electrical pulses for electroporation, regardless of the application, it is very important to provide complete reports to enable comparability and reproducibility of the results [4], [12]–[14].

A description of pulses and how the electrical pulses were measured is necessary. The researchers must provide all specifications of the measuring equipment, identify the point of measurement and state if the pulses were delivered and measured in each experiment. Additionally, parameters and complete time-domain waveforms of the pulses should be provided with an appropriate description of the electroporator and electrodes used. For commercial equipment, the name of the company and model should be specified. If the pulse generator is a laboratory prototype or specially manufactured unit, suitable description of the components, electrical configuration, measurement and data acquisition systems should be provided. Lastly, the electric field induced by the delivered pulses inside the biological load should be calculated and/or all data describing the electric field should be listed i.e., electrode shape and their position with respect to the treated sample/tissue.

Currently, we can say that we have a developing market for clinical electroporators and new electroporators designed for specific applications are coming up. However, the absence of industry, laboratory and medical specific standards may eventually become an obstacle for further development of approved electroporation devices and associated equipment. Papers calling for standardization for other applications of electroporation have already been published (e.g., for standardization of IRE techniques and protocols), in which authors propose a set of technical recommendations for the use of IRE for treatment of locally advanced pancreatic cancer [15]. Having a specific standard for each application will simplify the harmonization of all commercial, certified electroporators and improve the safety, quality and efficiency of these devices. Current problems like voltage drop during pulse delivery, unknown pulse parameters, insufficient electrical field, and non-comprehensive reports can be solved by stating limits and recommendations for voltage/current, energy, load, electrodes used, insulation and design.

The standard should define:

- maximum tolerances of generated pulses compared to expected values by considering the plasma membrane permeabilization of the load and technical limitations of electroporation device development;
- how to provide technical specifications of the device, together with conditions under which they are achieved, e.g., to define maximum amplitude of the pulses together with the pulse duration range and load resistance at which it can be achieved;
- pre-pulses (amplitude, pulse duration and exact timing regarding the preset sequence), if used;
- how to implement safety features like galvanic isolation, current, energy and voltage limitations, warnings if the pulse delivery was stopped or limited, or if any other unforeseeable event or malfunction has occurred;
- by which load the electroporators should be tested to ensure effectiveness at specific applications, or to ensure predictable operation or operation within tolerances (this can be quite challenging in case of delivery of nanosecond pulses as pulse reflections can occur due to the dynamics and variability of the biological load, i.e., conductivity increase due to electroporation);
- which electroporation cuvettes and electrodes should be used;
- maximum tolerances of the distances between the electrodes.

The standard should also consider procedures for different materials used and recommend a way of defining a treatment volume and an electric field distribution between the electrodes.

Recommendation for Electrochemotherapy Device Standardization

ECT is an established cancer treatment used in clinics [16] for safe and convenient treatment of cutaneous and subcutaneous tumors following standard operating procedure (SOP) [17], [18]. The pulses are delivered to target tissue via electrodes, which are considered to be medical accessories, used only in combination with a particular pulse generator (mostly used is the Cliniporator EPS02). If the electrodes are placed on the patient's skin (e.g., plate or non-penetrate electrodes), they are considered to be non-invasive medical accessories, used to treat cutaneous tissues. In cases when they are intended to be placed inside the patient's body (e.g., needle electrodes),

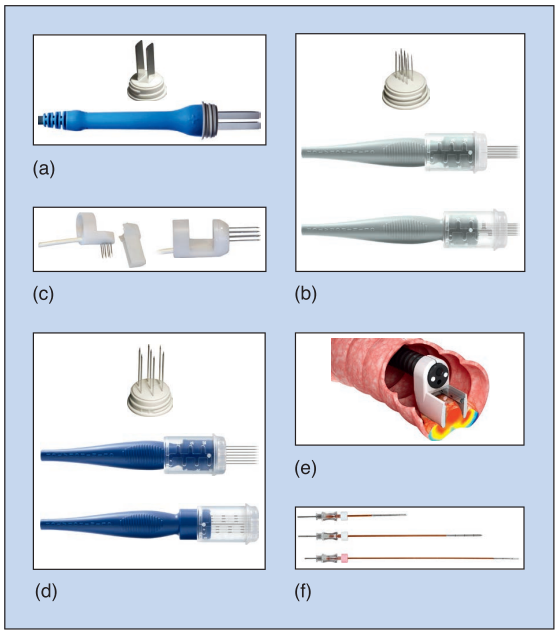

Fig. 3. Different types of electrodes (by IGEA S.p.A.). (a) Plate electrodes. (b) Linear (parallel needle) array electrodes (first top image), adjustable linear needle electrodes with needle-length adjustment with 5 mm increment (bottom two images). (c) Finger electrodes with orthogonal linear needles (left) and longitudinal linear needles (right). (d) Hexagonal needle electrodes (first top image), adjustable hexagonal configuration needle electrodes with needle-length adjustment in 5 mm increments (bottom two images). (e) Endoscopic electrode EndoVE (Endoscopic Vacuum Electrode) which is mounted at the head of an endoscope and utilizes a vacuum source to drag the tissue alongside with the electrode. (f) Individual (long) needle electrodes for variable electrode-geometry (from 2 to 6 electrodes with 16–30 cm long needle and active tip of 3 or 4 cm).

they are considered to be invasive and are used to treat deeper tissues.

The updated SOP [18] defines five types of electrodes (made of stainless-steel) that are commercially available (IGEA S.p.A, Carpi MO, Italy) and can be used together with the Cliniporator depending on the treated area:

Plate electrodes: with 8 mm gap in-between, used for superficial skin lesions (Fig. 3a).

Linear array electrodes: (parallel needle array) that have 2 arrays of 4 needles (with needle length of 10-, 20- or 30 mm), separated by 4 mm distance, used for smaller tumors (recommended to be used for tumors in the facial region) with local anesthesia (Fig. 3b).

Hexagonal needle electrodes: with needle length of 10-, 20- or 30 mm, used for treatment of larger areas, e.g., cutaneous metastases (Fig. 3d).

Finger electrodes: (longitudinal or orthogonal) with needle length of 5- or 10 mm, used for treatment of mucosal tumors, e.g., in the oral cavity (Fig. 3c).

Adjustable electrodes: (linear (Fig. 3b) or hexagonal (Fig. 3d)) allow adjustments in needle length (from 5 mm to 40 mm with 5 mm increments) for better support in treatments of tumors with heterogeneous size.

In addition, the endoscopic electroporation system EndoVe (Mirai Medical, Galway, Ireland) was developed to be used with the ePORE electroporation generator, which is also suitable for the Cliniporator (Fig. 3e). Furthermore, long freely-placeable needle electrodes (Fig. 3f) were introduced, and new minimally invasive laparoscopic expandable needle electrodes are being developed by IGEA S.p.A [3], [16].

Pulse parameters are defined in the SOP as a result of numerous previously conducted studies. For each pulse delivering, 8 square-wave pulses of 100 µs with pulse amplitude of about 1000 V (1000 V up to 1300 V) across an 8 mm distance between plate electrodes should be delivered at repetition rate of either 1 Hz or 5 kHz. ECT with pulse repetition rate of 5 kHz is mandatory for hexagonal needle electrodes because the treatment (delivering 8x12 = 96 pulses) with 1 Hz repetition rate would extend over a prohibitively long time and high-frequency (5 kHz) pulses reduce the number of contractions. Nevertheless, several applications may be needed to cover the whole tumor volume in a single session.

All electrodes which are commercially available and meant to be used with the Cliniporator are for single use for a particular patient and only for a single session (for one nodule or several similar nodules in the same patient). In a case of more nodules of different sizes, more than one electrode type may be needed for a particular patient in the same session.

The galvanic isolation of the output is preferably implemented in the power supply and not in the output module to have accurate measurement of the output signal. Output current and voltage are measured at the output of the pulse generator to implement current, energy and voltage limitations. The SOP should define the maximum expected current or minimal expected resistance of the load. The maximum current of the device should be 10% or 20% higher than the maximum expected current, which is 20 A for the Cliniporator EPS02. SOP defines the maximum treatment voltage as 1300 V/cm voltage-to-distance ratio times 8 mm which is equal to 1040 V. The maximum voltage is defined by the SOP and tolerances and is 1000 V for the Cliniporator EPS02. The maximum energy should be equal to the maximum treatment time, times maximum current, times maximum voltage.

Considering the SOP, for square wave pulses (described by the amplitude and the pulse duration t_{FWHM}, where FWHM is Full Width at Half Maximum, we propose the following tolerances:

- the pulse amplitude between 15% and 85% of FWHM should not rise over or fall below 110% or 90% of SOP amplitude (Fig. 4a);
- the FWHM should not be longer or shorter than ±8% of SOP FWHM;
- delivered number of pulses should be exactly the same as in the SOP and variations of this parameter are not allowed;

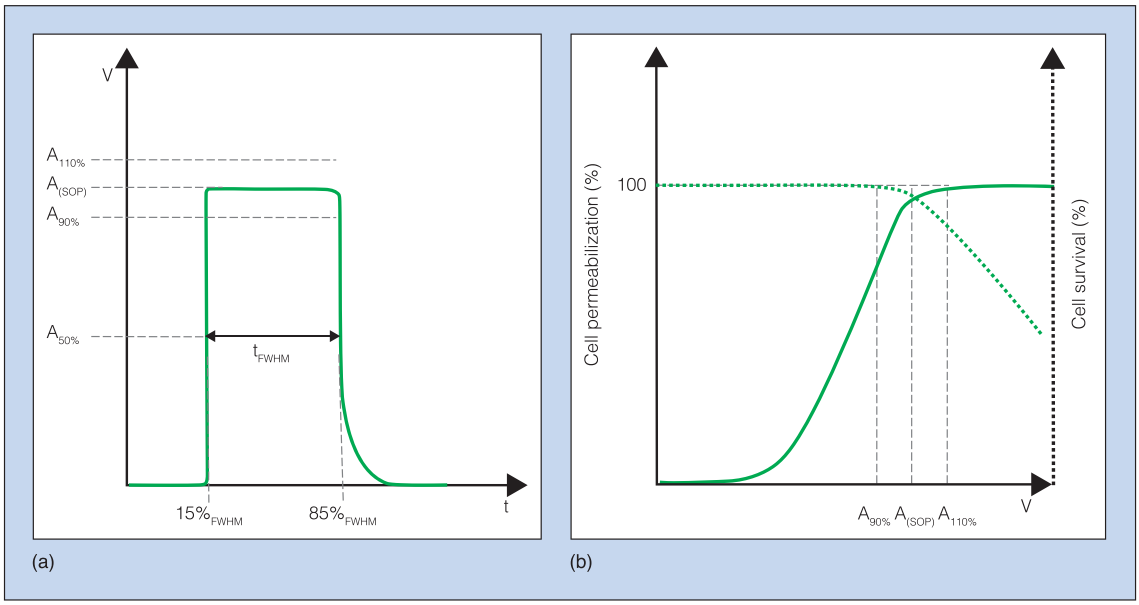

Fig. 4. a) Electroporation square wave pulse: pulse parameters and tolerances. b) Permeabilization curve (solid line) and cell survival (dashed line) with defined tolerances [19]. For amplitudes between 90% and 110% of the defined SOP amplitude will still be possible to achieve efficient treatment. With amplitudes values higher than 110%, cell survival will be increasingly lowered and can lead to IRE while with amplitudes values lower than 90%, the cell permeabilization will decrease and can lead to inefficient tumor treatment.

- pulse repetition rate may deviate from SOP pulse repetition rate (for both options) for maximum ±5%.

For successful ECT it is important to keep within these tolerances, as we calculated them to define the maximum deviations where it is still possible to achieve the desired effect. For example, higher (more than 110% of the amplitude) or lower (less than 90%) values of the SOP amplitude can lead to IRE or insufficient electric field for ECT, respectively. Based on the permeabilization curves (Fig. 2 from reference [19]), even for pulses with the lowest or highest defined tolerances (for pulse amplitude and pulse durations), it will still be possible to stay on the part of the permeabilization curve where the treatment will be efficient (Fig. 4b).

ECT devices should work within the tolerances on zero load and on electronic emulator of ECT load. Operation of the limitation should be tested by the device on power up and conformity by using electronic emulator of biological load.

All electrodes should be manufactured utilizing a biocompatible material, usually stainless-steel. However, materials tested by the requirements stated in the ISO 10993 series of standards for biological evaluation of medical devices that come into direct or indirect contact with biological tissues (parts -1, -5 and -10 are the most important) may be allowed.

Following the SOP, all electrodes need to be for single use. We propose the option of using multiple-use electrodes. In this case, clear instructions for electrode cleaning and maintenance after every treatment should be provided. Moreover, multiple-use electrode replacement on predefined intervals should be stated and provided in the instructions for use.

The allowed tolerances for the diameters of the electrodes, the distances between the electrodes and the length of the needles should also be defined.

Conclusions

With implementation of a specific standard for particular applications, electroporation devices will be safer, treatments more efficient and results more reproducible, which will allow faster and more straight-forward progress of electroporation as well as treatments and therapies based on electroporation. By implementing a particular medical standard for electrochemotherapy electroporation devices, the standard operating procedure will be improved which will result in better and more effective cancer treatment.

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3.3 Paper 3

Title: **Design, development, and testing of a device for gene electrotransfer to skin cells in vivo**

Authors: Aleksandra Cvetkoska, Janja Dermol-Černe, Damijan Miklavčič, Simona Kranjc-Brezar, Boštjan Markelc, Gregor Serša and Matej Reberšek

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Article

Design, Development, and Testing of a Device for Gene Electrotransfer to Skin Cells In Vivo

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Abstract: Gene electrotransfer (GET) is considered one of the most efficient, safe, reproducible, and cost-effective methods of gene therapy, in which a gene is delivered to the cells in the form of a plasmid DNA vector by a method known as electroporation. To achieve successful electroporation, cells must be exposed to sufficiently high electric fields generated by short-duration, high-voltage electrical pulses that result in a temporary increase in plasma membrane permeability. The electrical pulses are generated by pulse generators (electroporators) and delivered to the cells via electrodes (applicators). However, there is a lack of standardized pulse delivery protocols as well as certified clinical pulse generators and applicators for gene delivery. In this paper, the development of a new pulse generator, applicator, and pulse delivery protocol for GET to skin cells is presented. A numerical model of electroporated skin developed and tested for two electrode configurations and two different pulse delivery protocols is also presented. An alternative pulse delivery protocol was proposed. The developed pulse generator, applicator, and the proposed pulse delivery protocol were then used in vivo for GET to skin cells in mice. The results showed high efficiency of the proposed pulse delivery protocol for the purpose of GET in mouse skin cells. Specifically, electroporation with the developed pulse generator, applicator, and proposed pulse delivery protocol resulted in higher gene expression in skin cells compared to the currently used pulse generator, applicator, and pulse delivery protocol.

Keywords: electroporation; gene electrotransfer (GET); plasmid DNA; pulse generator; pulse delivery protocol

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1. Introduction

Gene therapy is one of the new and promising therapeutic approaches for the treatment of cancer, in which plasmid DNA vectors containing therapeutic genes are introduced into target cells to induce a therapeutic effect [1]. Gene delivery methods are divided into viral and non-viral methods based on the vectors that carry the information DNA [2]. Gene electrotransfer (GET), a non-viral delivery method, is considered one of the most efficient, safe, reproducible, and cost-effective methods [3,4]. GET allows the genetic material to be delivered directly into tissues (skin, muscle, or tumor) by a method known as electroporation [5–8]. To achieve successful electroporation, cells must be exposed to sufficiently high electric fields, which leads to a temporary increase in the permeability of the plasma membrane. Electroporation pulses are electrical pulses generated by pulse generators, also known as electroporators, and delivered to the cells (in the tissue) via electrodes (applicator) [9,10] as a necessary accessory part but separate medical device. The pulse parameters are usually set by an operator via a user interface. Electroporation has been shown to significantly increase the efficiency of DNA drug delivery [11]. Therefore, transdermal or intradermal GET is one of the most promising and widely used applications of skin electroporation [12]. However, the translation of skin electroporation into the clinic has been

slow and lags behind in vitro and in vivo studies [13,14]. One of the possible reasons is inadequate dosimetry, which impedes comparison of the pulse generators, applicators, and pulse parameters [15]. There is a lack of certified clinical pulse generators and applicators for gene delivery, as well as standardized pulse delivery protocols to enable translation to human applications. Many pulse generators currently in use do not meet their technical specifications and do not verify the delivered waveforms [16]. Different pulse parameters are used with varying success, which renders comparison between the results difficult. The equipment and pulse parameters are often inadequately reported, making the studies not comparable or reproducible. We believe that with adequate dosimetry, predictive modeling, and development of high-quality electroporation devices, the efficiency of skin electroporation treatments can be increased, allowing comparison between treatments and facilitating the translation into the clinics.

In this paper, we present the development of the new pulse generator, applicator, and pulse delivery protocol for GET to skin cells based on predictive modeling. First, we explain the developed numerical model of the electroporated skin, which allowed testing of different electrode configurations and pulse delivery protocols to achieve the best possible effect for gene delivery. Then, all the necessary requirements and recommendations for simpler design and development of a pulse generator for clinical use are listed and the treatment protocol is suggested. Based on the basic requirements for a medical device, we thus describe the design of the newly developed device for GET to skin cells. We also present the design of the newly developed applicator (noninvasive electrodes) for safe and easy delivery of the electrical pulses. Finally, we present the results of the performed in vivo study in mice and compare our results with the currently used pulse generator, applicator, and pulse delivery protocol.

2. Materials and Methods

2.1. Numerical Determination of the Optimal Electrode Configuration

Optimal electrode configuration and pulse delivery protocol for gene electrotransfer (GET) were determined numerically. The development of the optimal electrode configuration was based on two criteria:

1. Minimizing collateral damage by minimizing the volume of irreversible electroporation.
2. Maximizing gene transfer efficiency by maximizing the reversibly electroporated volume.

We developed a numerical model of skin, which allowed us to easily test different electrode configurations and pulse delivery protocols. Our skin model was based on multiscale analysis and was constructed according to [17,18], with sensitivity analysis performed as in [15]. The model consisted of eight different layers, also considering anisotropy of tissue conductivity. The electrical conductivity was a function of the electric field described by sigmoid, and the process of electroporation was modeled stationary and sequentially [19]. The thickness of the layers, their initial electrical conductivities, and the threshold values for the maximum increase in conductivity due to electroporation were based on [17,19,20] and are listed in Table 1.

Based on the previous knowledge, we selected and compared two different pulse delivery protocols: the classical [21] (Figure 1a) and the proposed alternative protocol, similar to [22,23], with addition of pulsing around the perimeter (Figure 1b). The main difference between the applicators, i.e., electrode configurations and pulse delivery protocols, is that the proposed alternative protocol does not include a central electrode. The arrows in Figure 1 indicate which electrodes are used and the order in which the pulses are delivered. In the classical protocol, the pulses are delivered between all adjacent electrodes, first in one direction and then in the opposite direction, i.e., with reversed polarity. In the proposed alternative protocol, the pulses are delivered first between adjacent electrodes on the rim, again switching polarity (Figure 1b, left). To compensate for the missing central pin, the pulses are then delivered between two opposite pairs of electrodes and with the polarity also switched (Figure 1b, right).

Table 1. Properties of each skin layer used in the numerical model. σ signifies the electrical conductivity; RE—reversible electroporation; IRE—irreversible electroporation.

Skin Layer	Layer Thickness	σ_x (S/m)	σ_y (S/m)	σ_z (S/m)	RE Threshold (V/cm)	IRE Threshold (V/cm)	Maximal σ Increase
Stratum corneum	20 µm	1.10×10^{-2}	1.10×10^{-2}	2.23×10^{-4}	400	1200	100×
Epidermis	0.1 mm	5.82×10^{-2}	5.82×10^{-2}	6.36×10^{-2}	400	1200	3.5×
Papillary dermis *	0.15 mm	7.19×10^{-2}	7.19×10^{-2}	7.19×10^{-2}	300	1200	3.5×
Upper vessel plexus	80 µm	4.22×10^{-1}	3.86×10^{-1}	3.86×10^{-1}	300	1200	3.5×
Supply layer	1 mm	3.12×10^{-1}	3.12×10^{-1}	3.19×10^{-1}	300	1200	3.5×
Deeper vessel plexus	0.1 cm	3.42×10^{-1}	3.28×10^{-1}	3.28×10^{-1}	300	1200	3.5×
Hypodermis *	0.5 cm	6.35×10^{-2}	6.35×10^{-2}	6.35×10^{-2}	300	1200	3.5×
Muscles	2 cm	1.57×10^{-2}	6.86×10^{-2}	1.57×10^{-2}	200 ** 80 **	800	2.5×

* The layers are isotropic and only one value for conductivity is given. ** The threshold value changes according to the direction of the applied electric field with respect to muscle fiber orientation, with the higher value for the perpendicular direction and the lower for the parallel direction.

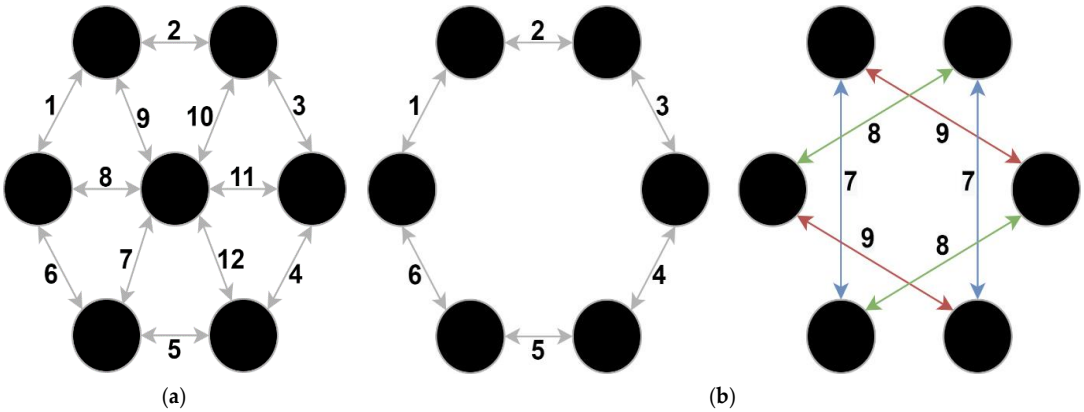

Figure 1. The order of pulse delivery for (**a**) the classical pulse delivery protocol and (**b**) the proposed alternative pulse delivery protocol. The numbers indicate the order of pulse delivery. The arrows indicate the direction of the applied pulse (anode -> cathode).

2.2. Requirements and Recommendations to Be Considered When Designing a Clinical Electroporator for Gene Electrotransfer to Skin Cells

2.2.1. Medical Device Regulation and Standards

A clinical pulse generator (electroporator) for GET to skin cells is considered a medical device for which patient and operator safety must be ensured under both normal and single-fault conditions. In addition, such a device must comply with medical device standards and meet the requirements of local medical regulations, e.g., Medical Device Regulation (MDR) 2017/745 in Europe or CFR (Code of Federal Regulations) Title 21 in the United States (US), in order to be sold on the market, e.g., certification mark (CE) in Europe or FDA (Food and Drug Administration) approval in the USA.

A clinical electroporator is classified as a Class IIa active therapeutic device, type BF (Body Floating). All technical documentation required for certification of the device should then be based on the established level of risk, i.e., classification class. The main standard to be considered when designing such a device is EN/IEC 60601-1: Medical electrical equipment—Part 1: General requirements for basic safety and essential performance. This

standard is a generally accepted criterion for medical electrical equipment, and compliance with this standard has become the main requirement for placing the medical electrical equipment on the market. According to the standard, the essential safety factors that should be considered in the design of the device are: limitation of voltage, current, and energy, limitation of leakage currents, adequate insulation according to the device class, and maintaining safe operation, quality, and efficiency even in the event of a single-fault condition. Electromagnetic compatibility requirements should be met according to the EN/IEC 60601-1-2 standard, while risk analysis should be performed according to the ISO 14971 standard. Other standards that should be considered in the development of a clinical electroporator for GET to skin cells are ISO 13485 for the quality management system, EN/IEC 60601-1-6 and ISO 62366 for usability, ISO 62304 and IEC 80002-1 for medical device software, and IEC 62311 for a battery-powered pulse generator [24].

2.2.2. User and Technical Recommendations

Portability of the pulse generator, i.e., that can be easily transported from one place to another (between different clinics or operating rooms), is often desired by operators. To enable/facilitate portability, the pulse generator must be battery-powered with a rechargeable battery. A battery level indicator is required to allow the operator to estimate the remaining operating time of the device. A pedal control or button on the applicator is necessary to arm and deliver the electrical pulses, so that the operator (clinician) can independently hold the applicator in the sterile field. A touchscreen is preferred (over keyboard and mouse) as the user interface to set pulse parameters manually or automatically (based on the treatment plan). The device should be able to generate the pulse parameters set by the operator (amplitude, pulse duration, pulse repetition rate, etc.) and be equipped with appropriate visual and audible alarm systems to alert the operator to low-risk or high-risk processes or events. Validation of the current and voltage of the output pulses is essential, as is the storage of treatment data for post-treatment analysis and quality control. The device needs to be designed in a way to allow easy maintenance and cleaning. Noninvasive, reusable electrodes must be made of biocompatible material (e.g., medical grade stainless steel), and designed to allow appropriate and safe cleaning.

The user and technical recommendations were determined based on the operator's needs and previous user experience with other pulse generators.

2.2.3. Recommended Treatment Protocol for Safe and Efficient Gene Electrotransfer to Skin Cells

It is recommended that the entire procedure is performed in one room, usually an examination room (in hospitals/clinics). The patient must be informed in advance that some contractions of the underlying muscle are to be expected, but that a local anesthetic should protect against pain, as the penetration of the electric field is not great. The appropriate amount of local anesthetic and plasmid DNA dose to be administered to the patient must be prepared [25]. The device must be in good working order, the battery charged before use, and the applicator must be connected to the device. Once the device is set and ready, the operator can select the pulse parameters. Experienced medical personnel should then perform the local injection of the plasmid DNA. The waiting time between the injection and the application of the electrical pulses is proposed to be between 30 s and 2 min [25]. While holding the handle of the applicator with one hand and lifting the area from the underlying muscle with the other hand (when possible), the operator should start the application of the electrical pulses using a control pedal connected to the device or by pressing the button on the applicator. Monitoring the delivered pulses is important to verify that the voltage and current delivered are consistent with the values set by the operator. After the treatment, the electrodes must be removed and discarded (single-use electrodes) or sterilized for the next use (reusable electrodes). The device then needs to be switched off and cleaned for the next use. If warnings and alarms occur during the treatment, they must not be ignored. In the case of suspicion or malfunction, the manufacturer must be contacted.

2.3. System Design

Figure 2 shows a block diagram of the system design for the device for GET to skin cells. Five different functional units were defined prior to development, colored differently depending on the task being performed: Graphical User Interface (GUI) and Control (yellow), Safety (orange), Pulse Generation (blue), Power (red), and Battery (green).

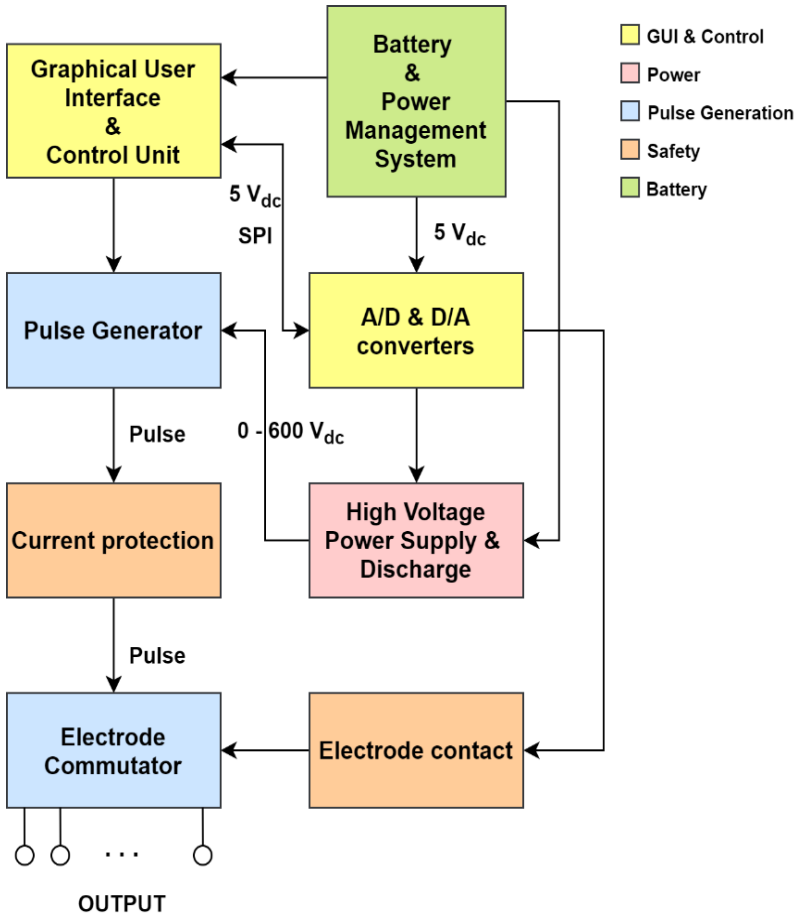

Figure 2. Block diagram of the device for GET to skin cells.

2.3.1. Graphical User Interface (GUI) and Control Unit

The GUI and Control unit (yellow, Figure 2) consists of a graphical user interface (GUI) and an isolation and conversion circuit with Analog-to-Digital (A/D) and Digital-to-Analog (D/A) converters. The GUI of the device was developed on a Raspberry Pi 3 (Raspberry Pi Foundation, United Kingdom) with a LogiPi FPGA circuit installed (Valent Fx, France), which was used as a control unit of the device. The parameters of the electrical pulses are entered into the device through GUI (Figure 3) developed using the GTK3 library (Genome Foundation, USA). The GUI is displayed on a SunFounder 10.1" 1280 × 800HDMI Touchscreen (Shenzen Headquartes, China).

The isolation and conversion circuit with A/D and D/A converters provides galvanic isolation of the control signals by optocouplers and enables control of the high-voltage (HV) power supply. The isolation ensures that the high voltage does not transfer to the low voltage part of the device in case of a fault in the high voltage part. The conversion

part of the circuit enables the digital control signal to be converted to an analog signal and the voltage to be measured with the A/D converter using the standardized SPI (Serial Peripheral Interface) protocol.

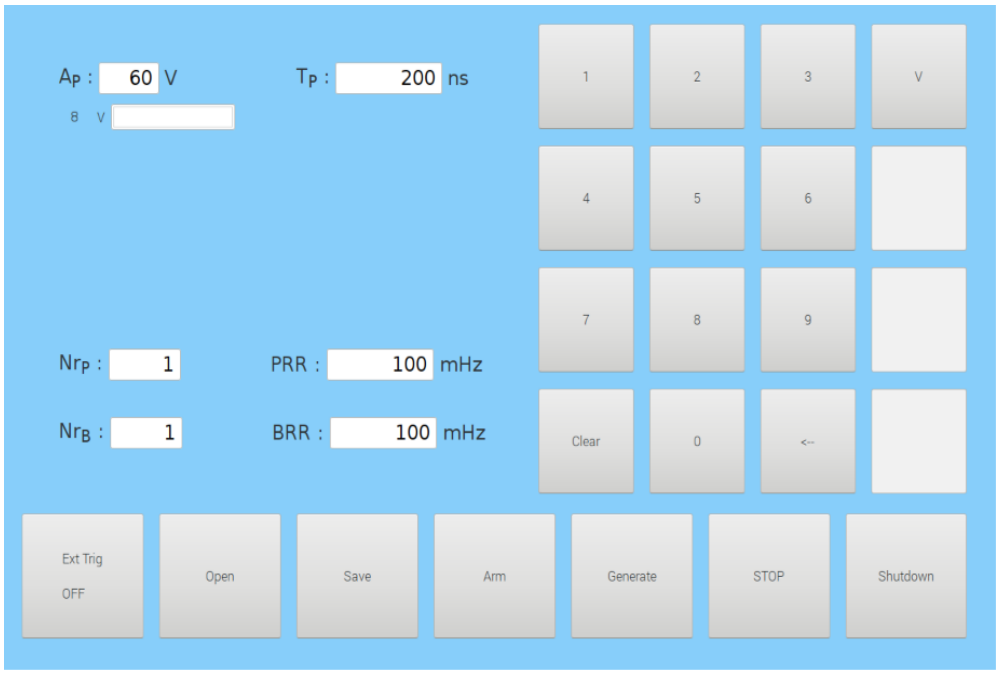

Figure 3. Graphical User Interface (GUI) of the device, displayed on the SunFounder 10.1″ touchscreen. The values of the parameters can be entered using the keyboard on the right side. A_P—amplitude of the pulse; T_P—duration of one pulse; Nr_P—total number of pulses, Nr_B—total number of bursts; PRR—Pulse Repetition Rate; BRR—Burst Repetition Rate.

2.3.2. Power

The Power unit (red, Figure 2) provides the power to the device. The high-voltage (HV) power supply consists of an HV DC-DC converter HRL3024S600P (XP Power, Kunshan, China), three HV capacitors B32774D0705K000 (7 µF, 1.1 kV; EPCOS, TDK Corporation, Tokyo, Japan) connected in parallel, and an HV fuse at the output 0090.0004 (4 A, 1 kVdc; Schurter, Lucerne, Switzerland). Thus, the HV power supply has a total capacitance of 21 µF, power of 30 W, and enables controlled power supply from 0 to 600 V.

2.3.3. Pulse Generation

The pulse generation unit (blue, Figure 2) is responsible for generating the electrical pulses and providing the correct voltage supply to the electrodes. The generator has three control inputs (pulse, stop, and discharge), low voltage and high voltage power inputs, and an output for electrical pulses. The pulse control signal can be used to raise the output to 560 V and lower it back to 0 V in 1–5 µs. The stop signal must always be present, otherwise the HV pulse will be turned off in less than 1 µs. The discharge control signal is used to discharge the HV capacitors.

The electrode switching circuit, i.e., the electrode commutator, switches the electrical pulses between the electrodes according to the selected pulse delivery protocol (Figure 1). The electrode commutator provides the output voltage of the generator to the electrodes in the correct sequence and transmits the output voltage to each individual electrode at the required moment according to the signals from the control unit. We used 14 HE24-1A83

reed relays (Standex, Salem, NH, USA), which can commutate the electroporation output signal to up to seven independent electrodes.

2.3.4. Safety

The safety unit (orange, Figure 2) provides protection in the case of overcurrent at the output of the device and verifies the electrical parameters of the generated HV pulses. The current limiter prevents the current and power from becoming too high when discharging the HV capacitors. In the event of high currents, which may occasionally occur during therapy, the current limiter does not stop the therapy, but only limits it to the maximum expected value of the current during therapy. This allows the therapy to proceed normally even if the current occasionally increases.

In addition, we developed a circuit to check the contact of the electrodes with the skin before delivering the electrical pulses, as this may increase the probability of successful delivery of the pulses. The skin electrode contact detector is designed to distinguish between three different impedance ranges between the electrodes to determine if the electrodes are in contact with the skin, i.e., if they have the appropriate impedance for the pulse generator. The first impedance range that can be determined by the circuit is too low impedance for the generator (no skin contact). The third impedance range that can be determined by the circuit is too high impedance between the electrodes and the skin (too high conductivity range). The impedance range between the first and the third range is the impedance range where the electrodes are in contact with the skin.

2.3.5. Battery

In order to have a rechargeable device that can be easily transported between different examination rooms, a battery unit (green, Figure 2) was added to the device. It consists of a 24 V battery power supply with a battery power management system. A level indicator was implemented to allow the operator to predict the remaining operating time of the device. For the power supply, six lithium cells 1850CA (BIPOWER Corp., Monterey Park, CA, USA) connected in series with an average voltage of 3.75 V were used. To monitor the performance of the battery system, the MAX17263 integrated circuit (Maxim Integrated, San Jose, CA, USA) was used. The battery management system was implemented on the MAX17263GEVKIT# development board (Maxim Integrated, USA).

2.4. In Vivo Experiments

2.4.1. Plasmid DNA

Plasmid pEGFP-N1 (Clontech Laboratories Inc., Mountain View, CA, USA), encoding enhanced green fluorescent protein (GFP), was prepared from Escherichia coli cultures using the Qiagen Endo-Free Plasmid Mega kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and diluted to a working concentration of 1 μg/μL. Plasmid concentration was determined using the Qubit DNA Broad Range kit (TFS, Waltham, MA, USA) using fluorometric quantification with the Qubit 4 Fluorometer (TFS, Waltham, MA, USA). Plasmid quality was assessed using the 260/280 nm ratio determined using the Epoch Microplate Spectrophotometer (BioTek, Bad Friedrichshall, Germany).

2.4.2. Mice

Female 10–12 week-old Balb/c (BALB/cAnNCrl) mice (Charles River Laboratories (Italy)) were used in the experiments. Mice were kept in a specific pathogen-free environment with a 12 h light-dark cycle at 20–24 °C and relative humidity of 55% ± 10%; food and water were provided ad libitum. The experiments were approved by the Ministry of Agriculture, Forestry and Food of the Republic of Slovenia (permission no. 34401-1/2015/43 and U34401-3/2022/11). The experimental procedures were performed in accordance with the guidelines for animal experiments of the EU directive (2010/63/EU) and ARRIVE guidelines.

2.4.3. In Vivo Gene Electrotransfer

Before in vivo gene electrotransfer (GET) of pEGFP-N1 both flanks of mice were shaved and depilated with hair removal cream (Vitaskin, Krka d.d). Considering randomization, each flank was assigned to a different experimental group. Before GET, mice were anesthetized with Isoflurane (Piramal Healthcare UK Limited, London, UK). A 29 G insulin grade syringe (CHIRANA T. Injecta, Stará Turá, Slovakia) was used to inject 25 µL of pEGFP-N1 intradermally at a concentration of 1 µg/µL. Immediately, i.e., within 30 s to 2 min after plasmid injection, the electrical pulses were applied. Two different pulse delivery protocols were used for plasmid delivery. The first was the low-voltage (LV) pulse delivery protocol with an amplitude-to-distance ratio of 170 V/cm (12 pulses, amplitude 60 V, duration 150 ms, pulse repetition rate 2.82 Hz) applied with the Cliniporator (IGEA s.r.l., Carpi, IT) through noninvasive multi-electrode array (MEA, Iskra Medical, Podnart, SI) consisting of six spring-loaded pins arranged in a hexagonal mesh (Figure 1b, first picture with only 6 arrangements) and spaced 3.5 mm apart, as this was found to be optimal [26,27]. The second was the proposed alternative pulse delivery protocol (18 sequences (Figure 1b) with 4 pulses, burst repetition rate 50 Hz, amplitude 560 V, duration of each pulse 100 µs, pulse repetition rate 5 kHz) applied with the pulse generation unit (Section 2.3, Figure 2) and the applicator described in Section 3.2.1 During GET a conductive gel (Gel G006 ECO, FIAB, Vicchio, Italy) was used at the point of contact between the electrodes and the skin to ensure good conductivity. The currents reached with the first pulse delivery protocol were 100 mA, while the currents reached with the second (proposed) alternative pulse delivery protocol were 500 mA.

2.4.4. Image Acquisition and Analysis

To determine in vivo transfection efficiency, mice were imaged with a fluorescence stereomicroscope (excitation: 470/40 nm, emission: 525/50 nm, SteREOLumar V.12, Carl Zeiss, Jena, Germany), equipped with an AxioCam MRc5 digital camera (Carl Zeiss), on days 1, 3, 5, and 7 after GET. The images were subsequently analyzed using FIJI [28]. On each image the transfected area was separated from the non-transfected area by determining the number of pixels with the intensity above the same threshold pixel intensity. From the determined transfected area, the mean fluorescence intensity of the pixels and the integrated density (product of area and mean fluorescence intensity) were determined.

3. Results

3.1. Numerical Determination of the Optimal Electrode Configuration

The modeling results show that the proposed alternative protocol yields an 8% larger reversibly electroporated volume which also penetrates deeper in comparison to the classical protocol. In addition, by avoiding the use of a central electrode, the damage by irreversible electroporation is reduced by 15% in the proposed alternative protocol compared to the classical protocol. The irreversible damage is mostly concentrated in the stratum corneum directly under the electrodes. The proposed alternative protocol is thus more successful in achieving deeper and more homogeneous reversible electroporated volume than the classical protocol, while collateral damage remains low, suggesting that gene electrotransfer should be more successful with the proposed alternative pulse delivery protocol than with the classical pulse delivery protocol. The electric field distribution and reversible electroporated volume of the classical and proposed alternative protocols 2 mm below the skin surface and as a side view between the electrodes are shown in Figure 4 (a and b for the classical, and c and d for the proposed alternative pulse delivery protocol).

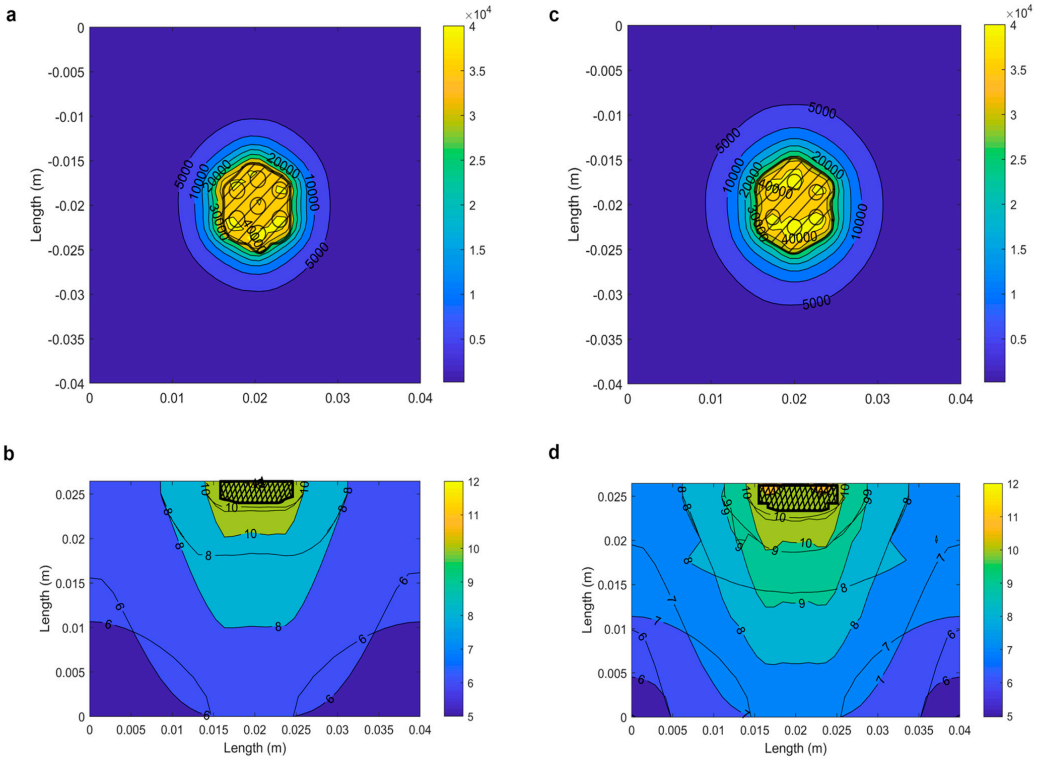

Figure 4. Electric field distribution for (**a**) the classical and (**c**) the proposed alternative pulse delivery protocol (V/m) 2 mm below the skin surface in the hypodermis, where the cells important for the immune response are located. The location of the electrodes is marked with circles. Side view of the natural logarithm of the electric field distribution (V/m) in the middle between the electrodes for (**b**) the classical and (**d**) the proposed alternative pulse delivery protocol. The shaded area shows the area of reversible electroporation. We chose the logarithmic representation as the electric field values differ for ranges and the differences would not be clearly seen otherwise.

3.2. System Design

3.2.1. Applicator—Electrode Development

Based on the results of the developed model and the proposed alternative pulse delivery protocol, we designed an applicator with six hexagonal rod electrodes without the central electrode (Figure 1b and red frame in Figure 5). The spacing between adjacent electrodes is 2.5 mm, while the distance between the centers of the opposite electrodes is 9 mm. The electrodes are 10 mm long (outside the housing) with rounded tips and are made of stainless steel 316L. They are intended for multiple use and can be taken off the applicator for easier cleaning and disinfection or for replacement after the determined usage. The geometry of the electrodes allows them to fit different areas of skin on the body, irrespectively of the curvature. The applicator has a built-in green warning light, which informs the operator that the applicator is in contact with the skin and, thus, the device is ready to generate the electrical pulses. In the handle of the applicator there is also a built-in button that is used to trigger the device directly from the applicator. This type of electrode allows for noninvasive pulse delivery with less pain and muscle twitching [29], while also allowing efficient gene electrotransfer.

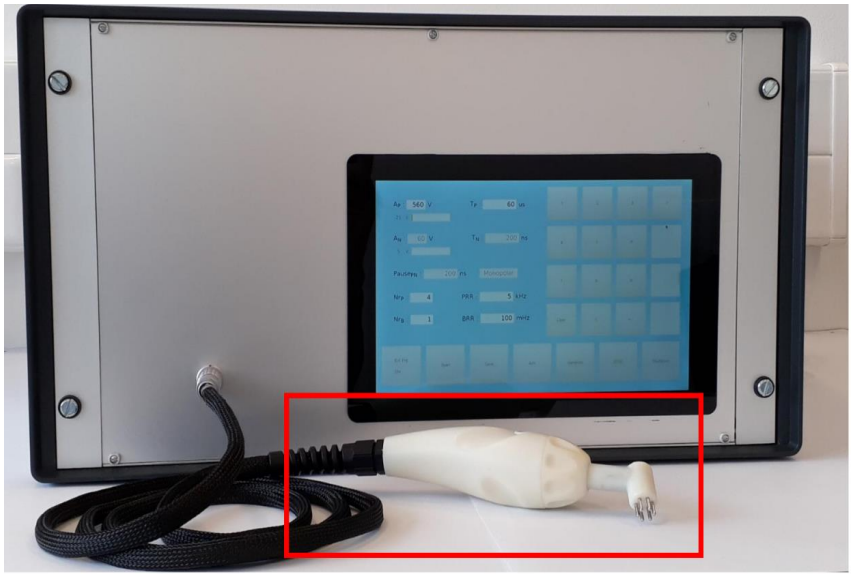

Figure 5. The completed device (pulse generator and applicator) for gene electrotransfer to skin cells. The applicator is shown inside the red frame.

3.2.2. Device Development

The development of the device started with the design of circuits to power all the other circuits in the device and isolate the low voltage signals at the applicator from the control unit. Then, we designed a circuitry that isolates the high voltage from the control unit (isolation and conversion circuit, A/D and D/A converters). We proceeded with installation of a 30 W high voltage power supply with 21 µF capacitance, which provides a controlled power supply from 0 to 600 V. A switching circuit between the electrodes (electrode commutator) that switches the electrical pulses between the electrodes according to the proposed alternative pulse delivery protocol was also developed. This circuit was connected to the applicator connector. Finally, we added a pulse generator and a current limiter into the housing and developed a graphical user interface displayed on a 10.1″ touchscreen. The completed device (pulse generator and applicator) for GET to skin cells is shown in Figure 5.

The pulse generator is capable of generating square wave electrical pulses from 80 to 600 V with a pulse duration of 10 µs up to 1000 µs at a pulse repetition rate from 0.1 to 5000 Hz.

3.3. In Vivo Experiments

To determine the efficacy of the newly developed pulse generator in combination with the new applicator and pulse delivery protocol for GET to skin cells (SmartGene—SMG), they were compared with a previously published pulse delivery protocol for GET to the skin using MEA electrodes and Cliniporator [26,27]. Both pulse delivery protocols successfully transfected mouse skin resulting in detectable EGFP fluorescence already on day 1 after GET, which persisted at least until day 7 after GET (Figure 6).

The newly developed pulse delivery protocol (SMG) outperformed the MEA pulse delivery protocol resulting in higher mean fluorescence intensity on all the examined days, indicating a higher level of EGFP expression in the transfected area (Figure 7A). Similarly, the SMG pulse delivery protocol showed a statistically significant increase in integrated density on days 3, 5, and 7 after GET, indicating that a larger area of the skin expresses the

transfected protein, resulting in more of the transfected protein being produced overall compared to the MEA pulse delivery protocol (Figure 7B).

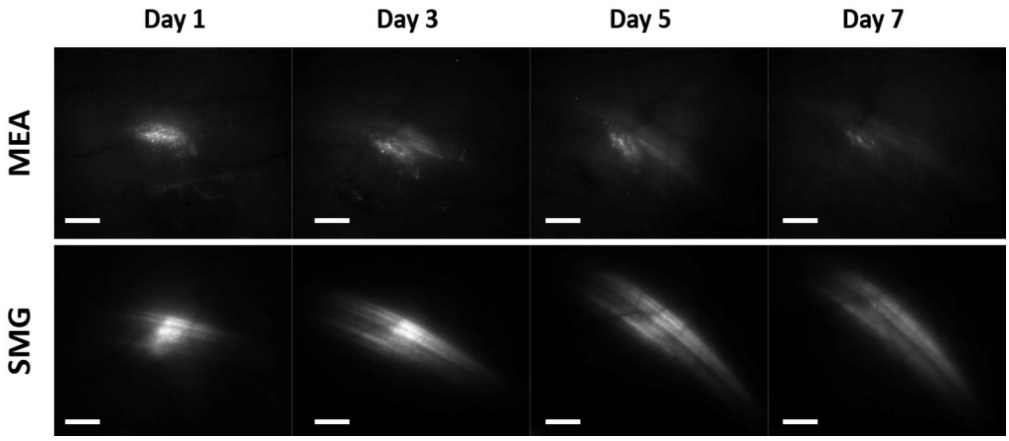

Figure 6. Fluorescence of EGFP protein following GET of the reporter plasmid pEGFP-N1 coding for EGFP in mouse skin. Representative images of EGFP expression in the skin of mice after GET using the multi-electrode array (MEA) electrodes and the previously published pulse delivery protocol: 12 pulses, amplitude 60 V, duration 150 ms, and pulse repetition rate 2.82 Hz ((**upper**) part of the figure); and the newly developed pulse generator in combination with the new applicator and pulse delivery protocol for GET to skin cells—SmartGene (SMG): 18 sequences with 4 pulses, burst repetition rate 50 Hz, amplitude 560 V, duration of each pulse 100 µs, and pulse repletion rate 5 kHz ((**lower**) part of the figure). Scale bar: 2 mm.

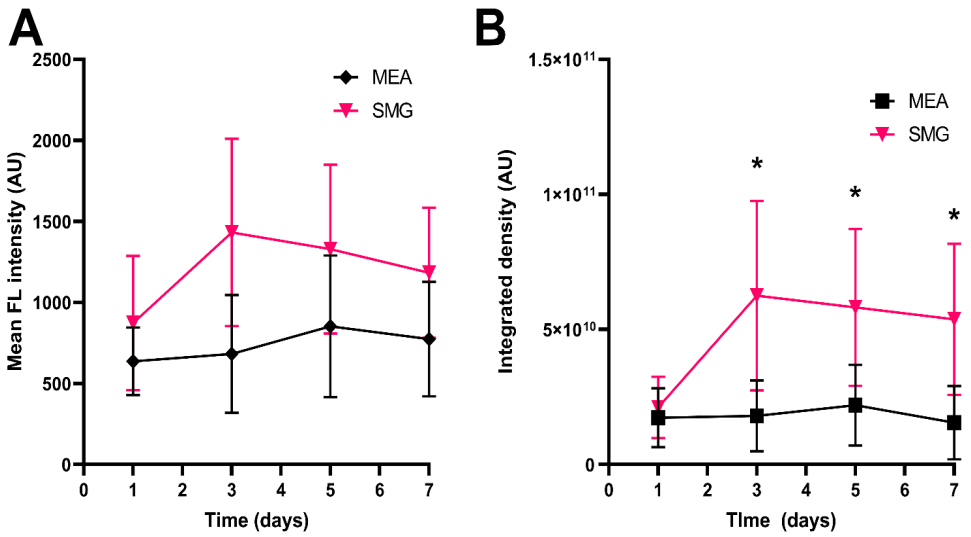

Figure 7. Expression of EGFP protein following GET of the reporter plasmid pEGFP-N1 coding for EGFP in mouse skin. (**A**) Mean fluorescence intensity of EGFP in the transfected skin after GET using

the multi-electrode array (MEA) electrodes and the previously published pulse delivery protocol: 12 pulses, amplitude 60 V, duration 150 ms, and pulse repetition rate 2.82 Hz; and the newly developed pulse generator in combination with the new applicator and pulse delivery protocol for GET to skin cells—SmartGene (SMG): 18 sequences with 4 pulses, burst repetition rate 50 Hz, amplitude 560 V, duration of each pulse 100 µs, and pulse repletion rate 5 kHz. (**B**) Integrated density of EGFP in the transfected skin after GET using the MEA electrodes and previously published pulse delivery protocol, and the newly developed pulse generator in combination with the new applicator and pulse delivery protocol for GET to skin cells—SmartGene (SMG). N = 5 (MEA) and N = 4 (SMG). Shown are the mean values ± SD. *—$p < 0.05$, *t*-test. The Shapiro–Wilk test was used to test for normal distribution of the data. A non-parametric *t*-test was performed only for Mean FL intensity (day 3) because a non-normal distribution was found for this point.

4. Discussion

The aim of our study was to design, develop, and test a new electroporation device (pulse generator and applicator) and a pulse delivery protocol that would maximize gene delivery. The design was based on the target tissue and the effect to be achieved, i.e., gene electrotransfer (GET) of skin cells, while following the previously determined user and technical requirements. We numerically determined the optimal electrode configuration and pulse delivery protocol. We proposed an alternative pulse delivery protocol, which proved to be more successful in achieving a deep and homogeneously reversible electroporated volume, with less damage due to irreversible electroporation than the classical pulse delivery protocol. This also suggests that GET will be more successful with the newly proposed alternative pulse delivery protocol than with the classical pulse delivery protocol. We also focused on the safety of the device and the requirements for clinical use, given the lack of pulse generators for GET that can be used in human studies and in the clinics. Therefore, we designed and developed a new pulse generator, and tested its operation on both a resistive load and in an in vivo gene electrotransfer study.

The results of the performed in vivo study showed that high expression levels of the transfected plasmid DNA proteins can be achieved with the newly developed pulse generator, applicator, and pulse delivery protocol for GET to skin cells in mice. When compared to the previously published pulse delivery protocol for GET to the skin using MEA electrodes [26,27], the newly proposed pulse delivery protocol achieved higher expression levels in the transfected area, as well as higher overall production of the transfected protein.

In developing the pulse generator, we followed the standard EN 60601-1: 2007: Medical electrical equipment—Part 1: General requirements for basic safety and essential performance. This standard is a generally accepted criterion for medical electrical equipment and compliance with this standard has become the main requirement for marketing of medical electrical equipment. Therefore, the pulse generator was tested with a certified and calibrated Fluke ESA620 electrical safety analyzer (Fluke Biomedical, Washington, USA) for medical devices in accordance with the medical standard EN 60601-1: 2007. The electrical safety report showed that the leakage currents are within the allowable leakage currents according to the standard. This means that even in the event of a single fault, the device will not cause harm to the patient.

However, the device is still not certified as a medical device under the Medical Device Regulation (MDR) 2017/745, although the electrical safety report showed that the device can be used safely. Additional testing by a notified body certified under the current MDR is required to assist us in resolving existing discrepancies, as we were not able to meet all the requirements of the other listed standards and prepare the technical documentation. In addition, we do not have a Quality Management System (QMS) for the procedures and processes required to develop and manufacture a medical device. Therefore, in order to proceed with the development of a clinical electroporator and later with the production, we need to establish QMS and prepare the technical documentation. Overcoming these obstacles will lead to the availability of a certified clinical electroporator for GET to skin cells that can be used with a standardized protocol for new in-human studies.

5. Conclusions

This paper presents the design and development of the pulse generator and applicator for gene electrotransfer to skin cells, following user preferences, technical recommendations and treatment protocol. The developed numerical model enabled testing of two different pulse delivery protocols and proposed an alternative pulse delivery protocol, which was then used in vivo for gene electrotransfer to skin cells in mice. The results showed higher mean fluorescence intensity and a statistically significant increase in integrated density after GET with the newly developed pulse generator and applicator for gene electrotransfer to skin cells along with the proposed alternative pulse delivery protocol, compared to the currently used Cliniporator, MEA electrodes, and pulse delivery protocol. However, the device for gene electrotransfer to skin cells and the proposed alternative pulse delivery protocol need further evaluation. In addition, the device needs to be certified as a medical device under the Medical Device Regulation 2017/745 in order to be safely used for new in-human studies.

Author Contributions: Conceptualization, M.R., D.M. and G.S.; methodology, M.R., J.D.-Č. and B.M.; software, M.R. and J.D.-Č.; validation, B.M., M.R. and D.M.; formal analysis, B.M., G.S. and D.M.; investigation, S.K.B., B.M., A.C., J.D.-Č. and M.R.; data curation, A.C. and B.M.; writing—original draft preparation, A.C., B.M., J.D.-Č. and M.R.; writing—review and editing, All authors; visualization, A.C., J.D.-Č. and B.M.; supervision, M.R., D.M. and G.S.; funding acquisition, G.S. and D.M. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The experiments were approved by the Ministry of Agriculture, Forestry and Food of the Republic of Slovenia (permission no. 34401-1/2015/43 and U34401-3/2022/11). The experimental procedures were performed in compliance with the guidelines for animal experiments of the EU directive (2010/63/EU) and ARRIVE guideline.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data that support the findings of this study are available within the article.

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Conflicts of Interest: The authors declare no conflict of interest.

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4 Conclusions

The muscle stimulation and pain study presented in Paper 1 (section 3.1) confirmed the hypothesis that the use of short (1 µs, 2 µs), bipolar high-frequency pulses with short interphase and interpulse delays reduces muscle contraction in healthy individuals as opposed to the currently used long monopolar pulses (8 pulses with pulse duration of 100 µs, delivered at 5 kHz pulse repetition frequency). Similarly, these pulse protocols reduce the pain sensation experienced during the delivery of the pulses. However, interphase and interpulse delays also play an important role in reducing muscle contraction and/or pain sensation, making the interplay between the pulse parameters more complex. Based on the chosen pain descriptors from the pain questionnaires, our results also suggest that the A-delta nerve fibers are predominantly excited compared with the C fibers. More A-delta fibers were excited/stimulated in each cluster of bipolar pulse protocols, suggesting that there is a higher involvement of A-delta nerve fibers in the transmission of nociception during the delivery short, bipolar high-frequency pulses. With this study, we also concluded that muscle contraction and pain felt during the delivery of the pulses are not always correlated. Indeed, pain is not necessarily elicited as a consequence of muscle contraction and vice versa. Therefore, higher pain indexes can be observed for pulse parameters that do not cause strong muscle contraction. One possible explanation for this is the gate-control theory of pain mechanisms [76–78]. This theory states that the activity of large fibers excites inhibitory neurons, thereby decreasing the transmission of pain information. When more large fibers (A-alpha and/or A-beta fibers) are activated compared with smaller pain fibers (A-delta and C fibers), people tend to experience less pain. Thus, a non-painful input (e.g., a touch/massage on a bumped area) can close the nerve "gates" to the painful input because the activity of the large fibers (in this case, A-beta) is increased. Thus, pain sensation can

be prevented or at least reduced (pain fiber activity is reduced) because not all pain signals reach the central nervous system. In our case, this would mean that with the stimulation of the muscle, a muscle contraction is produced that activates/excites the large fibers and thus reduces the excitation of the nociceptive (pain) fibers, i.e., the gates close. Therefore, with this study, we have also shown that the optimal range of pulse parameters can be extended, as some therapies require only certain conditions to be met (e.g., only reduced muscle contraction). This means that based on the particular application of electroporation, appropriate modifications of the pulse parameters can be made to reduce adverse effects and provide a safe, successful, and effective therapy. However, further evaluation of the presented bipolar pulse protocols with clinically relevant high-voltage amplitudes, also applied to different tissues (tumors, heart) and locations (deep, superficial), is necessary to confirm our results and the efficiency of the newly proposed high-frequency electroporation.

Nowadays, the industry of electroporation devices is growing even faster than before, considering the new technologies and the increased research intensity and knowledge. It also shows huge potential for further implementation in clinical practice. However, the translation of some applications of electroporation into the clinic (e.g., transdermal or intradermal gene electrotransfer (GET)) is still slow and lags behind in vitro and in vivo studies [79, 80], in spite of its great potential [24]. We believe that one of the possible reasons for this is inadequate dosimetry, which does not allow a thorough comparison between different research studies and results because different electroporators, electrodes (applicators), and pulse parameters are used [56, 81]. In addition, there is currently a lack of certified clinical electroporators and applicators on the market, as their certification/commercialization is complicated and long-lasting. As they are considered to be medical devices, clinical electroporators must meet medical-safety standards and comply with the requirements of local medical regulations, e.g., Medical Device Regulation (MDR) 2017/745 in Europe. We believe that many researchers/developers of electroporation devices are not aware of how extensive the documentation required for certification under the new MDR needs to be and how many safety standards and requirements must be followed. The lack of a particular medical safety standard for clinical electroporators further complicates this process by requiring other different safety standards to be followed.

Therefore, in Paper 2 (section 3.2), we have identified the safety standards that should currently be considered when developing clinical electroporators based on the requirements of the new MDR. In addition, we have defined the necessary safety measures to be considered in the development of such devices, based on the general standard for the safety of medical electrical equipment EN/IEC 60601-1:2007: "General requirements for basic safety and essential performance" and the accompanying safety standards that should be considered in establishing a particular standard for clinical electroporators in the future. We also proposed recommendations for the requirements that the particular standard for clinical electroporators should have to make the development and certification process for these devices more straightforward. Finally, in order to make electrochemotherapy even more widespread and safe in clinics, we determined the tolerances for pulse amplitude and pulse duration based on cell permeabilization curves, which may be introduced as an additional improvement for the current standard operating procedure. With this in mind, we have also proposed guidelines (procedural requirements) for a standardized treatment protocol for GET in the scope of Paper 3 (section 3.3), which may help in establishing a safe and efficient standard operating procedure even for this application of electroporation. Following these guidelines, as well as the requirements and safety factors based on the general standard EN/IEC 60601-1 presented in Paper 2, we designed and developed new safety measures for an electroporator and applicator for GET to skin cells, presented in Paper 3. The device was also tested in vivo for GET in mouse skin cells, and it was shown that electroporation with the developed device, applicator and proposed pulse delivery protocol resulted in higher gene expression in skin cells compared to the currently used Cliniporator, multi-electrode array electrodes (MEA) and pulse delivery protocol [82–84]. In the scope of the paper and for the purpose of this doctoral dissertation, new circuits were developed using Altium Designer to enable safe generation of the electroporation pulses. Isolation and conversion circuit was developed using A/D and D/A converters to galvanically isolate the control signals using optocouplers and to enable control of the high-voltage power supply. In addition, new safety measures were developed and tested to determine and limit high values of the output pulse. Since we were unable to measure the current with the selected current sensor, the current limiter was implemented separately based on the simulations performed in LT Spice. To verify whether the safety of such a device was improved, we tested

the electroporator with a certified and calibrated Fluke ESA620 electrical safety analyzer (Fluke Biomedical, Washington, USA) for medical devices according to the medical standard EN 60601-1:2007. The electrical safety report showed that the leakage currents were within the allowable leakage currents according to the standard (Table 3.1). This means that even in the event of a single fault, the device will not cause harm to the patient. However, the device is not yet certified as a medical device. There are still some discrepancies, as we have not yet been able to test and meet all the requirements of the other safety standards (e.g., electromagnetic compatibility) and prepare all the required technical documentation. We also do not have an implemented Quality Management System (QMS), which follows the ISO 13485 standard for the procedures and processes required for developing and manufacturing a medical device. In the future, it will be necessary to first establish a QMS at the beginning of the development phase, plan the usability engineering, and create a risk management plan. In addition, the technical documentation will need to be prepared in accordance with Annexes II and III of the MDR 2017/745 for marketing the device in Europe. Overcoming these obstacles will lead to the availability of a new certified clinical electroporator that can then be used with the current/proposed standard operating procedures for new, safe and efficient human studies.

5 Original scientific contributions

Reduced muscle contraction and pain sensation during high-frequency electroporation treatments

There is an increasing emphasis in the literature on developing new electroporation techniques that can reduce the muscle contractions during electroporation-based treatments. The use of bursts of very short (≈ µs) bipolar pulses instead of the relatively long monopolar pulses is mainly proposed. However, all the data obtained so far do not allow us to evaluate the reduction of pain. Therefore, we performed the first study on healthy volunteers[1] using different types of pulses: classical (monopolar with pulse duration of 100 µs, delivered at 5 kHz pulse repetition frequency) and bipolar with different pulse parameters (pulse duration, interphase and interpulse delays). Statistically significant differences were found between the protocols and they were grouped into five different clusters, i.e., bipolar pulse protocols with higher/lower muscle contraction response and/or higher/lower pain index. Additional measurements with extended interpulse delays and interchanged interphase and interpulse delays were also performed. In addition, pain descriptors selected from the questionnaires were used to analyze which pain fibers were more likely to be excited (A-delta or C fibers). The hypothesis that bipolar high-frequency pulses with a pulse duration of 1 or 2 µs reduce muscle contraction and pain sensation was confirmed. However, the interphase and interpulse delays play an important role in reducing muscle contraction and/or pain sensation. Thus, the interplay between pulse parameters is more complex. Pain is not necessarily elicited as a consequence of muscle contraction and vice versa. Therefore, the range of optimal pulse parameters can be extended depending on the requirements of the particular therapy.

[1]The study was approved by the National Ethics Committee of Slovenia (Doc. no. 0120-61/2020).

Preparation of a concept for standardization of electroporation devices for clinical use

The lack of a medical particular safety standard for clinical electroporators is an obstacle to the development of safe and efficient electroporation devices. Currently, there are few certified clinical electroporators on the market because the process of commercialization requires a great deal of documentation and effort. Researchers/developers of electroporation devices are often not aware of how extensive the technical documentation needs to be in order to be certified under the new Medical Device Regulation (MDR) 2017/745 and how many safety standards and requirements need to be met. Therefore, recommendations for simpler standardization have been proposed based on a review of the current electroporation application and associated equipment. The necessary safety features that a clinical electroporator should have were determined based on the general safety standard for medical electrical equipment EN/IEC 60601-1: "General requirements for basic safety and essential performance". The safety standards that currently need be followed based on the requirements of the new MDR were also identified. Based on the medical safety standards, guidelines were recommended for the preparation of a particular medical standard for clinical electroporators. In addition, tolerances of the pulse parameters for electrochemotherapy from the standard operating procedure were defined for easier operator guidance and improving/assuring the quality of electrochemotherapy.

Development of safety measures for electroporation device to protect the patient from excess output voltage, current or energy

A clinical electroporator must pass all safety tests in order to be accepted for further testing under the relevant standards and certification under the Medical Device Regulation (MDR) 2017/745 in Europe. This would ensure that it can be used safely on patients. Therefore, a new clinical electroporator and applicator for gene electrotransfer to skin cells with better safety performance were designed, developed and tested in an in vivo study, so that they can be certified under the new MDR in the future. We proposed technical and user requirements and recommendations that should be considered in the development of such a clinical electroporator and we followed them as much as possible during the development. Electrical insulation between the high- and low-voltage parts of the device was provided, as well as appropriate control of the high-voltage power supply. In

addition, new safety measures were developed and tested to detect high values of the output voltage, current or energy and limit them to the maximum expected value during the therapy. A new approach to limit the current was simulated in LT Spice and then implemented in the actual design of the device. This will ensure safe generation and delivery of electroporation pulses to the patient. The electroporator was tested with an electrical safety analyzer and was found to be safe in terms of leakage currents, as they were within the range of allowable values according to the general safety standard for medical electrical equipment EN/IEC 60601-1.

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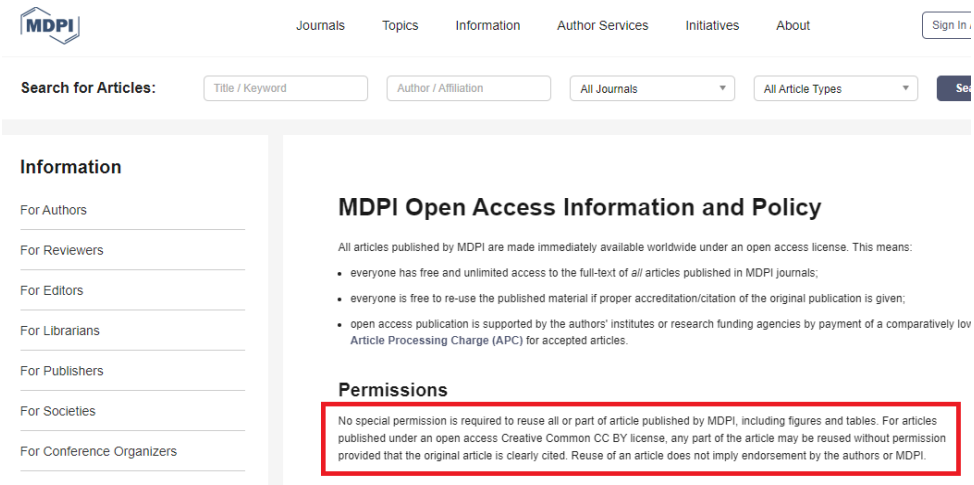

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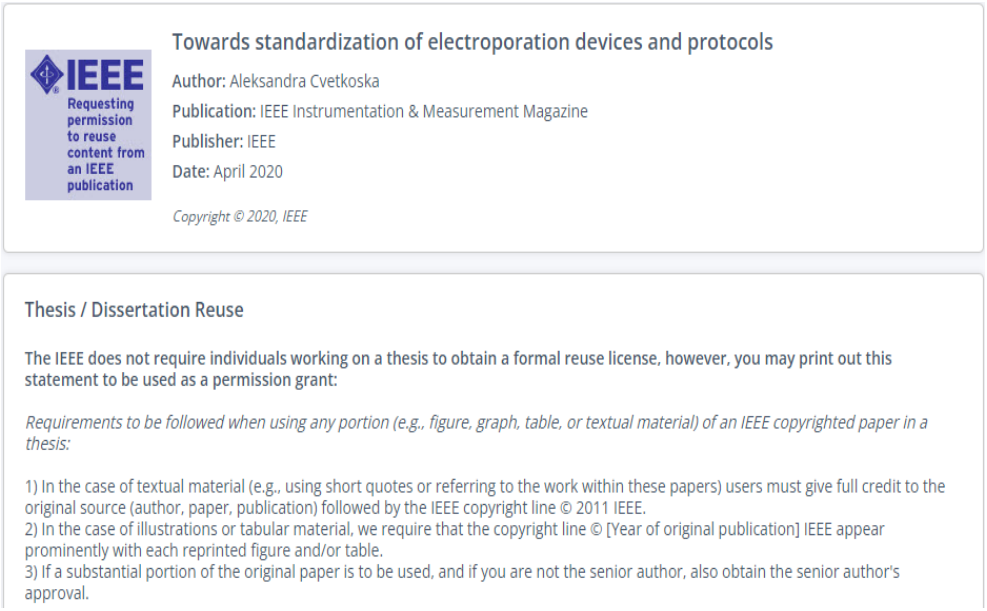

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